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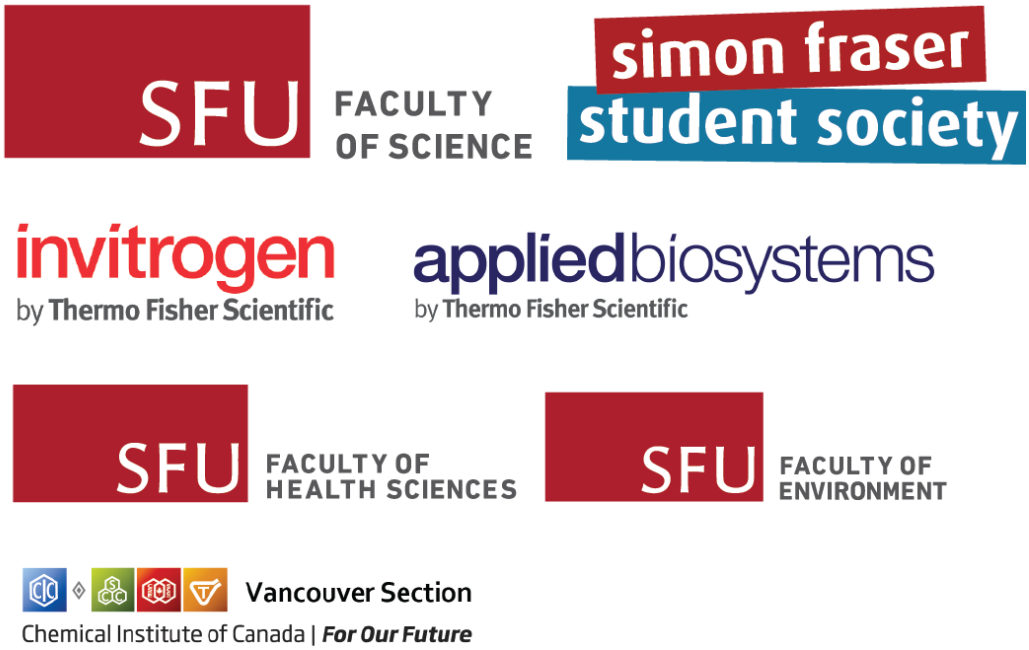

2016-17 Board of Editors

"For a research worker the unforgotten moments of [their] life are those rare ones which come after years of plodding work, when the veil over nature's secret seems suddenly to lift and when what was dark and chaotic appears in a clear and beautiful light and pattern."

- Gerty Cori

Foreword

Dear Reader,

We created the SFU Science Undergraduate Research Journal with goals that reached beyond the scope of a publication released once a year. We ambitiously put forward that we would foster a community of undergraduates who value research while engaging students in science communication and education. To cultivate such a community takes time and much more than a collection of papers published every so often. Reflecting on the past year, we are proud to have contributed to the broader undergraduate research community at Simon Fraser.

The past year marked the beginning of our series featuring undergraduate researchers on our blog. We met 28 young scientists from a broad range of departments. It was remarkable learning about these driven, insightful, and creative students diligently navigating the world of research. As editors, we were overwhelmed by the diversity and quality of undergraduate research quietly happening in pockets around SFU, and we are excited to introduce you to more undergraduate researchers in the coming year.

In September, we hosted the first annual SFU Science Undergraduate Research Poster Competition that brought together 17 researchers from six departments to present their research posters to the SFU community. Not only did the day remind us of the depth of research conducted by undergraduates, but professors and graduate students echoed our impression, approaching us to specifically remark on the calibre of the work presented.

The journal embodies the process of learning, whether it be as an editor, author, or reviewer. It continues to be an immeasurable pleasure to facilitate that process, and here's to more years (and journals) to come. With delight, we present the second edition of the SFU Science Undergraduate Research Journal.

Sincerely,

Emma Atkinson, Sean La, and Christine Wang

Executive Editors

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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*

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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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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 = $3003.90 \pm, 303.10$ & 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

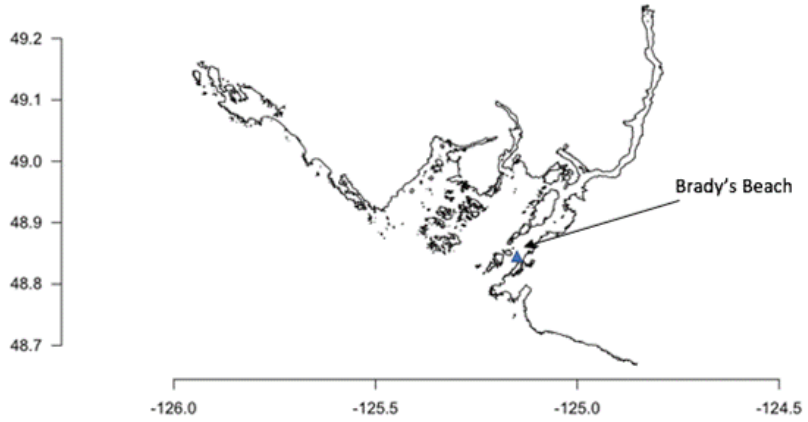

Figure 1: *Map marking the location of the collection site, Brady's Beach (48.8271°N, 125.1531°W) within the Barkley sound, BC area. The location is marked with a blue triangle.*

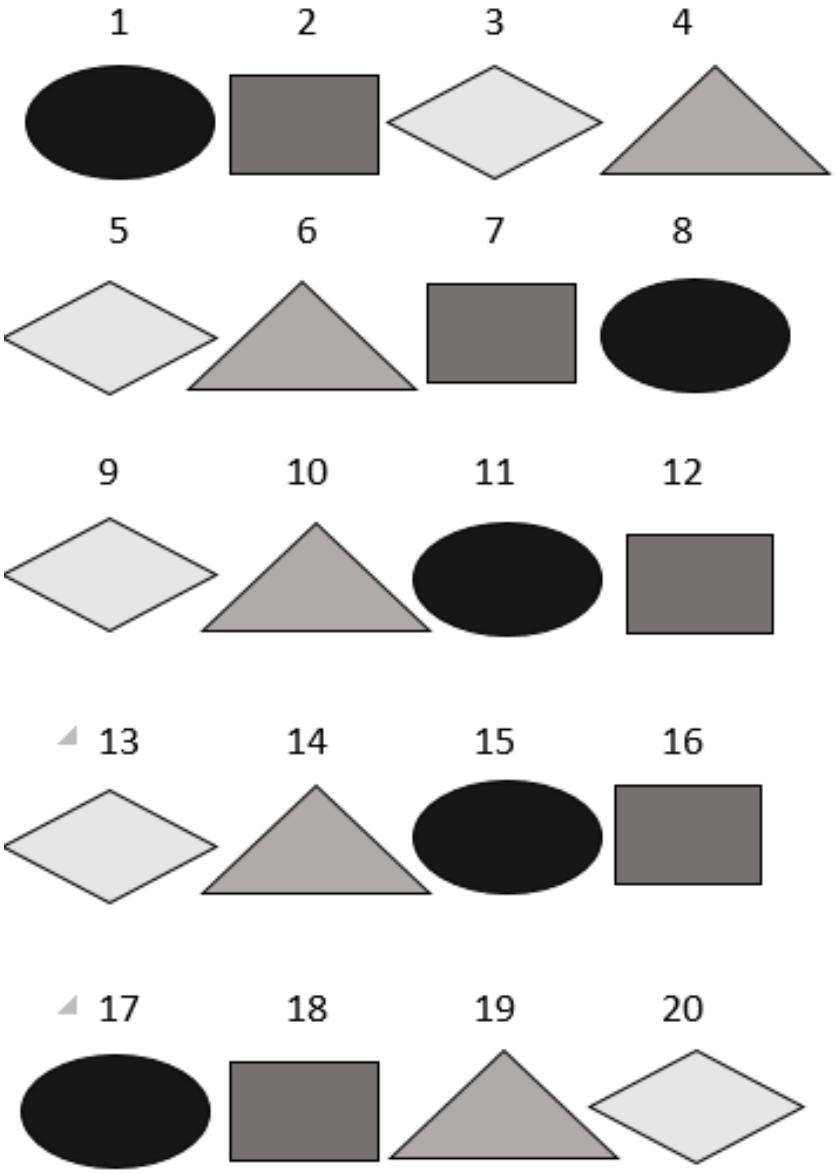

Figure 2: *Sample showing a subset (20 out of 120) trials used for each experimental trial. Each experimental setup consisted of four separate treatments, determined by a random number generator [39] regular sperm and egg (diamond), diesel sperm and diesel egg (circle), regular sperm and diesel egg (triangle),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

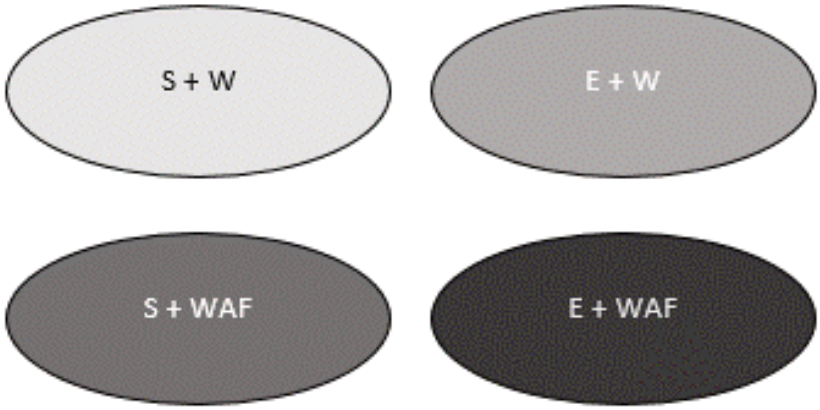

Figure 3: *Stock solutions used throughout each experimental trial: sperm and filtered sea water, egg and filtered sea water, sperm and water-accommodated fraction with diesel (WAF), egg and WAF. New stock solutions were created for each of the three trials. Egg and Sperm were evenly distributed between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

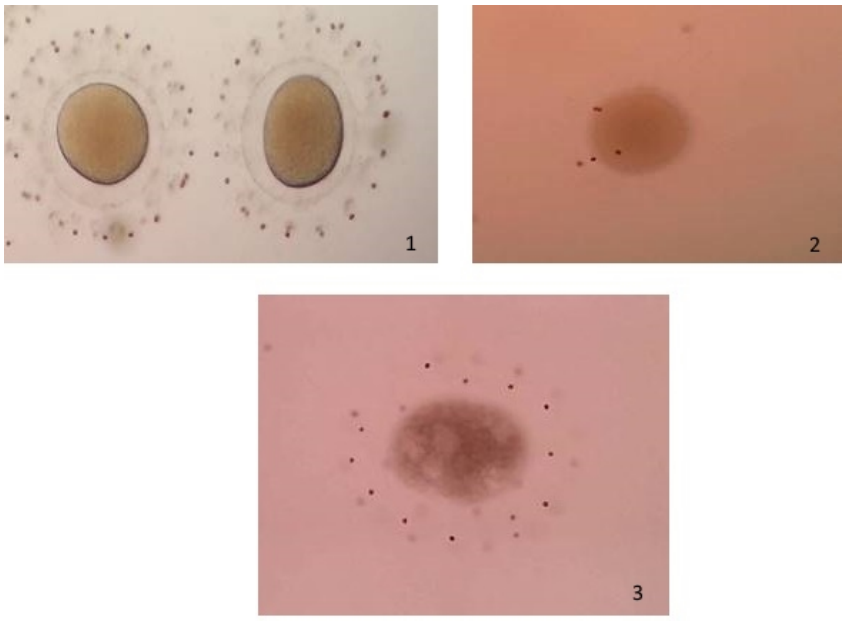

Figure 4: *Images comparing differences between a fertilized and unfertilized egg. 1) normal fertilized egg. 2) an egg that has lost its jelly layer. 3) an egg that contains missing portions within the egg itself. Image 2 and 3 are examples of unfertilized eggs, therefore, not included in final counts.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

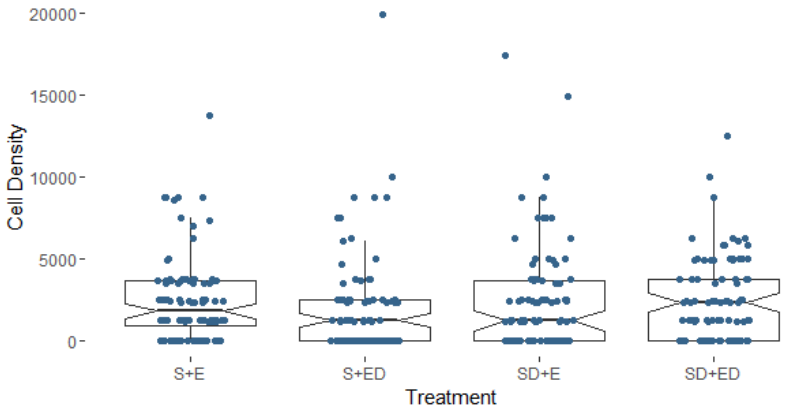

Figure 5: *Treatment vs. cell density among experimental trials pooled into one data frame. Data points represent raw data obtained with boxplots behind to better represent trends in data. Notches represent 95% confidence intervals of the median.*

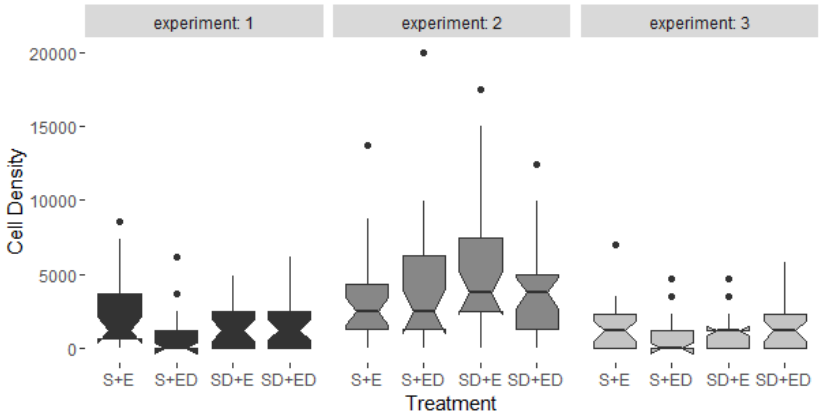

Figure 6: *Treatment vs. cell density box plots separating experimental trials (1,2,3) with 95% confidence intervals. Outliers are represented with dots above whiskers. Experiments 1,2,3 represent experimental trials.*

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Open the Cage: Handling, not captivity, affects escape responses of the red sea urchin, *Strongylocentrotus franciscanus* to the sunflower seastar, *Pycnopodia helianthoides*

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Abstract

The effects of handling and captivity of red sea urchins, *Strongylocentrotus franciscanus*, on its escape response to the predatory sunflower seastar, *Pycnopodia helianthoides*, were measured. One hundred and twenty four individuals of *S. franciscanus* were collected and subjected to 1 d, 2 d, 3 d and 4 d captivity treatments. Handling effects due to the tagging process were controlled for with 30 individuals. 1 d, 2 d and handling control treatments exhibited significantly lower escape responses compared to wild control organisms (all p values < 0.05). No effect of captivity time on escape responses was seen as handling controls did not differ from the captivity treatments. These results suggest that *S. franciscanus* would succeed after being reintroduced to the wild environment following captivity.

Keywords — Predator escape, captivity, *Strongylocentrotus franciscanus*

1. Introduction

Human populations have drastic effects on the natural world particularly through habitat change [1, 2]. These environmental changes, a portion of human-induced rapid environmental change (HIREC), can result in behavioural responses from various organisms [3]. Scientific studies often place their subjects in new environmental conditions to study their behavioural responses [4]. While captive environments offer opportunities for experimental manipulation, they may have effects on study organisms that magnify or dampen their intended responses. The environmental changes that scientists subject their study organisms to must be carefully acknowledged as well as the resulting effects that their reintroduction into native habitats may have [5].

Animals kept in captivity will quickly undergo behavioural responses similar to HIREC [6]. These studies often look at the evolution of captive animals over generations and therefore genotypes [6, 7], while relatively few studies look at the effects of short term responses (within one generation) of captive populations [4]. Changes in behaviours due to captivity can be caused by many stressors including proximity to humans and restricted movement [8]. These short term behavioural responses may result in lowered survival in the wild following reintroduction or

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negative consequences to the native environment such as the introduction of disease [9]. Handling, another common stressor during captivity, sometimes changes organism behaviour [10]. Understanding how quickly behavioural responses occur due to handling and captivity, along with the effects they have on survival in the wild, should impact reintroduction policy decision making.

Bamfield Marine Science Center (BMSC) recently proposed euthanizing collected organisms that are kept in captivity for more than 72 hours. BMSC has a strong history of animal care and takes in a wide variety of marine taxa but recognizes that the acquisition of study organisms disrupts the natural environment [4]. Among other reasons cited, the low probability of organism survival following reintroduction was an important factor in the decision making process [4]. The release of sport fish shows relatively low survival due to the aggressive nature in which the fish are caught and hatchery reared salmon often have difficulty recognizing predators [11, 12]. The Seattle Aquarium released a Giant Pacific Octopus successfully but there are few other studies that look into the reintroduction of invertebrates [4, 13]. It stands to reason that lower invertebrates, collected with less invasive procedures, would have a higher likelihood of survival following the reintroduction to their native environment [14]. Marine research stations need more evidence of reintroduction success to make well informed policy decisions.

Echinoderms are popular subjects for scientific research [14]. Forming a large portion of the specimens collected by BMSC, they are an appropriate study organism to educate changes to animal care policies [15]. Ordinary behavioural responses may change following captivity and could impact the ability of an echinoid to survive. Urchins have been known to exhibit escape responses in the presence of predatory sea stars [15, 16]. If responses are minimized due to increased stress, a symptom of captive and handled animals, lower survival could be expected [3]. *Strongylocentrotus franciscanus* is a common urchin to Barkley Sound that exhibits an escape response to *Pycnopodia helianthoides* [17]. The escape response of *S. franciscanus* in wild and reintroduced populations will be measured.

