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Cover Image Description

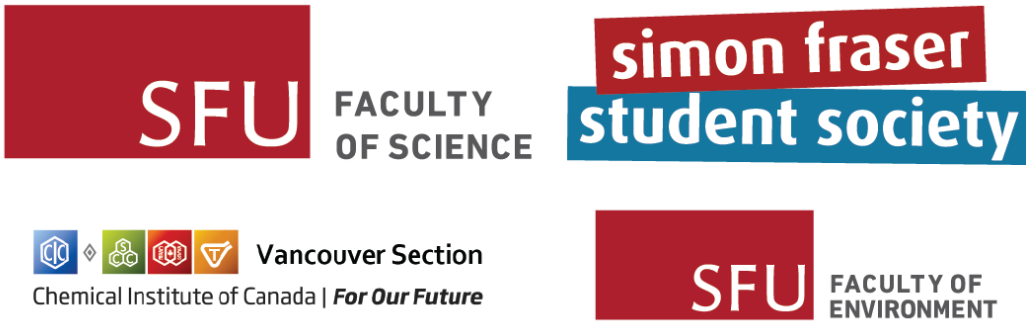
Named after the Michigan Cancer Foundation, the institute where it was first developed by Herbert Soule and colleagues, the MCF-7 cell line is an extensively studied human breast cancer cell line. It was derived in 1973 from a Caucasian woman with metastatic breast cancer and is the most well studied breast cancer cell line. Studies carried out with the MCF-7 cell line have yielded results that have played an essential role in expanding the horizons of breast cancer research. This image portrays an MCF-7 cell transfected with a plasmid that encodes Green Fluorescent Protein tagged Von Hippel-Lindau (VHL-GFP). VHL is a tumor suppressor gene, whose protein product functions as an E3 ubiquitin ligase that directs the degradation of hypoxia inducible factor (HIF), a transcription factor involved in regulating oxygen dependent gene expression. The purpose of this transfection was to observe the localization of VHL-GFP in human cancer cells under untreated, normal conditions. This image shows that VHL-GFP has nucleocytoplasmic disposition in untreated MCF-7 cells.

The image was taken and processed by using the new Zeiss super resolution fluorescence microscope at Simon Fraser University, Burnaby, Canada on July 18, 2019. The GFP tag was excited by exposing the cells to blue light, with a wavelength of 488 nm & observed using the 62$\times$ oil immersion lens.

Copyright policy:

Acknowledgements

Many hands contributed to production of this journal, and we are extremely grateful. Our thanks to the ongoing support of Kevin Stranack and the folks at the Public Knowledge Project for providing us with the online platform that keeps us organized, and the support to go with it. Thank you to all those at the Simon Fraser Student Society for guiding us through the financial and printing logistics with grace and generosity. Perhaps most importantly, our sincere thanks to the wonderful group of graduate students and professors who provided us with their time and expertise as peer reviewers this year. We are immeasurably grateful. Finally, a big thank you to the generous sponsors who provided us with the means to make this a reality:



2018-19 Board of Editors

"The most exciting phrase to hear in science, the one that heralds the most discoveries, is not 'Eureka!', but "That's funny..." "

- Isaac Asimov

Foreword

Dear Reader,

Scientific research is ultimately a venture into the unknown, with the goal of understanding all that surrounds us. To achieve this, passion, hard-work, and persistence are required. However, the truly remarkable thing about science is that anyone who has these qualities can help make progress in pushing the boundaries of the collective knowledge of humanity.

Since 2015, the SFU Science Undergraduate Research Journal (SURJ) has aimed to foster a community to help budding undergraduate researchers flourish, by providing many opportunities to showcase their work. Initially, and chiefly, this has been done through the publication of manuscripts written by undergraduate researchers at SFU, which have gone through a rigorous peer review process facilitated by SURJ. On top of the publication of the journal which caters to a more niche crowd, we have expanded to also run a blog which makes the research at SFU, and those who conduct it, more accessible to a general audience. One of our most successful and popular endeavours is our undergraduate research poster competition, where anyone can come and see some of the finest undergraduate research being done at SFU.

The many hours of physical, mental, and emotional work which go into a manuscript are something we at SURJ truly appreciate and understand. Each year we see many remarkable efforts and submissions that humble and motivate us. It is thus with great fervour that we work with authors, editors, and reviewers in the peer review process to prepare submissions for publication. When we look back at all we have accomplished, we are truly thankful to all the authors, peer reviewers, and editors past and present. We are also tremendously grateful to our sponsors – the Faculties of Science, Environment, and Health Sciences as well as the Chemical Institute of Canada – for their continued support. With much excitement and delight, we look forward to the future of the journal in the years to come.

Without further ado, we present to you, the fourth edition of the SFU Science Undergraduate Research Journal.

Sincerely,

Emily Leung, Olivia Tsai, Siobhan Ennis, and Anish Verma

Executive Editors

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Long-Term Effects of Salmon Subsidies on Terrestrial and Freshwater Invertebrate Communities

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Abstract

Pacific salmon (*Oncorhynchus* spp.) provide a flux of nutrients into terrestrial and freshwater food webs during the spawning season, which has been shown to positively increase the abundance and biomass of terrestrial and freshwater invertebrates. Previous research has shown that, in the immediate post-spawning period, salmon-derived subsidies (ie., resources produced outside the principal ecosystem) in the form of salmon carcasses provide a surplus of nutrients that can cause two-fold increases in the abundance and biomass of terrestrial invertebrates. Here, we quantify terrestrial and freshwater invertebrate abundance, biomass, and composition to determine if the salmon subsidy has a lasting effect on invertebrates into the pre-spawning period the following year. We hypothesize that if a lasting effect is present, the abundance and biomass of invertebrates collected at the salmon-bearing reaches will be greater when compared to those collected in the salmon-absent reaches. Terrestrial and freshwater invertebrates were collected and identified from above and below a salmon barrier in the pre-spawning season at two streams: Sugsaw Creek and Sarita Falls on the West Coast of Vancouver Island, BC. Abundance and biomass displayed a significant negative correlation for both terrestrial and freshwater invertebrates, demonstrating a bottom-up trophic level community. We collected a greater invertebrate abundance and biomass below the falls with the exception of terrestrial invertebrates at Sugsaw Creek. Outliers were noted for orders such as Diptera and Coleoptera. There was little difference in invertebrate diversity across any of the locations. Our results indicate that there is no yearly legacy effect of salmon subsidies on terrestrial and freshwater invertebrates into the pre-spawning season. The importance that nutrient transfer across ecosystem boundaries have on structuring community food webs has long since been demonstrated, and through our findings, we hope to contribute to the notion that salmon play key roles in structuring not only freshwater but terrestrial communities as well.

Keywords — Invertebrates, Trophic Levels, Community Ecology, Salmon Subsidy, Size Spectra

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1. Introduction

Anadromous salmon (*Oncorhynchus* spp.) return annually to freshwater streams and rivers for the spawning season in late summer, and transfer marine-derived nutrients to terrestrial and freshwater systems [1]. A considerable influx of salmon biomass can enter freshwater sources from the ocean [1] and provide a nutrient pulse to otherwise nutrient-limited areas, such as the temperate rainforests located along the coastline of the Pacific Northwest [2]. Salmon derived nutrients are transferred to the surrounding ecosystems through a variety of mechanisms. As salmon carcasses begin to accumulate in terrestrial and freshwater habitats, they effectively transport nutrients such as nitrogen and phosphorus to the surrounding riparian vegetation and soil communities [2, 3]. Deceased salmon are then colonized by carcass-specialist terrestrial invertebrates that are in turn consumed by organisms higher up in the food chain, effectively transferring energy between trophic levels [4, 5].

Marine subsidies represent a key nutrient source for terrestrial and freshwater ecosystems, and in both cases, contribute extensively towards structuring the community composition and food webs [2]. Communities where the main nutrient and energy source is smaller organisms found at the base of the food chain typically occur as a bottom-heavy trophic level pyramid [6, 7]. Species abundance within this type of community is thus limited by the amount of nutrients and energy that is available [2]. Nutrient limitation is determined by a multitude of factors including: primary production, temperature, ecological interactions, and the metabolic rate and body size of the organism. The invertebrate abundance-body size relationship within a community can be used to determine energy flow and productivity of the ecosystem [6]. The slope of the abundance-body size relationship represents the rate at which abundance changes with increasing body size [7]. The slope can be interpreted as the efficiency of energy transfer between trophic levels, or the rate at which energy is being lost as it is transferred from one trophic level to another. The y-intercept, on the other hand, represents the baseline productivity within the ecological system and is thus related to the abundance of smaller-bodied organisms found in lower trophic levels [7]. Both the slope and intercept can shift in response to local changes in nutrient availability occurring in the ecosystem [2]. An increase in the intercept would indicate an overall increase in the abundance and body mass for that particular community, implying that an increase in productivity occurred over the entire system as a result of the marine subsidy. A steeper or shallower slope would indicate that a specific trophic level (lower or higher respectively) - determined by body mass - is receiving more resources and is more highly subsidized relative to the other levels.

Salmon-derived nutrients that are transferred to terrestrial and freshwater ecosystems have been shown to influence the structure of invertebrate communities [2]. Salmon derived nutrients can alter terrestrial primary production (3) which, in turn, can alter the diversity and composition of riparian invertebrate communities [5]. Evidence for this is demonstrated by an increase in the abundance-body size intercept and thus the productivity of terrestrial invertebrate communities immediately following a salmon spawning event [8]. Alternatively, salmon redds disturb sediments and therefore may alter benthic freshwater invertebrate abundance and productivity [9]. While the long-term effects of salmon subsidies on invertebrate communities have been

well-documented in terrestrial ecosystems [2, 3], to our knowledge, these effects on invertebrate abundance and biomass have yet to be thoroughly studied in freshwater ecosystems. Thus, our study aims to add to the existing knowledge of the long-term effects of salmon subsidies in terrestrial invertebrate communities, as well as further explore these effects in freshwater invertebrate communities. Specifically, we test if there is a long-term change in either the slope or intercept of the abundance-body size relationship for invertebrates above and below salmon barriers indicating a legacy effect of marine subsidies. We examined the effects of marine subsidies on terrestrial and freshwater invertebrate communities by measuring: (i) biomass, (ii) abundance, (iii) slope and intercept of the abundance-body size relationship, and (iv) species diversity in the pre-spawning period, almost one year after the previous salmon return.

We aim to determine if the effects on invertebrate size and abundance from salmon-derived nutrients last into the pre-spawning period the following year, or if these effects occur strictly in the short term when salmon carcasses are present. Specifically, we ask: (a) if the terrestrial and freshwater invertebrate communities are size-structured, (b) if there is an observable difference in abundance or biomass between the control and salmon-bearing communities, (c) if there is a difference for terrestrial or freshwater communities in the slope or intercept between the control and salmon-bearing communities, and (d) if there is any difference between terrestrial or freshwater invertebrate diversity between the control and salmon-bearing locations. If a legacy effect of the salmon subsidy from the previous year is present on the invertebrate community, we expect an increase in the intercept and a shallower slope, indicating a net increase in productivity over the entire ecosystem as a result of the salmon nutrient subsidies. We would also expect that the size and abundance of both the terrestrial and freshwater invertebrates would be greater below the falls than above, since that is where salmon spawning occurs.

2. Materials and Methods

2.1. Site Descriptions

We sampled terrestrial and freshwater communities from two streams: Sugsaw Creek (48°50’15.80"N, -125°06’22.30"W) and Sarita Falls (48°54’10.4"N, -124°55’15.7"W), both located on the western coast of Vancouver Island, near Bamfield, British Columbia (Fig. 1). These salmon-bearing streams were chosen due to the presence of a waterfall at each location; serving as a physical barrier to the upstream migration of salmon (hereafter referred to as our control reaches). Sites below the waterfall represent areas that salmon are able to access (hereafter referred to as our salmon-bearing reaches). At Sugsaw Creek, the control site above the falls was relatively flat on either side. For the salmon-bearing site below the waterfall however, the riparian profile became very steep on both sides of the stream. The canopy cover over the stream at both sites was fairly dense. There was an increasingly steep slope on either side of the stream for both sites above and below the falls. For both sites, there was almost no canopy cover. For each location, we further measured several forest and stream characteristics above and below the waterfalls including: bankfull width, wetted width, canopy cover, thalweg depth, stream bank slope, and substrate sizes (Tab. 1).

Cutthroat trout (*Oncorhynchus clarkii*), as well as Chum (*O. keta*), Coho (*O. kisutch*), and Pink (*O. gorbuscha*) salmon have been found in Sugsaw Creek in late August [10], which were limited to the lower sections of the stream before the waterfall barrier. The species observed in Sarita River include Chum (*O. keta*), Chinook (*O. tshawytscha*), and Coho (*O. kisutch*) salmon [11, 12]. We chose these streams based on their close proximity, though considering that they contain different species, we are unable to assume that they experience similar amounts of salmon biomass during the spawning season. For both streams, we expect to observe similar effects on the abundance and biomass of terrestrial and freshwater invertebrates.

2.2. Sampling Methods

To determine size spectra relationships and assess the terrestrial invertebrate community found in the riparian forest surrounding these salmon barriers, we set up 18 pitfall traps [13] both above and below the falls at both streams. Pitfall traps were set up at Sugsaw Creek and Sarita Falls on July 12, 2018. At Sugsaw Creek we set the traps in a 3×3 grid with each trap approximately 1.5 m apart. We plotted the grids on either side of the stream both above and below the falls in locations with similar canopy cover and vegetation density. At Sarita Falls we set up 9×9 grids in a similar manner on one side of the stream both above and below the falls due to the steep and rocky terrain of the forest making it difficult to set traps on the other side. All traps were set within 20m of the stream on either side. We collected the traps after 48 hours and stored invertebrates in 15% ethanol.

We collected freshwater invertebrates along two transects both above and below the falls at both locations. Freshwater samples were collected at Sugsaw Creek on July 12, 2018, and at Sarita Falls on July 14, 2018. Each transect was within 100 metres from the falls and ran perpendicular to the stream's flow. We agitated stream sediment for two minutes in front of a Surber net sampler (30×30 cm quadrat, 1 m net length) that was placed facing upstream. At Sugsaw Creek we placed the Surber net at three different locations along each transect, while at Sarita Falls we placed the Surber net at four different locations due to the larger wetted width of this stream (Tab. 1). We stored collected invertebrates in 15% ethanol and also measured substrate sizes at each location where we took a Surber net sample (Tab. 1).

We classified all caught invertebrates to order using identification guides and literature [14, 15]. We counted the abundance of each order for each ecosystem environment (above or below the falls) and location (Sarita Falls or Sugsaw Creek). We then measured the overall dry mass for each invertebrate order and calculated a mean body mass in milligrams per individual as well as total biomass.

2.3. Data Analysis

Size spectra and abundance versus - body mass data were binned by invertebrate order and plotted to assess the fit of individual slopes and compare the above and below locations for both streams. To determine slope, intercept, and the net size spectra at each location, we fit linear regression models to each subset of the data (Sarita Terrestrial, Sarita Freshwater, Sugsaw Terrestrial, Sugsaw Freshwater; Tab. 2). To further

assess each dataset, we performed an analysis of covariance (ANCOVA) to examine the relationship between the slopes and intercepts of the individual location data above and below the falls (Tab. 3). Shannon-Wiener indices were calculated to assess diversity at each location. All statistical analyses were performed in R v3.5.1 [16].

3. Results

We caught and classified to order a total of 1,211 terrestrial and freshwater invertebrates above and below for Sugsaw Creek and Sarita Falls. This included a total of 14 terrestrial and 16 freshwater invertebrate orders. The most abundant terrestrial and freshwater order was Collembola and Diptera respectively.

Our terrestrial pitfall traps caught a total abundance of 87 invertebrates in the Sarita Terrestrial control reach, while a total abundance of 100 invertebrates was caught in pitfall traps in the salmon-bearing reach (Fig. 2A). This equated to 9442.2 mg in invertebrate biomass caught in the control reach, and 10203.7 mg in invertebrate biomass caught in the salmon-bearing reach (Fig. 2B). In the Sugsaw Terrestrial control reach, our pitfall traps caught a total invertebrate abundance of 78, and 35359.5 mg in invertebrate biomass, while we caught a total invertebrate abundance of 136 and 12209.0 mg in biomass in the salmon-bearing reach (Figure 2A and 2B).

During our freshwater Surber net sampling, we caught a total abundance of 275 invertebrates in the control reach compared to 294 in the salmon-bearing reach at Sarita Falls (Fig. 2C). This equated to 13631.7 mg and 17687.1 mg in invertebrate biomass collected in the Sarita Freshwater control and salmon-bearing reaches respectively (Fig. 2D). In the Sugsaw Freshwater control reach we caught a total invertebrate abundance of 49 and 15011.0 mg in biomass, while we caught a total invertebrate abundance of 192 and 16630.1 mg in biomass in the salmon-bearing reach (Fig. 2C and 2D).

We found negative relationships between abundance and body mass for both terrestrial and freshwater invertebrates at Sugsaw Creek and Sarita Falls (Fig. 3). Sarita Terrestrial (Fig. 4A), had a significant ($p < 0.05$) relationship between abundance and body mass ($R^2 = 0.90$ and 0.94 at the control and salmon-bearing sites), with slope values of -0.85 for control and -1.05 for salmon-bearing reaches (Tab. 2). However, the difference in slope was not significant ($p = 0.28$). Sarita Freshwater (Fig. 3B) had a non-significant relationship due to two outliers identified as Diptera larvae escaping the size spectrum ($p > 0.05$, $R^2 = 0.17$, and 0.02 for control and salmon-bearing sites respectively). The slopes were determined to be -0.36, and -0.18 for control and salmon-bearing respectively, but were not significantly different ($p = 0.73$) (Tab.2).

Sugsaw Terrestrial had significant slopes ($p < 0.001$, $R^2 = 0.57$; $p < 0.001$, $R^2 = 0.92$ for Sugsaw control and salmon-bearing sites, respectively). Slope values for the Sugsaw control and salmon-bearing sites were calculated to be -0.72 and -0.88 respectively (Fig. 3C). Sugsaw Freshwater slopes were also found to be significant ($p < 0.001$, $R^2 = 0.72$; $p < 0.001$, $R^2 = 1.00$ for the control and salmon-bearing sites respectively). Slope values for control and salmon-bearing sites were -0.92, and -1.02 respectively. The outlier at approximately 2.75 mg is noteworthy, which was determined to be Dipteran larvae (Fig 3D).

There was no statistical difference between the slopes or intercepts of the abundance body mass relationships in control reaches verses salmon-bearing reaches for either terrestrial (Fig. 3A and 3C) or freshwater (Fig. 3B and 3D) invertebrate communities at both Sarita and Sugsaw locations (Tab. 3).

There were minimal differences in the calculated Shannon-Weiner diversity index values between both terrestrial and freshwater invertebrate communities in control and salmon-bearing sites at both locations (Fig. 4). Salmon-spawning reaches generally had lower invertebrate diversity than control reaches. The largest difference was seen for Sarita Terrestrial (0.34), while the smallest difference was seen for Sugsaw Terrestrial (0.16). The location with the highest and lowest diversity of terrestrial invertebrates was the salmon-bearing sites at Sugsaw Creek (1.82) and Sarita Falls (1.17) respectively. The location with the highest and lowest diversity of freshwater invertebrates was the control site at Sarita Falls (1.92) and salmon-bearing site at Sugsaw Creek (1.66) respectively. The only invertebrate community which saw a higher diversity in the salmon-bearing sites was in the terrestrial invertebrates we collected at Sugsaw Creek.

4. Discussion

Our study has shown that during the pre-salmon spawning season, terrestrial and freshwater invertebrate communities below a salmon barrier are not differentially structured compared to invertebrate communities above a salmon barrier (Fig. 3). This implies that the salmon subsidization to invertebrate communities that occurs during the spawning season [2] is immediate and does not last into the pre-spawning season of the next year. However, there are a few orders of invertebrates which did not follow this trend. For instance, freshwater Diptera at both locations showed higher abundances than expected both above and below the fall barriers (Fig. 3B & 3D). This observation would be expected during the salmon-spawning season because fly larvae are carcass-specialists [2]. As this study was conducted during the pre-spawning period, before the carcasses are available, escape of the size spectra cannot be explained by salmon subsidies, especially as the control region does not receive salmon subsidies. The peak in Dipteran larvae may possibly be explained by their life cycle. These larvae emerge in the summer and the latter portion of the spring [2]. We may have captured this emergence event during the time of our sampling which may account for the large quantity of these larvae in our samples.

We obtained a greater abundance and biomass for all of the salmon-bearing sites with the exception of Sugsaw Terrestrial. The high biomass obtained for Sugsaw Terrestrial relative to the salmon-bearing site was contrary to what we initially predicted. A likely explanation may be due to the fact that the pitfall traps for Sugsaw control were the sole traps that were set in a flat area near the stream. For the other three locations (Sugsaw salmon-bearing and Sarita control and salmon-bearing), pitfall traps were set directly into a steep slope overlooking the stream to ensure that they were within 20 m of it in order to guarantee that the collected invertebrates were representative of the riparian community.

The steepness of the terrain has a significant effect in determining the composition, biomass, and density of the nearby riparian vegetation [17, 18], which in turn will

affect biological stream components such as nutrient flow, exchange of organic and inorganic matter, and the movement of organisms [19]. These factors may affect the species composition, biomass, and abundance of terrestrial invertebrates that are found in the area. The Sugsaw control site may have provided a more favourable habitat for terrestrial invertebrates due to the flatness of the slope and dense riparian vegetation in this area. The other locations experienced substantially lower values for biomass, which may be because the traps were set along a steeper gradient and in more open areas.

Alternatively, another aspect of the environment that may have been a factor includes canopy cover. The abundance of terrestrial invertebrates and specifically the input of such invertebrates into stream systems is highest in closed-canopy areas [19]. The greater degree of canopy cover found at Sugsaw Creek may account for the high biomass found at the control site.

An additional observation to make note of is that the control site for Sugsaw Terrestrial was the only site where a slug (Stylommatophora) was found in addition to a substantial number of beetles (Coleoptera). Beetles can be found in areas where there is lots of vegetative foliage [20], which may explain the spike in beetle abundance for the control location at Sugsaw Creek.

Abundance-body mass relationships serve to demonstrate the effects of salmon subsidies on the ecosystem. Although our findings found no statistically significant differences in slope or intercept, we do see observable changes in slope amongst the abundance-body mass plots. We observe a steepening of the slope below the falls (compared to the control group above the falls), at Sarita Terrestrial, Sugsaw Terrestrial, and Sarita Freshwater (Fig. 3). This suggests that smaller and more abundant individuals are being preferentially selected over the larger ones, and thus smaller and more abundant individuals comprise a larger portion of the trophic pyramid than expected. Our results indicate that there are no observable overall increases in the intercept. This suggests that the effects of salmon nutrient subsidies are transient, occurring during and immediately after the salmon run when carcasses are available, and slowly diminishing until the effects are no longer observable in the invertebrate population. For the freshwater invertebrates, additional reasoning may be that they have either already been consumed by the salmon directly or lost through the subsequent bioturbation as the salmon construct their spawning redds [21].

The difference in slope and intercept between freshwater and terrestrial communities at our sample sites were negligible. Observed differences in orders such as Coleoptera and Diptera that escaped the size spectrum serve as the only distinguishing factor. We predicted that there would be a difference between both habitats because of the various impacts that salmon have, depending on the ecosystem. As the spawning season approaches, salmon entering freshwater streams subject invertebrates to bioturbation (the disturbance of sediments) and therefore disrupt their natural habitats [22, 23, 24]. They also consume these organisms directly, which is another factor that is not experienced by terrestrial communities. Since both ecosystems are impacted by salmon in unique ways, differences between them were expected. However, both communities seem to have been equally unenriched by salmon subsidies in the pre-spawning season, suggesting that there is no long term effect on either habitat from the previous spawning

season.

Freshwater invertebrate diversity was similar between control and salmon-bearing reaches during the pre-spawning season. This is expected as salmon density has been shown to have weak effects on freshwater invertebrate diversity, which is instead predominantly impacted by stream characteristics, such as streambed substrate size [25]. Both streams had significantly similar substrate sizes (Sarita: $p = 0.19$; Sugsaw: $p = 0.64$) between control and salmon-bearing sites at both locations (Tab. 1), which could also explain why there was little difference in freshwater invertebrate diversity. We also showed that terrestrial and freshwater invertebrate diversity was minimally higher for the control site at every location, the only exception being for Sugsaw Terrestrial, where a greater invertebrate diversity was seen in the salmon-bearing site.

Forests surrounding both Sugsaw Creek and Sarita Falls have experienced logging activities in the past, which would have contributed to alterations in freshwater and terrestrial habitats, further impacting the organisms that live within these areas. In Sarita specifically, extensive logging activities have occurred in the 1950s and 1960s [11], and even more recently within the past year. This expansive logging could potentially impact invertebrate communities in both terrestrial and freshwater ecosystems and may explain the results obtained for invertebrate diversity. For instance, reduction of riparian forest due to logging can result in reduced canopy cover, nutrient inputs, habitat complexity and input of woody debris into the stream [11]. This can result in degraded freshwater habitats and potentially alter freshwater invertebrate diversity. The extent of the logging below the falls seemed to extend closer to the stream at both locations, thus making the aforementioned effects more prominent below the falls which may explain why the lower diversity was observed in the freshwater invertebrates at Sugsaw Creek and Sarita Falls.

Investigating the relationship between abundance and body size in terrestrial and freshwater invertebrates can provide insight into community structure and energy flow between trophic levels in different ecosystems [2]. The mechanisms through which marine subsidies may impact the size spectrum relationship of freshwater and terrestrial communities has not been extensively researched. Through this study, however, insight can be gained into the possible long-term effects that salmon subsidies from the previous spawning season may have on invertebrate communities. Shifts in local species abundance or body mass due to a nutrient subsidy may lead to changes in local community structure and trophic cascades. This may also have implications for organisms located higher up in the food chain such as predaceous invertebrates or vertebrate consumers [8].

5. Conclusion

Through abundance-size spectra analysis, we confirmed the presence of a size-structured ecosystem with a bottom-heavy trophic pyramid. We did not observe significant intercept increases during the pre-spawning season, indicating that salmon subsidy effects last only during and shortly after the spawning season. Even though our results show that there is no legacy effect, a multi-year study period conducted periodically throughout the year would further our understanding. This way, direct

comparisons could be made between pre-spawning and spawning events. By examining how salmon abundance may vary on a year to year basis, greater understanding could be obtained regarding how exactly salmon subsidies affect invertebrate abundance and biomass. Additionally, the short-term effects that these nutrient pulses have on community structure in different habitats (terrestrial and freshwater) would be studied. Since freshwater ecosystems are impacted differently by salmon through factors like predation and bioturbation the instant they enter the streams to spawn, we may expect to observe delayed effects for the terrestrial system. It would be interesting to note when exactly such effects start to fade and how long after until the observable differences in the scaling relationship (abundance and body size) for invertebrates between above and below the falls shifts back. Further research could focus on the specific effects that the added nutrient subsidy in the form of salmon carcasses would have on the surrounding riparian vegetation, and how this could further influence the scaling relationship for invertebrates.

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7. Tables and Figures

Table 1: *Stream characteristics for locations above and below the waterfall barriers at Sugsaw Creek and Sarita Falls.*

Site	Location	Coordinates	Distance Between Sites (m)	Bankfull Width (m)	Wetted Width (m)	% Canopy Cover	Thalweg Depth (m)	Slope Left Side	Slope Right Side	Number of Traps	Average Intermediate Substrate Size (cm)
Sugsaw Creek	Above	48°50′15.80"N, -125°06′22.30"W	156.6	5.6	3.3	35	0.23	10	10	18	3.25
	Below	48°50′23.69"N, -125°06′8.51"W		13.4	8.7	0	0.11	32	28	18	3.6
Sarita Falls	Above	48°54′9.09"N, -124°55′12.17"W	189.2	31	29	0	0.76	15	22	18	5.54
	Below	48°54′9.78"N, -124°55′20.51"W		49	37	0	0.51	28	30	18	4.36

Table 2: *Summary table of linear regression models performed for each regression line plotted in Figure 3.*

Method	Location	Ecosystem	Slope	R^2	P-Value
Simple Linear Regression	Sarita Above	Terrestrial	-0.85	0.90	1.12e-3
	Sarita Below	Terrestrial	-1.05	0.94	3.17e-4
	Sarita Above	Freshwater	-0.36	0.17	0.27
	Sarita Below	Freshwater	-0.18	0.02	0.67
	Sugsaw Above	Terrestrial	-0.72	0.57	6.99e-3
	Sugsaw Below	Terrestrial	-0.88	0.92	1.112e-5
	Sugsaw Above	Freshwater	-0.92	0.72	4.08e-3
	Sugsaw Below	Freshwater	-1.02	1	1.132e-15

Table 3: *Summary table of ANCOVA results from each location sampled including interaction parameters. Note: If body mass*above.below is significant, the slopes are significantly different, and if above.below is significant, the intercepts are significantly different.*

Method	Ecosystem	Parameter	Test of	P-Value
ANCOVA	Terrestrial	body mass	Slope	4.89e-8
		above.below	Intercept	0.27
		body mass*above.below	Slope	0.18
	Freshwater	body mass	Slope	5.14e-4
		above.below	Intercept	0.58
		body mass*above.below	Slope	0.49

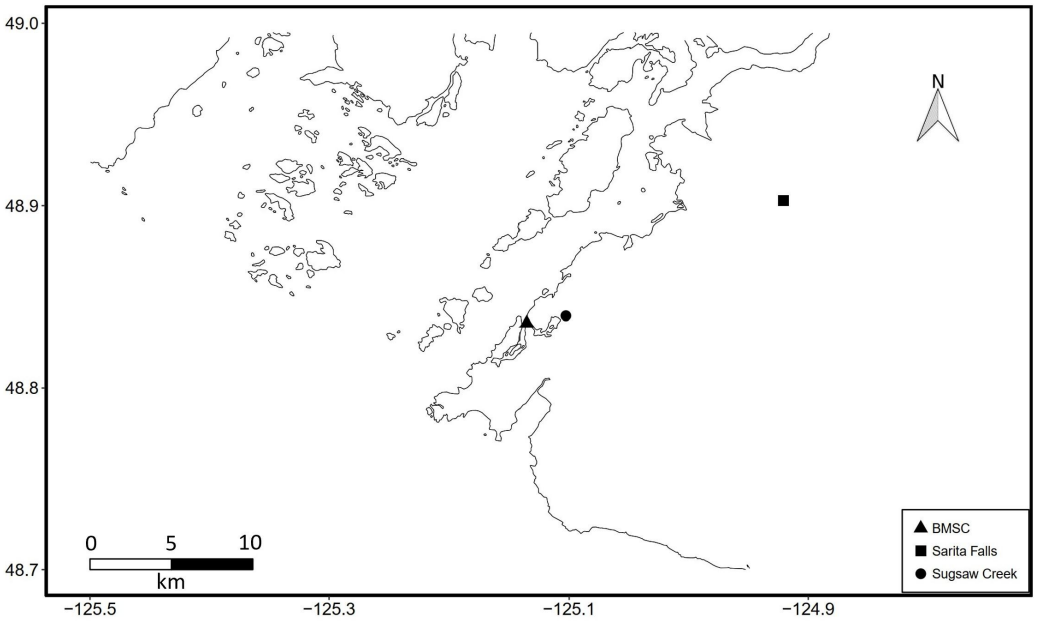

Figure 1: *Site map of Barkley Sound, Vancouver Island, BC. Depicted are the sites that were sampled above and below waterfalls barriers at Sugsaw Creek and Sarita Falls. Also depicted is Bamfield Marine Sciences Centre (BMSC) for reference. Map was generated using R v3.5.1.*

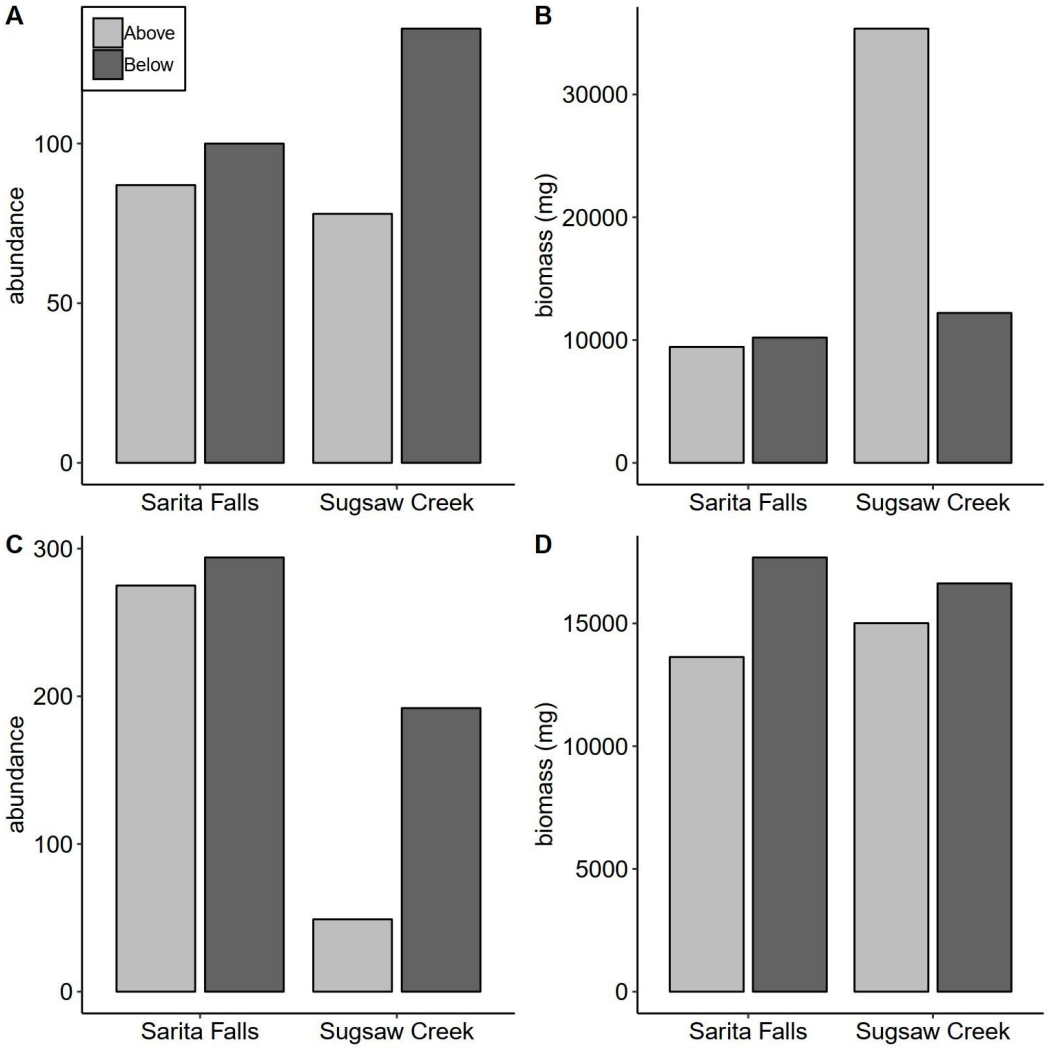

Figure 2: *Total abundance and biomass values from invertebrates captured in Surber net and pitfall traps plotted against location. (A, B) Terrestrial, (C, D) Freshwater. Dark bars indicate below the falls (salmon-bearing) and light bars indicate above the falls (control).*

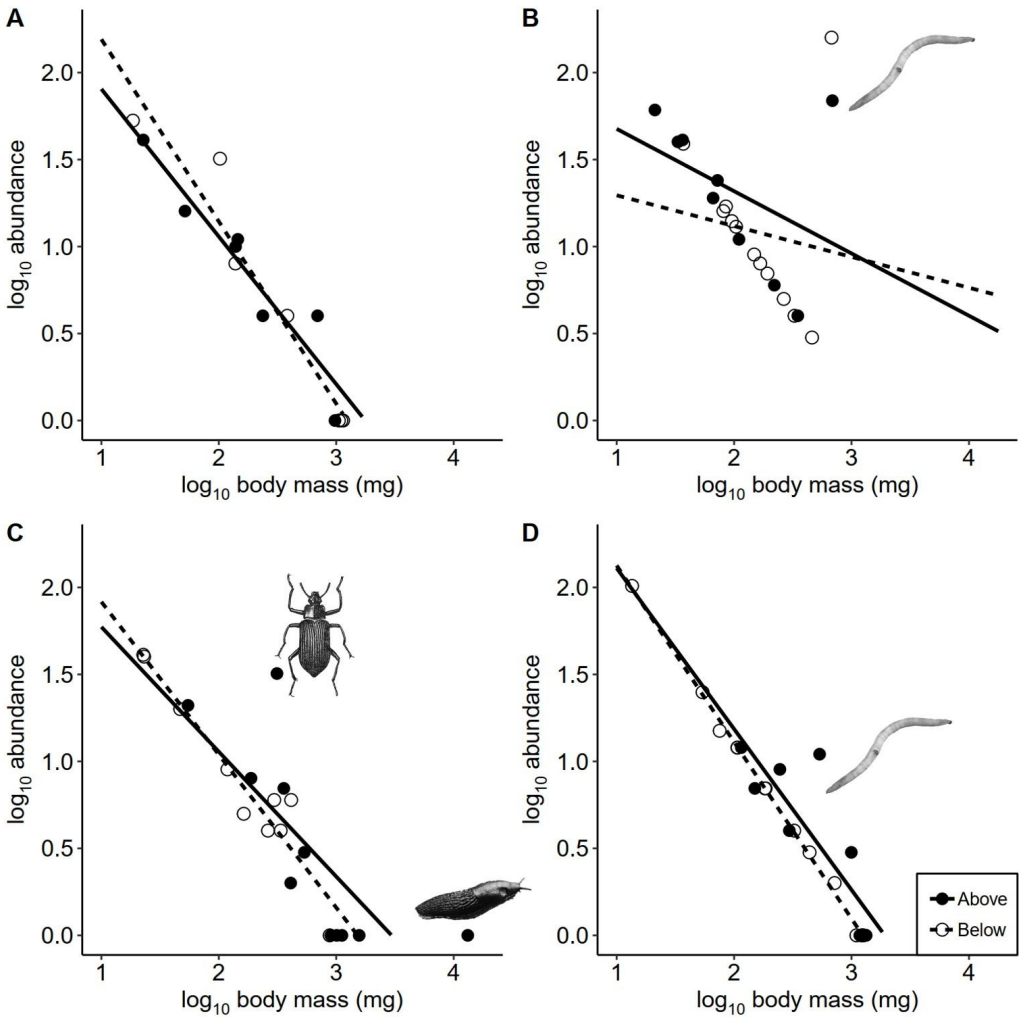

Figure 3: *Log_{10} abundance plotted against log_{10} body mass of freshwater and terrestrial invertebrate species. (A) Sarita Terrestrial, (B) Sarita Freshwater, (C) Sugsaw Terrestrial, (D) Sugsaw Freshwater. Locations above the falls are denoted by solid lines/filled circles, locations below the falls are denoted by dashed lines/empty circles. Images of invertebrates emphasize outlier Orders. Clockwise: Diptera larvae, Stylommatophora, and Coleoptera.*