The primary predictions are:

1. Initial escape responses, the distance covered in 10 seconds following contact with *P. helianthoides*, decreases as time in captivity increases.

2. Escape response strength, the total distance covered in 30 seconds following contact with *P. helianthoides*, decreases as time in captivity increases.

3. Initial escape responses will be negatively affected by handling.

4. Escape response strength will be negatively affected by handling.

2. Materials and Methods

Five study sites were marked with anchored buoys, separated by 10 meters, along a transect running parallel to the coast at a depth of 8 meters at Aguilar Point near Bamfield, British Columbia (48.839444 125.141111; Figure 1). The bottom substrate was mostly a mixture of boulder and sand with little evidence of a dominant macro

algal species at the time of survey. Five teams of two divers conducted the experiment between May 11 and May 17, 2016.

One hundred and twenty four individuals of *S. franciscanus* were collected from depths ranging from 6 to 9 meters at Aguilar Point by SCUBA over five collection dives. These urchins ranged in test diameter size from 23 to 92 millimetres. They were brought back to BMSC and placed in flowing salt water tanks for their captivity treatments. Urchins were not fed while in captivity. Urchin test diameters were measured with calipers and individuals were given a random treatment. 1 day captives ($n = 31$) were kept in the laboratory for a minimum of 24 hours, 2 day captives ($n = 29$) for a minimum of 48 hours, 3 day captives ($n = 30$) for a minimum of 72 hours and 4 day captives ($n = 34$) for a minimum of 96 hours. Individuals were identified by treatment group with the placement of two small (1 cm x 1 cm) rubber hosing tags on their spines. This was done by inserting a plastic pipette through a small hole in each tag and letting it off at the base of the spine after it was covered by the pipette. Once marked individuals satisfied a treatment requirement they were deployed by SCUBA equally at the anchor of the five study sites. Additionally, 30 *S. franciscanus* were subjected to a handling control treatment. These individuals were brought to the surface where they were tagged and redeployed equally among the study sites. Data were also collected on wild controls ($n = 57$) when time permitted following the underwater testing of treatment groups. Wild controls were haphazardly chosen by testing the third specimen encountered that satisfied the test diameter requirement of 3 to 9 cm during a standard roving survey.

Behavioural response data were collected the day following deployment. Predator escape response was induced by placing an individual of *S. franciscanus* on a flat surface and gently touching the tube feet on one side with *P. helianthoides*. The distance travelled along a standard transect tape was measured at 10 seconds, 20 seconds and 30 seconds following contact with the seastar.

Data were analyzed using a non-linear mixed effects model to describe the initial response (distance traveled within 10 seconds following contact) and response strength (total distance travelled within 30 seconds following contact) of *S. franciscanus* to contact with *P. helianthoides* using the R package "nlme" [18]. The fixed effect was treatment group while dive teams were included as random effects to account for variation between dive teams and the sites that they collected their data. All analyses were conducted in R [19].

3. Results

Initial predator escape responses did not vary significantly between captivity treatments and handling controls (Figure 1, Table 1). Response strengths, measured as total distance travelled in 30 sec, did not differ significantly between captivity treatments and handling controls (Figure 2, Table 2).

Wild urchins moved significantly faster in the first 10 seconds after contact with a seastar than all other urchins, except in four day captive treatments (Figure 1, Table 3).

Wild urchins moved greater distances over 30 seconds than urchins that had undergone handling processes (Figure 2, Table 4).

4. Discussion

Initial predator escape response and predator escape response strength appeared to be unaffected by length of captivity, which contradicts our first two predictions. This suggests that the amount of time *S. franciscanus* is subjected to captivity should not be a factor for determining whether an individual should be released back into the wild or euthanized. Instead, it appears that urchins that had been handled had weaker predator escape responses compared to the wild controls, which supports our third and fourth predictions. This suggests that the handling of wild urchins should be considered for reintroduction policies.

4.1. Responses to captivity

No significant differences in initial predator response nor predator response strength due to captivity time was found. Often behavioural responses change with captivity due to increased stress as an organism adjusts to its new environment [6]. Contrary to many other studies on organisms such as bony fishes and other vertebrates, these results suggest that captivity has little to no effect on the stress of *S. franciscanus* over the first 96 hours of captivity [20]. This might be due to the a high quality captive environment of the flow-through salt water system of BMSC, which can simulate the natural underwater environment of *S. franciscanus*. It could be expected that other, less advanced, captive environments might produce a negative effect on predator escape responses. Policies regarding the reintroduction of collected organisms should not be concerned about the captivity time of *S. franciscanus*, and possibly other echinoids, at BMSC.

4.2. Responses to handling

Urchins that experienced any handling prior to behavioural testing had weaker responses to predation than wild caught individuals. The process of collecting urchins, bringing them to the surface, placing tags and redeploying them appears to have put stress upon individuals. It is not clear from the study what portion of methodology changed the response of an individual to the presence of a predator. Three mechanisms are proposed by which this could occur: rapid changes in environmental pressure, tagging and increased detachment occurrences.

The process of bringing *S. franciscanus* from depth to the surface could have a negative impact on escape response. Many fish species cannot survive the process of being brought to the surface so it is possible that a rapid change in pressure could affect other organisms [21]. However, the impact would likely not be as significant in echinoids as they have a water vascular system that does not allow for large expansions of air within the body cavity [22]. For this reason, changes in environmental pressure during handling is the least likely mechanism for which predator escape responses change.

The tags put onto *S. franciscanus* may have negatively affected the predator escape as tagging has been shown in other studies to affect behavioural responses [23]. The placement of the rubber hosing sometimes resulted in the breaking of urchin spines

and may restrict spine and tube feet movement. Two small tags however cover very little of the urchins surface area and obstructed few, if any, of urchins spines and tube feet. Furthermore, there appeared to be little tag rejection during visual roving surveys of the study organisms. Since it is common for echinoids to autotomize appendages that are obtrusive or injured, it is likely that our tagging techniques were relatively unobtrusive [24].The tags may have had a slightly negative impact on predator escape responses but not enough to explain the significantly different results between handled individuals and wild controls.

All individuals that had previously been handled underwent more detachment processes than wild caught individuals. When *S. franciscanus* feels threatened by detachment its natural response is to grip tighter to the substrate with its many tube feet [25]. If divers were not capable of conducting a swift detachment procedure, it is likely that many tube feet and spines were injured. Since tube feet and spine condition are critical components of escape response speed, increasing detachment occurrences should have a strong negative impact on escape response [26, 27].

Furthermore, urchins have been shown to have a tremendous ability to regenerate tube feet [28]. This may explain the increased predator escape response by the four day captives as this treatment group did not show significantly weaker predator responses compared to the wild controls. If increased days in captivity means more opportunity for the regeneration of tube feet, it is possible that more days in captivity could be beneficial for predator responses when compared to handling controls. Further experiments would need to be conducted over a longer period of time to see what long term effects captivity would have on predator escape response. It appears that this mechanism has the largest impact on predation escape response and suggest its further study.

It appears that the handling process had a negative impact on predator escape response while captivity appeared to have no effect. The lack of ecologically relevant changes to escape response due to captivity should not affect reintroduction. Thus, BMSC review their reintroduction policy for animal care to account for the handling of organisms and continue to review the effects of captivity on the reintroduction of wild organisms to their native habitat.

5. Acknowledgments

Thanks to my terrific teaching team of Isabelle Côté, Siobhan Gray and *Fiona franciscanus*. Many thanks to the beautiful boating bunch of Stove Johnston, Mother Rachure, John and Janice for looking after us on the high seas. Thanks to Owen and animal care for keeping a watchful eye on our captives. Gracias SciDive 2016, especially Spice and the Greek, for dealing with a gypsy soul like myself. To close, I have one last thing to say: M A L A K A

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6. Appendix

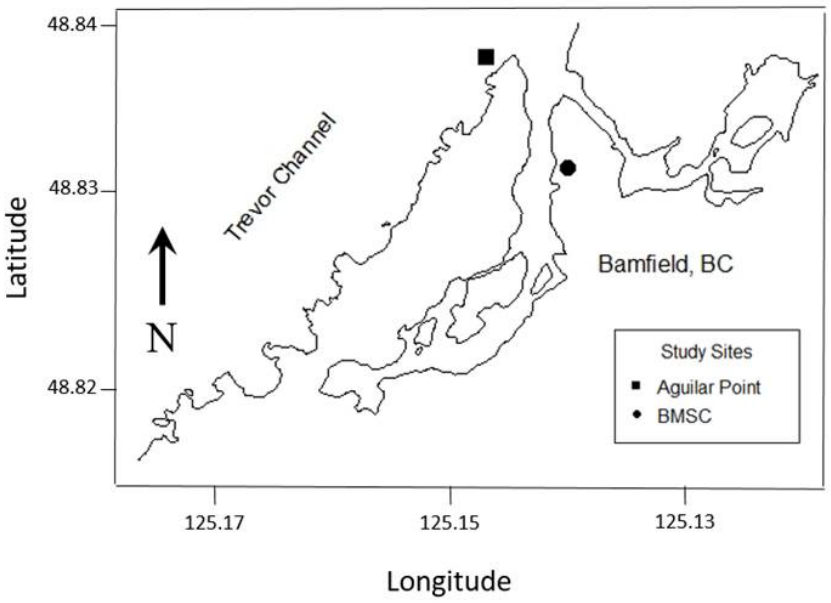

Figure 1: *Map of study areas in and around Bamfield, British Columbia.*

Table 1: *Model predictions and experimental observations of C. maenas and H. nudus distributions among good and poor patches ($N = 3$, averages ± 0.33 absolute error).*

	Experimental Group				
	Day 1	Day 2	Day 3	Day 4	WC
t-values	-0.243	-0.059	-0.581	0.916	2.179
p-values	0.808	0.953	0.562	0.361	0.031*
n	31	29	30	34	57

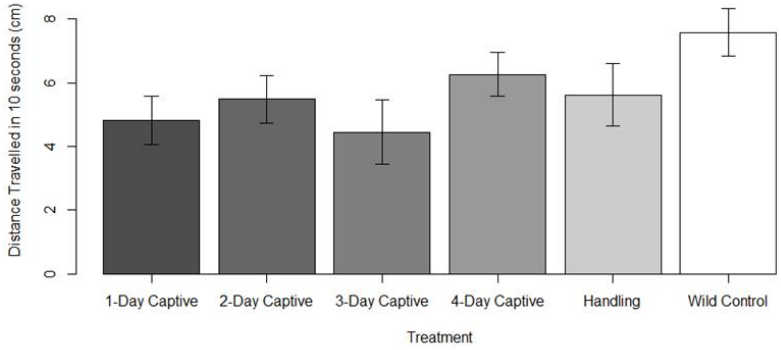

Figure 2: *Initial response values for captivity treatments, handling and wild control S. franciscanus. Wild control urchins travelled significantly further than handling control, 1 day captive, 2 day captive and 3 day captive treatments. Mean initial response distances are shown ±1 SE. Sample sizes are shown in Table 1 and Table 3.*

Table 2: *Summary of non-linear mixed effects model for mean predator escape strength of red sea urchins in different captivity treatments with HC as the baseline. Fixed effects are treatment groups and random effects are dive team. HC is handling controls, WC is wild controls and n is the sample size.*

	Experimental Group				
	Day 1	Day 2	Day 3	Day 4	WC
t-values	-0.794	-0.571	0.058	1.206	1.905
p-values	0.428	0.568	0.954	0.229	0.058
n	31	29	30	34	57

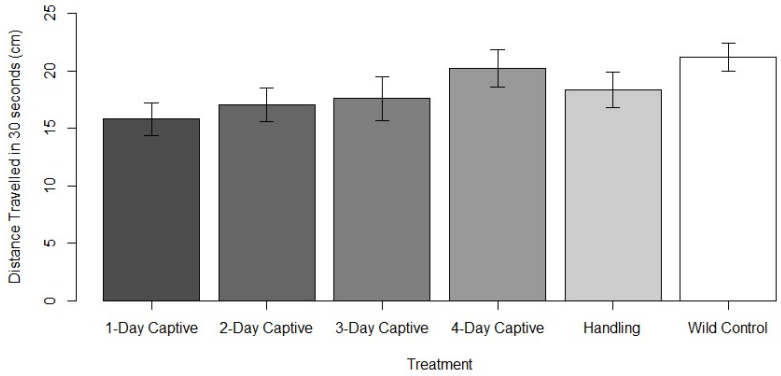

Figure 3: *Response strength values for captivity treatments, handling and wild control S. franciscanus. Handling control urchins travelled significantly further than 1 day and 2 day captivity treatments. Mean distance travelled are shown ±1 SE. Sample sizes are shown in Table 1 and Table 3.*

Table 3: *Summary of non-linear mixed effects model for mean initial escape response of red sea urchins in different captivity treatments with WC as the baseline. Fixed effects are treatment groups and random effects are dive team. HC is handling controls, WC is wild controls and n is the sample size.*

	Experimental Group				
	Day 1	Day 2	Day 3	Day 4	WC
t-values	-2.544	-2.288	-2.959	-1.275	-2.179
p-values	0.012*	0.023*	0.003**	0.204	0.031*
n	31	29	30	34	30

Table 4: *Summary of non-linear mixed effects model for mean space response strength of red sea urchins in different captivity treatments with WC as the baseline. Fixed effects are treatment groups and random effects are dive team. HC is handling controls, WC is wild controls and n is the sample size.*

	Experimental Group				
	Day 1	Day 2	Day 3	Day 4	WC
t-values	-2.895	-2.592	-1.924	-0.638	-1.905
p-values	0.004**	0.010*	0.056	0.524	0.058
n	31	29	30	34	30

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The Effects of a Static Magnetic Field on the sensitivity of *Escherichia coli* to Ampicillin, Streptomycin and Nalidixic Acid

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Abstract

The goal of this study was to determine if we could increase the sensitivity of *E. coli* to antibiotics by exposing it to a static magnetic field (SMF). In this study, we exposed 12 mL aliquots of *E. coli* culture to a 19.5 mT SMF for 300-, 100-, 30-, or 10-minute intervals at 36 °C, then performed a disk diffusion assay using ampicillin, streptomycin and nalidixic acid. We looked for differences in the zones of inhibition (ZI) between SMF exposed and unexposed E. coli to quantify changes in antibiotic sensitivity. We found that exposure to SMF for 300 minutes' results in a significantly larger ZI for ampicillin, and SMF exposure of 30-minutes reduced the ZI of streptomycin. Our results suggest that exposure to a 19.5 mT SMF can change *E. coli*'s susceptibility to ampicillin and streptomycin. This knowledge may be relevant to developing alternative treatments against infectious bacteria.

Keywords — Static Magnetic Field, *Escherichia coli*, Antibiotic Sensitivity

1. Introduction

Exposure to electromagnetic fields affects bacterial cell growth, viability, and proliferation [1]. Rod-shaped bacteria such as *E. coli* are highly susceptible to electromagnetic fields [2]. Static magnetic fields of strengths varying from 450mT to 3500mT were applied to *E. coli* and with the use of both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) cell surface damage was observed [3]. The effects of an SMF were found to be dependent on the magnitude and the duration of exposure [4].

A relationship between the exposure to a SMF and a bacteria's sensitivity to different antibiotics is recognised [5, 6]. This relation between magnetic fields and antibiotic sensitivity can play a key role for treatment of antibiotic resistant bacteria. Medicine is facing a crisis as more virulent strains of bacteria are becoming resistant to common antibiotics [7]. *Salmonella enterica* subsp. *enterica serovar Hadar* grown in a liquid nutrient broth was exposed to a SMF (200 mT) during its growth phase for 12 and 24 hour

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periods, which increased its sensitivity to the antibiotic gentamicin [5]. This result was not observed with all antibiotics and a mechanism of how an SMF interacts with a bacterium to increase its sensitivity to specific antibiotics has not been established. In our study, we used antibiotics with a particular mode of interaction to infer which biological mechanisms a SMF affects to stimulate increased response to specific antibiotics.

We chose the three antibiotics ampicillin, streptomycin and Nalidixic acid because of their different mode of action. Ampicillin is part of the β-lactam family of antibiotics, and inhibits peptidoglycan synthesis in bacteria (a critical component of the outer membrane of bacteria). Thus, cell surface damage caused by a SMF can increase *E. coli*'s susceptibility to ampicillin. Streptomycin, like gentamicin is an aminoglycoside antibiotic and inhibits translation by binding to the 30s ribosomal subunit [8]. We chose streptomycin because of its similarity to gentamicin thus we can determine whether the effects of from an SMF is correlated to the mode of action of aminoglycosides. Lastly Nalidixic acid is a quinolone which inhibits DNA gyrase resulting in inhibition of nucleic acid synthesis [9].

The primary objective of our study is to learn if the application of a weak SMF (19.4-19.5 mT) can result in an increased sensitivity to ampicillin, streptomycin or Nalidixic acid. By exposing *E. coli* to a 19mT SMF for the durations of 300, 100, 30 and 10 minutes our secondary objective becomes to pinpoint at what duration of exposure is needed to observe a significant increase in antibiotic sensitivity. We hypothesize that an increase in antibiotic sensitivity to ampicillin and streptomycin will be observed after *E. coli* has been exposed to a SMF of 19.4-19.5 mT for the greater time periods of 300 and 100 minutes but not for 30 and 10 minutes because it does not allow enough time for the SMF to significantly alter the *E. coli*.