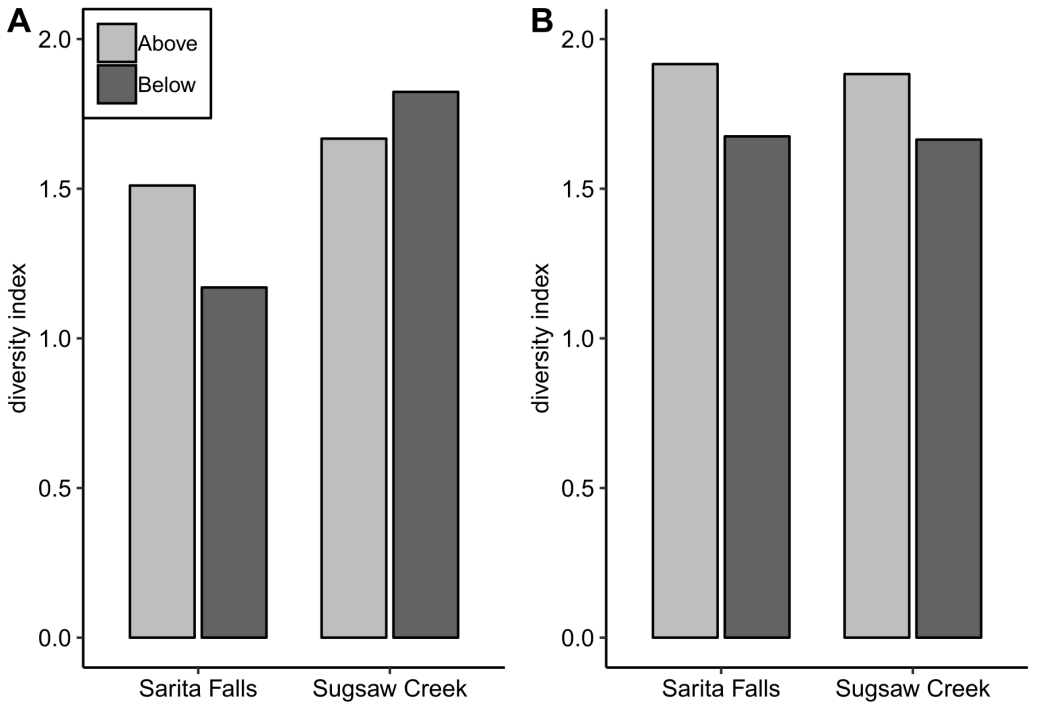

Figure 4: *Shannon-Weiner Diversity Index values of (A) terrestrial and (B) freshwater invertebrate communities above (light grey) and below (dark grey) salmon barriers at both Sugsaw Creek and Sarita Falls.*

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Ammonium Excretion Rates of Giant California Sea Cucumbers (*Apostichopus californicus*)

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Abstract

Nitrogen is a key nutrient used to support the growth of primary producers. In the ocean, it can be supplied in various ways, including through decay, upwelling, nitrogen fixation, and via animal excretion. There is a possibility that ambient nitrogen availability in nutrient-rich cold waters could be enhanced by animal-mediated nitrogen production. Here, we used giant California sea cucumbers, Apostichopus californicus, to explore animal-mediated nitrogen contribution in temperate oceans by holothurians, asking specifically if (a) nitrogen levels decrease with increasing distance from sea cucumbers, and (b) larger sea cucumbers excrete ammonium at higher rates. We analyzed ammonium concentrations using fluorometry water samples collected internally, near (3-5 cm) and far (1 m) from sea cucumbers at Reed Point Marina, with average ammonium concentrations of 2.79 µmol (SD = 0.75 µmol) and 3.21 µmol (SD = 1.28 µmol) respectively. There was no significant difference in ammonium concentrations near and far from sea cucumbers. Ammonium concentrations in the two internal samples obtained were ~ 7 times higher (22.96 ± 2.12 µmol) than the ambient samples, suggesting a strong dilution of sea cucumber nitrogen upon excretion. There was a strong positive correlation between excretion rate and sea cucumber mass ($R^2 = 0.79$). Based on our findings we are still unsure of the ecological impacts of sea cucumber fishing, which is becoming more prevalent in the northeast Pacific, as we can only see that giant California sea cucumbers at an individual scale do not contribute a significant amount of available nitrogen. Further research needs to be conducted as the role of a species in its environment is key to learning the potential ecological repercussions of removing it.

Keywords — Deposit feeders, Holothurians, Nutrient cycling, Nutrient availability, Producer productivity

1. Introduction

A debated dichotomy in ecology is whether primary producers are regulated mainly by top-down effects (i.e. by consumers), or bottom-up forces (i.e. resource availability) [1]. Primary production is affected by consumers, which are kept in check by predators (top-down effects) and are impacted by resource availability (bottom-up forces). However, debates on which mechanism has the greatest effect on primary producers (and the rest of the food web) have begun to shift towards

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hypotheses of how these mechanisms work together [1]. Nitrogen and phosphorus are common growth-limiting nutrients for photosynthetic organisms in marine, freshwater, and terrestrial environments [2]. Nitrogen and phosphorus are equally limiting in freshwater and terrestrial environments, but nitrogen is the most limiting nutrient in coastal marine environments [2]. Nitrogen is supplied to the ocean in various ways and is used to support primary producer nutrient requirements, primarily via nitrogen fixation, decay, upwelling, and animal excretion. Nitrogen can be provided to the ocean surface through both nitrogen fixation [3, 4], and ocean upwelling [5]. Ocean upwelling supplies nitrogen to the ocean surface created by the decay of both autotrophs (predominately phytoplankton) and heterotrophs [5], as well as from animal excretion [6].

The effects of animal-mediated nutrient contributions on primary producer productivity will differ across tropical and temperate regions due to differences in ambient light and nutrient availability [3]. Tropical waters of moderate depth cannot heat all the way through, which produces different water density layers. This reduces nutrient cycling in the tropics as mixing will only occur fully during the winter. This creates much lower nutrient levels in the upper layer of tropical waters, compared to cooler temperate waters which have the same density throughout the year and are able to mix constantly [7]. Due to this, nitrogen is more often limiting for autotrophs in the tropics than in temperate oceanic regions [7]. Animals will therefore likely play an important role in recycling nutrients back to autotrophs at low latitudes, since nitrogen levels are lower in the tropics [2, 7]. However, our understanding of how important excretion is for nutrient recycling in temperate waters is immensely limited. Given that temperate waters have higher nutrient levels than the tropics, the contribution of animal-mediated nitrogen should be relatively less important in temperate waters than in tropical waters.

The amount of nitrogen excreted by animals, and hence the importance of this source of available nutrients, will also depend on body size as metabolism and waste production scales proportionally with body size. Smaller species have a higher metabolism and thus a higher excretion rate per unit mass. Therefore, biomass consisting of smaller organisms will contribute more nutrients than an equal biomass of much larger animals [8, 9]. Also, within-species excretion rates scale allometrically with body mass (e.g., zooplankton, 11). However, the extent that mass alters excretion rates can vary in different species. For example, the per-gram excretion rates of smaller freshwater fish could be less than, equal to, or higher than those of larger individuals in the same species, demonstrating that both body size and taxonomy control excretion rates [8].

Sea cucumbers (class Holothuroidea) are model organisms for exploring the importance of animal-mediated nitrogen excretion in the ocean as multiple species of holothurians can be abundantly found in both temperate and tropical waters. Holothurians digest organic materials contained in sediments (i.e., deposit feeding) and then release waste as inorganic nitrogen in the form of ammonium, along with small amounts of phosphorus in the form of phosphate [10, 11]. Ammonium is mainly excreted from the respiratory trees through the anus and, to a lesser extent, through the body wall [10]. In the tropics, sea cucumber nitrogen excretion is of similar magnitude to the amount of nitrogen fixed by cyanobacteria. Holothurian excretion in the tropics enhances water nutrient levels, which positively impacts benthic microalgae productivity [10]. There

is no comparable study of the possible impacts that sea cucumber-mediated nitrogen contributions have in richer temperate waters.

In this study, we used giant California sea cucumbers, Apostichopus californicus, to examine the importance of animal-mediated nitrogen excretion in temperate waters. Giant California sea cucumbers are the largest sea cucumbers found in northeast Pacific waters and are often locally abundant. Our specific objectives were twofold. First, we estimated the differences in nutrient levels within, near, and far from sea cucumbers. We predicted that nutrient levels would decrease with distance from sea cucumbers. Second, we investigated whether mass and excretion rates were correlated, anticipating a positive association. This pilot study is important because echinoderms, such as sea cucumbers, are an emerging fishery in the northeast Pacific [12]. Our findings will begin to address the potential ecological impacts of removing sea cucumbers, which have been so far overlooked.

2. Materials and Methods

We collected giant California sea cucumbers on September 7th, 2017 at Reed Point Marina, Burnaby, British Columbia, Canada (49.2913°N, 122.8823°W). Samples were collected near the boating docks, at the interface between the rocky reef area extending from shore and the sandy/muddy sediment, at approximately 3-4 m in depth. There was minimal water current near the sample site as the day was calm, there was minimal boat traffic that was located far enough away to cause negligible water disturbance, and the site was surrounded by docks that dampened water movement.

From the surrounding areas of seven randomly selected sea cucumbers, a SCUBA diver collected 14 water samples into individually numbered, 1 L Ziploc bags. Sea cucumbers were spaced at least 1 m apart to reduce any changes to the concentration of excreted ammonium surrounding them due to SCUBA diver turbulence (from fin kicks) while collecting samples from another sea cucumber. One 'near' sample was taken near each sea cucumber, approximately 3-5 cm from the animal, and a 'far' sample was collected roughly 1 m away. Each of the seven sea cucumbers were then brought up using a mesh bag, one at a time, and placed into holding troughs with flow-through seawater. Sea cucumber locations were roughly mapped to ensure that water samples were labelled with the same number as the relevant sea cucumber.

We first subsampled the 'near' and 'far' water samples. To do this, we cleansed a plastic syringe by withdrawing and expelling 20 mL of seawater from one of the bagged samples. We then withdrew a further 60 mL and pushed it through a 0.45 µm filter paper, of which the first 10 mL were expelled to cleanse the syringe tip (of any small organisms) and another 10 mL to cleanse a darkened 250 mL bottle. The darkened bottle was capped, shaken, and emptied. The remaining 40 mL of filtered seawater was then injected into the labelled bottle. We repeated this process for the other 13 samples. The filter paper was replaced when the syringe started to get blocked by filtered substances; it was changed approximately every two subsamples.

To obtain background (pre-incubation) levels of nitrogen, nine 8 L Ziploc bags were labelled and filled with seawater (of various volumes), which was filtered through a manifold with a 0.7 µm glass fibre filter. From an 8 L Ziploc bag, we took one 40

mL subsample of pre-excretion water and placed it into a 250 mL darkened bottle, repeating the above syringe and bottle cleansing procedures. This process was repeated for the other eight samples.

To obtain samples of sea cucumber excretion, we placed the seven sea cucumbers collected into the 8 L Ziploc bags filled with filtered seawater, as described above. We placed the sealed bags, seven of which contained the sea cucumbers and the other two had filtered seawater (controls, without a sea cucumber), into the flow-through holding troughs. After the incubation period of at least 30 minutes, we subsampled the seawater in all bags by the procedure described earlier. One 40 mL post-incubation subsample was collected from the seven bags containing sea cucumbers (which remained in the bag during sampling) and from the first control bag. We then took seven 40 mL subsamples from the second control bag to later be spiked with nitrogen to produce a standard curve for fluorometry.

Following post-incubation subsampling, each sea cucumber was removed from its holding bag and held vertically, out of the water, for at least one minute to encourage drainage of body cavity water. We collected this water and obtained two water samples from the two sea cucumbers that expelled a large amount of water. Sea cucumbers were then weighed on an electronic balance (to the nearest 2 g). There was probably weighing error as five of the seven sea cucumbers did not properly drain when held.

After the sea cucumbers were removed from the post-incubation 8 L Ziploc bags, the volume of seawater in the nine bags was measured in a large graduated cylinder. These volumes were used later in nitrogen concentration calculations.

In the laboratory darkroom, we estimated nitrogen levels with fluorometry, using the seven 40 mL seawater subsamples taken from the second control bag. To create a standard curve, six of the control bottles were spiked with 100, 300, 400, 500, 800, and 1200 µmol/L of NH_4^+, respectively. Post-incubation subsamples from the seven sea cucumbers and the two samples of sea cucumber internal water had nitrogen levels that were too high for the fluorometer to read. From these bottles, samples were taken and diluted using the pre-incubation first control bottle. Dilution was done in 1:4 ratios, using 500 µL of the high nitrogen level samples to 2000 µmol/L of the pre-incubation first control sample. Fluorescence values from diluted samples were multiplied by five to adjust for the dilution.

All sample bottles (near/far/internal and pre/post incubation samples) were shaken and then subsampled using an adjustable pipette and expelled into a mini cuvette to be used in a fluorometer. This occurred three times for each bottle and the mean fluorescence value of the three subsamples was calculated.

We accounted for the different volumes of each of the nine 8 L post-incubation bags by multiplying the concentration of nitrogen by the volume of water in the bag. This gave us the correct micromoles of nitrogen for each post-incubation sample. We also accounted for the differences in incubation times by expressing excretion rates of the seven sea cucumbers as µmol/h.

To allow conversion of sea cucumber fluorescence values to excretions rates, we created a standard curve by constructing a linear model relating the mean fluorescence values and respectively known nitrogen concentrations from the seven (second) control bottles. We used the regression equation of the standard curve to calculate ammonium

concentrations of the sea cucumber samples.

Analysis of ammonium concentrations within, near, and far from the sea cucumbers was carried out in R (version 3.3.1). This was done using two linear models, one including distance while the other model was null (with no distance), which were compared with a log-likelihood test. Ammonium concentration was the response variable, distance (near and far) the fixed effect, and our sea cucumbers were used as a random variable. We obtained only two internal samples, which precluded formal analysis; however, we plotted their mean and 95% confidence interval to allow comparison with the near and far samples.

The association between excretion rate and mass was examined using a linear model with log excretion rate against log mass. Data points were plotted using a scatterplot with a best fit line.

3. Results

3.1. Effect of distance on nutrient levels

Distance from sea cucumbers did not alter ambient ammonium concentration (log-likelihood test, $p = 0.13$; Fig. 1).

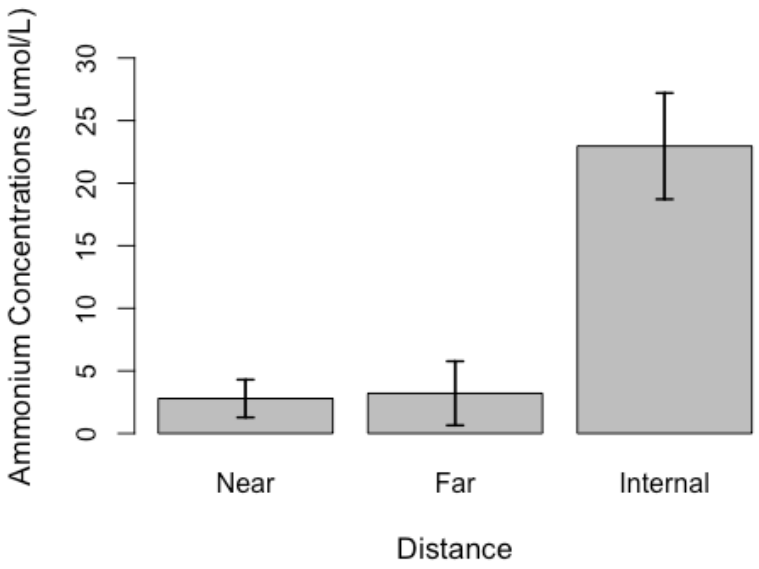

Figure 1: *Average ammonium excretion concentrations taken at three distances (internal, near (3-5 cm), and far (1 m)) from giant California sea cucumbers at Reed Point Marina. Bars are means with 95% confidence interval error bars. Near and far distances had a sample size of $N = 7$ sea cucumbers each, and internal was $N = 2$.*

3.2. Excretion rate in relation to sea cucumber mass

There was a strong positive correlation between ammonium excretion rate and sea cucumber mass; larger sea cucumbers had a greater ammonium excretion rate compared to sea cucumbers of lower mass ($p = 0.005$, $R^2 = 0.79$, $N = 7$; Fig. 2). Rounded, untransformed excretion rates (µmol/h) from smallest to largest sea cucumbers: 12, 7, 22, 30, 27, 41, 39.

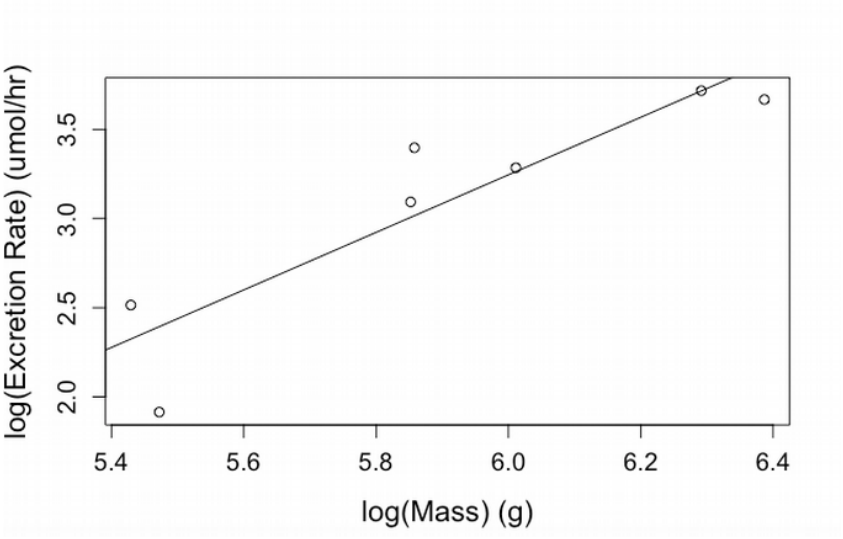

Figure 2: *Ammonium excretion rates of giant California sea cucumbers in relation to their mass at Reed Point Marina. Data points are single animal measurements ($N = 7$), represented on a log-log plot with a best fit line. Excretion* $= 0.492(Mass) + 2.310$.

4. Discussion

We used giant California sea cucumbers to further understand animal-mediated nitrogen contributions in temperate waters by holothurians. We observed no difference in ammonium levels near (3-5 cm) and far (1 m) from sea cucumbers at Reed Point Marina, but ammonium levels in internal samples were noticeably higher. This means that distance (outside of the sea cucumber) is not a factor in determining ammonium levels in our samples. We also found that larger sea cucumbers excrete more ammonium than smaller sea cucumbers. With further research, it would be interesting to determine if the extensive removal of holothurians by over-fishing could still have an impact on nitrogen recycling in temperate waters and thus cause a detectable change in primary producer productivity.

Distance is not a factor in determining ambient nitrogen levels as high nitrogen levels within sea cucumbers are immediately diluted once excreted. This was shown in our near and far samples as there was no significant change in ammonium concentration. We need to collect samples farther than 1 m away from sea cucumbers to ensure that ammonium concentrations do not, in fact, decrease past that point. However, our results contrast with the effect of distance as a factor altering ammonium concentrations

in the tropics. In warm water, holothurian excretion increases water nitrogen levels directly behind the sea cucumbers [10]. Water expelled by one tropical sea cucumber can increase ammonium concentration for a short time over an area of approximately 0.2 m^2 per hour [10]. There is no comparable study of the impact that sea cucumber-mediated nitrogen contributions might have in richer temperate waters.

As predicted, larger sea cucumbers excrete more ammonium than smaller sea cucumbers. This conclusion is consistent with the results found for two other species located in the tropics, *Holothuria atra* and *Stichopus chloronotus* [10]. However, we did not investigate the intraspecific variability of per-gram excretion rates. For example, the per-gram excretion rates of smaller freshwater fish can be less than, equal to, or higher than those of larger individuals in the same species, demonstrating that both body size and taxonomy control excretion rates [8]. Whether the same variability exists within temperate sea cucumber species is unknown.

Mass is not the only factor that determines nitrogen excretion rates of sea cucumbers. Both the species of sea cucumber and temperature changes throughout the year contribute to differences in ammonium excretion rates. Ammonium excretion rates differed between two species of tropical sea cucumber, *H. atra* and *S. chloronotus*, meaning that different sea cucumber species can have different excretion rates [10]. In addition, metabolism is altered by temperature, which in turn affects ammonium excretion rates, meaning that excretion rates are higher in the summer than in the winter [10, 8].

It appears that temperate sea cucumbers do not excrete enough nitrogen to make a detectable increase in primary producer production. In general, the effects of animal-mediated nitrogen contributions on primary producer productivity differs between tropical and temperate regions due to differences in ambient light and nutrient availability [3]. In the tropics, holothurians released 0.52 to 5.35 mg m^{-2} day^{-1}, which is the same order of magnitude as the nitrogen fixation rate of cyanobacteria (0.5 to 5.6 mg N m^{-2} day^{-1}) [10]. This creates a short-term nutrient enhancement in the surrounding water and sediment, which then increases benthic microalgae productivity [10]. This shows that nitrogen released by holothurian excretion in tropical waters is a significant process for nutrient recycling. Given that temperate waters have higher nutrient levels than the tropics [2, 7], contributions of animal-mediated nitrogen should be relatively less important in the former than in the latter. Our results suggest that individual giant California sea cucumbers probably make a negligible contribution to the surrounding nitrogen levels, and thus might not affect nearby primary producer productivity to a great extent. However, holothurians are locally abundant in northeast Pacific waters, so the sum of all sea cucumber excretions might have an impact on the overall nitrogen levels in the ocean. More data is needed to test this idea.

There are many studies on the cascading effects of removing consumers, i.e. top-down effects [13]. However, research is more limited on the bottom-up effects, i.e. nutrient availability, on community organization [13]. Based on findings by Uthicke [10], we know that sea cucumber excretion in tropical waters is a significant process for nutrient recycling. Eliminating nitrogen-releasing species could create a detrimental effect on marine ecosystems, as nitrogen fixation and decay alone may not be able to supply enough available nitrogen to support primary producer survival and growth. However, the removal of numerous temperate sea cucumbers through over-fishing may

only make a minor difference on primary producer survival as our results suggest that individual giant California sea cucumbers probably make a negligible contribution to the surrounding nutrient levels.

5. Acknowledgments

I would like to thank Dr. Isabelle Côté for the opportunity to work with sea cucumbers and her guidance in the writing of this paper. Fiona Francis for designing the experiment and for setting up the R script. Mr. Rod MacVicar for the use of the docks and holding tanks at Reed Point Marina. And finally, a thanks to the anonymous reviewers whose advice made this paper better.

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The Effect of Sustainably-Sourced Waste Food Diets on Yellow Mealworm Larvae (*Tenebrio molitor*)

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Abstract

As the world's population continues to increase, the demand for protein-rich foods will increase commensurately as well. Currently, a large amount of deforestation, pollution, climate change, and other deleterious global phenomena occurs to meet current demand. Deforestation in particular is most concerning as large amounts of land is used to produce food for livestock instead of people. With respect to the latter, current food production systems result in a large amount of food that goes to waste. As such, using insects to address these issues is a promising avenue of research. Insect species use less space and water to grow, have significantly reduced feeding costs, and produce fewer emissions than traditional livestock (e.g. beef cattle). In addition to these benefits, a variety of insects such as flies, have the potential to consume items like waste food without any detriment to their health or growth. This project assessed the feasibility of feeding Tenebrio molitor (*T. molitor*) larvae three diets - oatmeal, waste food, and animal protein/brewer's spent grain. Population numbers, mortality rate and pupation rate at each stage of the *T. molitor* lifecycle were recorded for each diet. Low mortality among other observed metrics suggest that *T. molitor* can grow on a waste food diet.

Keywords — Entomophagy, Waste-Food, Sustainability, Yellow Mealworm

1. Introduction

With the world's population projected to reach nine billion by 2020 [1], there is an ever-increasing demand for land and for protein-rich foods. Currently, global issues such as deforestation, pollution and climate change are due in part, to this demand. Maintenance of agricultural crops leads to land and water degradation via the use of pesticides, fertilizers, and animal waste [2].

Globally, the agricultural industry is responsible for approximately 50% of methane emissions and 60% of nitrous oxide emissions [3]. With respect to a rising population, the increased demand for protein-rich foods will dramatically increase these negative effects [4, 5].

A large amount of land will be required to feed these livestock as well and indeed, according to Cassidy et al [6], "in developing countries with high rates of increasing animal product demands, a greater proportion of cereals are being directed to animals."

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This land will be required in the years to come to sustain an increased population either with food or housing.

Insect species such as yellow mealworm beetle larvae (*Tenebrio molitor*), hereafter referred to as *T. molitor*, are a realistic solution to these problems. Compared to other livestock species, raising insects as a source of food features significant benefits.

Firstly, compared to cattle ranching, insects produce fewer emissions during their growth. On average, cattle produce 67.8 kg of CO_2 per kg [7] in contrast to *T. molitor* which produce 2.2 g of CO_2 per kg [8]. Secondly, insects require significantly less water. For example, 22,000 L of water is required to produce 1 kg of beef whereas insects like *T. molitor* do not directly require a water supply [9]. Lastly, insect protein can be grown in a small area [10] and with further research could be used to potentially supplement the diet of livestock [11, 12] as well as humans [13]. This nutritional density means that the land previously used to grow feed crops for livestock could be repurposed for housing, solar panels or even be reforested.

The ability of many insects to consume a variety of products from low nutrient waste food [8, 14, 15] to manure is also important. Globally, waste food contributes 3.3 Gtonnes of CO_2 into the environment every year [16]. This waste food could instead be used to feed different insect species which in turn could be used as a high-quality protein source. This would effectively close the loop between food production and food waste.

With respect to the objectives of this research project, we chose *T. molitor* larvae as our model organism. For research purposes, they were they easy to procure and required very little maintenance.

The objectives of this research project were as follows. First, we wanted to see if *T. molitor* could successfully grow on a waste food diet. As such, we used three different diets during this experiment - oatmeal, waste food and animal protein/ brewer's spent grain.

The oatmeal diet is what most home and commercial growers feed to mealworms and it served as our control diet. We hypothesized that the animal protein/brewer's spent grain diet would be higher in protein and starch and therefore be more nutrient dense than the other diets.

Second, to evaluate if these three diets influence mortality rate, and/or life stage transition (i.e. pupation) rates. Lastly, to explore novel possibilities of applying the potential of insects in our communities. For example, previous studies have shown that mealworms are capable of safely breaking down Styrofoam [17, 18] (Fig. 1). Additionally, the shells of the mealworm beetle, which contain chitin, can be used to create a biodegradable plastic substitute [19] (Fig. 2) among other novel products [20]. For the purposes of our research we wanted to explore ways in which the community or local companies could contribute and make a difference.

2. Materials and Methods

All *T. molitor* were obtained from Wild Birds Unlimited in Surrey, B.C., Canada. Their supplier, Little Fish Company, is also located in Surrey, B.C., Canada and typically supplies *T. molitor* to be fed to pet fish or reptiles.

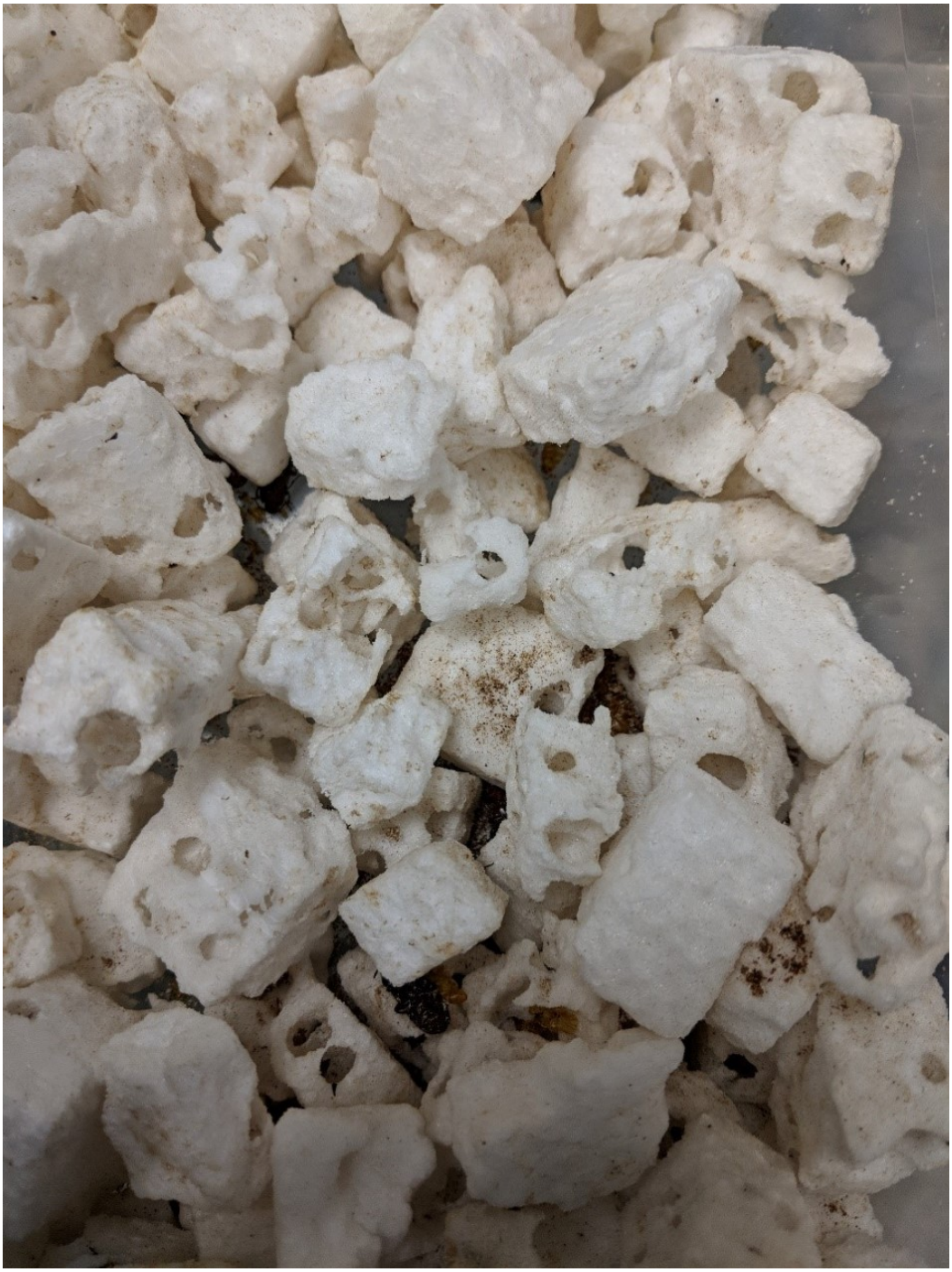

Figure 1: *Styrofoam that has been consumed by T. molitor. This photo was taken in our lab.*

Despite looking at a number of research papers, there were no established methods to raise *T. molitor* larvae for research purposes. As such, the methods outlined below were developed by our team.

Figure 2: *A chitosan based plastic substitute made from T. molitor beetle shells. On Top: The finished biodegradable plastic substitute. On Bottom, from left to right. a) Breaking down T. molitor beetle shells by grinding. b) Removal of organic matter. c) Deprotonation of T. molitor beetle shells. d) Evaporating excess liquid to form chitosan sheets.*

The research lab in which the *T. molitor* were grown in maintained an average temperature of 22 degrees Celsius during the study period. Each life stage was raised in food safe Sterilite containers. Initial density consisted of 500 *T. molitor* per container and the first generation was raised on the control diet to allow for acclimatization and to improve the research methodology.

Post generation one, 3000 *T. molitor* were randomly assigned into three different diet groups. Each group consisted of two containers each containing 500 *T. molitor* and were manually checked to ensure that density did not exceed this number. In cases where this did occur, the excess was added to a new container.

As *T. molitor* continued to develop past the larvae stage, they were separated into

their respective life stages - pupae and then beetles. The beetle eggs were collected and the container monitored until new *T. molitor* larvae were seen. In general, the stages of the *T. molitor* lifecycle occur at set times.

All life stages were raised in a dark environment and containers were removed only for data collection or maintenance procedures. The removal times did not exceed more than one hour.

To create consistent food pellets for each diet the raw components of each diet were processed separately albeit in the same fashion (Tab. 1). Oatmeal did not require dehydration but was otherwise processed in the same fashion.

Table 1: *Diet processing procedure.*

Dehydration	Each diet component was dehydrated to prevent formation of mold and pathogens.
Pulverize	Dehydrated diet components were ground to a fine powder. This ensured that each pellet was consistent.
Binding Agent	Potato starch was added to bind components together. It was chosen as it had little to no nutrition.
Baked	The components were mixed together with water and baked at a low temperature until hard.

We were able to collaborate with several supermarkets and a local brewery for waste food and spent grain respectively. To mimic real world conditions, where waste food composition would be continually changing, a new batch of waste food was obtained biweekly. As the waste food provided varied in composition between batches, we recorded the composition of each batch as well. In the event that a *T. molitor* group being fed on waste food was affected, we could see which specific batch was used.

Ingredients for each diet were sourced locally, with the support of community partners (Tab. 2). Carrot slices (30 g per container) were included in each container and served as a water source.

Table 2: *Diet composition.*

Diet Type	Diet Composition	Source
Control	Oatmeal	Supermarkets
Waste Food	Waste Food	College cafeteria, personal homes, supermarkets
Animal Protein and BSG	Animal Protein and Brewer's Spent Grain	Supermarkets, Faculty Brewing: Local brewery located in Vancouver, British Columbia

Containers were checked at the end of each data collection session. Old feed was removed and replaced with 500 g of fresh feed along with 30 g of fresh carrot slices.

Due to logistical issues we were only able to start data collection at the end of generation two - October 23, 2017. In this period, the population of *T. molitor* for each diet was around 200 larvae and new hatchings were being introduced.

Data was collected every Monday, Wednesday, and Friday for six months (Tab. 3), with minimal exceptions due to holiday closures or scheduling conflicts.

Table 3: *Recorded metrics.*

Metric	Rationale
Number of alive/dead *T. molitor* larvae	Track population numbers/mortality
Number of new *T. molitor* larvae added	Population growth
Number of alive/dead *T. molitor* pupae	Track pupation rate

3. Results

From October 23, 2017 to November 20th, 2017 the increase in population size was due to *T. molitor* larvae being added (Fig. 3). Compared to the oatmeal diet, the *T. molitor* on the waste food and animal protein/brewer's spent grain diets were able to introduce more new larvae into the population (Fig. 6).

Figure 3: *Impact of diet on T. molitor larvae population from October 23, 2017 to March 16, 2018.*

From November 20th, 2017 to January 1st, 2018, population size started to decrease through a combination of *T. molitor* death and pupation (Fig. 4 and Fig. 5). The *T. molitor* on the oatmeal diet displayed a sharp drop in population and there was a corresponding spike in both mortality and pupation rate. After November 20th, the population of this group increased due to new larvae hatching. In contrast, the animal protein/brewer's spent grain group showed a more consistent population decrease with mortality and pupation increasing towards the end of this period. Interestingly, the waste food population was able to maintain the larval stage more consistently compared to the other two diets. While there was a small decrease in this group's population due to *T. molitor* death, the pupation rate of the waste food group decreased as well.

From January 1st, 2018 to January 31st, 2018 all three populations displayed a sharp drop in population. Both pupation rates and mortality rate increased concurrently during this period as well. Around January 31st, 2018, the population sizes of all three diets was as the lowest. The population of the waste food and oatmeal diets were similar and were slightly higher in density compared to the size seen start of the generation. In contrast, the animal protein/brewer's spent grain diet had the lowest

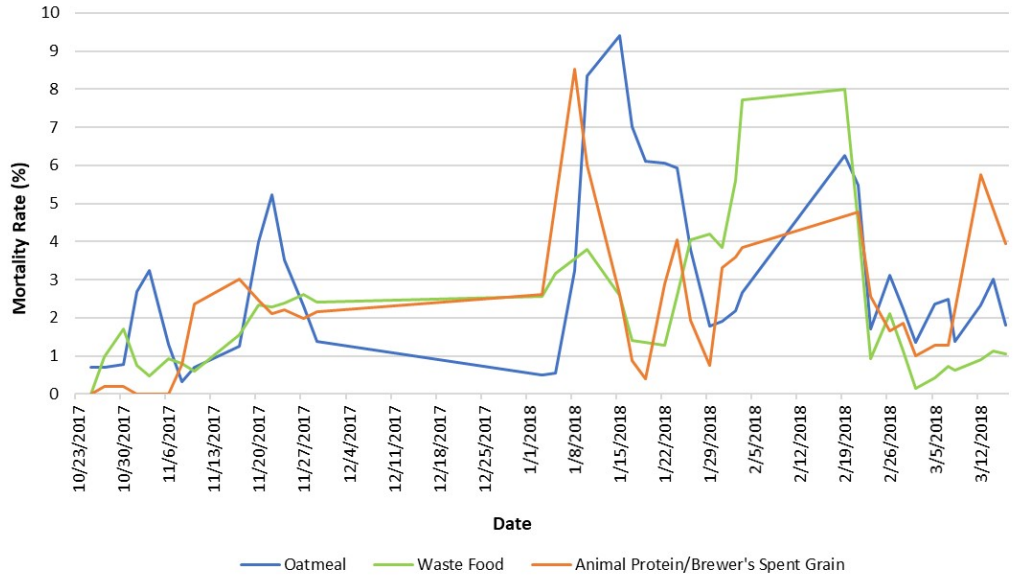

Figure 4: *Impact of diet on T. molitor larvae mortality rate from October 23, 2017 to March 16, 2018.*

population size.

From January 31st, 2018 to February 10th, 2018 the next generation of *T. molitor* larvae emerged and were added. Unlike the previous generation, the waste food diet was the only diet to have the largest population size.

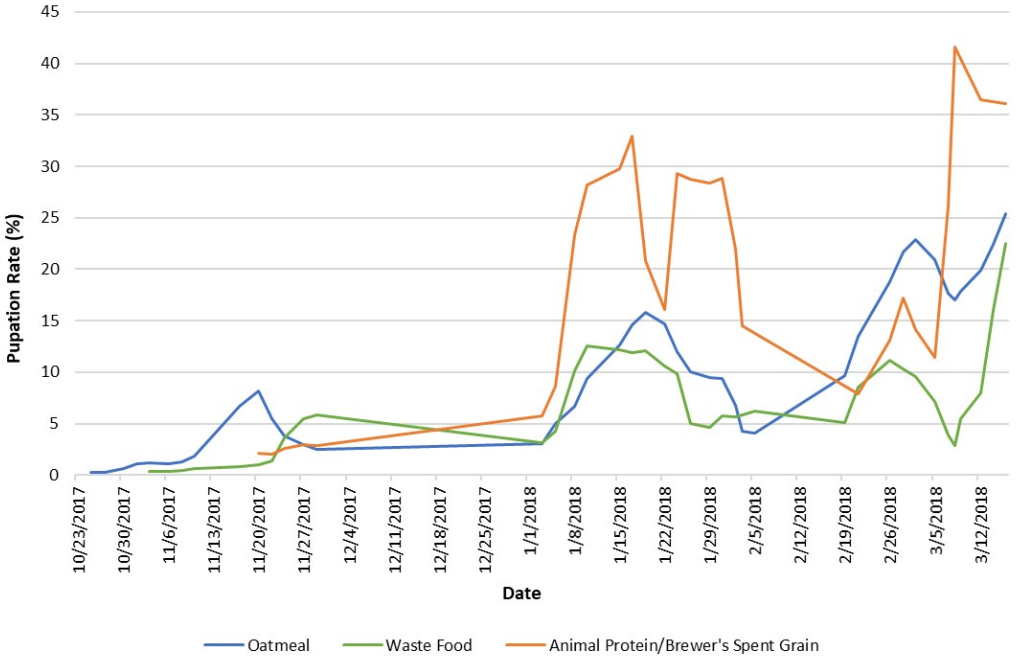

Figure 5: *Impact of diet on T. molitor larvae pupation rate from October 23, 2017 to March 16, 2018.*

The oatmeal diet produced roughly the same number of new larvae as the previous generation while the animal protein/brewer's spent grain diet was the lowest. This was interesting as the animal protein/brewer's spent grain diet had the highest pupation rate compared to the other two diets.

From February 10th, 2018 to February 20th, 2018 - the conclusion of the experiment, all *T. molitor* populations decreased in size. During this period the animal protein/brewer's spent grain diet again had the highest pupation rate however the mortality rate for all three diets was much more consistent.