2. Materials and Methods

2.1. SMF Apparatus

Our SMF-generating setup consisted of eight independent vertically-positioned 15 cm long copper solenoids (3720 turns per metre), each connected to a Xantrex XT 15-4 power source using cables and alligator clips. The setup is illustrated in Figure 1. The power supply produced a current of 4.16-4.18 A and voltage of 8.5-8.7 Volts. We calculated the produced SMF to be between 19.4 and 19.5 mT using the formula:

$$B = \frac{\mu_0 NI}{L} = \frac{\left(4\pi \times 10^{-7} N/A^2\right)\left(3720 \frac{turns}{m}\right)\left(4.16A\right)}{0.15m}$$

For each coil, we positioned a 250 mL Erlenmeyer flask such that the bottom of the flask was 5 mm above top end of each solenoid. Each flask was positioned on the south pole of the SMF.

2.2. Experimental Groups

2.2.1 SMF exposed *E. coli*

We exposed 12mL of a liquid culture of *E. coli* to a 19.5 mT SMF by suspending it above a copper solenoid in an Erlenmeyer flask (Figure 1). The liquid culture was suspended

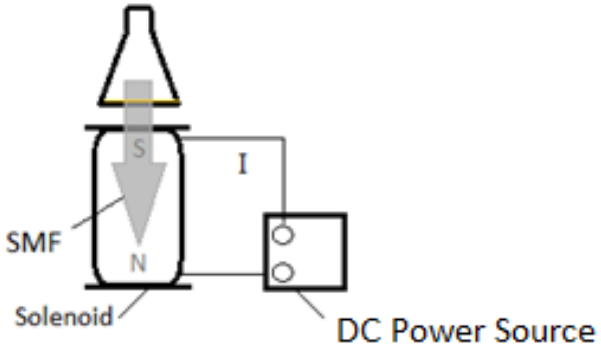

Figure 1: *SMF Apparatus. A diagram of how a 19.5 mT SMF was produced and applied to E. coli. The Erlenmeyer flask was suspended 5 mm above a solenoid that was attached to a DC power source to produce SMF. Each flask was positioned above the South pole of the SMF.*

in Erlenmeyer flasks rather than test tubes to create a thin uniform film of bacteria with a greater surface area to maximize oxygen availability to the bacteria. We tested exposure times of 300-, 100-, 30-, and 10-minutes, using fresh new liquid cultures (LB) for each time period. For each exposure time 8 replicates were tested simultaneously, with each flask suspended above its own solenoid independent from the others.

2.2.2 SMF unexposed *E. coli*

Liquid culture of *E. coli* that was not exposed to the 19.5 mT SMF was prepared in the same way as exposed *E. coli* and kept in 12 mL volumes in Erlenmeyer flasks. For each exposure time (300-, 100-, 30, and 10-minutes) we put 8 replicates in a water bath for the duration of the SMF exposure time.

2.3. Controlling for temperature

Preliminary experiments informed us that the resistance in the solenoid and circuit produced enough heat to increase the temperatures of the test broths to 36 °C. Thus we decided to place the unexposed groups in a Fischer Scientific ISOtemp 210 (product of USA) water bath of 36 °C for same amount of time the *E. coli* was exposed to the SMF (300-, 100-, 30-, or 10-minutes).

2.4. Testing antibiotic sensitivity

We quantified the sensitivity of *E. coli* to an antibiotic using disc diffusion assay and comparing the zone of inhibition (ZI) of the SMF exposed *E. coli* to the SMF unexposed

E. coli. We prepared a plate (diameter: 100 mm, height: 15 mm) for each replicate with 20-mL of BD® Mueller-Hinton II Agar (Lot#: 4216798, France). Immediately following the SMF exposed or unexposed time trial, 100μL of the liquid *E. coli* culture from each flask was spread onto a separate agar plate with a sterile glass rod. After the bacteria was applied, we placed a filter paper disc- cut from VWR® Grade Blotting Paper (catalogue #28298-020, Canada) using a Staples® Adjustable Hole Punch (Canada). We prepared solutions of ampicillin, streptomycin, and Nalidixic acid (all from Sigma Aldrich®, USA) for use in the disk diffusion assay at concentrations of 10 mg/mL. Four disc were placed on each plate with either 10μL of ampicillin, streptomycin, Nalidixic acid or distilled H_2O pipetted onto a disc. We incubated the plates face down for approximately 20 hours, enough time for the *E. coli* to grow into a visible lawn on the plate. We then measured the maximum diameters of the ZIs using a caliper.

2.5. Liquid Culture Preparation

We prepared 128 test tubes, each containing 6 mL of BD® Mueller-Hinton broth (Lot#: 4216798, France). Each broth was then inoculated with *Escherichia coli ATCC 11303*. This was done by dragging an inoculating loop across a 1x2mm lawn of the bacteria, which was then dipped into one of our test tubes containing 6 mL of broth, and spun between the fingers for two seconds in order to dislodge the bacteria. This process was repeated for each of the 128 test tubes. The test tubes for the 10-, 30-, and 100-minute trails were incubated on a Barnstead MaxQTM orbital shaker for 18.25 hours at 180 rpm and a temperature of 37 °C. The broths used for the 300-minute trial was incubated for a 40-hour period at a temperature setting of 27.5 °C because the lab was inaccessible, thus we reduced the temperature for this extended time to have similar concentration to the other time trials. After, the cultured broths were transferred to a 250 mL Erlenmeyer flasks, such that two 6-ml test tubes were poured into each flask resulting in a volume of 12 mL of liquid *E. coli* culture per flask.

2.6. Statistical analysis

To determine any significant differences between the ZIs of SMF exposed and unexposed *E. coli* a 2-sample unpaired *t*-test was done ($\alpha = 0.05$). All *p*-values less than 0.05 is reported in our results, *p*-values greater than 0.15 were not reported within our results. In addition to the unpaired *t*-test, the average ZI of each time trial was taken and plotted with 95% confidence intervals.

3. Results

We wanted to determine the effects on antibiotic sensitivity of *E. coli* after exposure to a 19.5 mT SMF. We exposed the SMF to liquid *E. coli* culture to varying time periods of 300-, 100-, 30- and 10-minutes. We performed a disc diffusion assay and used the diameter of the zone of inhibition (ZI) to quantify the sensitivity of *E. coli* to ampicillin, streptomycin, and Nalidixic after SMF exposure. We compare the ZI of SMF exposed bacteria to the ZI of unexposed SMF *E. coli*.

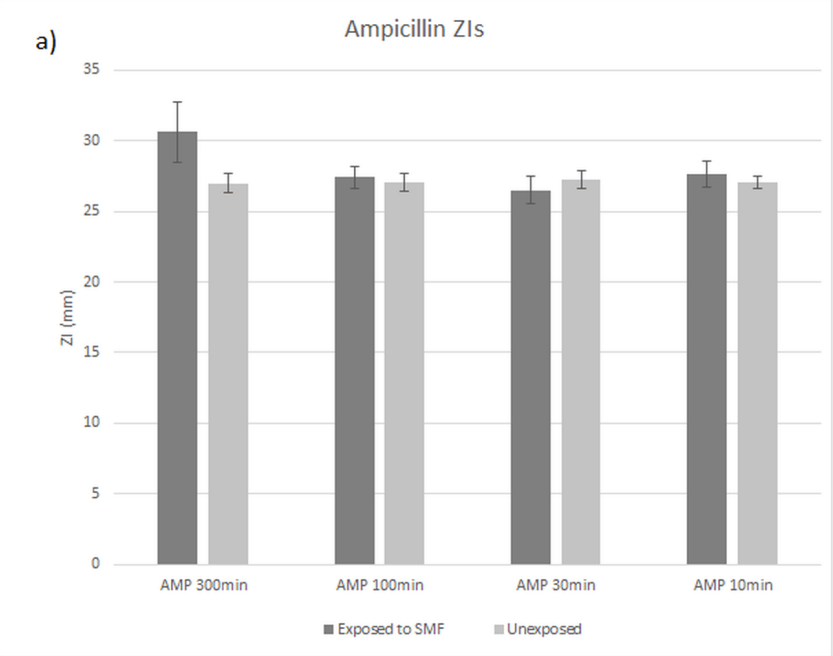

Figure 2: *The average Zones of Inhibition (ZI) of ampicillin at the different time trials. A comparison is made between E. coli that was exposed and unexposed to a SMF. 95% confidence intervals are presented to compare data; a significant difference was observed between the ZIs of ampicillin (300-minute SMF exposure) and streptomycin ZIs (30-minute SMF exposure).*

The average ampicillin ZI of the 300-minute trial is significantly larger in the SMF exposed *E. coli* than the SMF unexposed *E. coli* with a p-value of 0.0043 (Figure 2). We found no significant differences between the SMF exposed and unexposed *E. coli* for the 100-, 30-, and 10-minute time trials (all p-values > 0.1).

The average streptomycin ZI for the 30-minute time trial was significantly smaller in the SMF exposed *E. coli* than the SMF exposed *E. coli* with a p-value of 0.0026 (Figure 3). But no statistical significant difference was observed for any of the other exposure times, all p-values obtained were greater than 0.1, thus not reported in this paper.

There was no significant difference in the ZI's of Nalidixic acid measured in any of the SMF exposure times in comparison to *E. coli* unexposed to a SMF (Figure 4). All p-values for each time trial was greater than 0.1 and thus not reported in our paper. Additionally, no ZI was observed for the negative control discs with distilled H_2O.

4. Discussion

Disc diffusion assay can be used to indicate a bacterium's susceptibility to an antibiotic. An antibiotics concentration is inversely related to the distance from the disc. From the paper disc antibiotic diffuse outward, with the concentration dropping as the distance from the disc increases [10]. A larger ZI in the SMF exposed bacteria indicates that a lower concentration of antibiotic is required for a bactericidal effect on the *E. coli*. Our data suggests that *E. coli*, after an exposure of 300 minutes to a 19 mT SMF, is more

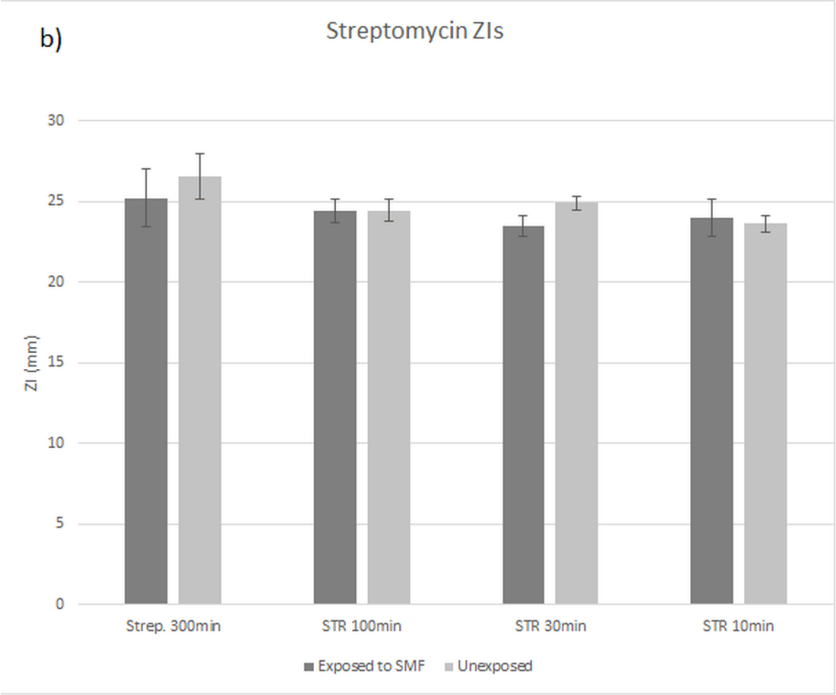

Figure 3: *The average Zones of Inhibition (ZI) of streptomycin at the different time trials. A comparison is made between E. coli that was exposed and unexposed to a SMF. 95% confidence intervals are presented to compare data; a significant difference was observed between the ZIs of ampicillin (300-minute SMF exposure) and streptomycin ZIs (30-minute SMF exposure).*

sensitive to ampicillin than *E. coli* that was not exposed to any SMF (Figure 2).

We formulated two possible explanations for the significantly larger ZI for ampicillin after 300-minute exposure to an SMF in comparison to the control groups. Damage from the SMF decreased the ability of the *E. coli* to defend itself from incoming ampicillin thus increasing its susceptibility to ampicillin. After *E. coli* is exposed to a SMF, SEM and TEM images showed cell surface damage [4]. SMF exposure has been shown to induce a stress on a bacterium [11, 12]. A second possibility is a change in membrane symmetry, ion concentration, pH and other biological changes from a SMF exposure [12, 13, 14]; could have resulted in an increased efficiency of ampicillins mode of action or membrane penetration. Increased uptake of ampicillin would result with more antibiotic entering the cells therefore increasing *E. coli*'s sensitivity to it. If the application of an SMF to a bacterium can increase the influx of an antibiotic into the cell, this knowledge can be used to develop a strategic treatment against resistant bacteria. To confirm these changes further studies to determine the relationship between SMF exposure times and ZIs with greater exposure times, such as 400- and 600- minutes. A progressive trend following 300 minute SMF exposure time will confirm these differences. We further recommend for future studies to be done to test any changes in antibiotic susceptibility on resistant bacteria.

No differences in ZI's between SMF exposed and unexposed *E. coli* were observed for ampicillin at shorter exposure times of 100-, 30-, and 10-minutes (Figure 2). Ji et

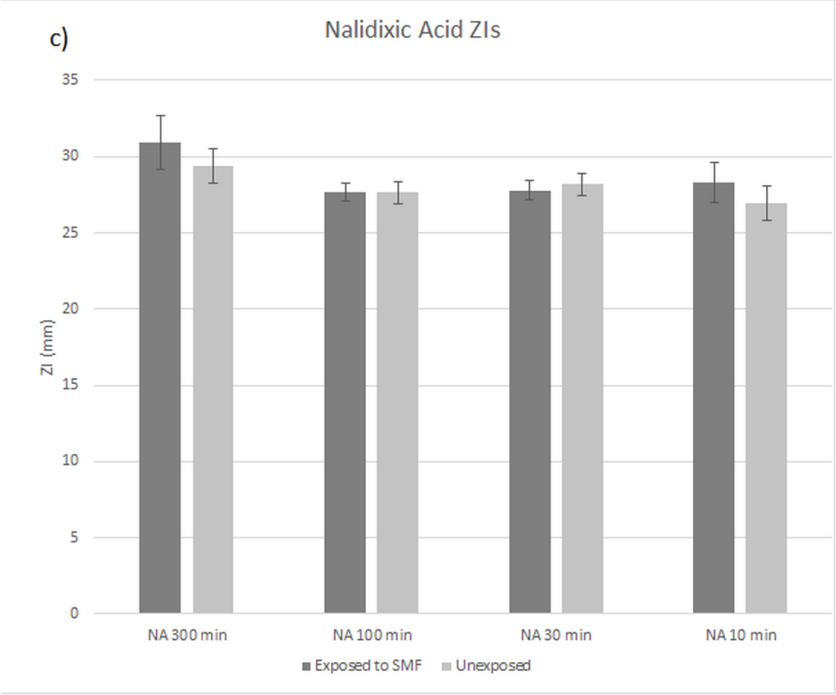

Figure 4: *The average Zones of Inhibition (ZI) of Nalidixic acid at the different time trials. A comparison is made between E. coli that was exposed and unexposed to a SMF. 95% confidence intervals are presented to compare data; a significant difference was observed between the ZIs of ampicillin (300-minute SMF exposure) and streptomycin ZIs (30-minute SMF exposure).*

al. (2009) determined a time dependence on the effects of a SMF on *E. coli* viability, they found the number of colony forming units (used to quantify viability) dropped exponentially as the exposure time increased to a 450 mT SMF. Our results suggest that 100-, 30-, and 10-minutes isn't a long enough exposure time for a 19 mT SMF to cause any observable effect on the *E. coli*'s susceptibility to ampicillin.

The study demonstrated that a 19 mT SMF does not change *E. coli*'s sensitivity to streptomycin for 300-, 100- and 10- minutes (Figure 3). These results contradict our hypothesis that streptomycin would have an increased bactericidal effect on *E. coli* exposed to an SMF. *Salmonella enterica*, a Gram-negative bacterium, displayed an increase in sensitivity to gentamicin after 12 and 24 hours of exposure to a 200 mT SMF [5]. We hypothesized a larger ZI for streptomycin because of its similarity to gentamicin, but our data shows that there was no change in *E. coli*'s sensitivity to streptomycin after 300-minute exposure to a 19mT SMF. SMF exposure didn't strongly affect *E. coli* susceptibility to streptomycin in the way it did to gentamycin (in Salmonella). This is evidence that SMF induced susceptibility is not correlated with aminoglycoside antibiotics like streptomycin and gentamycin. Additionally, our hypothesis needs to be investigated with the same conditions with gentamycin. It is very probable that the differences in our experimental design would account for this difference between these two studies. A repetition of these experiments done with gentamycin would establish if this is relevant to the antibiotic mechanism.

Our experiment obtained some inconsistent results for the ZI's of streptomycin. After an exposure time of 30-minutes, the unexposed SMF had a significantly larger ZI than the treatment group for streptomycin (Figure 3), possibly indicating an increased resistance to streptomycin when *E. coli* is exposed to a 19.5 mT SMF. This difference in the ZI would be expected to be observed at the higher SMF exposure times for streptomycin as well but this pattern did not occur within our results. A variation in ZIs is shown in the unexposed samples for streptomycin. These values should remain consistent between the different exposure times. This can be due to the application of streptomycin to the disc, or inconsistent concentrations of streptomycin applied to the discs. This variation is not seen with the other antibiotics used in this study.