Although the experiment concluded on February 20th, 2018 data collection continued until March 16th, 2018. Due to construction noise and reduced maintenance during this time period, the results are likely not accurate.

Overall, mortality never exceeded 10% of the total population. The oatmeal diet experienced the highest overall mortality rate (14.1%) on January 15, 2018 and had the highest average mortality rate ($3.0\% \pm 2.9\%$). Diets 2 and 3 had lower average mortality rates ($2.2\% \pm 2.5\%$ and $2.2\% \pm 2.0\%$).

With respect to the pupation rate, the animal protein and brewer's spent grain diet was much higher than the other two diets. This diet displayed the three highest pupation rates (27.7% on January 3, 2018, 21.2% on January 8, 2018 and 21.0% on January 15, 2018).

Over the study period, new larvae hatched from eggs produced by *T. molitor* beetles. Compared to the other two diets hatching numbers were low for the animal protein/brewer's spent grain diet. (Fig. 6).

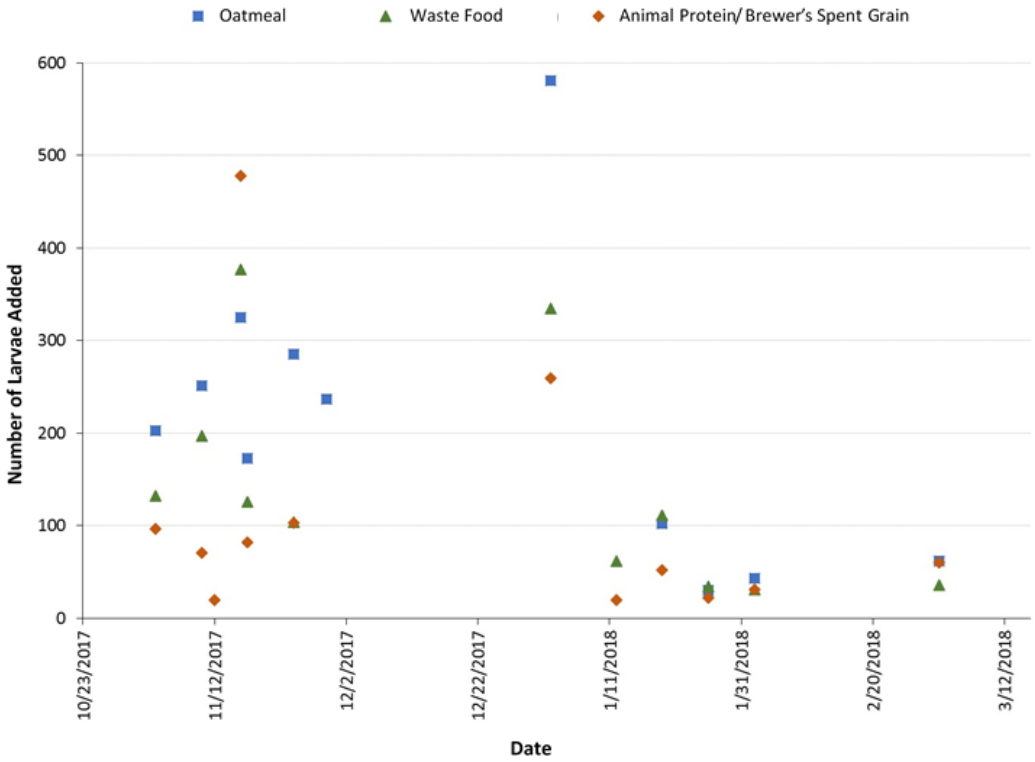

Figure 6: *Impact of diet on T. molitor larvae pupation rate from October 23, 2017 to March 16, 2018.*

4. Discussion

The goal of this experiment was to see if *T. molitor* could successfully grow on a waste food diet. In addition to serving as an environmentally friendly source of protein, this would allow for large amounts of agricultural land to be repurposed.

Throughout the course of this experiment *T. molitor* were able to sustain populations. However, there were differences for each group that provided insight into the effectiveness of each diet.

With respect to the other two diets, the animal protein/brewer's spent grain displayed the largest differences. During the first generation, this diet was able to introduce a similar number of new larvae as the waste food diet. However, unlike the waste food diet, the population of this group decreased over time. In contrast, the waste food diet, had a more consistent population with a drop only seen during pupation.

During the transition to the next generation, the animal protein/brewer's spent grain diet displayed much higher pupation rates than the other diets. This increased rate did not translate into a larger population size as this diet group also had an increase in larval mortality. This could suggest that the animal protein/brewer's spent grain diet lacks key nutritive components that are needed as *T. molitor* develops from new to adult larvae. The brewer's spent grain could be suspect as many of the complex carbohydrates would have likely been extracted during the brewing process.

To see if the possible lack of carbohydrates was affecting these metrics we looked at the oatmeal diet. While the mortality rate for this diet was similar to the animal protein/brewer's spent grain diet, the oatmeal diet had a pupation rate comparable to the waste food diet.

The high mortality and low pupation rate seen in the oatmeal diet was interesting as this group was able to produce a higher population at the start of the second generation. We have two theories why this may be the case. The first is that diet affects the ability of the *T. molitor* pupae to transition into the final life stage of beetles. Despite a high pupation rate, if a fewer number of beetles are present then this would impact the number of offspring produced. The second is that diet affects the ability of *T. molitor* beetles to produce eggs or for the eggs to hatch into new larvae. It could also be that results we saw are due to a combination of both theories.

Based on the population increase seen in generation one, none of the diets appeared to affect the new mealworms added. However, the start of generation two saw marked differences with respect to population size. Both the oatmeal and the animal protein/brewer's spent grain diets were able to add new larvae to the population. However, the oatmeal diet population increase occurred over a longer period of time - approximately two weeks. In contrast, the increase seen in animal protein/brewer's spent grain diet occurred in less than a week. As such, it seems that diet plays a role in the long-term development of *T. molitor*.

With respect to long term growth, it would appear that the waste food diet minimizes the risks seen with the other two diets. Although mortality and pupation rates were similar to the oatmeal diet, the overall population of the waste food group was higher. As such, even though the waste food diet changed biweekly, there is evidence to suggest that *T. molitor* would do well on it.

While these results are promising they are not definitive and further research is

required. In retrospect, we realize that gathering data on *T. molitor* beetles and eggs would have been valuable. As such a task would have increased the timeline of our experiment, we were unable to do so. We would also recommend that future studies be longer in length as we were only able to collect just over one generation's worth of *T. molitor* data.

Additionally, future studies that cover feed consumption and feed conversion ratios would also be insightful. We did measure the amount of feed consumed as well each weight of each *T. molitor* colony, however the scales we used were not precise enough. For future studies, we recommend using scales that are accurate to 0.01 g to measure both. We also found that the food pellets absorbed a significant amount of water weight between data collection intervals. Storing the pellets with a desiccant would help to alleviate this issue.

Lastly, experiments that also incorporate research into the nutritional aspects of each diet type would help to shed further light. It is possible that a combination of diets or a sequential feeding of the diets may perform better than the diets on their own. Researching each component of the diets, in addition to studying when each diet may perform best (e.g. at each life stage) merits further study.

The results of our research strongly suggest that *T. molitor* can be raised on a variety of different diets. However, diets that are varied in composition will likely result in more robust *T. molitor* populations.

5. Conclusion

There is evidence that raising *T. molitor* on a waste food diet is feasible, however further research is needed. As such, this research is a promising first step in using insects to address global issues like deforestation, waste food and climate change.

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Modifications to Bacterial Growth Conditions and Mini-Prep Procedure for Maximizing DNA Yield

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Abstract

Plasmid DNA purification from *Escherichia coli* (*E. coli*) bacterial cells is essential for standard molecular biology experiments such as transfection, polymerase chain reactions, and restriction enzyme digests. To improve yield, we have modified the growth broth, miniprep protocol precipitation step and elution buffer to optimize DNA yield and purity. We hypothesized that cells under the condition of Terrific Broth (TB), ethanol, and double distilled water (ddH_2O) will generate the highest DNA yield and purity. Six experimental conditions were used to test for growth differences between TB and Lysogeny Broth (LB). The quality of DNA samples that were isolated using ethanol and High Salt Wash Buffer (HBC) were compared, as was the elution quality using Tris-EDTA (TE buffer) and ddH_2O. The DNA concentration and purity were measured, and *E. coli* cells grown in TB with no mini prep modifications had a significantly higher DNA yield. This suggests that TB should be used in place of LB for transformed bacterial amplification when large quantities of plasmid are required.

Keywords — Terrific Broth, bacterial growth, plasmid isolation, plasmid purification

1. Introduction

Plasmid DNA purification from bacteria cells removes cellular components such as proteins, lipids, and the bacterial chromosome. A successful purification that gives a high yield DNA is beneficial for further experimental uses, such as transfection, restriction enzyme digests and polymerase chain reaction experiments. The media used for bacterial culture, the buffer used in miniprep procedures, the size of spin columns and the pH of the eluent can all influence DNA yield and purity. The quality of isolated DNA can also be compromised by nuclease activity, free radical oxidation and UV light exposure [1]. In the process of purifying plasmid DNA from bacterial cells, it is also important to keep in mind that the lipopolysaccharides in the membrane of bacterial cells, called 'endotoxins', can impair purity and disrupt later experiments [1].

Liquid media are used to grow bacterial culture from single colonies of bacteria that have been transformed with plasmid DNA. Growth media contains tryptone, a source of nucleic and amino acids, which provides bacteria with nutrients that support cell division and maturation [2]. Lysogeny broth (LB) is used most commonly for

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Escherichia coli (*E. coli*) because it promotes proliferation and gives consistent plasmid DNA yields [2]. Another growth medium that contains higher yeast extract compared to LB is Terrific broth (TB). TB contains glycerol and potassium phosphates to provide the bacterial cells with an additional carbon source and prevent premature cell death due to inhospitable pH values [2, 3]. In a recent study by Wood et al. [3], *E. coli* growth in TB gave the highest yield of plasmid. A greater yield resulting from TB was found to be solely caused by the higher concentration of yeast in the media [3].

A miniprep begins with inoculation of bacteria into liquid media followed by overnight culture. Once the bacteria are harvested, a resuspension buffer that contains RNAse A is added. Afterwards, an alkaline lysis solution is added to the bacterial cells that contain sodium hydroxide which breaks down the phospholipid bilayer and disrupts hydrogen bonds between DNA bases. As a result, plasmid DNA will exit the bacterial cell [4]. A neutralization solution is added to preserve the plasmid DNA and promote the precipitation of the bacterial DNA [4]. HBC buffer is then added, to reduce the solubility of the DNA in water, as it contains guanidine hydrochloride and isopropanol [5]. Guanidine hydrochloride is a strong chaotropic agent that affects the hydrogen bonds in water molecules, so the DNA can easily bind to the silica filter in the column [1, 6]. However, isopropanol may not be the best precipitant because sucrose and salts are less soluble in isopropanol and may precipitate and contaminate the DNA [7, 8]. Ethanol also contains chaotropic properties which allow DNA to bind to the silica filters without disrupting the solubility of salt [9].To further purify the DNA from RNA and protein residue, a wash buffer is required prior to elution. For the final step of the miniprep, the DNA must unbind from the column so that it can be collected. For plasmid DNA purification methods, such as those found in the the Mira-prep [6] or Omega BioTek protocol [10], the elution step calls for the use of water at a neutral pH or a common elution buffer like Tris-EDTA, which serves to prevent oxidation of DNA by free radicals as well as prevents DNA degradation by DNAses [1].

The plasmid pCDH1.2_EF1_vGpH_ELKS1_shRNA ("pCDH1.2) is classified as a high copy number plasmids because it is derived from a pUC vector [11]. High copy number plasmids have hundreds of plasmid DNA molecules per cell and thus give high DNA yields when extracted from bacterial cells by utilizing plasmid miniprep kits [3]. In an upper-division cellular physiology lab course with an expected enrollment of 40-70 students per semester, it is essential to develop a protocol that would result in high DNA yields while also being time-efficient and straightforward for novice bench scientists. The purpose of this experiment is to modify the procedure from the OmegaBioTek E.Z.N.A Plasmid Miniprep Kit I lab manual to enhance the yield of the plasmid pCDH1.2.

In this experiment, we modify three factors to potentially maximize DNA yield of the miniprep procedure and compared the results to the standard miniprep protocol. TB was used for bacterial amplification instead of LB, HBC buffer was modified with ethanol and Tris-EDTA (TE) buffer was substituted with ddH_2O with a pH of 7.0 to investigate whether they are equally sufficient for DNA elution. With these modifications, we hypothesize that DH5α *E. coli* bacteria cells grown in TB with miniprep substitutions using ethanol as a buffer and water as an eluent will produce the highest DNA yield compared to our control condition of using LB, HBC buffer, and

Tris-EDTA buffer.

2. Methods

The miniprep protocol was adapted from weeks 3 and 4 of the BPK 408W Lab Manual [12]. The growth media were Terrific Broth (TB; 2.4% (w/v) yeast extract, 2% (w/v) tryptone, 0.4% (v/v) glycerol, 10% (v/v) Phosphate buffer) and Lysogeny Broth (LB; 0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl). The bacterial cells were inoculated into 18 mL of TB or LB, with either broth containing 100 µg/mL ampicillin. After being grown in a shaking incubator overnight, the samples were divided into six different experimental conditions as seen in Table 1. Purification of the plasmid DNA was completed by following the Omega BioTek E.Z.N.A Plasmid Miniprep Kit I protocol [10]. Then, either HBC buffer or ethanol was added to the columns containing plasmid DNA followed by a wash buffer. Finally, the DNA was eluted with either ddH_2O or TE buffer.

2.1. Experimental Miniprep Procedure

Omega BioTek E.Z.N.A Plasmid Miniprep Kit I was used for plasmid DNA purification from the bacterial cells (refer to Figure 1 for general experimental procedure). The Omega BioTek protocol [10] recommends using an endA negative strain of *E. coli* such as DH5α for plasmid isolation. DH5α cells were transformed with pCDH1.2 and grown overnight in either 18 mL LB or TB with 18 µL of ampicillin (100 µg/mL) on a shaking incubator at 37°C. The following day, 1 mL of the culture was transferred to the appropriate 1.5 mL Eppendorf tubes for each condition and spun at 10,000 $x\ g$ for 1 minute. The media was decanted and another 1 mL of culture was added for a total of 2 mL per tube, which was subsequently spun again for another minute. 250 µL of resuspension buffer was added and the cells were vortexed followed by 250 µL of lysis buffer. The tubes were inverted fifteen times and left to incubate for 3 minutes. 350 µL of a neutralization solution was added and the tubes were inverted until a white precipitate formed. The tubes were then spun for 10 minutes at 10,000 $x\ g$. The lysate was then transferred to mini-columns, spun for 1 minute at 13,000 $x\ g$ and the filtrate discarded. 450 µL of HBC buffer or ethanol was added to the columns and they were spun again at 13,000 $x\ g$. Wash buffer was added in two intervals at volumes of 700 and 400 µL and the columns were spun for 1 minute at 13,000 $x\ g$ after each time the wash buffer was added. Empty columns were spun at 13,000 $x\ g$ for 2 minutes to ensure the column was dry. TE buffer was diluted in water (1:10) and heated to 55°C. 55 µL of TE buffer or ddH_2O was added and incubated for 1 minute. The DNA was eluted from the column and they were spun at 13,000 $x\ g$ for 1 minute, and the eluate was reapplied to the column and subsequently spun again for another minute. A Thermo Scientific^{TM}NanoDrop Lite Spectrophotometer was blanked with 1 µL of TE buffer or water. 1 µL of the plasmid DNA was placed on the spectrophotometer to obtain the DNA concentration and the A_{260}/A_{280} ratio.

2.2. Experimental Conditions

The experiment consists of six total conditions: control, terrific broth, water eluate, ethanol, terrific broth/ water eluate/ ethanol, and terrific broth/ TE buffer/ ethanol condition (refer to Table 1).

Table 1: *Experimental Conditions and Miniprep Modifications.*

Condition	Control	Water Eluate	Ethanol	(TB)	TB Water Ethanol	TB, TE Buffer, Ethanol
Bacterial Growth Media	LB			TB		
Wash Reagent (450 µL)	HBC	HBC	Ethanol	HBC	Ethanol	Ethanol
Elution Reagent (55 µL)	TE	Water	TE	TE	Water	TE
Number of Trials	3	3	3	3	3	2

TE:10 mM Tris-EDTA pH 8.5
HBC: Guanidine Hydrochloride; pH 3.0-5.0
Water: Double-distilled

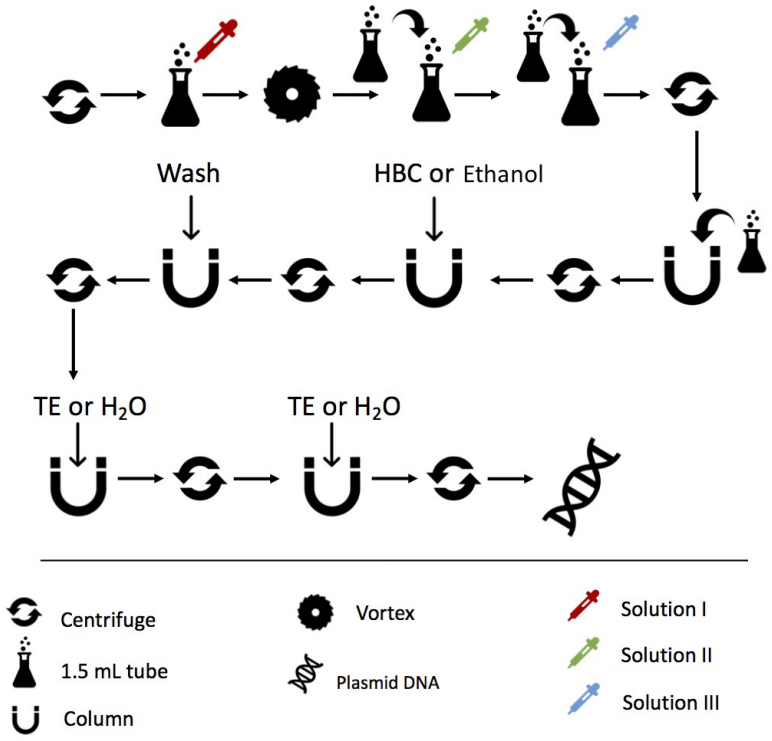

Figure 1: *General experimental mini-prep procedure. The procedure outlines modifications that give rise to the 6 experimental conditions in Table 1. A legend of the symbols is shown at the bottom of the figure.*

3. Results

The bacterial cells transformed with plasmid pCHD1.2 and amplified in TB with no miniprep modifications had the highest DNA yield (8.52 ± 0.59 µg) when compared to the other 5 experimental conditions (Fig. 2). The average A_{260}/A_{280} ratio for condition 2 was also equivalent to the control condition, 1.77 ± 0.05 and 1.76 ± 0.15 respectively. In contrast, pCHD1.2 DNA precipitated with ethanol (condition 4) had the lowest DNA yield (2.85 ± 0.44 µg). The TB, water and ethanol (condition 5) had a higher DNA yield (6.14 ± 0.41 µg) compared to the control condition (4.76 ± 1.60 µg). The sixth condition, TB, TE buffer and ethanol, produced the highest purity with a A_{260}/A_{280} ratio of 1.85 ± 0.01. To test for a quantitative significant difference between our conditions and the control, an Analysis of Variance (ANOVA) test with the Tukey-Kramer method was used ($\alpha < 0.05$). There was a significant increase in the DNA yield when LB was replaced with TB. As shown in Fig. 2, these increases in DNA yield are only significant for the TB condition. The ethanol condition (condition 4) had the lowest DNA yields (2.85 ± 0.44 µg). The plasmid DNA was pure in all six conditions (Fig. 2). In general, we would expect for high copy number plasmids such as pCDH1.2 a yield of 15-25 µg for 5 mL of starting culture grown in LB [10].