No difference in *E. coli*'s sensitivity to Nalidixic acid was observed when comparing the ZIs for all exposure times (Figure 4). This is congruent with previous studies which found that after 12 and 24 hours' exposure to a 200 mT SMF there was no difference in Salmonella enterica susceptibility to Nalidixic acid [5]. Previous studies showing no effect of an SMF on a bacterium's susceptibility to Nalidixic acid [5, 6] verifies the differences observed in the other antibiotics are due to the exposure to the 19.5 mT SMF because no difference was measured in the susceptibility to Nalidixic acid.

From our results, we can conclude that SMFs affect *E. coli*'s sensitivity to certain antibiotics by its interaction with the cell membrane and protein translation because of the changes observed with ampicillins and streptomycin's ZIs. Our study shows that this interaction is dependent on the exposure time to the SMF, other variables such as temperature and SMF magnitude can possibly lower the time required to increase *E. coli*'s susceptibility to these antibiotics. Also, our research shows that this change can be observed with only half the SMF exposure time than previous studies that detect this change after 12 hours to a much stronger SMF (200 mT) [5]; this can be used to impose these changes in a more efficient manner with fewer resources to produce a SMF. Our study highlights the importance of understanding the effect of magnetic fields on bacteria, and could contribute to developing alternative treatment to combat antibiotic resistant bacteria.

5. Conclusion

Our results show that an SMF can have three different effects on *E. coli*'s sensitivity to different antibiotics. *Escherichia coli* had an increased sensitivity to ampicillin after 300-minutes of SMF (19.5 mT) exposure, this can be further investigated to determine if this can be observed in other gram-negative and antibiotic resistant bacteria. The opposite effect was measured after 30-miuntes of exposure to a SMF (19.5 mT) for streptomycin. A decrease in streptomycin sensitivity is inconsistent with the response to other antibiotics of the same class, thus this warrants further investigation of SMF influence on *E. coli* and other bacteria.

Investigating whether exposure to low strength SMFs can produce changes to antibiotic susceptibility in bacteria is useful because of the increasing challenges to treat resistant strains of bacteria. Furthermore, knowing more about low SMF would be useful in medical therapies because high SMF could present more risk to the patient.

6. Acknowledgments

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Variational Junction Conditions in $F(T)$ Gravity

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Abstract

We consider variationally permissible junction conditions in extended teleparallel gravity. The general junction condition requires continuity of the normal component of the boundary term at the junction hypersurface. We show that in the spherically symmetric case, if continuity of the tetrad and spin connection are assumed, both Synge's and ISLD boundary conditions of GR are obtained. We analyze both the static (R-Domain) and Time only (T-Domain) dependent scenarios. With the assumption that $F(T)$ is continuously differentiable, both conditions are obtained in the $F(T) \neq T$ case.

***Keywords* —**

1. Introduction

In 1915 Einstein introduced the scientific community to general relativity. One of the key assumptions in the model was that the theory was torsion free. Since then many other models of gravity have been proposed that use curvature to explain how masses move and affect spacetime. There are models that use both curvature and torsion to explain our observations, the most familiar of these is the Einstein-Cartan theory. Interestingly, it is also possible to construct models that are described in terms of a function of only the torsion. These torsion only theories are called $F(T)$ gravity theories. Rather than masses curving space, masses twist space, this is to say translational defects occur when parallel transporting vectors. This concept is notoriously difficult to visualize in more than one dimension. Imagine a hand crank that moves an object along a spiral path. Once the crank has returned to it's original position, the object is it moving has returned to the same angular position but it is now radially displaced. This is to say that the actual position of the object and the 4 dimensional space it is embedded in are no longer the same thing. In the special case where the model is linear in the torsion scalar, it is equivalent to general relativity except for the boundary term. This is called the Teleparallel equivalent of General Relativity. Both this case and the more complicated case where the Lagrangian consists of a non linear function of the torsion scalar will be examined.

The concept of gravity requires both intrinsic and extrinsic curvature. Intrinsic curvature is a property of a surface that does not change as you deform it without stretching. This is the reason that Mercator projections of the globe fail, they are taking an image on a sphere, and trying to project it onto a flat surface. A sphere has positive

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intrinsic curvature and a flat object has 0 intrinsic curvature. Extrinsic curvature is a property of the specific embedding of a surface. A cylinder has 0 intrinsic curvature, it is indistinguishable from a piece of paper. One interesting implication of this is that an observer on the surface can determine the intrinsic curvature of their space, but not the extrinsic curvature.

Two important boundary conditions arise in general relativity, and it is an interesting question whether or not these conditions can be recovered in $F(T)$ models of gravity. The first is the ISLD condition, which is that continuity of the extrinsic curvature implies continuity of the equations of motion. The second condition, Synge's condition, is that continuity of the normal component of the boundary term implies the continuity of the equations of motion. We show which of these conditions are recovered in the various cases we examine.

2. Theory

Curvature can best be quantified as an angular defect in a vector that has been parallel transported on a surface. The usual example is taking a vector pointing tangent to a sphere and moving it in a circuit, as shown in fig(1). Here we are parallel transporting a vector from point P back to point P.

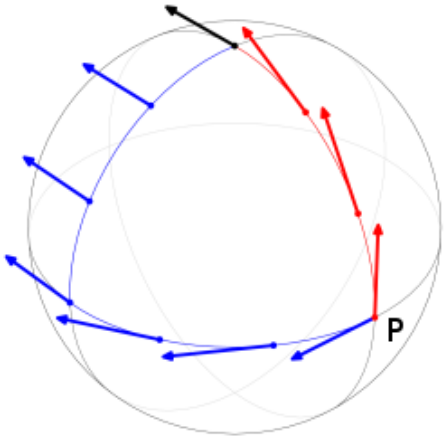


Figure 1: *In a space with curvature but no torsion, we can see that there is a 90° angular difference between the red and blue vectors at point P when parallel transported around a closed loop.*

Torsion is a similar property that manifolds can have. It is best imagined as a twisting around the path of parallel transport. In a surface that has non zero torsion, the path that a vector is transported over matters. Consider a square path on a surface with torsion, moving from one corner to the opposite corner along one path will result in an translational difference compared to moving via the opposite path, see fig(2). As an example moving 4 steps up and 4 steps to the right gets you to the point (3.95,4) and moving 4 steps right and then 4 up gets you to the point (4,3.9)

To understand the objects that build the theory, we need some language from differential geometry. A fibre bundle is a space that appears to be a product space, but

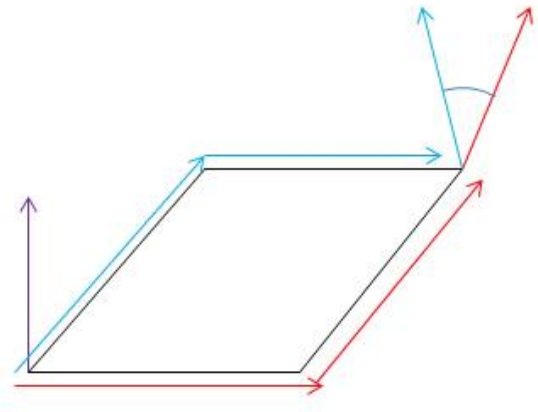


Figure 2: *In a space with torsion but no curvature, we can see that travelling along equivalent paths has produced an angular difference between the red and blue vectors.*

might have some other topology globally. This is similar to a the idea that a space can be locally flat, but have curvature globally. Möbius strips and Klein bottles are familiar examples of fibre bundles.This leads us to the concept of a vector bundle. A vector bundle is an invertible homomorphism that takes a set of vectors from one space to another. A class of these bundles is tangent bundles, which maps a set of vectors to its tangent space. Note that we are assuming that spacetime is a smooth manifold, which is required for the definition of a vector bundle to work.

A connection on a fibre bundle is a device that defines the notion of parallel transport on that bundle.This arises out of the problem of defining rates of change as one vector space approaches another. This is the same concept that is usually called a gauge in physics. In GR the Christoffel connection is used specifically because it is torsion free. This allows the model to be built up using the Ricci tensor. In F(T) theories the Weitzenböck connection for a similar reason: it is curvature free.

Tetrads are a set of four vectors that map the 4D tangent space of each point of a differentiable manifold. The Latin letter indices indicate that the vector set is in the orthonormal tangent space, and the Greek indices specify the point in Lorentz space to which the tangent space is attached. The original F(T) arose from the issue of Lorentz non-covariance. There are accelerating frame effects in gravity that require a special choice of tetrad to avoid. However we can make the theory covariant by adding a correction term. This term is found by turning the gravity off, and seeing what fictitious forces remain. The correction term is then added to remove these non zero terms in the torsion tensor. The benefit of the covariant formulation is that we can use a much larger class of tetrads, including ones that permit time variation. When we operate in the spherically symmetric model we have to choose a very specific tetrad, the diagonal tetrad, to arrive at the relatively simple equations of motion that model produces.

We can now introduce the metric. The metric tensor takes as input two tangent vectors on a surface, and outputs a real valued scalar. With the metric we can define a distance function that measures how far away two points are from each other on our surface. We are specifically using a positive definite, or Reimannian manifold, this

results in the useful property that the shortest distance between two points, called a geodesic, is how far an observer would need to travel to go from point a to point b.

The metric can be built from certain tetrads as well. We call these tetrads metric compatible. Later on we will choose specifically these metric compatible tetrads when we are developing the theory. In the spherically symmetric covariant case we can choose our tetrads to just be the square root of the metric. In the non covariant case we need to be a bit more careful with our choice.

The torsion scalar is built out of curvature free Weitzenböck connection. The Weitzenböck connection is built from any non-trivial tetrad as follows

$$\mathring{\Gamma}^{\rho}{}_{\mu\nu} = h_a{}^{\rho}\partial_{\nu}h^{a}{}_{\mu}. \tag{1}$$

Here $\mathring{\Gamma}^{\rho}{}_{\nu\mu}$ is the Weitzenböck connection and $h^{a}{}_{\mu}$ is the tetrad. Note that the significance of the $\circ$ is to differentiate between the Christoffel connection and the Weitzenböck connection. We can then construct the torsion tensor ($T^{\rho}{}_{\mu\nu}$) which by definition is given by the antisymmetric part of the connection.

$$T^{\rho}{}_{\mu\nu} = \mathring{\Gamma}^{\rho}{}_{\nu\mu} - \mathring{\Gamma}^{\rho}{}_{\mu\nu} \tag{2}$$

We can define the contortion tensor ($K^{\mu}{}_{\nu a}$) as follows:

$$K^{\mu}{}_{\nu a} = \frac{1}{2}\left(T^{\nu\mu}{}_{a} + T_{a}{}^{\mu\nu} - T^{\mu\nu}{}_{a}\right). \tag{3}$$

From this we can write the superpotential ($S_{a}{}^{\mu\nu}$) which when contracted with the torsion tensor gives us the torsion scalar.

$$S_{a}{}^{\mu\nu} = \frac{1}{2}\left[K^{\mu}{}_{\nu a} + h^{\mu}_{a}T^{\beta\nu}{}_{\beta} - h^{\nu}_{a}T^{\beta\mu}{}_{\beta}\right]. \tag{4}$$

In the covariant theory there is a slight adjustment to the Torsion tensor. This arises because we need to correct for the fictitious forces produced by accelerating frames.

$$T^{a}{}_{\gamma\delta} = \tilde{T}^{a}{}_{\gamma\delta} + \omega^{a}{}_{b\gamma}h^{b}_{\delta} - \omega^{a}{}_{b\delta}h^{b}_{\gamma}. \tag{5}$$

Here ω is the spin connection.

To express the action, we need to define a function of that torsion, $F(T)$. We are going to assume that this function is continuous and differentiable. This implies that' $F'(T)$ exists and is continuous.

The covariant action fully expressed is

$$\begin{aligned} S = \int h\,\delta h^{c}_{p}\Big[h^{-1}\partial_{\beta}\Big(hS^{\alpha\rho}{}_{c}\Big)f'(T) - h^{\kappa}_{c}\,T^{\zeta}{}_{\alpha\kappa}\,S_{\zeta}{}^{\rho\alpha}f'(T) \\ -\omega^{a}{}_{c\sigma}S_{a}{}^{\rho\sigma} + S^{\alpha\rho}{}_{c}\,\partial_{\beta}(T)f''(T) + \frac{1}{4}h^{\rho}_{c}f(T)\Big]d^{4}x \\ + \int_{\partial B} f'(T)S_{c}{}^{\alpha\rho}\,h\delta h^{c}_{\rho}\,\hat{n}_{\alpha}\,d^{3}x. \end{aligned} \tag{6}$$

Note what if the ω terms are zero we have the non-covariant action. In our boundary term, we have a normal vector. This normal is defined as outwardly pointing, and we are going to only consider cases where the field is orientable. This is to say that the outwardly pointing normal is uniquely defined.

3. Non-Covariant Spherically Symmetric F(T) Gravity

In the non-covariant case we are limited to a class of tetrads that have vanishing internal spin connection. Choosing a tetrad is a non-trivial process. All tetrads need to be metric compatible, this is to say that

$$h^a_\mu \, h_{\nu a} = g_{\mu\nu}. \tag{7}$$

Here the matrix representing

$$g_{\mu\nu} = \begin{bmatrix} A(r)^2 & 0 & 0 & 0 \\ 0 & -B(r)^2 & 0 & 0 \\ 0 & 0 & -r^2 & 0 \\ 0 & 0 & 0 & -r^2\sin(\theta)^2 \end{bmatrix}$$

Now our task is to find a tetrad that is metric compatible but also has vanishing internal spin connection. Note that in the covariant case we don't have to worry about the internal spin connection and we can choose a simpler tetrad. In the non covariant case the matrix representing the tetrad we use is

$$h^a_\mu = \begin{bmatrix} A(r) & 0 & 0 & 0 \\ 0 & B(r)\sin(\theta)\cos(\phi) & r\cos(\theta)\cos(\phi) & -r\sin(\theta)\sin(\phi) \\ 0 & B(r)\sin(\theta)\sin(\phi) & r\cos(\theta)\sin(\phi) & r\sin(\theta)\cos(\phi) \\ 0 & B(r)\cos(\theta) & -r\sin(\theta) & 0 \end{bmatrix}.$$

The spherically symmetric case is arguably the most important special case. By choosing the diagonal tetrad and choosing our second index to be radial, we arrive at our equations of motion. Here $A(r)$ and $B(r)$ are the metric functions.

$$E^1_1 = \frac{(4r(B(r)-2)A_r(r) + A(r)(r^2B(r)^2F(T) + 4B(r) - 4))}{2r^2A(r)B(r)^2} \tag{8}$$

Since in all cases the tetrad is assumed to be continuous, we can note a few facts. Both Synge's condition and the ISLD condition are sufficient to make the boundary term continuous and continuity of the boundary term implies both conditions. Synge's condition is that the continuity of the boundary term implies that the radial pressure is continuous, and in the $F(T) = T$ case that radial pressure is only dependent on the tetrad and the metric functions. The ISLD condition is the same in all cases: continuity of the boundary term implies continuity of the extrinsic curvature.

In the $F(T) \neq T$ case we get a more complicated equation of motion:

$$E^1_1 = \frac{(4r(B(r)-2)A_r(r) + 4A(r)(B(r)-1)F'(T) + F(T)A(r)r^2B(r)^2}{2r^2A(r)B(r)^2} \tag{9}$$

Again we recover both Synge's and the ISLD condition. Noting that the equation of motion can be solved for $F'(T)$, we see that continuity and differentiability of $F(T)$ implies continuity of $F'(T)$.

4. Covariant F(T) Gravity

For the remainder of the thesis we will be working with the covariant model, as it allows us to use a diagonal tetrad, and all results shown for covariant hold for non covariant.

4.1. Timelike

In the time like boundary condition we contract the superpotential in the boundary term with a normal pointing in the radial direction. Because we are in the covariant frame, we can pick the diagonal tetrad:

$$h^a_\nu = \begin{bmatrix} A(r) & 0 & 0 & 0 \\ 0 & -B(r) & 0 & 0 \\ 0 & 0 & -r & 0 \\ 0 & 0 & 0 & -r\sin(\theta) \end{bmatrix}$$

This tetrad is clearly metric compatible. To get vanishing inertial effects, we need to choose a specific omega. The way to find this ω is to set $\alpha(r)$ and $\gamma(r)$ equal to 0. This in effect turns gravity off. We then set each component of the Torsion tensor to be 0 and solve out for the components of ω. We arrive at a very sparse omega:

$$\omega^{12}{}_2 = 1, \omega^{13}{}_3 = \sin(\theta), \omega^{23}{}_3 = \cos(\theta)$$

The spin connection is antisymmetric in its first two indices, leaving us with a total of 6 non zero entries.
With the spin connection calculated, we can turn the gravity back on, and look at the relevant equation of motion.

$$E^1_1 = \frac{(4r(B(r)-2)A_r(r) + 4A(r)(B(r)-1))F'(T) + F(T)r^2(B(r))^2A(r)}{2r^2(B(r))^2A(r)} \tag{10}$$

For our extrinsic curvature we get terms with $A(r)$,$B(r)$ and $A_r(r)$.

$$K^1_1 = 0, K^2_2 = \frac{r}{\sqrt{B(r)}}, K^3_3 = \frac{r\sin^2(\theta)}{\sqrt{B(r)}}, K^4_4 = \frac{A_r(r)}{2\sqrt{B(r)}} \tag{11}$$

We also get these terms in our boundary term, which is the integrand in the last term of equation 6.

$$F^{'}(T)S_a{}^{r\nu}\hat{N}_r \tag{12}$$

$$2,[1,2] = \pm\tfrac{r(A(r)B(r)-A_r(r)r-A(r))}{2A(r)}$$

$$3,[1,3] = \pm\tfrac{r\sin^2(\theta)(A(r)B(r)-A_r(r)r-A(r))}{2A(r)}$$

$$4,[4,1] = \pm\tfrac{A(r)^2(B(r)-1)}{r}$$

Since our boundary is a rank 3 tensor with 64 entries, it is not represented in full. Fortunately it is very sparse. Above are the only non-zero entries. The tensor is also anti symmetric in it's last two indices. The $3[1,3]$ notation indicates that the positive term is in position $3,1,3$ and the negative in $3,3,1$. You'll note that equation 10 is the exact same equation we arrived at in the non covariant formulation. We are able to recover both Synge's condition and the ISLD condition as we were in the non covariant case.

4.2. Spacelike

For spacelike boundaries, we contract the boundary term with a normal pointing in the time direction. Our metric gets adjusted slightly to look like:

$$g_{\mu\nu} = \begin{bmatrix} A(t)^2 & 0 & 0 & 0 \\ 0 & -B(t)^2 & 0 & 0 \\ 0 & 0 & -t^2 & 0 \\ 0 & 0 & 0 & -t^2\sin(\theta)^2 \end{bmatrix}$$

We can again choose the diagonal tetrad:

$$h^a_\nu = \begin{bmatrix} A(t) & 0 & 0 & 0 \\ 0 & -B(t) & 0 & 0 \\ 0 & 0 & -t & 0 \\ 0 & 0 & 0 & -t\sin(\theta) \end{bmatrix}$$

We follow the same procedure for determining omega and we end up with:

$$\omega^{02}{}_2 = 1, \omega^{03}{}_3 = \sin(\theta), \omega^{23}{}_3 = \cos(\theta)$$

The relevant equation of motion in this case is different:

$$E^0_0 = \frac{(4t(A(t)-2)B_t(t) + 4B(t)(A(t)-1))F'(T) + F(T)t^2(A(t))^2B(t)}{2t^2(A(t))^2B(t)} \tag{13}$$

It is clear that this is a similar equation to the timelike boundary, the only difference is that $r \to t$ and $A \leftrightarrow B$. The extrinsic curvature is slightly different, but still contains the terms $A(t)$,$B(t)$ and $B_t(t)$.

$$K^1_1 = \frac{B_t(t)}{2\sqrt{A(t)}}, K^2_2 = \frac{t}{\sqrt{A(t)}}, K^3_3 = \frac{t\sin^2(\theta)}{\sqrt{A(t)}}, K^4_4 = 0. \tag{14}$$

As does the the boundary term:

$$F'(T)S_a{}^{tv}\hat{N}_t \tag{15}$$

$$1,[4,1] = \pm\frac{B(t)^2(A(t)-1)}{t}$$

$$2,[4,2] = \pm\frac{t(B(t)A(t)-B_t(t)t-B(t))}{2B(t)}$$

$$3,[4,3] = \pm\frac{t\sin^2(\theta)(B(t)A(t)-B_t(t)t-B(t))}{2B(t)}.$$

We again recover the ISLD condition and Synge's condition.

4.3. Mixed Boundary Conditions

In the case of mixed boundary conditions, we are no longer able to recover either the ISLD condition or Synge's conditions.The equations of motion contain partial derivatives of the metric functions with respect to both r and t that do not show up in either the boundary term or the extrinsic curvature.

5. Conclusion

To summarize, by varying the action with respect to the tetrad, we were able to show that the ISLD condition is recovered in the spherically symmetric case of torsional gravity with both timelike and spacelike boundaries. This is true for actions containing an arbitrary function for Torsion. Furthermore we were able to show that Synge's condition is recovered in the case where the action is linear in torsion, and, under the assumption that $F'(T)$ is continuous, non linear as well. We were not able to recover either condition in mixed boundary conditions.

5.1. R

$$Eom_1^1 = \frac{(4r(B(r)-2)(A_r(r))+4A(r)(B(r)-1))(F^{'}(T)+F(T)r^2B(r)^2A(r)}{2r^2B(r)^2A(r)} \tag{16}$$

Extrinsic Curvature

$$K_1^1 = 0, K_2^2 = \frac{r}{\sqrt{B(r)}}, K_3^3 = \frac{r\sin^2(\theta)}{\sqrt{B(r)}}, K_4^4 = \frac{A_r(r)}{2\sqrt{B(r)}} \tag{17}$$

Boundary Term

$$F'(T)S_a{}^{rv}\hat{N}_r \tag{18}$$

5.2. T

$$Eom_4^4 = \frac{(-4t(A(t)-2)(B_t(t))-4B(t)(A(t)-1))(F^{'}(T)+F(T)t^2A(t)^2B(t)}{2t^2A(t)^2B(t)} \tag{19}$$

Extrinsic Curvature

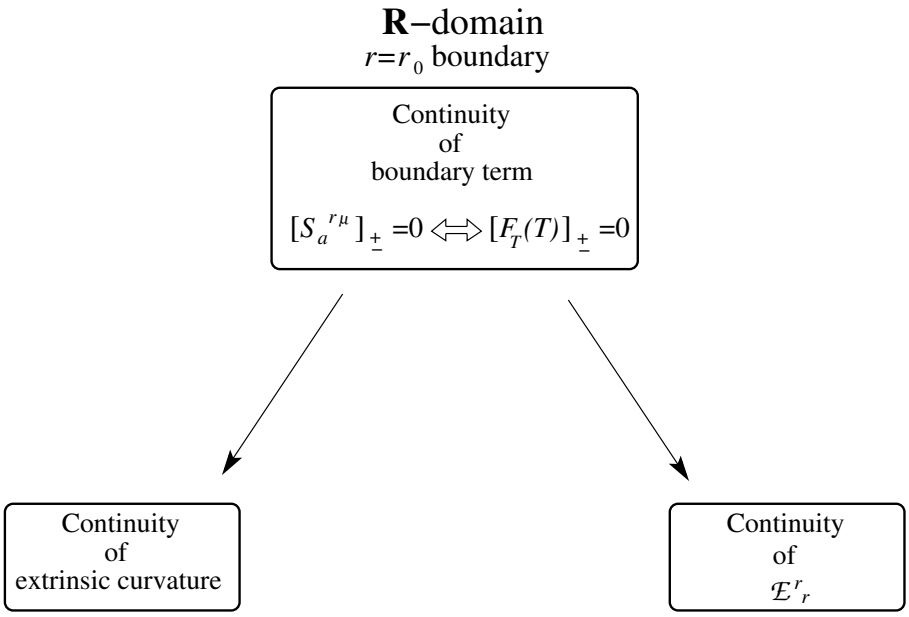
$$K_1^1 = \frac{B_t(t)}{2\sqrt{A(t)}}, K_2^2 = \frac{t}{\sqrt{A(t)}}, K_3^3 = \frac{t\sin^2(\theta)}{\sqrt{A(t)}}, K_4^4 = 0 \tag{20}$$

Boundary Term

$$F'(T)S_a{}^{tv}\hat{N}_t \tag{21}$$

5.3. Implications

For both cases, if the EOM is continuous, the boundary condition and the extrinsic curvature are. If the extrinsic curvature is continuous the EOM is too. Continuity of the normal boundary term also implies continuity of the equation of motion in the the direction of the component the boundary is dependent on.



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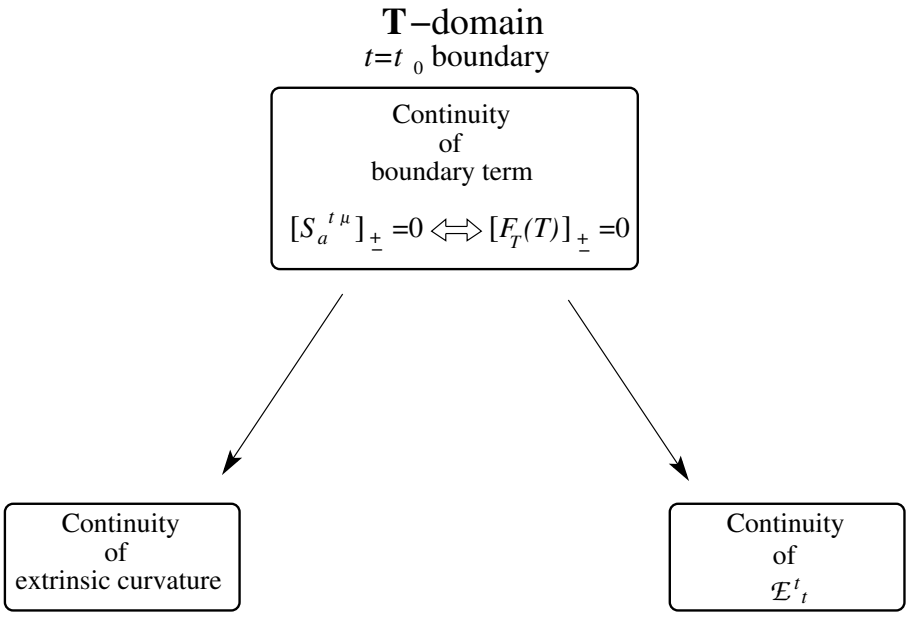

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The effects of *phloroglucinol* on *Tegula herbivory*

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Abstract

Primary producer's strategies for defending themselves against herbivores has manifested in many ways. Chemical defense is one strategy that many plants have utilized to become less palatable to herbivores. The production of secondary metabolites such as tannins in terrestrial plants and phlorotannins in marine algae are hypothesized to have a deterring effect on herbivore grazing. Phloroglucinol (1,3,5 - trihydroxybenzene) is the monomer of all phlorotannins found in brown algae (Division *Phaeophyceae*). The monomer can be arranged in a variety of different ways for a variety of functions. Research on the function of phloroglucinol as the active deterrent of herbivory has conflicting results. To address this, two snail species from the genus *Tegula* were used to determine if the presence of phloroglucinol reduced herbivory. We exposed *Tegula funebralis*, an intertidal species and *Tegula pulligo*, a subtidal species to experimental seaweed plates with varying concentrations of phloroglucinol. The two *Tegula* species were selected as closely related representatives from different tidal heights to see if habitat played a role in phloroglucinol tolerance. We measured consumption and preference of experimental plates with different concentrations of phloroglucinol. Consumption was measured directly and preference was determined using a Y-maze. Our study shows that there is no deterring effect of phloroglucinol on either of the two *Tegula* species. The function of phloroglucinol may not have any implications in defense against *Tegula* herbivores. The production of phlorotannins in kelp species could be a general stress response without a specific stressor activating the production, and many compounds likely carry out chemical defense.

Keywords — Phlorotannins, Chemical defence, Algae, Preference, Y-maze

1. Introduction

Herbivory is at the core of all ecosystems and is essential to support higher levels of life. In response to herbivory, many prey organisms have evolved defensive strategies to reduce their losses. Defensive strategies can take many forms, including or combining mechanical, chemical, constituted or induced mechanisms. Induced chemical defenses occur when the prey organism responds directly or indirectly to grazing with the increased production of a chemical deterrent [1]. Examples of induced and constituted chemical defenses are well known and described in vascular

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terrestrial plants [2], while examples of chemical defenses in marine primary producers are less understood.

Phlorotannins are a marine example of a chemical defense that is seen only in the division Phaeophyceae or the brown algae [3]. Phlorotannins are polymers of phloroglucinol (1,3,5 - trihydroxybenzene) and can have different structural forms [4]. Phlorotannins are secondary metabolites that have many speculated functions such as, protection from ultraviolet (UV) radiation [5, 6], reduction of localized phytoplankton levels, reduction of fouling organisms [7], increased wound repair [4], and chemical herbivore defense [3, 8, 9]. The simplest form of phlorotannin is the monomer phloroglucinol. The functions of different forms of phlorotannins are not well understood and it is unclear as to whether phloroglucinol is active in minimizing herbivory. It has been disputed whether phlorotannins function as a chemical defense against herbivores [10].

Concentration and allocation of phlorotannins vary in tissues of the same individual [7], between different life stages of conspecifics [9], and throughout populations [11]. Closely related brown algal species from the same geographical area can have varying amounts of phlorotannins [3, 8]. Species from different climates can also have a variety of phlorotannin concentrations. Temperate brown algae tend to have higher amounts of phlorotannins compared to tropical species [3] and kelp of the Pacific Northwest have been found to have less phlorotannins than Australian kelp species [8].

Research of phlorotannins and phloroglucinol as herbivore deterring compounds, similar to phlorotannin, allocation research, yields conflicting results. Steinberg (1988) found that different amounts of phlorotannins and polyphenolics mixed into agar plates had similar herbivore deterrence, while Deal et al. (2003) found that in *Fucus vesiculosus*, galactolipids rather than phlorotannins deterred urchin herbivory. These discrepancies in the literature suggest that there is a wide variety of responses both by brown algae to produce phlorotannins and herbivores being deterred by phlorotannins [4]. Studies that use the same herbivore-seaweed relationships also have different results, as reviewed in [4]. With that, experiments investigating the effects of phlorotannins, polyphenols, and phloroglucinol on herbivory must further investigate and consider what is involved in this complex interaction.

Phloroglucinol has been tested specifically for its involvement in deterring herbivory and has had inconsistent results. Steinberg (1988) found that the monomer did not deter herbivory by the intertidal snail *Tegula funebralis*. In contrast, Pereira et al. (2015) measured the distance *Tegula trindentata* traveled in response to the addition of an agar plate containing phloroglucinol and found that on average, the snails moved farther away from plates containing higher concentrations of phloroglucinol [12].

To contribute to the research into whether phloroglucinol is the active molecule of the herbivore deterring properties of phlorotannins, we exposed two gastropod grazers to varying phloroglucinol levels to determine if there is an effect. Two snails of the genus *Tegula* were selected. *Tegula funebralis*, an intertidal species and *Tegula pulligo*, a subtidal species. These species were selected for their relatedness and differing habitats. Since phlorotannins and phloroglucinol offer protection to seaweeds against UV radiation [5, 6], we suspect that intertidal herbivores such as T. *funebralis*, would be more resilient to phloroglucinol exposure. Subtidal herbivores such as *T. pulligo*

would be less resilient to phloroglucinol as there is less need for protection from UV radiation at greater depths. We sought out to determine if increasing concentrations of phloroglucinol results in deterrence from food choices containing phloroglucinol and if it reduces grazing by *Tegula* species. We hypothesize that both species of *Tegula* will prefer food sources lacking phloroglucinol and that grazing will be reduced in both species at higher concentrations of phloroglucinol. In addition, the intertidal species, *T. funebralis*, may be less affected by the presence of phloroglucinol compared to *T. pulligo*.

2. Materials and Methods

2.1. Animal Collection and Preparation

One hundred *Tegula funebralis* specimens were collected from Aguilar Point in Bamfield, B.C. on November 8th, 2016. Ninety-three *Tegula pulligo* specimens were collected from Bamfield Inlet on November 10th, 2016. Snails were kept in groups of 20 in five liter containers, the same as those used in the consumption experiment.

2.2. Animal Preparation

Seventy-five snails of each species were starved for 90 hours before starting the consumption experiment. Of those not selected for consumption trials, 12 snails of each species were starved for 14 days prior to the preference experiment. The weight of each snail was recorded before beginning the consumption trials and then randomly assigned a treatment container. Snails used for the preference trials were randomly assigned but not weighed.

2.3. Agar Plate Preparation

We prepared agar plates infused with seaweed (*Macrocystis pyrifera*) and isolated phloroglucinol. The phloroglucinol was added to 100 mL of distilled water, five grams of seaweed and 2.5 grams of bacteriological agar. The five grams of seaweed consisted of 2.5 grams of blade tissue, and 2.5 grams of stipe tissue. The use of equal parts of both stipe and blade tissue was to account for any variations in the natural levels of phlorotannins in kelp. The seaweed was blended in water and then heated to a gentle boil. The agar and phloroglucinol were added to the mix and then immediately poured into six 60mm x 15mm sterile polystyrene Petri Dishes. Treatment concentrations of phloroglucinol were as follows, control was 0 mg/mL, low was 8 mg/mL, medium was 12 mg/mL, high was 17 mg/mL, and very high was 22 mg/mL (as per Steinberg, 1988). Each treatment was prepared separately. The agar plates were left to sit for three hours until the agar set and were stored in a fridge at 6 °C until experiments could start ($\sim$48 hours). Plates weighed approximately 18 grams. Plates were then soaked in seawater for 12 hours to allow the plates to absorb water and sink. Plates were weighed again after soaking to be able to determine amount consumed by the snails.

2.4. Experiment 1: Consumption

One agar plate was secured to the bottom of a clear, 5 L testing container with a small amount of hot glue. Each container was randomly assigned three weighed snails and a sea table. Five replicates of each treatment and a control replicate (without snails) were done. This resulted in 25 containers with snails and 5 control containers without snails (a total of 30). This was done for both species for a total of 60 trials. All the snails used were starved for 90 hours before starting the experiment. The snails were left in the containers with the pre-weighed agar plates for five days. When done, each agar plate was removed, reweighed and used to determine the amount of agar consumed by the snails.

$$\text{wet mass before} - \text{wet mass after} = \text{amount consumed}$$

The amount of agar consumed was then divided by the mass of the three snails used in the trial. This determined the consumption to biomass ratio for the trial.

$$\text{amount consumed}/\text{total biomass} = \text{con : biom ratio}$$

2.5. Experiment 2: Preference

A Y-maze made of corrugated plastic cardboard was used. The maze was set up in a sea table with water flow. Each trial had one control plate and one treatment plate. Three concentrations of phloroglucinol were used as treatment plates. A control with 0 mg/mL, a low with 8 mg/mL, and a high with 17 mg/mL of phloroglucinol were used. The seawater flowed over each plate and toward the proximal end of the maze. Three zones were distinguished in the Y-maze. Zone 1 referred to the center, Zone 2 referred to the left arm and Zone 3 referred to the right arm. Before commencing a trial, a control plate and treatment plate (either control, low or high) was randomly assigned to either Zone 2 or Zone 3 and glued in the distal end of the Y-maze. Once water was flowing, a snail was placed at the proximal end of the maze. The 12 *T. funebralis* and 12 *T. pulligo* used for these trials were starved for 14 days. We were limited in the availability of *T. pulligo* so 12 snails was the largest sample size attainable.