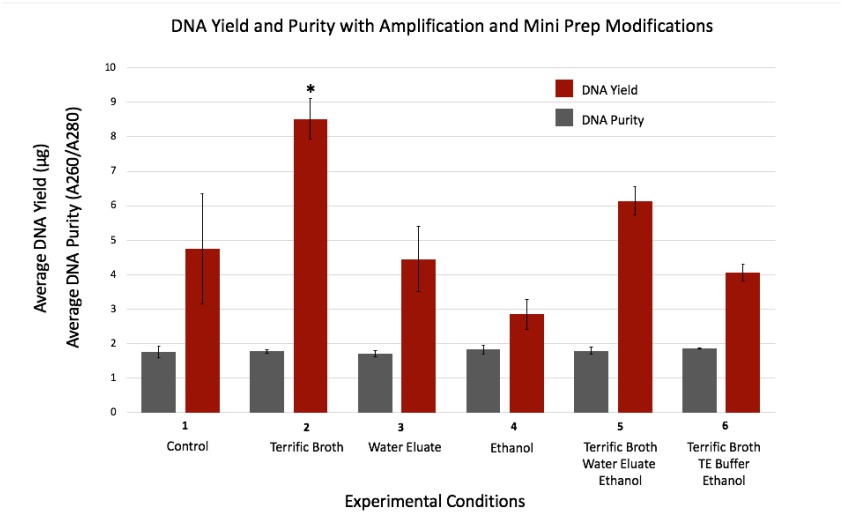

Figure 2: *DNA Yield with Amplification and Mini Prep Modifications. Bar graph of average DNA yield (ng) from all six experimental conditions. Error bars represent standard deviation and significance is based on these error bars. n=6 for conditions 1-5 and $n = 4$ for condition 6. * = significant increase compared to control condition.*

4. Discussion

4.1. DNA Purity

The pCHD1.2-transformed *E. coli* cells grown in TB had the same DNA purity when compared to the control. The purity of our DNA yield was determined by the A_{260}/A_{280}

ratio, in which a value of 1.75 -1.95 is accepted as pure. Values greater than 1.95 suggests RNA contamination while less than 1.7 suggests protein contamination [6]. All six conditions had ratios ranging between 1.71-1.85, indicating acceptable purity of the plasmid isolation. Our hypothesis was refuted as the condition containing three modifications (TB broth, ethanol, and water) did not generate the highest DNA yield. Based on the data obtained from the DNA yield and purity of the fourth condition (HBC buffer substituted with ethanol), we speculate that the volume of ethanol used was not sufficient at precipitating our plasmid DNA. According to BiteSizeBio [9], higher volumes of ethanol are required to produce similar results to isopropanol. In addition, another DNA precipitation protocol recommended using ice-cold ethanol over isopropanol; however, in our protocol the 95% ethanol was at room temperature [8]. Colder temperatures of ethanol may result in an increased isolation yield as it promotes the flocculation of nucleic acids [9]. Our data also showed that TB conditions had a higher purity than the control, which makes the TB amplifications protocol suitable for experiments such as transfection, restriction enzyme digests, and polymerase chain reaction.

4.2. DNA Yield

In comparing the amount of DNA purified from cells grown in different media, we demonstrated that *E. coli* cells grown in TB had the highest DNA yield compared to the control condition. This is due to the larger amounts of nutrient found in TB when compared to LB broth. TB contains five times more yeast, two times more tryptone than LB, and the presence of glycerol. This extends bacteria growth and plasmid amplification by supplying *E. coli* bacteria cells with larger amounts of amino acids. One study found that when the same amount of yeast extract was added to LB as TB, similar DNA yields resulted [3]. Therefore, the high yeast content of TB is likely the key factor in achieving higher yields.

When considering the plasmid isolation reagents, the DNA yields were lower than the control when HBC buffer was substituted with ethanol and when TB and ethanol were substituted with LB and HBC buffer respectively. Based on these results, it is likely that ethanol contributed to a lower DNA yield in both conditions 4 and 6. Therefore, ethanol may not be an acceptable alternative for HBC buffer in the plasmid DNA miniprep procedure. We propose that TE buffer may have contributed to a higher DNA yield than water because DNA is more stable at an alkaline pH, and TE buffer has a higher efficiency at dissolving DNA than water [13]. In general, our DNA yields across all six conditions were not as high as expected. According to the product manual for the E.Z.N.A Plasmid Mini Kit I [14], for 5 mL of inoculated culture high copy number plasmids have an expected yield of 15-25 µg. This could be due to poor cell lysis, overgrown culture or low elution efficiency. The protocol warns against leaving the lysing solution for more than 5 minutes however, due to our many samples it could be possible that the solution what left on too long. The cells also may not have been dispersed evenly before adding Solution II or Solution II had to be left longer for a clear lysate. Finally, another potential explanation is that the cultures were incubating for almost 18 hours which is more than the 16-hour maximum time recommendation [14]. In future experiments, tight regulation of time restrictions and appropriate temperature

and pH of solutions will hopefully result in the expected DNA yields.

4.3. Other Considerations

With further experimentation in mind, plasmid DNA storage is also crucial as these experiments may not be performed simultaneously. According to Oxford Gene Technology [15], DNA stored in Tris-EDTA buffer were stable at 2-8°C for 8 years, but DNA stored in water experienced degradation. Furthermore, Tris-EDTA chelates magnesium and other divalent metals which suppresses DNA degradation by DNAses. Therefore, the usage of Tris-EDTA for elution may be more suitable if long term storage is required. Further research should test whether larger volumes of ethanol and TB modifications will result in a higher DNA yield and purity. The cost of TB and LB are both 62 dollars per litre on ThermoFisher [16, 17], therefore there are no cost restraints for using TB.

4.4. Endotoxin Contamination

With a higher plasmid DNA yield, the presence of endotoxin may affect processes such as transfection. Endotoxin is found on the cell wall of *E. coli* bacteria and is produced during plasmid purification [18]. When bacterial cells are lysed, they release a large amount of endotoxin to their surroundings. Endotoxin forms large micelles that are negatively charged and can be purified along with plasmid DNA [18]. This could lead to a higher yield because the endotoxin is being co-purified with the DNA. On the other hand, this toxin is toxic to mammalian cells and can result in a reduction in cell viability and transfection efficiency. There are currently photometric tests available that can identify the presence of endotoxins in purified plasmid DNA samples [18]. There is also an 'Endo-Free' version of the plasmid purification kit that was used in this experiment that includes an endotoxin removal step in the purification process [19].

5. Conclusion

TB broth is more efficient at promoting bacterial growth and plasmid amplification than LB. The DNA miniprep procedure with TB generated equivalent purity as the control. Therefore, TB should be used in substitution of LB in bacterial growth for plasmid amplification. Further research is still required to understand the effects of endotoxin on mammalian transfection, and the effects of TE buffer and water on DNA degradation.

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A Review of Altered Neurophysiology and Connectivity of the Brain in Autism Spectrum Disorder and its Impact on Common Symptoms

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Abstract

Autism Spectrum Disorder (ASD) is a developmental condition which refers to a broad range of abnormalities and challenges with social skills, cognitive abilities, and behaviour. The prevalence of ASD is increasing, yet we currently do not have a clear neurodevelopmental understanding of this complex disorder. A narrative review is done on the topic of abnormal functional connectivity in ASD and its relation to some of the symptoms and consequences which have been observed in the literature. Thus, no formal process was used to carry out this review and no attempt was made to conduct a statistical analysis of the data. The purpose of this review is to integrate the findings related to the topic in a meaningful and logical manner. The review will also discuss related topics such as white matter connectivity, anatomical and pathological findings, in addition to biochemical changes observed in the ASD brain. Findings from studies discussed such features and changes in the ASD brain using different brain imaging methods. A review of the literature guides us to the conclusion that there is no single causal gene or exclusive physiological factor for ASD. Furthermore, we find that there is also no universal case of under- or overconnectivity in autistic brains. Instead, we observe variation in abnormal connectivity between different brain areas, which are theorized to be associated with some of the commonly reported symptoms in ASD.

Keywords — Autism Spectrum Disorder, Neurodevelopment, Neuroatypical, Functional Connectivity, Structural Connectivity, Neuroanatomy, Neurophysiology, Neurochemical, fMRI, EEG, MEG

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition which is characterized by difficulties in social communication and repetitive and/or restricted behaviours and/or interests. Over the years, research has allowed for advancements in reasonably accurate diagnosis. One popular diagnostic technique is the Autism Diagnostic Observation Schedule (ADOS), which is an assessment of communication and social behaviour for individuals who may potentially have ASD or

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other pervasive psychological and neurodevelopmental disorders. The test involves the administration of standardized activities organized into four modules, based on the participant's age and language development. The examiner would then observe the presence or absence of key behaviours associated with ASD and formulate a score used to make a diagnosis [1]. This is just one example of several other diagnostic techniques which clinicians and researchers may find helpful, yet there is still a large gap in finding clear biomarkers that would ideally provide more reliable and valid diagnoses. A biomarker is any objective indicator that could be accurately and reproducibly measured to determine the medical status of an individual [2]. Autistic individuals do not present any particular physical appearances; unlike other developmental disorders, such as Down Syndrome and Cornelia de Lange Syndrome. On the other hand, autistic individuals have been shown to share relatively subtle distinct facial features which have only been reported in males [3]. A recent study by Tan and colleagues [4] found that increased facial masculinity in ASD correlated with greater communication difficulties based on the ADOS. Indeed, genetic and epigenetic factors could also serve as biomarkers for diseases and illness. Individuals can have a genetic predisposition to ASD [5, 6, 7]. However, there is a high volume of candidate genes which may be involved [6] which by definition makes ASD a polygenic condition. Furthermore, this introduces another dimension of complexity and results in ASD to not have a clear genetic basis or any obvious biomarker(s) that would be easily associated.

More interestingly, ASD subjects have a wide spectrum of different complications and characteristics, yet there are unifying symptoms which are hallmarks of the condition, such as impaired communication and social behaviour, restricted range of interests, and repetitive behaviour [8]. ASD is often diagnosed after infancy and its occurrence is reported in all racial, ethnic, and socioeconomic groups, where delays in diagnosis were found in certain cultural groups [9, 10]. The prevalence of ASD is greater in males as indicated by a study which found that males were four times more likely than females to be diagnosed with the condition [11]. There is an increasing prevalence of ASD, but we have yet to identify a clear pathophysiologic basis of the condition. Research to fill this gap has shown promising findings and has led to many paths for further and much-needed investigations.

A useful theory to provide some foundational explanation to the mechanism of ASD and its associated symptoms is studying the functional connectivity of the autistic brain. Functional connectivity of the brain refers to the synchronization of activity between different areas of the brain. In other words, this parameter qualitatively indicates how collaboration occurs between different cortical regions and structures, and how much of it is taking place. This idea links neurophysiological information, obtained through brain imaging modalities, and histological findings with behavioural observations. Thus, it can serve as an effective path to have a better understanding of the disorder. Researchers from different branches of neuroscience and psychology, as well as the engineering sciences, have been led to discover that there seem to be distortions in functional connectivity for subjects diagnosed with ASD, compared to their neurotypical (NT) counterparts [12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22].

A battery of psychological tasks have been used to test and measure participants' cognitive and motor performance, all while using brain imaging modalities such as

functional magnetic resonance imaging (fMRI) [23, 24, 12, 25, 13, 26, 27, 14, 15, 28, 16, 29, 30, 31], magnetoencephalography (MEG) [32], and electroencephalography (EEG) [33] to collect data pertaining to cortical activity, primarily, but not limited to, functional connectivity. Authors also make use of complex computational models and cognitive architectures [34, 17] to connect their results with theories about how the brain systematically operates. Other forms of data which some authors point to include histological [35, 36, 37, 38], structural connectivity [24, 27, 18, 39], anatomical [40, 41, 42, 43, 44, 45], and biochemical abnormalities [46, 47] observed in ASD. In this narrative literature review, we will discuss some of these studies and their findings with a greater emphasis on the role of functional connectivity in several of the most common complications seen in ASD. The rationale for this review paper is to provide readers with insights about the biomedical findings of ASD with an emphasis on neurophysiological findings.

2. Functional Imaging Results

The neurobiology of ASD is being extensively studied to find some notable evidence for differences in functional and structural connectivity between the brains of individuals with ASD versus NT controls [48]. One of the earliest suggestions directs our attention to fMRI results which support the claim that there is reduced long-distance connectivity between the frontal and posterior regions in the autistic brain [12, 25, 13, 26, 15]. This influential idea is known as the underconnectivity theory of autism. In their review article, Just and colleagues [34] refer to numerous fMRI studies which show such reduced inter-regional communication as mentioned above in ASD participants during several cognitive tasks. The fMRI studies show impaired activation and synchronization in the frontal and posterior regions, which can be linked to the observed decrease in performance in a wide range of cognitive tasks such as language comprehension [25], executive functioning [12], social processing [28, 29, 49], working memory [15, 28], advanced inhibition [26, 30]. and visuospatial processing [23]. Just [34] suggests that poor capabilities with these cognitive tasks, especially those involving Theory of Mind and executive functioning, serve as evidence for the theory of underconnectivity in the frontal lobe of the cerebral cortex due to the great demand of that specific region for carrying out these tasks.

Furthermore, a computational model was used to demonstrate what the functional connectivity would look like in individuals with ASD under an executive functioning task known as the Tower of London. The computational model showed supporting results for the underconnectivity theory, and moreover, Just and colleagues [34] elaborated on two concluding cases. Firstly, the authors concluded that there is constrained "communication bandwidth" [34] between the frontal and posterior regions of the cortex, where communication bandwidth is described by the authors as the optimal rate of information transfer along a neural path. Secondly, this difficulty with proper communication leads to the need for the parietal area to function without central input from the frontal area to complete a given task. Thus, this will lead to the adaptation of having a greater parietal autonomy. This is considered by the authors as a more comprehensive theoretical model of the autistic brain and its implication in the decreased functional

connectivity as noted above. Nevertheless, there is increasing evidence hinting that there is more to discover, as other studies exhibit cases of both underconnectivity and overconnectivity (hypoconnectivity and hyperconnectivity, respectively) [19].

A study by Redcay and colleagues [16] looked at whole-brain functional connectivity in NT adolescents and ones with ASD, aged 14 to 20 years, using graph theory and resting-state functional connectivity magnetic resonance imaging (fcMRI). They observed increased functional connectivity between the right lateral parietal region and prefrontal regions in the ASD group. Building upon this, Delmonte and colleagues [18] found abnormally high functional connectivity of frontostriatal connections in adolescents with ASD. In terms of the default mode network, decreased connectivity was reported between anterior and posterior default mode sub-networks in adolescents with ASD [31]. Local overconnectivity in posterior occipital and temporal areas in addition to local underconnectivity in posterior cingulate and medial prefrontal regions were found in adolescents with ASD, using a voxel-based approach to measure functional connectivity between a particular voxel and its nearest neighbours [14]. It is important to note that fcMRI studies show diverse findings in connectivity with respect to specific regions and different states, such as resting state versus task state, measured in participants [48]. This variation in under- and overconnectivity patterns across different regions of the cortex is expected since there is strong evidence that the autistic brain does show structural and functional differences compared to a typically developing brain.

Functional connectivity may also change depending on age; thus, the study of differences in this phenomenon through a developmental aspect deserves adequate attention. Uddin and colleagues [20] present evidence which exhibits overall overconnectivity in the brains of younger children afflicted with ASD. Another study utilized functional near-infrared spectroscopy to look at the same topic but specifically looked at infants at one year of age who were not diagnosed with ASD but were a high-risk group for the disorder. As early as three months into their life, these selected infants showed overall overconnectivity compared to the control group, but this difference was no longer present in the later months to follow [21]. More recent evidence found that high risk infants showing abnormal connectivity at six months of age were more likely to be diagnosed with ASD and show greater symptom severity, such as repetitive behaviour and restricted social-communication skills [50, 51, 52].

3. Structural Imaging Results

Functional connectivity can also be better understood by investigating the structural or anatomical connectivity of the brain. Diffusion tensor imaging (DTI) is an effective way to accomplish this. Specifically, DTI can be implemented to study white matter tracts and their connectivity [48]. White matter is of special interest due to its broad and vital role in supporting neurons, particularly in neural communication within the cortex and periphery. Making a link between functional and structural connectivity may be difficult, as illustrated by Delmonte and colleagues [18], whose study was mentioned above for reporting overconnectivity between the striatum and frontal cortex during the resting state. However, they were unable to find evidence for differences

in structural connectivity of the frontostriatal tracts via DTI. The authors suggest that the observed overconnectivity may be due to functional reorganization in the autistic brain instead of an anatomical abnormality. On the other hand, one cannot ignore other anatomical deviations in the autistic brain, and their potential role in atypical functional connectivity. Cortical connectivity in adults with ASD was found to be affected by intracranial volume [39]. The authors believe there is a possibility that this consequence of constrained long-range connectivity is manifested by the early brain overgrowth in ASD. A significant relationship was found between abnormal white matter structure and distortions in functional connectivity during the completion of a visuospatial task [27]. Using fMRI and high angular diffusion MRI, the change was more notably recorded in the connections between the left occipital lobe and five regions in the left hemisphere. An influential study by Deshpande and colleagues [24] used fMRI and theory of mind task to make some interesting findings regarding connectivity differences in ASD. Up to 19 cortical pathways were found to be reliable in distinguishing participants with ASD from those who were NT. These cortical pathways were found to be altered in the autistic group in terms of effective connectivity. Effective connectivity is the direct influence of one brain area over another [48]. The difference in effective connectivity between the two groups was prominent enough to allow a 95% accuracy rate in predicting experimental participants from controls. Aside from its remarkably high accuracy with predicting effective connectivity, one can appreciate the authors' choice to use three complementary metrics: functional connectivity, white matter connectivity, and effective connectivity.

4. Biochemical Findings

The neurochemistry of the brain can be non-invasively studied in vivo through proton magnetic resonance spectroscopy (1H MRS) or also commonly known as nuclear magnetic resonance (NMR) spectroscopy. Using similar principles as MRI, this analytical approach has been used to study the concentration of brain metabolites as biomarkers for various neurological and psychiatric conditions, such as ASD [46, 47].

Friedman and colleagues [46] used an advanced form of 1H MRS, known as proton echo-planar spectroscopic imaging (PEPSI), where the relative concentrations of selected chemicals could be obtained from multiple brain regions all at once, rather than having to do it repetitively for each of the single three dimensional spaces in the brain (ie. a single voxel approach). The goal of the study was to quantitatively measure and analyze the concentrations of N-acetylaspartate (NAA), creatine plus phosphocreatine (Cre), choline (Cho), and myoinositol (mI) in addition to relaxation times of 45 children from the ages of 3 to 4 years [46]. The participants were children with ASD, NT, or had some form of delayed neurodevelopment. The authors found a decrease in NAA, Cre, Cho, and mI concentrations for the ASD group, compared to the NT subjects in several brain regions such as the cingulate gyrus, white matter tracts associated with the corpus callosum as well as the frontal and parietal lobes, gray matter associated with the temporal lobe, and subcortical structures such as the thalamus and the insula. These reductions in NAA, Cre, Cho, and mI concentrations for the ASD group were reported to be subtle, such that it would not support their hypothesis of increased neuronal

density in ASD and its role in faulty apoptosis (cell death) or synaptic pruning. However, it is important to note the role NAA has in the synthesis of lipid by oligodendrocytes, the glial cells that provide myelination for the axons of neurons in the central nervous system (CNS) [53]. Thus, this observed chemical imbalance may be implicated in the abnormal functional connectivity reported in ASD; especially in the cases of the underconnectivity reported in the frontal and parietal regions [12, 25, 13, 26, 15] with regard to the reductions of NAA in the white matter tracts of the frontal and parietal lobe [46]. Levitt and colleagues [47] conducted a similar study using 1H MRS and MRI, but in a single voxel approach, for several brain regions and subcortical structures. They included 22 children with ASD, who were in an older and broader age range (5 to 16 years of age) and had 20 age-matched NT controls. Comparing the ASD subjects with the NT controls, there was no significant difference in NAA concentrations, but significant differences were found in Cho concentrations [47], which is contrary to Friedman's findings. Cho levels were found to be reduced by 27.2% in ASD subjects in the inferior anterior cingulate and increased by 19.1% in the right caudate nucleus [47]. Cre levels were increased in the right caudate nucleus by 21% but also reduced by 17.9% in the body of the left caudate nucleus and right occipital cortex [47], which is consistent with Friedman's findings. With that said, Levitt did observe relatively larger reductions in Cre [47]. In summary, the authors were able to show that differences in metabolites exist between ASD and control subjects.