We ran four trials of each treatment with each species of *Tegula* for 30 minutes. Time spent in each zone was recorded. The snail's preference (control or treatment) was established by which tray they arrived at or by which zone they spent the most time in. All snails tested made a choice. Preference was recorded at the end of the trial. The maze was rinsed between trials to prevent any influence from mucus trails left by previous snails.

3. Results

3.1. Consumption

Consumption to biomass (con:biom) ratios were calculated and negative ratios, resulting from an increase in plate mass, were disregarded for our analysis. Causes for the negative consumption ratios, which indicate an increase in weight are discussed later in this manuscript. There was a total of 4 negative con:biom values (3 from *T. funebralis*

and 1 from *T. pulligo trials*). Mean con:biom ratios for *T. funebralis* trials were 0.105 for control, 0.069 for low, 0.080 for medium, 0.159 for high, and 0.113 for very high. Standard deviations of these means were 0.089, 0.073, 0.040, 0.108 and 0.113 respectively. The mean consumption ratio for T. pulligo trials were 0.112 for control, 0.163 for low, 0.110 for medium, 0.088 for high, and 0.122 for very high (Figure 1). Standard deviations of these means were 0.049, 0.025, 0.059, 0.044 and 0.083 respectively. These ratios represent the average mass of agar consumed per unit of snail biomass.

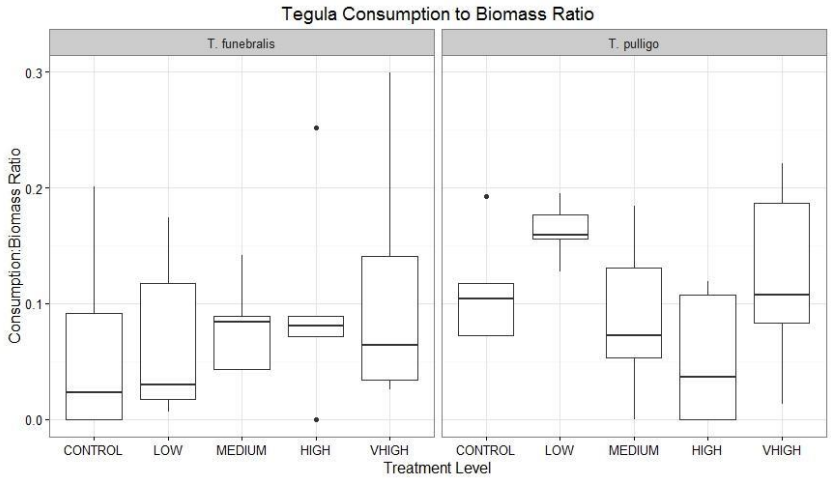

Figure 1: *The consumption:biomass ratio for each treatment level in the consumption experiment. VHIGH = Very High treatment.*

	T. funbralis		*T. Pulligo*	
	treatment	control	treatment	control
Zero	2	2	2	2
Low	2	2	3	1
High	3	1	3	1

Table 1: *The choice made by each snail in each trial run in the Y-maze. Zero = Control treatment (0 mg/mL), Low = Low treatment (8 mg/mL), and High = High treatment (17 mg/mL).*

3.2. Preference

All snails tested made a choice of either the treatment or the control. *Tegula funebralis* chose the treatment 50% of the time in control trials, 50% of the time in low trials, and 75% of the time in high trials. *Tegula pulligo* preferred the treatment 50% of the time in control trials, 75% of the time in low trials, and 75% of the time in high trials (Figure 2).

The time spent in the treatment zone of the Y-maze was averaged for each treatment and species. *Tegula funebralis* spent 49.92% of the time in treatment zone during control trials, 38.54% of the time in the treatment zone during low trials, and 52.43% of the time in the treatment zone during high trials. *Tegula pulligo* spent 40.89% of the time in

treatment zone during control trials, 35.53% of the time in the treatment zone during low trials, and 46.52% of the time in the treatment zone during high trials.

3.3. Analysis

Results of statistical analysis using linear models to fit consumption data returned no effect of concentration, species, or any combination of the two factors on con:biom ratios (p-values> 0.05). A chi-squared test was used to determine the independence of phloroglucinol concentration from preference choice. The results from the test indicate that concentration of phloroglucinol was independent of choice (p-value$= 0.586$, $\chi^2 = 1.066$).

4. Discussion

Statistical analysis of results from both experiments found no effect of phloroglucinol concentration or species on consumption or choice. In the consumption experiment, con:biom ratios for increasing phloroglucinol concentrations in each species of *Tegula* were not significantly different from one another and showed no trend. There was neither an increase nor decrease in consumption by either species; therefore the phloroglucinol monomer did not affect the amount of *Tegula* grazing. There was no effect of species on the amount of agar consumed or con:biom ratios. These results suggest there is no habituation in higher intertidal snails due to increased phloroglucinol from UV radiation. The amount of the agar consumed was low in all trials. This could be due to the short duration of trials and short starvation time prior to the experiment. The negative con:biom ratios that were disregarded in our analysis suggest that there may have been inaccuracies in the measuring techniques or unforeseen increases in agar mass that were not considered, such as absorption of water by the agar or bacterial growth on the agar.

In the preference trials, choice was independent of concentration, but there was a noted affinity for the phloroglucinol treatment plates over the control plates. More snails of both species chose phloroglucinol plates over control plates in the high concentration treatment (75% for both *T. funebralis* and *T. pulligo*). Both species on average spent a large proportion of time in the treatment zone during both low and high trials. This might suggest that phloroglucinol as a monomer could attract Tegula to the seaweed, influencing food choice. The preference experiment would benefit from more replicates.

Both the consumption and preference experiments had unexpected results. Our result of a slight preference for phloroglucinol may suggest that its presence in seaweed may be an indicator of food to *Tegula* species. The lack of effect of phloroglucinol concentration on consumption suggests that phloroglucinol does not affect the amount consumed by *Tegula* species. Considering both results, the amount of grazing is likely not affected by the presence of phloroglucinol, but in food sources may influence *Tegula* food choice. Seaweed that produces large amounts of phloroglucinol may attract herbivores resulting in greater overall herbivory on that individual. Since phloroglucinol may promote herbivory, it is fair to speculate that it it does not occur in high levels naturally. It is likely that phloroglucinol must be polymerized into phlorotannins or polyphenols for any herbivore deterring properties to arise. There are

multiple configurations of phlorotannins with many functions and benefits [4]. Further research of phlorotannin configurations and their function would aid in determining the influence of phlorotannins on herbivory.

Our results suggest there is more involved in herbivore deterrence than simply the monomer phloroglucinol. There are a number of potential alternatives to phlorotannins and phloroglucinol as herbivore deterrents in marine algae. An alternative is that phlorotannins and phloroglucinol may affect different species differently. Similar to our study, Steinberg (1988) found no effect of phloroglucinol on *T. funebralis* herbivory, but found that phloroglucinol did affect Echinoid herbivores such as *Strongylocentrotus purpuratus*. Another alternative is that there are other compounds responsible for herbivore deterrence. Deal et al. (2003) used bioassays to separate compounds and suggests that galactolipids are responsible for herbivore deterring properties of seaweeds. As phlorotannins are difficult to isolate and quantify [10], they are often tested as an extracted concoction of potentially confounding compounds. Deal et al. (2003) suggests that galactolipids are a confounding chemical that is extracted with phlorotannins and has likely influenced previous research. This raises the notion that other bioactive metabolites may be uninvestigated and have unknown properties within phlorotannin extracts.

Phlorotannin production in kelps is often observed in stressed individuals [5]. The production of these compounds appears to be in direct response to stressors like herbivory, UV radiation, and others. The stress response could be a general one that is initiated as an attempt to lessen the stress, regardless of its source. If phlorotannin production is a general response to stress, it would be difficult to determine which stressor the phlorotannins most effectively reduce.

Chemical defenses are not rare in the marine environment. Additional examples of chemical defenses are demonstrated in the Genus *Desmarestia*. These brown algae are capable of producing sulfuric acid that can contribute up to 18% of the algae's dry mass [13]. The acid produced by these algae can dissolve mouth parts of urchins and deter herbivore grazing [13]. Other brown algae in the Order *Dictyotales*, can produce a diterpenoid alcohol that has shown to greatly decrease herbivore grazing [14]. Understanding the relationship and interactions between primary producers and herbivores is essential to understanding the fundamental ecology of any ecosystem.

5. Conclusion

The function of phloroglucinol as the active molecule in phlorotannin herbivory deterrence is not clear from our experiments. The monomer appears to have no deterring effect on the amount a herbivore will consume. Phloroglucinol may attract herbivores to food sources but more conclusive evidence is required. Extracting phlorotannins that are free of confounding compounds from seaweeds is essential to determining the specific function of each phlorotannin, and determining phloroglucinols role in the seaweed.

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Physiological mechanisms mediating the trade-off between survival and reproduction in birds

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Abstract

Despite an abundance of evidence in support of a trade-off between survival and reproduction in birds, we still have a limited understanding of the physiological mechanisms allowing this trade-off to persist. In this review, I discuss three physiological pathways hypothesized to mediate the trade-off between survival and reproduction in birds: 1) baseline corticosterone, 2) oxidative stress, and 3) immune function. While I found evidence in the literature in support of each of these pathways as a mechanism mediating this trade-off, there are still many questions that remain unanswered for each of the proposed mechanisms. In particular, given that these mechanisms are unlikely to be mutually exclusive, there is currently a lack of research incorporating interactions between mechanisms. I suggest that future research should include multiple mechanisms and their interactions as a sufficient effect size of the trade-off between survival and reproduction may only be detected when these are considered.

Keywords — life history, costs of reproduction, corticosterone, oxidative stress, immune function, reproductive investment

1. Introduction

The trade-off between survival and reproduction is one of the central tenants of life history theory and is documented in many taxa including fish, insects, mammals, reptiles, and birds [1, 2]. The resource allocation hypothesis postulates that in an environment with finite resources an investment of resources in reproduction has the capacity of reducing survival because these resources can no longer be directed towards self-maintenance [2]. In birds, there is strong, but some contradictory (see [3]), evidence for this trade-off with the costs of reproduction arising from several breeding stages not limited to chick-rearing and incubation [3]. Despite the impressive amount of evidence in support of this trade-off, far less is known of the physiological mechanisms responsible [1, 4]. In this review I first discuss three of the proposed physiological pathways mediating the trade-off between survival and reproduction in birds: 1) baseline corticosterone, 2) oxidative stress, and 3) immune function. After, I discuss the need for future research to focus both on pathways of individual mechanisms and potential interactions between mechanisms.

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2. Baseline corticosterone

The hormone corticosterone (CORT) may be able to mediate the costs of reproduction through its role in mobilizing energy stores [5]. Controlled by the hypothalamic-pituitary-adrenal (HPA) axis, CORT serves the primary function of maintaining adequate glucose and free fatty acid levels in the blood through stimulation of gluconeogenesis and lipolysis. The baseline levels of CORT maintained in the blood stream are pivotal to survival as baseline CORT levels have downstream effects on the levels of e.g. glucose, free fatty acids, hematocrit, reproductive hormones, immunosuppression, oxidative stress, and telomere length, many of which have been linked to survival in vertebrates [6, 7, 8, 9]. CORT values rise during extended periods of increased activity or stress to mobilize energy stores, potentially during reproduction, and thus baseline CORT may serve as a mechanism of mediating the costs of reproduction [5].

Evidence in support of CORT as a mediator of the trade-off between survival and reproduction through mobilization of energy stores for current resource demand is equivocal. The Cort-Fitness Hypothesis proposes that CORT levels should increase with declines in reproductive success because CORT is involved in response to environmental challenges and increased environmental challenges cause reallocation of resources from reproduction to respond to these challenges [10]. However, studies testing the Cort-Fitness Hypothesis present contradictory results with no relationship between CORT and reproductive success [11], a positive correlation [12], and the predicted negative correlation [11, 13]. For example, non-manipulated European starlings (*Sturnus vulgaris*) raising offspring had higher baseline CORT levels compared to those not raising offspring [14]. In contrast, chronically stressed barn swallows (*Hirundo rustica*) had increased glucocorticoid levels associated with declines in reproductive success through the production of lower quality offspring [15]. One potential explanation for these conflicting results is the use of inconsistent or inadequate metrics to measure baseline CORT.

While most studies measure the total level of CORT in circulation as a metric of baseline CORT, this may be an insufficient metric because hormone effects are modified by binding proteins in the blood [16]. Specifically, CORT binds to corticosteroid-binding globulin (CBG), which functions to transport CORT to various tissues via the circulatory system. There is now substantial evidence demonstrating that the biologically active fraction of CORT consists only of the portion that is free i.e. not bound to CBG. This suggests that the measurement of free CORT is more biologically relevant than the measurement of total CORT, despite its less frequent use in the literature [9]. The discrepancy across studies in measuring total CORT vs. free CORT levels may be able to explain some of the contradictory results discussed above. Additionally, the effects of CORT depend on cell-specific receptor mechanisms such as mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). If the effects of CORT are mediated by the availability of these receptors, then a measurement of their availability would also be meaningful to include when assessing the impact of CORT [17]. Finally, measurements of upstream mediators of CORT production such as Corticotrophin Releasing Hormone (CRH) and Adrenocorticotropic Hormone (ACTH) may also be relevant if either are the limiting factor in determining circulating levels of CORT [18]. Considering a wider range of metrics involved in the CORT stress response may allow us to deepen our

understanding of the mechanisms by which CORT mediates the trade-off between survival and reproduction.

The inability of the Cort-Fitness Hypothesis to reliably make predictions may also suggest that CORT plays a more complex role in responding to current resource demand in mediating the trade-off between survival and reproduction. In response to an inconsistent relationship between CORT and reproductive success across breeding stages in tree swallows (*Tachycineta bicolor*), where females with heavier clutch masses had lower CORT levels early in breeding whereas females raising broods with the greatest mass had elevated CORT levels, Bonier et al. [19] proposed that the Cort-Fitness Hypothesis be modified to the Cort-Adaptation Hypothesis. According to this hypothesis, when an individual makes a decision to reproduce, a negative relationship between baseline CORT and reproductive investment is expected because individuals facing less environmental stress can invest more in reproduction. After an individual invests heavily in reproduction CORT levels should positively correlate with reproductive success because this increased investment in reproduction requires increased CORT levels to allocate energy to reproduction [19]. This hypothesis is able to explain the variation in the relationship between reproductive investment and CORT across time within individuals and the results are reproducible across several other species [20, 21, 22]. For example, European starlings (*Sturnus vulgaris*) increased baseline CORT levels in preparation of reproductive investment and thus used CORT as a mechanism to manage investment decisions across reproductive attempts to maximize fitness [21]. Love et al. [15] concluded that CORT is able to mediate the trade-off between reproduction and survival in birds through management of reproductive investment annually to optimize lifetime reproductive success. This suggests that CORT mediates this trade-off through management of long-term reproductive investment in addition to current resource demand, and future research should be directed towards understanding mechanistically how CORT mediates future vs. current resource demand. Additional work may also focus on linking changes in CORT levels to changes in survival (although some studies have already done this, see [23]). Given that CORT is believed to influence survival indirectly via its downstream effectors (e.g. glucose, free fatty acids, oxidative stress, immunosuppression, telomere length etc.), it may be beneficial for future studies to include measurements of these downstream effectors in addition to measurements of total and free CORT [9].

3. Oxidative stress

Oxidative stress has the capacity to mediate the trade-off between survival and reproduction in birds through its production as a result of metabolic demand and association with aging [24]. Aerobic species, including birds, use oxygen for efficient energy release during metabolic processes, resulting in the production of reactive oxygen species [19, 20, ROS]. These ROS function as signaling molecules [25], but are also highly unstable and cause damage to membranes, proteins, lipids, and DNA. Oxidative stress occurs when antioxidant molecules are unable to fully neutralize ROS resulting in damage to biomolecules [26, 27]. This mechanism may be used to mediate the costs of reproduction as an increase in energy demand during reproduction results in an

increase in production of ROS and, if not enough antioxidants are produced, an increase in biomolecule damage occurs, contributing to aging and hence survival [24, 28].

Quantifications of the trade-off between reproduction and survival using antioxidant measures have produced indefinite results [24]. Many studies observe the predicted negative relationship between reproductive effort and antioxidants or the overall antioxidant capacity of the blood [29, 30, 31, 32, 33]. For example, male zebra finches (*Taeniopygia guttata*) with experimentally enlarged brood sizes experienced decreases in antioxidant enzyme activity [33]. However, the relationship between antioxidants and survival remains less clear with some evidence for a positive relationship between antioxidant capacity and survival [34, 35] and other evidence for no association between antioxidant status and survival [36, 37]. Theoretically, antioxidant status should correlate positively with survival if higher levels of antioxidants result in less oxidative stress and delayed senescence. It is important to note however, that measures of antioxidants do not provide a direct measure of oxidative stress as an increased level of antioxidants may be coupled with an increase in ROS, resulting in no change in oxidative damage. Thus to get a more accurate measure of oxidative stress an additional measure of the amount of ROS is necessary [24]. Several studies provide support for an oxidative cost of reproduction when oxidative stress is measured as the ratio of reactive oxygen metabolites to antioxidants, however few studies have used this approach [37, 38] and there are also results from it suggesting no oxidative cost of reproduction [39]. This inconsistency in results suggests that antioxidants may have a more complex relationship with the costs of reproduction or are an inadequate measure of oxidative stress.

Another approach to measuring oxidative stress, via direct measurement of oxidative damage to biomolecules, supports oxidative stress as a mediator of the trade-off between survival and reproduction. For example, a non-manipulative study of Florida scrub jays (*Aphelocoma coerulescens*) found an increase in oxidative damage to proteins in males post-breeding [40]. This suggests that reproduction increased oxidative stress, likely through an inability of antioxidants to match increases in ROS. Further support for this exists as increased oxidative damage, resulting from increased reproductive effort, is associated with no change in antioxidant status [41] and increased levels of antioxidants during reproduction are associated with no change in oxidative damage [36]. Interestingly, a recent meta-analysis by Blount et al. [42] revealed that while oxidative damage is frequently positively associated with reproductive effort in females, breeding females paradoxically have lower oxidative damage compared to non-breeding females. This suggests that the costs of reproduction may be mediated by 1) increases in oxidative damage associated with reproductive effort and 2) the cost of mechanisms utilised by females to minimise such damage i.e. oxidative shielding [42]. Further work is needed to understand how significant the cost of diminishing oxidative damage is for females and if it, alone and combined with the effects of oxidative damage, can have a significant impact on survival.

The inconsistent use of metrics to measure oxidative stress is a significant limiting factor in this field. The number of studies that address direct oxidative damage is limited and of the ones that do, there is not a wide enough range of tissues being measured as damage may be tissue-specific [43]. Thus while there is evidence in support

of oxidative stress as a mediator of the trade-off between survival and reproduction, this support should be interpreted with great caution [43]. That results depend on the measure of oxidative stress used suggests that we have little scope for how this mechanism mediates the costs of reproduction and that future research is needed measuring a wider range of tissues and including measurements of ROS, antioxidants, oxidative damage, and repair mechanisms to improve our understanding [44].

4. Immune function

Immune function is hypothesized to mediate the trade-off between survival and reproduction in birds through an energetic trade-off [45, 46, 47]. Reproduction and immune defenses are both energetically costly such that an increase in reproductive effort should lead to a decrease in immune function resulting in increased susceptibility to parasitism and thus reduced survival [48, 49]. Two predictions resulting from this model are that a positive relationship should be observed between reproductive effort and levels of parasitism and a negative relationship between reproductive effort and immune response [50]. Evidence supporting these predictions is mixed with some studies supporting both of the predicted relationships [51, 52], some providing no support for an effect of reproduction on parasitism [53], and others providing no support for an effect of reproduction on immune function [54]. However, a meta-analysis on this topic by Knowles et al. [50] found an overall weak, but well supported positive effect of reproductive effort on blood parasite levels and a moderate negative effect of reproductive effort on immune responsiveness. Additionally, inconsistencies in results were largely dependent upon experimental design and the length of time between manipulation of reproductive effort and measurement, with results suggesting that immunosuppression increases with the length of the chick-rearing period. For example, Skylarks (*Alauda arvensis*) handicapped with additional weight experienced no change in immune function during their first brood, however immune function during their second brood was able to predict return rates the following year [55]. Inconsistent results may also be explained by variation in metrics used to measure parasitism and immune responsiveness.

Similar to the CORT stress response and oxidative stress, there are a variety of metrics that have been used to document immune function in birds. Moreover, a single metric is unlikely to capture the complexity of immune function [56]. Parasitism can be measured with 1) prevalence (the proportion of the population that is infected) or 2) parasitaemia (the number of parasites in an infected host). Weaker effect sizes have typically been seen in studies using prevalence as a measure of parasitism [50]. This may be because studies were not long enough to capture changes in prevalence due to long periods of prepatency in hosts (i.e. the parasite is present, but not at high enough levels to be detected). Further, prevalence may be more dependent on environmental factors that influence host exposure and transmission. Alternatively, immune function can also be measured as the immune response to a novel antigenic challenge (e.g. sheep red blood cells). But there is no consistent novel antigenic challenge used across studies and it is unclear how an immune response to a novel antigen relates to host-parasite interactions in the host' natural environment [50]. Further, depending on the metric

used to measure immune response (e.g. leukocyte proportions), different measurements may have bias towards innate or adaptive immunity. While innate immunity requires investment early in life, adaptive immunity is developed later in life upon encountering novel pathogens and is therefore more likely to play a larger role in explaining the trade-off between reproduction and survival. It is possible that studies using metrics that better capture adaptive immunity will be more likely to detect an energetic trade-off between survival and reproduction.

There is currently no consensus of the currency that may allow immune function to mediate the trade-off between survival and reproduction. Evidence in support of an energetic cost is weak as the 5-15% increases in metabolic rate associated with an immune response are insignificant compared to the 300-400% increases during breeding [57]. While this may be important for a highly resource-limited individual, other explanations should be considered such as an adaptive suppression of the immune system during breeding to protect against harmful autoimmune responses [58] or increased damage to immune cells due to oxidative stress [57]. However, evidence supporting these mechanisms is also limited and often contradictory [57]. For example, male great tits (*Parus major*) rearing enlarged broods had increased parasite levels and decreased resistance to oxidative stress but no relationship between the two, suggesting independent mechanisms [59]. Overall this suggests we know very little of how immune function can limit the trade-off between survival and reproduction.

Regardless of the mechanism linking reproductive effort and immune function, one remaining question is whether changes in immune function can contribute significantly to changes in survival. While there is general support in the literature for a link between immune response and survival [60] the relationship between parasite infection and survival is less clear [50]. This may be because wild birds caught are more likely to be in the chronic infection phase which is rarely associated with survival effects [61, 62] or because the increases in parasite levels are insufficient alone to account for reduced survival because the costs of reproduction are felt along multiple pathways [59]. Thus while there is support for immune function as a mediator of the trade-off between reproduction and survival, further research is necessary both to understand the mechanism mediating the trade-off between reproduction and immune function as well as how immune function alters survival.

5. Conclusion

Despite the strong evidence in support of a trade-off between survival and reproduction in birds, we still know very little of how the proposed mechanisms are able to mediate this trade-off [1, 4]. While baseline CORT, oxidative stress, and immune function are supported mediators of this trade-off, our understanding of their mechanisms is limited. One obstacle impeding our progress in understanding the mechanisms behind all three physiological pathways is the inconsistent use of metrics. Advances in this field will likely be limited until adequate metrics are implemented universally. It is also clear that each of the proposed pathways does not act independently from the rest. For example, the effects of CORT on survival are mediated through immunosuppression [63, 64] and oxidative stress [64]. Similarly, immune response may be reduced during

reproduction due to increased damage to immune cells from oxidative stress [57]. Future research should not be limited by studying each of the mechanisms as mutually exclusive pathways. Instead, there is a need to focus both on pathways of individual mechanisms and potential interactions between mechanisms, as sufficient effect sizes may only be detected when multiple mechanisms and their interactions are considered. While some researchers have attempted to study multiple mechanisms together, e.g. male great tits (*Parus major*) rearing experimentally enlarged broods had increased parasite levels *and* decreased resistance to oxidative stress [59], the number of studies utilizing this kind of approach is limited. Where this approach may be particularly valuable is in studying the effect of potential physiological mediators of this trade-off on survival. For example, experimentally increasing levels of parasitism may not allow for detection of a significant effect on survival if multiple pathways interact with immune function to produce a significant survival effect. Instead, levels of parasitism could be manipulated in conjunction with levels of glucocorticoids and oxidative stress and the effect on survival measured. While there are drawbacks to this approach e.g. increased experimental design complexity, the current state of knowledge in this field requires the advancement to potentially more complex experimental designs to further our understanding of the mechanisms mediating this pivotal trade-off.

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Enlightened: The connection between circadian rhythms and depression

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Abstract

Most life on earth has evolved intricate clock systems to anticipate and adapt to the dynamic demands of a geophysical day. Rhythms can be found across the life kingdom and on several levels of organization – from genes to cells to organisms. Circadian rhythm is a tightly-regulated biological process that is both endogenously driven and entrained by environmental cues. Physiological processes, such as hormonal secretion, the stress system, and mood follow observable circadian rhythms. The hypothalamic-pituitary-adrenal (HPA) axis is an important stress system in humans. Circadian dysregulation in the HPA axis have been implicated in various metabolic diseases and depression. This paper presents a critical review of the current literature on the pathogenetic link between circadian regulation of the stress system and affective disorders.

Keywords — circadian rhythms, depression, light, hypothalamic-pituitary-adrenal (HPA) axis, stress, corticotropin-releasing-hormone (CRH)

1. Introduction

Most life on earth has evolved internal body clocks in-tune to their environment. Such body clocks are endogenously driven and modulated by external time-cues called "zeitgebers", or "time-givers" [1]. Zeitgebers could be patterns of light and darkness, temperature, humidity, and food intake [1]. The term "circadian rhythm" means "circa" (about) and "diem" (a day). Circadian rhythms are pre-adapted time-keeping programs that emerge from a synchronized network of internal body clocks; the cycles are intrinsically driven and persist in the absence of environmental stimuli. Circadian rhythms are ubiquitous across almost all life on earth, from cyanobacteria, [2] fungi, [3] to humans [4]. Humans living in temporal isolation demonstrate robust cycles of sleep-wakefulness, core body temperature, and urinary output [5]. Circadian clocks serve two important functions: first, they allow the organism to anticipate and respond appropriately to changes in the environment. This allows the organism to maximize and exploit resources within a particular temporal and spatial niche. Secondly, circadian clocks allow synchrony of various endogenous biochemical processes to facilitate metabolism. Most human physiological and behavioural processes follow circadian cycles. As such, it stands to reason that any disruptions in the endogenous time-keeping machinery may manifest in both psychological and physical disorders.

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In most vertebrates, circadian systems are hierarchically organized with the master pacemaker neurons located in the hypothalamic suprachiasmatic nucleus (SCN). The SCN is made up of approximately 10 000 neurons situated above the optic chiasma [6]. Research shows that destruction of the SCN abolishes overt circadian rhythms in most mammals [7]. In vivo studies demonstrate that isolated SCN neurons are able to maintain robust circadian cycles that slightly deviate from a geophysical day (∼24 hours), which suggests that the circadian rhythm in the SCN is intrinsically generated [8]. The SCN is entrained to light and detects photic information through specialized cells in the retina [9]. Two other inputs modulate the activity of SCN: Serotoninergic (5-HT) pathway from the raphe nuclei [10] and melatonin secreted from the pineal gland [11].

Disturbances in circadian rhythms have been linked to various metabolic diseases, such as Alzheimer's disease, [12] type 2 diabetes, [13] and cancer [14]. Patients with affective disorders tend to suffer from an array of dysregulated circadian rhythms, such as hormone secretion, sleep-wake cycle, and body temperature [9]. Such cycles are intimately linked to the stress system - the hypothalamic-pituitary-adrenal (HPA) axis - which also demonstrates a circadian rhythmicity in humans [15]. Taken together, this suggests that affective disorders such as major depression and atypical depression may involve abnormalities in the circadian regulation of the HPA axis [16]. This paper reviews the current literature on the relationship between circadian regulation of the stress system and its relationship to mood disorders.

2. Depression, Stress, and Light

Depression is a heterogeneous disorder with a complex interplay of symptoms. In particular, depression can be classified into two subtypes: major depression and atypical depression [9]. Major depression, or classic depression, features symptoms such as early-morning wakening, insomnia, lack of appetite, weight loss, idiopathic pain, anxiety, rumination, and pessimistic thinking [16]. In contrast, atypical depression features hyperphagia, hypersomnia, and increased sensitivity to interpersonal rejection [16]. Two symptoms: sleep and appetite, are diametrically opposed in the two disorders. Patients with major depression exhibit a diurnal variation in mood which is usually worst in the mornings, and then gradually improves as the day winds on [9]. Patients with atypical depression experience an opposite pattern; mood is usually elevated in the morning and steadily declines with the night [9]. Although the two depressive disorders are conceptualized separately, an individual may exhibit symptoms from one or the other [16]. Evidence also suggests that the two disorders may not be conceptually distinct, but share an underlying pathophysiology of abnormalities in circadian regulation of the HPA axis [16].

Light is a potent zeitgeber that entrains the SCN, the "master clock" in the human body. The master clock is responsible for orchestrating the alignment of subsidiary clocks found in peripheral tissues, such as the liver, spleen, and kidneys [17]. In addition to synchronizing sleep-wake cycles, the SCN is also involved in the regulation of the stress system - the HPA axis. The HPA axis is a rigidly controlled process that involves a complex network of neuronal and endocrine systems. Acute activation of the HPA

axis enhances stress response behaviours such as enhanced cognition, better attention, energy mobilization, and greater blood flow to the limbs [18]. Chronic activation of the HPA axis, however, can lead to pathological symptoms such as decreased immune function, osteoporosis, type 2 diabetes, poor cognition, memory deficits, and depression [18]. The HPA axis is modulated by corticotropin-releasing hormone (CRH), a hormone that responds to stress, injury, and light [19]. Although it is not yet clear whether a disrupted circadian clock leads to a dysregulated HPA-axis or vice versa, it has been demonstrated that chronic activation of the HPA axis precipitates depressive behaviours such as hypervigilance and stereotyped thinking [20].

CRH is a peptide hormone made up of 41 amino acids. CRH is the primary hypothalamic driver of the HPA axis. Other than a stress response, the HPA axis also plays a role in immune function, metabolism, growth, and reproduction [20]. Light, stress, or injury stimulates the release of CRH from the hypothalamus, which in turn triggers the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then stimulates the production of glucocorticoids from the adrenal cortex. The glucocorticoids negatively feedback on the anterior pituitary and hypothalamus modulating the duration and strength of axis activation. CRH acts as a major metabolic controller and has many neuroendocrine functions, either acting directly or by acting on the HPA axis [20]. Abnormalities in the HPA axis have been implicated in both major depression and atypical depression. In particular, evidence suggests that major depression may involve hyperactivity of the HPA axis whereas atypical depression is characterized by a hypoactive HPA axis [21, 22]. Patients with major depression have increased cortisol in the saliva, plasma, and urine; and an increased size of the pituitary and adrenal glands [23, 24]. Conversely, patients with atypical depression have lower CRH levels in their cerebrospinal fluid relative to healthy controls [25]. Another study has demonstrated that patients with atypical depression have lower serum cortisol compared to control subjects [26]. Indeed, the psychomotor symptoms of atypical and major depression are opposite - whereas the former is marked by psychomotor impairment and leaden paralysis, the latter is characterized by general restlessness and agitation. Since CRH is implicated in alertness and arousal, CRH deficiency may underlie atypical depressive symptoms such as heaviness of limbs, fatigue, and hypersomnia [22]. In humans, cortisol levels follow a circadian rhythm in which it declines steadily throughout the day, reaches nadir around midnight, and then peaks just before wakening [27]. In patients with major depression, the temporal pattern of cortisol secretion is disrupted. Studies indicate that plasma cortisol and norepinephrine (NE) are phase-advanced in patients with depression relative to healthy subjects [28]. In other words, noradrenergic neurotransmitters and their metabolites reach their peaks earlier in patients with depression than healthy controls. Patients with depression also have elevated levels of cortisol prior to sleep onset [28], which possibly contributes to complaints of poor sleep. The continuous elevation of NE levels in depressed patients may also lead to neurovegetative symptoms, preservative thinking, and anxiety [29]. As NE is an arousal hormone that prompts stress response behaviour, patients with major depression may be in a chronic state of hyperarousal and chronic stress. As such, the diurnal variation in depressive symptoms may be attributed to abnormal circadian regulation of stress hormone secretion.

In addition to sleep-wake cycles, CRH also plays a role in feeding behaviour. CRH is anorexigenic [17]. Indeed, patients with major depression experience weight loss and anorexia, whereas patients with atypical depression exhibit weight gain and hyperphagia [22]. Interestingly, light therapy, a treatment traditionally used for seasonal affective disorder (SAD), has been found effective to treat non-seasonal unipolar depression in adjunct to antidepressants [30]. As light is a potent zeitgeber that triggers the release of CRH, light therapy may augment the blunted CRH levels observed in patients with atypical depression. Although the exact mechanism is still unclear, chronotherapies that help to realign an individual's circadian cycles may be an invaluable approach for correcting abnormal activation of the stress system, since abnormal activation is a core component of depression pathophysiology.

3. Sleep Rhythms and Affective Disorders

Sleep-wake cycles are one of the most overt rhythms studied in humans [9]. Sleep disturbance is also a symptom common across mood disorders. Light inhibits production of melatonin, a neurohormone that promotes sleep. Melatonin is produced by the pineal gland; its levels steadily increase with darkness [1] and is used as a signal of light/dark duration in species with seasonal behaviour. Indeed, dim light onset of melatonin is considered one of the most useful marks of circadian phase position in humans [31]. Sleep disruption is one of the most common complaints in patients with depression. According to Almeida and Pfaff , up to 63% of depressed patients report trouble falling asleep, staying asleep, and experience early morning awakening [32]. Abnormal cortisol levels and melatonin secretions were detected in depressed patients [33]. Whereas levels of cortisol gradually decrease throughout the day for a healthy individual, cortisol levels stay high for a patient with major depression [33]. Depressed patients also have elevated nocturnal core temperatures compared to healthy controls [34]. Since the SCN oversees both the sleep-wake and body temperature cycles, abnormalities in biorhythms may stem from deficits in the master pacemaker rather than isolated rhythmic outputs. In bipolar disorders, abnormalities in sleep cycles also predict the onset of mood episodes: insomnia precedes and persists during manic episodes, whereas both insomnia and hypersomnia precipitate depressive symptoms [35]. Seasonal shifts in light and darkness can also trigger manic episodes in bipolar patients and depressive episodes in patients who have seasonal affective disorders [35]. This suggests that it is not the absolute level of light, but the relative shifts in light exposure that may be responsible for the onset of mood disorders, particularly those with a seasonal variation.

Notably, a growing body of research shows that one way antidepressants such as SSRI and mood-stabilizers work by regulating circadian rhythms. Lithium functions by targeting the enzyme glycogen synthase kinase-3B (GSK3β), an enzyme implicated in the regulation of circadian clocks [36]. Lithium has also been shown to lengthen the period of circadian rhythms in rodents [37]. Valproate, another mood stabilizer used in bipolar disorder, alters the expression of circadian genes in the amygdala [38]. Although the exact mechanisms remain to be explored, there is some evidence to suggest that the therapeutic benefits of these medicine stem from regulating the

endogenous clock system.