5. Pathological Findings

It is now widely accepted that ASD is a multifactorial disorder [54], meaning that ASD is dependent on various genetic and environmental factors. As with most multifactorial health complications, there are several genes which are involved in susceptibility and resistance to the development of ASD, in addition to significant influence by environmental factors [54]. Furthermore, ASD primarily involves the brain and increased brain size is the most prevalent trait regarding abnormal cortical structure [54]. This brain overgrowth does not result in damage to subcortical structures and spaces. This phenomenon is not specific to a certain brain area, although some authors have found the most significant increase to be in the frontal lobe [41, 43]. Some cases involve overgrowth in the cerebellum as well [40, 45] but increased size of the cortex seems to be supported more soundly [44]. More interestingly, white matter volume has been shown to be disproportionately larger in ASD subjects [38]. Casanova and colleagues [42] attribute this to the distortions in the structure and number of minicolumns which would have an impact on their circuitry. Minicolumns are organizational units consisting of layers of connected neurons arranged in vertical columns which cumulatively comprise the cortex, especially the superior area of the brain responsible for higher cognitive functioning which is known as the neocortex.

A comprehensive review by Goldberg and colleagues [55] argues that most of the neuroimaging findings of increased brain size may not be reliable since these experiments have not been replicated and do not make a proper effort to control extraneous variables. In terms of increased cerebral size, the author suggests that overgrowth in the temporoparietal region is the only appropriately replicated finding,

rather than enlargement of the frontal region, which is more frequently reported [55].

So far, no definite conclusions have been drawn about a cellular pathological feature in ASD. However, postmortem histological studies have shown atypical differences in the minicolumns of autistic brains [36, 37]. One of the most consistent changes in morphometry is the decreased width of minicolumns. This finding was verified by Buxhoeveden and colleagues [35] in another experiment involving a quantitative technique, known as Gray Level Index (GLI), to analyze stained tissues from postmortem ASD samples. GLI is the ratio of the area covered by stained cell bodies to those that are not stained. The initial analysis carried out by the authors was measuring the peaks of stained gray matter areas to find the column width. Other modifications of the GLI method [37] all led to the same result of a reduced minicolumn width, in addition to an increase in the number of minicolumns and a decrease in the size of neurons [5, 36]. Given the hypothesized importance of these minicolumns in the development and organization of the neocortex and white matter [36], it is no surprise that abnormal brain growth would be seen in ASD. These columns of pyramidal neurons are connected to each other through regional and global white matter tracts, such as those in the corpus callosum [54]. Reduction in the width of minicolumns and the size of their constituting neurons will be accompanied by an increase in the number of columns, which is linked to an increase in local and global neural fibre projections [56]. The former is more commonly observed because long white matter tracts are more physically and metabolically taxing [54]. However, by favouring shorter local projections, short-range functional connectivity is enhanced while long-range functional connectivity is not [54]. This would then direct back to the findings of decreased frontal and posterior connectivity as previously mentioned above [12, 25, 13, 26, 15]. Therefore, a distorted minicolumnar hypothesis may have an integral role in the changes in regional and long-range functional connectivity in ASD.

6. Discussion

There are an increasing number of studies being done on ASD participants who are on the high functioning end of the spectrum. Although the recent discoveries in ASD research are exciting, they also raise the concern of a bias towards high-functioning individuals within the ASD population, therefore inevitably neglecting research on the more severe or low functioning autistic individuals.

The approach of incorporating two or more neuroimaging techniques is key for this domain of research. Each tool has its advantages and drawbacks over the other. MRI experiments, which are done in various forms, are highly popular and some may argue that they are the most prolific, but they are also in fact very costly [16]. MRI techniques used in this field of research allow excellent spatial resolution but poor temporal resolution. However, EEG and MEG provide a different window of studying functional connectivity through having a high temporal resolution and moderate spatial resolution. Moreover, they are also not affected by motion artifacts which can introduce errors in connectivity measurements taken in fcMRI experiments [48]. With that said, EEG data was used to support the claim of both underconnectivity and overconnectivity in ASD [33], and interestingly, MEG results also led to observing different abnormalities

in brain oscillations in ASD samples and their family members who share half of their genome [32].

Genetic and cellular studies may lead to promising discoveries regarding atypical functional connectivity in ASD, but such studies face many obstacles such as difficulty with in-vivo cellular experiments with ASD participants, ethical issues since the syndrome is only found in humans and the demand for large sample sizes needed for completing meaningful genomic studies. Ultimately, the future of ASD research holds much potential in terms of experiments which involve a combination of different imaging approaches. Additionally, future studies should continue to consider analyzing more than one connectivity or physiological index as this approach can lead to more fruitful findings regarding the neurodevelopment of ASD and its link to reported symptoms and reliable biomarkers.

7. Conclusion

Investigating the literature makes it very clear that ASD exemplifies a complex neurodevelopmental disorder with a neurological and genetic basis that manifests itself in diverse psychological and physical symptoms and complications. One overarching topic is the distortions that have been reported in functional connectivity. Similar to our understanding that there is no single causal gene or environmental factor for ASD, we find that there is also no single region in the cerebrum where under- or overconnectivity is shown in individuals with ASD. Rather, there seems to be a mixture of atypical connectivity between different brain regions which can be associated with some common ASD symptoms. For instance, the observed frontal underconnectivity may be associated with deficits in executive functioning and language learning [28, 34, 49]. Mixed patterns of underconnectivity with the frontal area and overconnectivity in the cerebellum [22] may be related to even less frequent symptoms such as altered motor behaviour and coordination. Functional connectivity can also be used to account for the advantages observed in some cases of ASD. For instance, considering the important role posterior areas have in perceptual learning and processing [16, 34], one can note the consistency between improved perceptual functioning in ASD with the findings of overconnectivity in posterior regions, namely the temporal, parietal, and occipital areas, in addition to the proposed theory of greater parietal autonomy [14, 34].

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Are We There Yet? How CAR T-Cells Came to Be and Where They'll Take Us

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Abstract

Chimeric Antigen Receptor (CAR) T-cells have shown immense promise for the treatment of blood cancers. Fundamentally, CAR T-cell therapy involves the redirection of the immune system's natural response against pathogens towards the body's own cancer cells. The structure of the CAR allows the circumvention of the major histocompatibility complex, thereby allowing CAR T-cells to exhibit toxicity toward a chosen antigen. Advancements in CAR structure have improved CAR T-cell expansion and potency, also giving rise to a subset of engineered T-cells that can deposit cytokines into solid tumours. However, at this time the overall scope of CAR T-cells as a therapy is limited. Solid tumours are difficult to treat with CAR T-cells due in part to lack of appropriate target antigens, physical barriers to their efficacy, and a hostile tumour microenvironment. Toxicity is also an impediment to their clinical application. In this review, the molecular and physiological basis of CAR T-cells is outlined and areas for future research are briefly explored.

Keywords — Cancer, Chimeric Antigen Receptor T-Cells, Immunotherapy, Tumor Microenvironment, Toxicity

1. Introduction

CAR T-cells are genetically engineered T-cells designed to attack the cancerous cells of the patient from whom they are derived. To manufacture them, the patient's T-cells are modified outside the body to contain an antibody-derived receptor, such that the cytotoxicity of the T-cell can be directed towards any tissue that expresses the corresponding antigen. As of 2018, only two CAR T-cell therapies are available on the market. Both are intended to treat relapsed or refractory cancers - those that have either returned after previous improvement or have not responded to conventional treatment. Tisagenlecleucel (Kymriah), which is specific for the antigen CD19, is used in the treatment of B-cell precursor acute lymphoblastic leukemia (ALL) in children and young adults [1]. Axicabtagene ciloleucel (Yescarta), also specific for CD19, has been shown to be effective in the treatment of large B cell lymphoma in adults [2]. Both have shown promising short-term results: in an international Phase 2 study of tisagenlecleucel, 83% of patients experienced overall remission within 3 months after a one-time infusion, with overall remission being defined as complete remission with or without complete hematologic recovery that lasted for at least 28 days and was confirmed by laboratory tests [1]. A Phase 2 trial of axicabtagene ciloleucel had similarly notable results. After at least 6 months, 54% of patients had experienced complete response, and another 28% had experienced partial response [3].

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2. The Immune System

When a foreign body is recognized by the immune system, the innate immune response is activated. The term "foreign body" generally refers to external pathogens that an individual may encounter but, in the case of cancer, the desired target of the immune response may be the body's own cancerous cells. The innate immune response is so called because it consists of a set of genetically programmed responses towards such foreign particles [4]. This reliance on genetics imposes an important limitation: due to the finite number of genes that can be coded for in the human germline, the specificity of the innate immune response is lacking [5]. If the innate immune system is overwhelmed, the adaptive immune response is activated [4].

There are several important varieties of cells that constitute the adaptive immune system. The effector cells of the adaptive immune response are known as lymphocytes [4]. Sub-lineages of lymphocytes include B-cells and T-cells [4]. When they encounter a foreign body, B-cells secrete soluble immunoglobulins known as antibodies [4]. Conversely, the receptors of T-cells are restricted to the cell surface and are known, simply, as T-cell receptors [4]. The ligands of antibodies and T-cell receptors are known as antigens [4].

One essential distinction between the innate and adaptive immune responses is how foreign bodies are recognized. Antibodies and T-cell receptors are not derived directly from the genome, but rather from genes that undergo recombination and modification during lymphocyte development [4]. These receptors are therefore not bound by the genetic constraints that the receptors of the innate immune response face and can be made in billions of versions [6]. Each of these unique receptors can bind just one ligand, therefore allowing lymphocytes to recognize foreign bodies with a high degree of specificity [4].

However, T-cells, the vehicle for CARs, face their own limitations. A properly functioning immune system can distinguish "self" cells from "non-self" cells, a principle in part carried out by major histocompatibility complex (MHC) molecules. MHC molecules display fragments of proteins from a cell, either endogenous or pathogen-derived, on the cell's surface in a peptide-MHC molecule (pMHC) complex [7]. Developing T-cells first encounter MHC molecules in the thymus, where they present the T-cells with a variety of self-derived peptides [8]. If the T-cells bind to the self pMHC complexes with high specificity, they are instructed to perish, as they pose the rise of coordinating an attack against the body's own tissues [8]. Conversely, T-cells that bind to the self pMHC complexes with low to medium affinity will survive [8]. This selection allows vast variation within the body's T-cell population while minimizing the risk of autoreactivity [8]. While this distinction between self and non-self is vital for healthy immune function, it prevents standard cytotoxic T-cells from destroying the body's own cancer cells. However, CAR T-cells can harness the cytotoxicity of T-cells for this very purpose by bypassing the MHC. Rather than recognizing pMHC complexes using a T-cell receptor, the addition of a CAR allows direct recognition of antigens due to the structure of the receptor. CARs have four main parts: an extracellular, antibody derived domain used for antigen recognition; a transmembrane domain; a spacer or hinge domain; and an intracellular domain that plays a role in T-cell activation [9]. By using an extracellular domain of antibody origin, the CAR can recognize any cell-surface

antigen for which there is a corresponding antibody, including but not limited to those associated with MHC molecules. Furthermore, these antigens that CARs recognize can comprise many different structures, from peptides to inorganic compounds to carbohydrates, as opposed to T-cell receptors, which recognize exclusively peptides [10].

3. CAR T-Cell Structures

The two CAR T-cell therapies currently on the market, tisagenlecleucel and axicabtagene ciloleucel, are second-generation CARs of a four-generation series. T-cells with chimeric receptors that allowed antibody-type specificity in recognition of antigens were developed by Kuwana et al. in 1987, and in 1993 Eshhar et al. directed these principles towards cancer cells, calling the modified T-cells "T-bodies" [11, 12]. T-bodies, now known as first-generation CARs, utilize a CD3ζ T-cell activation domain [12]. The cytotoxic effect of these CAR T-cells is lacking due to their inability to produce sufficient levels of interleukin-2 (IL-2) [13]. As such, to achieve therapeutic results, exogenous cytokines are administered concurrently [14]. Cytokines, such as IL-2, are a general class of small, soluble molecules that influence how cells interact with each other [15]. IL-2 specifically plays a role in T-cell expansion, a process in which T-cells proliferate and differentiate from naïve T-cells into effector T-cells capable of cytotoxicity [16]. Clinical data suggests that the efficacy of CAR T-cells is influenced by the infused cells' ability to expand in vivo [17]. The magnitude of the T-cells' expansion is also related to the number of T-cells that will persist after the pathogen is eradicated, known as memory T-cells [18]. Therefore, an added benefit of treating cancer using T-cells is that any memory T-cells generated also have the potential to provide long-term tumour immunosurveillance against the cancer [19]. Due to the vital role that IL-2 plays in the expansion of CAR T-cells, the need to supply exogenous cytokines is unfavourable if a different CAR design could eliminate this requirement. Krause et al. accomplished this in 1998 by modifying T-cells to express a CD28-like receptor as a costimulatory domain that recognized a ganglioside found on the surface of many tumours [20]. These modified T-cells, now known as second generation CARs, were able to secrete their own IL-2 [20]. For modern second-generation CARs, CD28 or 4-1BB are often the costimulatory domains of choice [21]. This costimulation confers stronger persistence and therefore more potency in general [22]. Research performed by Savoldo et al. in which first- and second-generation CAR T-cells with the same antigen recognition domain were administered to the same patient showed that second-generation CARs demonstrate improved expansion and persistence relative to first-generation CARs [23]. Third-generation CARs, following the logic that one costimulatory domain notably improves the performance of the engineered T-cells, contain two costimulatory domains [24]. More research is warranted on third-generation CARs. So far, results in mouse models have been promising [25]. However, early human trials have shown lackluster results [26].

4. CAR T-Cells and Solid Tumours

The fourth-generation of CARs are specifically aimed towards solid tumours [27]. The need for a specific subset of CARs able to treat solid tumours is due to the many barriers that T-cells face when it comes to treating cancers outside the realm of hematologic malignancies. A primary challenge is identifying tumour-associated antigens (TAAs) suitable for attack by CAR T-cells. Such TAAs must fulfill two crucial criteria: their expression must be consistent on the surface of tumour cells, but low or absent on normal tissues [28]. Part of the success of CAR T-cells in treating B-cell malignancies such as those targeted by tisagenlecleucel and axicabtagene ciloleucel can be attributed to the TAA that is targeted: CD19. CD19 is expressed on both cancerous B-cells and non-cancerous B-cells, meaning it violates the latter of the two criteria for TAAs outlined above [28]. Attack of non-cancerous cells can lead to B-cell aplasia, the depletion of B-cells in the patient's blood [29]. Although the expression of CD19 on non-cancerous cells is not optimal in terms of its quality as a TAA, the corresponding aplasia can be counteracted via intravenous administration of a mixture of antibodies, known as intravenous immunoglobulin (IVIG) [29]. While the mechanism by which IVIG supports the immune system is not well understood, it involves processes such as interaction with the cytokine network, activation of B- and T-cells, and provision of otherwise depleted antibodies [30]. Overall, the administration of immunoglobulin likely acts to recapitulate the role of antibodies in the homeostasis of a functioning immune system [30].

The search for solid tumour TAAs has been intensive and remains ongoing. TAAs that have undergone exploration in clinical trials include Human Epidermal Growth Factor Receptor 2 (HER2), carcinoembryonic antigen, the diganglioside GD2, and interleukin 13 receptor α [28]. In addition, a recently published Phase I trial in which epidermal growth factor receptor (EGFR)-specific CARs were used was notable as far as CAR T-cell therapy for solid tumours goes, with ten out of seventeen participants achieving stable disease following infusion [31]. Although some success has been seen in trials focused on the aforementioned TAAs, the broad applicability of CAR T-cells for solid tumours is still limited in part by the identification of appropriate CAR targets.

The use of CAR T-cells for the treatment of solid tumours also presents a more literal barrier to their efficacy. As opposed to the case of hematologic malignancies where the bloodstream contains an admixture of both cancerous cells and T-cells, in the case of solid tumours CAR T-cells must navigate from the blood to the tumour site in a process known as trafficking. Chemokines, which are cytokines that induce chemotaxis, play a role in recruiting lymphocytes to the tumour [32]. For instance, chemokines such as exodus-2 and macrophage-derived chemokine are found to be expressed at higher levels in ovarian tumours containing T-cells than in those without [32]. Ideally, chemokines expressed by the solid tumour recruit lymphocytes with the corresponding chemokine receptor, however in some cases the chemokines produced by the tumour do not correspond to receptors on the T-cell [33]. In addition, the expression of chemokines involved in migration of effector cells varies among tumours, and in some cases the amount of ligand produced is not enough to promote efficient trafficking of effector cells [34]. To some extent, this heterogeneity in the chemokines produced as well as their variable amounts can help account for the inefficient trafficking of leukocytes to

the tumour site [35]. Other structural aspects of solid tumours also prevent lymphocyte trafficking. In normal tissue, pericytes support the endothelial tissue of capillaries and venules, however in tumours they are often loosely attached or absent altogether [36]. This lack of structural support leads to leaky vessels and therefore inconsistent blood flow, which may impede lymphocyte trafficking [35]. Given that irregular blood flow inhibits lymphocyte trafficking, increased blood flow would seem favourable to tumour infiltration by effector cells. However, overexpression of substances that promote angiogenesis in solid tumours, such as vascular endothelial growth factor (VEGF), can result in reduced trafficking [37]. When VEGF is produced in high levels, adhesion molecules are downregulated [37]. These molecules, such as intercellular cell-adhesion molecule-1, play a role in transmigration of the T-cell across the wall of the blood vessel into the tumour site [38]. As such, when their expression is suppressed, T-cell trafficking is reduced despite increased blood flow [39]. To circumvent the issue of trafficking, injection of CAR T-cells into the tumour itself or the surrounding region has been considered. Three clinical trials are currently active that are exploring the use of regional injection to treat mesothelioma and ovarian, lung, breast, and head and neck cancers and are projected to conclude in 2019 [40, 41, 42].

Even if CAR T-cells successfully make their way into the solid tumour, the tumour microenvironment (TME) itself generates conditions unfavourable to antitumour activity. CAR T-cells in the TME face hypoxia, which promotes glycolysis and therefore the production of lactic acid [28]. In a sufficiently acidic environment, the proliferation of T-cells and the production of cytokines that promote it is reduced [43]. Immunosuppressive soluble factors in the TME also pose a challenge. For instance, prostaglandin E2 is produced by tumour cells, and impedes T-cells' ability to proliferate by suppressing their expression of IL-2 [44]. Fourth-generation CAR T-cells provide a tailored approach to solid tumours by addressing the reduced expression of cytokines in the TME that are favourable to anti-tumour activity [27]. Physically, these specialized CAR T-cell are comprised of an ordinary CAR T-cell along with an expression cassette that is reactive to nuclear factor of activated T-cell (NFAT) proteins [45]. An expression cassette is a portion of vector DNA that includes a gene and a promoter [46]. Union of the T-cell's CAR with the target antigen results in the activation of the NFAT-responsive promoter, and the corresponding gene on the expression cassette is then transcribed [45]. This gene typically codes for a cytokine or chemokine that promotes inflammation such as interleukin-12 (IL-12), and when deposited in the targeted tissue by the CAR T-cell, can recruit additional immune cells to the site [45]. This concept is especially helpful with respect to the phenotypic heterogeneity of the cells found in solid tumours: by attracting an assortment of immune cells, even cells not expressing the relevant TAA can be targeted [45]. IL-12 in particular has also been shown to increase the cytotoxicity of T-cells themselves [47]. In general, the therapeutic use of IL-12 is limited by the toxicity that accompanies its systemic administration, thus making fourth-generation CAR T-cells an appealing mechanism for local deposition of IL-12 and other cytokines into the tumour itself [48].