Lastly, light therapy has been found to reduce depression in institutionalized older adults who usually lack sufficient access to natural lighting [39]. Intentional light deprivation, or "dark therapy", has also been found to treat patients with bipolar disorder, [40] possibly by altering the length of photoperiods. In summary, the evidence suggests that misalignment of endogenous clocks to external time cues may play an important role in the onset and etiology of affective disorders, including but not limited to depression.

4. Conclusion

Most life on earth has evolved intricate clocks to regulate physiological processes and behaviour. Organisms have evolved clock systems to anticipate regular patterns of light and darkness and to coordinate physiological processes accordingly. Emerging research suggests that abnormalities in circadian clocks may underlie the etiology of various affective disorders, such as depression, SAD, and bipolar disorder. Although it is not yet clear whether abnormalities in circadian rhythms cause affective disorders or vice versa, evidence suggests that chronotherapy may be an invaluable tool for treating mood symptoms with temporal variation. Possible therapeutic interventions include pharmacotherapy that target the circadian system which establishes consistent sleep-wake schedules in patients diagnosed with affective disorders and ensures consistent light exposure during appropriate times.

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A Literature Review: The Efficacy of MDMA-Assisted PTSD Psychotherapy

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Abstract

Researchers have proposed that 3,4-methylenedioxymethampethamine (MDMA), an illicit psychedelic drug with widespread recreational use, may be a beneficial therapeutic adjunct in PTSD treatment due to its unique mood altering and prosocial effects. A growing body of clinical evidence points to MDMA-assisted psychotherapy as a promising alternative for individuals who have found current PTSD treatments unsuccessful. Within the past decade, several studies integrating MDMA administration with psychotherapy have seen clinical PTSD symptom reductions. Despite this, the therapeutic application of MDMA remains contentious. A range of in vitro experiments as well as studies in drug users and animal models have associated MDMA with adverse health consequences, including psychiatric distress, cognitive decline, and neurotoxicity. However, it remains a challenge to parse out whether these negative side effects are truly applicable in a clinical setting, where a chemically unadulterated and standardized dose is provided during a limited number of sessions. In this literature review, I will summarize the recent clinical trials on MDMA-assisted psychotherapy, point out the limitations of this research, examine potential adverse health effects, and outline important topics for future exploration.

Keywords — MDMA, post-traumatic stress disorder, drug-assisted therapy

1. Introduction

Post-traumatic stress disorder (PTSD) is a mental illness involving intrusive thoughts and flashbacks which can develop after an experience of severe trauma, such as armed conflict, natural disaster, or sexual violence [1]. Using DSM-V criteria, global estimates of lifetime prevalence range from 1.3% [2] to 8.8% [3], and the illness causes a considerable public health and economic burden [4, 5]. Successful first-line psychotherapeutic treatment options for PTSD include cognitive behavioral approaches and eye movement desensitization and reprocessing (EMDR) [6, 7, 8]. Options for pharmacotherapy include selective serotonin reuptake inhibitors, which have shown success in reducing PTSD symptoms but are not considered as effective as psychotherapy [9, 10]. Critically, a portion of individuals remain non-responsive to both forms of intervention [11, 12, 13, 14, 15], and this has motivated researchers to develop other treatment options [16, 17, 18].

A promising candidate for drug-assisted psychotherapy is 3,4-methylenedioxymethamphetamine (MDMA), a ring-substituted amphetamine

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[19]. This drug possesses unique psychoactive and mildly hallucinogenic properties, causing users to feel euphoria, increased intimacy, and empathy [20, 21, 22, 23]. These psychoactive effects make MDMA a popular drug for recreational use [24, 25], and it is illicitly sold under slang names such as "ecstasy" and "Molly". Because MDMA is classified as a Schedule I controlled substance, the application for MDMA use in treatment for PTSD therapies has been controversial [17]. However, researchers have launched efforts to meet such challenges–the Multidisciplinary Association for Psychedelic Studies (MAPS) is a non-profit organization dedicated to researching and developing the therapeutic potential of psychoactive drugs [26]. Researchers collaborating with MAPS have completed the first clinical trials testing the efficacy of MDMA-assisted psychotherapy. In the following review, I will provide an outline of these clinical trials, highlight the potential limitations to the studies, and point out future topics for exploration. Also, I will attempt to provide a balanced view of the long-term negative sequelae associated with MDMA use.

2. Psychoactive Effects of MDMA

MDMA produces a suite of unique psychoactive effects, including positive mood, increased arousal, and greater empathy. This last quality places the drug within the class of enactogens–psychoactive agents that heighten feelings of intimacy, sociability and communication [16, 17, 18, 19]. Researchers hypothesize that MDMA induces such changes by increasing levels of serotonin and–to a lesser degree–norepinephrine and dopamine [27, 28]. This perhaps results in increased emotional tolerance when addressing traumatic experiences and memories [29, 30]. Additionally, MDMA has been found to elevate serum oxytocin, a neurohormone associated with social affiliation and facilitation of interpersonal trust [31, 32]. However, it is unclear whether this elevation in oxytocin actually contributes to the subjective effects of MDMA as several studies have found no association [33, 34]. Brain imaging in humans has revealed that MDMA also attenuates perceptions of threat by decreasing activity in the left amygdala [35]. Combined, these positive subjective effects seem to make MDMA a powerful adjunct for therapy. However, MDMA also produces effects that may be counterproductive towards therapeutic intervention. The 5-6 hour "high" experienced with MDMA is followed by a period of neurochemical depletion characterized by low mood and lethargy [36, 37]. And while the drug can help reduce anxiety regarding social interaction [38], numerous studies report that the drug can actually increase general anxiety, an effect common to other stimulants [39]. Despite such drawbacks, several clinical trials have found that MDMA-assisted psychotherapy can help decrease PTSD symptoms in individuals resistant to other treatment approaches [40, 41].

3. MDMA as a Therapeutic Adjunct

Prior to its classification as an illegal substance in the 1980s, MDMA was subject to growing interest in its potential therapeutic use. Alexander Shulgin, a pioneer in the field of experimental psychedelic research, promoted its seemingly beneficial psychedelic effects, which led to its wide application by clinicians and therapists [16]. Prominent

therapists Greer and Tolbert conducted MDMA-aided music therapy sessions, notably highlighting both the positive and negative effects experienced by their patients [42]. In a typical session, 75-150 mg of MDMA was administered to an informed volunteer and sometimes followed up with a smaller dose to prolong the effects of the drug. From these case studies, it was hypothesized that MDMA helped extinguish feelings of fear, allowing the participants to become more comfortable communicating traumatic experiences. Researchers have emphasized that the psychotherapeutic component of this treatment should not be overlooked–they argue that it is the interaction of MDMA with therapy that produces enduring results, not simply administration of the drug itself [29]. While the criminalization of MDMA largely halted such therapeutic studies for more than decade [30], the growing need for PTSD treatment alternatives has motivated current research to investigate the safety and efficacy of MDMA as a therapeutic adjunct.

4. MDMA Clinical Trials for PTSD

In 2008, Bouso et al. carried out the world's first government approved clinical trial designed to test the safety and efficacy of MDMA-assisted therapy [43]. Funded by MAPS, the study planned to administer relatively low doses (50 or 75 mg) of the drug to a small sample of women with chronic PTSD. Originally, the double-blind, randomized trial included 29 women, but political pressure led to the cancellation of the study when only 6 subjects had been treated, making it difficult to conduct meaningful statistical analysis. All participants who had been dosed with MDMA ($n = 4$) during therapy showed higher improvement than those given placebo ($n = 2$), with greatest PTSD symptom reduction seen in the single individual given the highest dose of MDMA (75 mg) and intermediate symptom reduction in the three individuals given a slightly lower dose (50 mg). Though limited by the small sample size, these findings indicated that administration of MDMA in conjunction with psychotherapy could be conducive to PTSD treatment.

A key paper by Mithoefer et al. [40] confirmed the findings of the Bouso et al. [43] study, while presenting an appreciably larger sample size. Also sponsored by MAPS, this American study employed a similar double-blind methodology, assigning 20 participants (14 female / 3 male) to either an MDMA group ($n = 12$) or a placebo group ($n = 8$). The experimental sessions, which included a balance of quiet introspection and therapeutic discussion, were 8-10 hours long and were ended by an overnight stay so health condition could be monitored. The dosage of MDMA in this study was considerably higher than the earlier study–at least 125 mg of MDMA was administered to each participant in the MDMA group. Thus, participants experienced more noticeable side effects, including elevations in blood pressure, heart rate, and body temperature. Critically, this undermined the double-blinded aspect of the study design: both participants and therapists easily recognized whether the sessions were medicated with drug or placebo. Elevated physiological measures seemed to reduce to baseline at the end of each session, and although some participants reported jaw tightness, nausea, loss of appetite, and impaired balance, the side effects were not serious or long-lasting. Neurocognitive tests conducted two months after the experimental sessions revealed

that MDMA intake did not have adverse effects on cognition. The researchers found that PTSD symptoms decreased for both the experimental and placebo groups, but the MDMA-administered group showed significantly greater improvement, with a symptom reduction of 83.3% compared to 25% (placebo) two months after the experimental sessions. Later, when the blind was removed and placebo participants were given the option to partake in MDMA-assisted therapy sessions, they saw similar levels of improvement.

To determine if these results would be consistently maintained, the researchers also conducted a long-term follow-up around 3.5 years after their original study's exit date [44]. All subjects who received concurrent MDMA administration and psychotherapy were enrolled in this follow-up. PTSD symptoms did not show a statistical increase during this period, demonstrating that participants saw a retained improvement after MDMA-assisted therapy. Additionally, all participants reported that the experience had imparted some degree of benefit on their lives. None of them reported any harm or dependence issues after taking MDMA, nor did they self-report any evidence of neurocognitive decline. However, one participant did admit to taking MDMA again in a "quasi-therapeutic" environment as an attempt to recreate the effect of the experimental sessions. This type of self-medication may be concerning, and its implications are discussed later. Additionally, while there was a lack of evidence for cognitive decline, objective assessments were not used to confirm the participants' favourable self-reports. The researchers also pointed out a major confounding factor–out of the 19 subjects enrolled in the follow-up, 8 were still in psychotherapy and 12 were taking psychiatric medications. This may have played a role in sustained symptom improvement, but the small sample size prevented statistical comparison between individuals who were undergoing continued treatment and those who were not. Despite its limitations, this follow-up demonstrated the promising treatment durability of MDMA-assisted psychotherapy.

In 2013, Oehen et al. [41] attempted to replicate the findings of the Mithoefer et al. [40] study. To increase the strength of the double-blind design, an "active" placebo of 25 mg MDMA was used (producing similar but milder effects compared to the 125 mg dose). Again, MDMA showed no serious adverse health effects when administered in a clinical setting. However, even though the full-dose group showed a decrease in PTSD symptoms as compared to the "active" placebo group (which showed no symptom reductions), the effect was not statistically significant. On average, those given the full-dose of MDMA saw symptom scores decrease by only 23.5%, a marked difference to the original 83.3% decrease found by Mithoefer et al. The researchers suggested cultural differences, therapist differences, and uneven sampling as potential explanations for these discrepancies.

Currently, MAPS has several ongoing clinical studies underway. In October 2016, researchers in the U.S. completed a study involving American veterans, firefighters, and police officers with chronic, treatment-resistant PTSD [45]. Additionally, a small, pilot study in Canada has recently finished experimental sessions [46]. The publication of these results will help determine whether MDMA-assisted therapy yields statistically significant PTSD symptom reductions.

5. Possibility of Adverse Health Sequelae

The short-term effects of MDMA are well-characterized in a clinical setting. Most dosages range from 0.75-1.5 mg MDMA/ kg body weight (up to 150 mg per session) and can cause elevations in body temperature, heart rate, and blood pressure [21, 23, 27]. Participants of such studies have reported adverse effects such as nausea, anxiety, tight jaw, and loss of appetite [40, 41, 47]. Importantly, these effects seem to be tolerable and do not last beyond several days [40, 41, 47].

While the MAPS-sponsored clinical trials showed a lack of persisting adverse health effects in participants [40, 41, 44], there is a large body of research associating MDMA with various long-term harms [36, 48, 49, 50, 51, 52, 53]. The drug has been linked to psychiatric distress, cognitive decline, and neurotoxicity [36, 51, 52, 53]. However, much of this research has been done in vitro, in animal models, or in a naturalistic or retrospective manner on drug users [36, 48, 49, 50, 51, 52, 53, 54, 55]. It remains a challenge to parse out whether these findings remain relevant to MDMA use in a clinical psychotherapeutic setting.

Studies conducted on drug users have found that MDMA may be implicated in a range of adverse psychological effects such as impaired cognition [37, 56] and sleep quality [52, 57]. At least one study has linked MDMA use with downstream depressive symptoms [39], but other research has not confirmed these findings [58, 59]. Thus, our understanding of negative psychological consequences remains equivocal. Common criticism for research done on drug users is that it fails to account for polydrug use and ignores the unpredictable composition of "ecstasy" [55, 60], the recreational form of MDMA which often contains adulterants that range from benign fillers to other illicit psychoactive substances [61, 62, 63]. Controlling for polydrug use, a 2014 retrospective study with a considerable sample size ($n = 997$) found that ecstasy polydrug users struggled more with impaired memory and sleep, increased impulsiveness, and greater prevalence of depression, in comparison to non-ecstasy polydrug users [50]. Despite rigorous controls for various polydrug use, this study is still limited by potential sampling bias and the questionable content of ecstasy tablets.

A range of in vitro, animal, and drug user studies have associated MDMA with neurotoxicity [49, 53, 64]. However, it is not clear whether these potential harms are applicable to the context of MDMA-assisted psychotherapy, where the dosage of the drug may be considerably lower, and the treatment consists of only a few medicated sessions [65]. Again, the confounding factors of polydrug use and potential adulteration of ecstasy come into play when interpreting research done on ecstasy drug users. Even so, a recent review of neuroimaging studies done on moderate ecstasy users in fact showed no evidence of associated structural or functional brain alterations [66]. Many questions about possible long-term adverse effects remain unresolved, and additional randomized studies in humans are necessary before further conclusions can be drawn.

6. Potential for Drug Abuse

The possibilities of abuse and dependence must be fully elucidated if MDMA is to be submitted for clinical application. It is not fully understood whether the drug

is physically addictive [67, 68, 69]. In animal studies, rats have been found to self-administer MDMA [70], though the motivation to do so is lesser compared to other drugs such as cocaine [71]. When generalizing such studies to humans, additional factors such as polydrug use and mental illness may complicate our understanding. In a case study report, Jansen [72] noted several situations where MDMA use met the criteria for substance dependence. One of the subjects in this case report had a PTSD diagnosis and was taking up to 25-30 tablets of MDMA every weekend as a way to alleviate emotional numbing and increase feelings of social empathy. His use of MDMA had caused him to critically neglect his financial livelihood as he had resorted to selling personal belongings to buy MDMA. Though a case like this illustrates an extreme, it demonstrates a potential complication for MDMA-assisted therapy. But perhaps, a more realistic scenario of potential abuse is brought to light by the study participant who reported seeking MDMA in a quasi-experimental setting outside of the study [44]. This type of self-medication may pose risks since health professionals are not present to monitor physiological condition. Also, if this behavior is repeated, it may lead to a pattern of abuse unconducive to the purpose of the treatment.

7. Study Limitations and Future Directions

The weakness of the double-blinded methodology in the Mithoefer et al. [40] study was a notable limitation. However, it is hard to determine if this factor confounded the results in any way. In the Oehen et al. [41] study, the blinding method was improved by replacing the placebo group with an active placebo group, whose participants received enough MDMA (25 mg) to produce some of the drug effects, but not enough to successfully aid treatment. Indeed, PTSD symptom scores actually saw a slight, non-significant increase in this group.

Another major limitation found across many of the studies is the disproportionate gender ratio of the participants–all the participants in the Bouso et al. [43] study and a majority of participants in the Mithoefer et al. [40] and Oehen et al. [41] studies were female. This may have played a role in the treatment success as the psychoactive effects of MDMA have been found to be more intense in women than men [73]. Critically, the participants in the U.S. veterans study [45] are mostly male, and publication of these results may help explore potential sex differences and increase the generalizability of the current results.

So far, MDMA-assisted PTSD therapy has not employed gold-standard psychotherapy approaches such as cognitive behavioural therapy or EMDR. MAPS plans to pilot a MDMA-assisted therapy trial integrating cognitive behavioural conjoint therapy [74]. Given the success of cognitive behavioural approaches [6, 7, 8], such directions are of great interest.

Notably, all MDMA-assisted PTSD therapy trials have been funded by the same source. Lack of funding from separate government and pharmaceutical agencies may be justified by the controversial nature of psychoactive drug research as well as the expired patent on MDMA. However, in the future, it may be helpful to see PTSD symptom reductions confirmed by research unassociated with MAPS.

8. Conclusion

Within the past decade, important findings have suggested the efficacy of MDMA as a therapy adjunct for individuals living with treatment-resistant PTSD. The therapeutic potential of this enactogen stems from its ability to elevate mood, enhance trust, and reduce perception of threats. However, our knowledge of long-term adverse effects is inconclusive. Although research has often associated MDMA with harmful sequelae such as psychiatric distress, cognitive decline, and neurotoxicity, the relevance of these studies in a psychotherapeutic context is debatable. Several MAPS clinical trials are either ongoing or awaiting publication. If the promising initial results of MDMA-assisted psychotherapy are confirmed, this treatment option may offer a transformative experience for individuals living with treatment-resistant PTSD.

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