5. Toxicity

When it comes to the clinical application of CAR T-cells, the toxicity that accompanies their administration is a primary concern. A variety of toxicities due to CAR T-cell treatment have been documented, with cytokine release syndrome (CRS) being the most common [49, 50]. CRS is caused by an increase in serum cytokines, such as interferon-γ and interleukin-6 (IL-6), that are released by CAR T-cells following their activation upon engagement with an antigen [51]. Symptoms are varied and can include fever exceeding 40.0°C, nausea, vomiting, mental status changes, and seizures [52]. The severity of CRS can range from mild to severe and is defined on a scale of 1 to 5 [52]. Grade 1 CRS requires only symptomatic treatment, such as antiemetics or medications to reduce fever [52]. CRS is defined as Grade 4 when symptoms become life-threatening and the patient requires more extensive medical assistance, such as use of a ventilator [52]. Grade 5 CRS results in death [52]. The severity of CRS correlates with extent of the patient's disease at the time of infusion, with a higher disease burden corresponding to more severe CRS [53]. In a Phase 1-2a study of tisagenlecleucel for the treatment of relapsed or refractory ALL, CRS occurred in 88% of patients [54]. Severe CRS, defined as CRS requiring respiratory or hemodynamic support, occurred in 27% of patients [54]. A Phase 1 trial of axicabtagene ciloleucel demonstrated a similarly high incidence of CRS. 93% of patients experienced CRS, with 13% of patients experiencing Grade 3 or higher, including 1% of patients whose CRS was fatal [3]. In both cases, tocilizumab, an IL-6 receptor antagonist, was used to manage the syndrome [3, 54]. IL-6 is a cytokine that promotes inflammation and of which high levels correlate with severe CRS [55]. Tocilizumab reduces signalling by binding to either soluble or membrane bound IL-6 and preventing its engagement with the corresponding receptors, thereby reversing symptoms of even life-threatening CRS [56]. CAR-T-cell-related encephalopathy syndrome (CRES), also described more generally as neurological toxicity, includes symptoms such as delirium, seizures, inability to speak, muscle spasms, and confusion [51]. Although the cause or causes of CRES are not yet clear, it has been suggested that elevated cytokine levels or direct effects of CAR T-cells on the central nervous system may play a role [57]. On-target off-tumour toxicity is another concern, especially with regards to the identification of new TAAs for attack by CAR T-cells. As mentioned previously, the ideal TAA is expressed consistently on cancer cells and at low levels, if at all, on normal tissues [28]. On-target off-tumour toxicity occurs when CAR T-cells attack the aforementioned normal tissues that express the relevant TAA, which has resulted in symptoms ranging from B-cell depletion to death [51]. In a study examining the efficacy of HER2-targeting CAR T-cells for the treatment of solid tumours, a patient with metastatic colon cancer developed respiratory distress, low blood pressure, decreased heart rate, and gastrointestinal bleeding, leading to her death [58]. It has been suggested that this fatality was due to the high magnitude of the dosage of CAR T-cells, which engaged with HER2 molecules found in pulmonary tissue, subsequently releasing inflammatory cytokines that led to severe CRS and death [58]. Further research using HER2-targeting CAR T-cells at a lower dose has shown underwhelming results, with only four out of seventeen patients showing stable disease following treatment [59]. However, these trials have also shown minimal toxicity, with only one patient experiencing a fever that was alleviated with ibuprofen [59].

6. Avenues for Future Research

Due to the recent nature of the literature surrounding CAR T-cells, a great deal of further research is needed. The theoretical long-term effects have not yet been observed. Certain blood cancers have demonstrated potential resistance to CAR T-cells via lineage switches, a process in which the phenotype of cancer cells changes [60]. In the case of leukemia, treatment with CD19-targeting CAR T-cells can cause antigen loss, and this plasticity may render CAR T-cells ineffective with respect to long-term immunosurveillance [60]. In addition, the use of lentiviruses as a vector may pose risks to the patient should vector DNA be inserted into the T-cell, potentially causing the T-cell to become malignant in a process known as insertional oncogenesis [61]. The locus at which the majority of insertional oncogenesis takes place is silent in T-cells, thus making the risk of mutagenesis low, however future research is warranted [57]. The management of toxicity is also an area of high importance. Suicide genes, or genes that allow for the destruction of CAR T-cells within the patient's body should toxicity occur upon infusion, have been proposed [57]. For example, the herpes simplex thymidine kinase allows modified T-cells to be killed by Ganciclovir, an antiviral medication [62]. However, because the herpes simplex thymidine kinase tends to provoke an immune response, these cells may be rejected [57]. Another avenue that would make targeted CAR T-cell death possible is the expression of an antigen by the T-cell for which there is a corresponding monoclonal antibody [63]. For example, a CAR T-cell expressing CD20 could be destroyed by rituximab [63].

7. Conclusion

Ultimately, a balancing act on the part of researchers is what is needed to make CAR T-cells applicable to a wide variety of clinical situations. Mitigating toxicity while increasing the potency of CAR T-cells, with respect to solid tumours in particular, will widen their use beyond just blood cancers that express CD19.

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Software Daemons, Their History and Use in Modern Computing Science as an Answer to the Turing Test

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Abstract

Any sort of Technology can be divided into categories based on the amount of control a human has over it- Open Loop, which works only when a command is given to it, like a washing machine; Closed Loop, which uses feedback, such as a thermostat that regulates temperature within a house based on the temperature it detects on the outside; and Adaptive or Autonomous, where the control system 'learns' and adapts based on information it receives. For instance, Parker Atlas, Boston Dynamics' Humanoid bot that can sense and jump onto objects,[1] can be said to be an autonomous system. However, between Closed Loop control systems and Adaptive Technology, there exists a subset of sorts- Bots: A system that performs a series of pre-defined functions and has the potential to learn from its environment and perform more advanced actions within its predefined parameters. In this review, I look at the history of these pieces of code, from Socrates' Daemon, to the Child Machine; and examine the applications these bots could currently have in Computing Science in general and as an answer to the Turing Test in Specific.

Keywords — Programming, Computing Science, Daemon

1. Introduction

The British mathematician Alan Turing proposed the Turing Test as a replacement for the question "Can machines think?" in his 1950 Mind article 'Computing Machinery and Intelligence'[2]. Since then, Turing's ideas have been widely discussed, attacked, and defended over and over. At one extreme, Turing's paper has been considered to represent the "beginning" of artificial intelligence (AI) [3] and the Turing Test has been considered its ultimate goal. At the other extreme, the Test has been called useless, even harmful [4]. In between are arguments on consciousness, behaviorism, the 'other minds' problem, operational definitions of intelligence, necessary and sufficient conditions for intelligence-granting, and so on [5, 6, 7, 8].

In multitasking computer operating systems, a daemon is a computer program that runs as a background process, completing automated tasks without a need for interaction from the user. Named after Maxwell's Daemon, these computer programs could be used to perform or optimise practically any repetitive task [9].

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If carefully unpacked, the word daemon uncovers a provocative and useful dualism [10]. It is of more than academic importance that we learn to think clearly about the actual cognitive powers of computers, for they are now being introduced into a variety of sensitive social roles, where their powers will be put to the ultimate test: In a wide variety of areas, we are on the verge of making ourselves dependent upon their cognitive powers [11]. The key to these cognitive powers lies in the functions that these computers perform in the background, running calculations, deciding the priority of tasks, deciding the best way to allocate memory etc.; all of which it does through daemons and other repeating functions. It is through these daemons that computers can even hope to achieve the cognitive powers that humans have.

As machine learning aims to address larger, more complex tasks, the problem of focusing on the most relevant information in a potentially overwhelming quantity of data has become increasingly important. For instance, data mining of corporate or scientific records often involves dealing with both many features and many examples, and the internet and World Wide Web have put a huge volume of low-quality information at the easy access of a learning system [12, 13]. Similar issues arise in the personalization of filtering systems for information retrieval, electronic mail, netnews, and the like [14].

In the following review, I will attempt to provide an outline of their many applications, highlight the potential limitations to the studies, and point out future topics for exploration. I have considered several research articles and Theses associated in favour of the usage of bots and daemons in AI and some against. Thus, I will attempt to provide a balanced view of the long-term benefits and risks associated with bot use.

2. The Origin of Species

It is said that one of Socrates's friends took council of the Oracle of Delphi and inquired the name of the wisest person in the world, and the Oracle replied "Socrates" [15]. When Socrates found out about this, he tried to prove her wrong, and started on a life-long quest to search out wisdom in others by asking them questions - a method known as maieutics [16], an attempt to 'give birth' to the latent truth inside a person. Thus was Socrates' journey prompted [17].

Socrates's death came at the hands of the Athenian polity, when they charged him for asebeia (impiety) on two counts: corrupting the youth of the city (through his maieutics) and failing to acknowledge the gods of the city and introducing new gods. They gave him the choice of exile or death, and Socrates chose death by wilful consumption of a poisonous hemlock beverage [18]. It is easy to focus on the fact that he was sentenced to die because of his philosophizing, but we cannot dismiss the latter half of his sentence of impiety, that he failed to acknowledge the gods of the city and attempted to introduce other gods [19]. Why would they accuse Socrates of this? What did it mean?

"They are the envoys and interpreters that ply between heaven and earth, flying upward with our worship and our prayers, and descending with the heavenly answers and commandments, and since they are between the two estates, they weld both sides together and merge them into one great whole. It is only through the mediation of the spirit world that man can have any intercourse, whether waking or sleeping, with the

gods. And the man who is versed in such matters is said to have spiritual powers, as opposed to the mechanical powers of the man who is expert in the more mundane arts."

- Plato while speaking about Socrates' Daemon in 'The Symposium' [20]

Socrates had a bot. Not in the literal sense, of course. But Socrates had a non-human helper, or so he claimed. He called this entity a daemon. Socrates' Daemon had many real, hard-coded linguistic and symbolic links with today's bots. It was Intelligent and ready to offer advice based on the situation without prompting, seemingly performing background functions without Socrates' interference.

In his 1867 thought experiment, "Maxwell's Demon" [21], James Clerk Maxwell attempted to show that thermodynamics is not strictly reducible to mechanics. Maxwellian Demons are mechanical devices that carry out measurements on a thermodynamic system, manipulate the system so as to extract work from it, and erase all records of the measurement outcomes [22]. If successful, they decrease the total entropy of the universe, thereby violating the Second Law of Thermodynamics [23]. Monitoring the speed at which the various molecules bounced around the chambers, the daemon could tell which specific molecules contained high or low energy states. Sliding a door open at intervals, he could separate the molecules into two different groups based on relative energy level [24].

Neither inward oracle nor false god, Maxwell's Daemon was hardly evil. He was merely a little otherworldly helper; wouldn't it be grand to be able to have little helpers fulfilling our wishes and doing our bidding? Maxwell's Daemon represented wishful thinking on a grand scale; until computer science.

3. The Imitation Game

The opening sentence of Turing's 1950 paper declares "I propose to consider the question, 'Can machines think?' [2] The paper provides a philosophical framework for answering this question. These 7 sections are briefly summarised below. The Imitation Game Often referred to as the "Turing test", this is a form of parlour game involving a human interrogator who alternately questions a hidden computer and a hidden person in an attempt to distinguish the identity of the respondents. The Imitation Game is aimed at providing an objective test for deciding whether machines can think.

Critique of the New Problem. Turing discusses the advantages of the game for the purposes of deciding whether machines and humans could be attributed with thinking on an equal basis using objective human judgement.

The Machines Concerned in the Game. Turing indicates that he intends digital computers to be the only kind of machine permitted to take part in the game. Digital Computers. The nature of the new digital computers, such as the Manchester machine, is explained and compared to Charles Babbage's proposals for an Analytical Engine.

Universality of Digital Computers. Turing explains how digital computers can emulate any discrete-state machine.

Contrary Views on the Main Question. Nine traditional philosophical objections to

the proposition that machines can think are introduced and summarily dismissed by Turing.

The Child Machine. In the final section of the 1950 paper Turing addresses the motivation and possible approaches for such endeavours [25, 26, 27]. Turing goes on to discuss three distinct strategies which might be considered capable of achieving a thinking machine. These can be characterised as follows: 1) AI by programming, 2) AI by ab initio machine learning and 3) AI using logic, probabilities, learning and background knowledge [25, 28].

In the next sections we discuss various phases of AI research as it has been conducted over the past half century.

4. The Chinese Room Problem

The argument and thought-experiment now generally known as the Chinese Room Argument was first published in a paper in 1980 by American philosopher John Searle. It has become one of the best-known arguments in recent philosophy. It is one of the best known and widely credited counters to claims of strong artificial intelligence (AI)—that is, to claims that computers do or at least can (someday might) think. According to Searle's original presentation, the argument is based on two key claims: brains cause minds and syntax doesn't suffice for semantics [29].

Searle's Chinese Room experiment parodies the Turing test and echoes René Descartes' suggested means for distinguishing thinking souls from unthinking automata [30].

Its target is what Searle dubs "strong AI". In case of strong AI, Searle says, "the computer is not merely a tool in the study of the mind, rather the appropriately programmed computer really is a mind in the sense that computers given the right programs can be literally said to understand and have other cognitive states" [31]. Searle contrasts strong AI with "weak AI". In case of weak AI, computers just simulate thought, their seeming understanding isn't real understanding (just as-if), their seeming calculation is only as-if calculation, etc.

Searle asks us to imagine that a man is seated in a sealed room with 2 doors: one allowing input from one source outside the room (in the form of a slot) and one allowing output to the source outside the room (also in the form of a slot). The input from the outside source are Chinese squiggles that have been printed on card, but to the man in the room they are nothing more than incomprehensible gibberish (since he does not know the first thing about Chinese). The man is told that upon receiving the input squiggles, he must open a heavily-indexed reference book, wherein he must scrupulously track down the squiggle he received and find the matching squiggle of another sort. Once the man finds the matching squiggle, he must record it on an output piece of card and send it back through the output door's slot. Unknowingly the man has just performed some sort of translation that is altogether opaque to his understanding [32].

To the outside source, the Chinese room as a whole, is a sort of system and is being treated as a subject of a Turing test. The interested parties of the outside source are typing in questions in Chinese and receiving answers in Chinese. If the Chinese room

is of good quality, then it should be possible to convince the interested parties that the room, or something inside it, is intelligent, thus suggested that the room, or something inside it, could pass the Turing Test. Searle suggests that this is an error, as the man in the room does not have any conscious states that exhibit and sort of understanding of the questions that he receives. To him it is all just squiggles. Certainly it might simulate intelligence impressively, but Searle suggests that this is precisely the problem, since it means only that we have an automata that is extremely good at fooling our test [33].

5. NLP and the ELIZA Effect

ELIZA was a computer program written by Joseph Weizenbaum of MIT University in the late 60s which is considered to be the first chatterbot, i.e. a program that can partially mimic a human in a conversation with a human [34]. In many ways ELIZA is has provided insights not just into what a serious NLP (Natural Language Processing) system should achieve but also has provided a lot of insight into human reactions to computer systems which look like "intelligent" systems but are not so. ELIZA was not meant to be an AI system, it was meant to be a toy or a parody system. ELIZA was first implemented in the SLIP language (Symmetric List Processor), a language incensed by Weizenbaum himself as an extension to FORTRAN but with better functionality to process doubly linked lists [35]. For its time, ELIZA was revolutionary in many aspects, as interfaces were not really common in the computers of the late 60s due to the absence of personal computing and thus the idea of interactive computation had not arisen yet or entered into popular fancy. Even though the perceived intelligence of it was an illusion (and a very bad illusion) the fact remains that it was the first genuine human machine interface (pretending to be a human – intelligent machine interface) attempting to use natural language [36].

As Weizenbaum discovered, many subjects who experimented with ELIZA got emotionally attached to it. Many did so despite Weizenbaum's informing them that there is no intelligence involved and that in fact ELIZA is not 'answering' them but only regurgitating a hardcoded script.

ELIZA was clearly just a daemon. It simply took input from a user and gave a previously stored vague response. It was a background process with no intelligence to speak of; the software equivalent of a ticket machine. However, the way Weizenbaum applied these processes into her code made her appear to be a form of Machine Intelligence. Another example of such a process would be Usenet's Serdar Argic.

6. Serdar Argic

In 1991, Usenet's culture and history discussions suffered under a flood of huge swaths of repetitive propaganda concerning the supposed Armenian murders of Turks in 1918 (history shows that the killing was the other way around), coming from a poster named Serdar Argic at a site known as zuma.UUCP [37].

Serdar responded to, seemingly, every and any Usenet post he could find that mentioned Turkey or Armenia, even in newsgroups that had nothing to do with either country. The poster was generally harangued with such phrases as "your

criminal Armenian grandparents" (even if the poster happened to be of a non-Armenian ethnicity) and with over-the-top subject headings such as "The Self-Admitted Crook and Liar", "The Criminal SDPA-ASALA Grandparents of The Gum Brain", or "A mouthpiece for the fascist x-Soviet Armenian Government". This was usually followed by a lengthy essay concerning the alleged Armenian mass murders [38].

Some participants tried to argue with Argic, but that only made matters worse as he replied to each post with more harangues, along with successively more hysterical accusations concerning secret Armenian conspiracies. Some watched in amusement, and some even wrote parodies mocking the overwrought style of the posts [39]. But the amusement quickly turned to annoyance when it became apparent that the sheer volume of Serdar Argic posts was overwhelming the discussions on the hardest-hit newsgroups.

It quickly became apparent, however, that his responses didn't have much intelligence behind them. For one thing, they followed a distinct repeating pattern. For another, Argic did not appear to distinguish between the nation and the bird: posts containing references to Thanksgiving turkey were as likely to become targets as posts discussing Turkey's foreign policy [32].

Over time, a consensus built on the Usenet community: Serdar Argic was not a person, but a computer program which scanned the news articles and responded to any article that contained certain words, plugging in the name of the article's writer ("John Sugaharo's criminal Armenian grandparents") and other random phrases. Because of the robotic nature of the responses, this program was promptly dubbed "the zumabot" [40].

Serdar Argic clearly wasn't a well-developed example of Artificial Intelligence. However, shrouded in the anonymity of the net, he might well have been some crazed man incensed by the Armenian Genocide. Is this not exactly what passing the Turing Test would mean? And if so, couldn't a more advanced daemon having ELIZA like qualities give us a clear answer the the Turing Test?

7. The Future of Bots: The Child Machine

Turing closes the Mind paper with the following statement: "We can only see a short distance ahead, but we can see plenty there that needs to be done." As the present article indicates, Turing's vision was far from myopic. Indeed, he foresaw many of the key issues which dominated Artificial Intelligence research over the last fifty years [2].

In his writings on intelligence and machinery, Turing often employs analogies. One analogy he states explicitly and calls the "guiding principle" of his investigation into "possible ways in which machinery might be made to show intelligent behavior" is "the analogy with the human brain" [3, 41, 42].

The analogy may not be precise, but it is pretty clear: humans undergo education processes for a portion of their lives (which Turing estimates at about the first twenty years of their lives), and their behavior after that is very much affected by the education they have received, even though they still receive other interference – most of the time, in fact. The point is to approximate the human process of education with some analogous process suitable for machines [3]. The major points of his proposal are that,

on analogy with a human's life, we plan for these three stages of a machine: first, there is the infant stage of a machine, which is a machine that has not been educated and is at least partly unorganized. It need not be a blank slate, but it is important that large amounts of its behavior are undetermined [2]. This is followed by the child-machine stage, during which the machine is educated. The first stage of education is to get the machine to a point where "it could be relied on to produce definite reactions to certain commands." Education involves a teacher who is intentionally trying to teach or modify the machine's behavior to effect some specific kinds of behavior. The machine's behavior is in flux during this time [3].

Even if the machine is given the means to educate itself using some kind of program during the child-machine stage, there is still oversight and monitoring by a teacher of sorts who checks up on its progress and intervenes if necessary. The machine that results when education is ended is supposed to behave in a way that can be predicted "in very broad outline" by someone familiar with how it has been educated — but its behavior might not, in fact probably will not, be fully predictable. Finally, there is the adult-machine, which is still capable of learning, but is also capable of quite complex behavior without additional intervention.

Thus, daemon qualities, if applied to a regular Machine Learning Process would be capable of so much more. Indeed, I believe that this would open up the door to a wide field of possibilities within the realm of Artificial Intelligence.

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