MSURJ

McGill Science Undergraduate Research Journal

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About mSURJ

mSURJ, the McGill Science Undergraduate Research Journal, is a student-founded, student-run initiative that benefits the McGill University research community. Our mission is to encourage, publish, and promote student research in all scientific disciplines.

Though the journal officially began in September 2005, this issue marks our re-launching as one of the first undergraduate journals in North America to select articles on a competitive, peer-review basis. Selection criteria include clarity, organization, and style; presentation and interpretation of results; motivation of the study based on literature; and contribution to advancing the field of study. The mSURJ editorial board handles the peer-review process by selecting one suggested reviewer and one anonymous reviewer, and adding its own review.

In addition to the journal, mSURJ boasts a variety of other forums to support our mission, including public lectures, writing and editing workshops, and information resources on-line.

Beginning next year, mSURJ will publish issues twice annually. Prospective student contributors and student editors are encouraged to contact the editorial board or visit our website – msurj.mcgill.ca – to learn more.

ON THE COVER

'Proportion Manifesto'
Designed by DEBBIE GELTNER

This composition portrays Leonardo Da Vinci's Vitruvian Man over a nautilus shell pattern. The Electron micrograph and microscope slide of spirochete-like cells in blood culture were provided by Dalius J. Briedis, Associate Professor in Microbiology and Immunology. The spiral reflects both the Golden Ratio and the Fibonacci sequence simultaneously, symbols of proportional perfection present in countless aspects of the known universe.

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Welcome to mSURJ

Dear Reader,

I ask you to reflect on the great achievements humankind has unveiled. The Manhattan Project brought nuclear energy to a new level; the Apollo Mission put a man on the moon; the human genome project mapped our DNA. We marvel at these achievements because they demonstrate scientific progress which was once thought impossible. Understanding how they led to turning points in world history is not obvious. The first step is to appreciate what it means to communicate science effectively.

It is unlikely that an article in this journal will make newspaper headlines. Scientific investigation is a lengthy process, and academia is only one part. We hope this journal inspires students to delve deep into their field of study and come up with original contributions. If our goal is to help humanity and the earth on a grand scale, we must share the importance of their work with a wider audience. This journal is a step in that direction.

Communicating science in clear, concise, and effective writing is difficult for junior and senior researchers alike. Challenges such as global warming have spurred a great deal of interest, but little progress has been made towards finding workable means to mitigate greenhouse gas emissions, let alone on agreeing on a strategy for climate stabilization. Many scholars have proposed hypothetical roadmaps for clean energy technologies, drawing on science and engineering as well as economics and international politics. This issue must be presented in an honest, straightforward manner if the public is to appreciate the urgency of this problem. Then, work towards a solution to the climate change energy challenge might usher in a new turning point in world history.

Science wears many hats. It can be elegant, complex, and beautiful. Ancient Greeks found solace in discovering the union of chaos and order in the natural world. Today, fascination with the human brain has inspired philosophical and neurobiological interpretations of approaches to consciousness. In scientific research of impending global crises or transcendent, abstract phenomenon, sharing one's ideas is equally important.

mSURJ, the McGill Science Undergraduate Research Journal, was launched last year as a student-founded, student-run initiative to benefit the McGill University research community. Our mission is to encourage, publish, and promote student research.

The journal gives undergraduate students experience in articulating and refining their ideas. They learn writing and editing academic articles, and have the opportunity to publish in a peer-reviewed journal which draws from the McGill community. mSURJ prides itself on being one of the first undergraduate journals to select articles on a competitive, peer-review basis. In this process, we unite student researchers, student editors, faculty supervisors, graduate students and faculty reviewers.

Our research and review articles represent significant forays into a wide range of disciplines. One article explores the electrocommunication behaviour of electric fish, while another deals with lung injury and immune response regulation. Our feature articles, in contrast, are short interest pieces with a different narrative style. By presenting a wide variety articles, we hope to give our readers a broad scientific perspective and to encourage scientific discourse within and between disciplines.

On behalf of the entire mSURJ Editorial Board, I thank you for supporting this initiative and applauding our student contributors.

Verily veritas,

Daniel Spitzberg
Editor-in-Chief
BSc 06, McGill School of Environment

Acknowledgements

The re-launching of mSURJ would not have been possible without the support of many individuals. With the help of our donors, advisors, and committee members, we are proud to present a new and exciting initiative that will be a permanent part of undergraduate life at McGill University.

First and foremost, we thank Professor Martin Grant, Dean of the Faculty of Science. We appreciate our very generous gift from the Dean's Discretionary Fund as a gesture of confidence in mSURJ.

We also thank all of our donors from within the Faculty of Science and the McGill community for their generous support.

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Science Undergraduate Society
SSMU Campus Life Fund

Mr. Victor Chisholm, Undergraduate Research Officer, provided mSURJ with crucial advice on administrative matters on countless occasions. We thank him for assisting us in improving our operations and establishing our permanence.

Our thanks go to Professor Frédéric Guichard, Department of Biology, for his guidance and welcome enthusiasm during the process of developing the mSURJ guidelines and for refining our peer-review process. Mr. Louis Houle, Schulich Science and Engineering Librarian, has generously provided us with information and instruction on publishing and distribution etiquette.

We also extend thanks to Professor Linda Cooper, Faculty of Science and Redpath Museum, who has recently joined mSURJ with great interest. Professor Cooper delivered a lecture to aspiring undergraduate researchers entitled "Writing, Editing, and Publishing in Science", an important extension of our mission, and will advise mSURJ editors and contributors in the future.

We also wish to thank more than two dozen professors and graduate students who graciously offered their time to review students' article submissions.

Finally, we wish to thank the students whose dedication made mSURJ possible. Every year, mSURJ invites undergraduate students to submit manuscripts for publication and to apply for positions on the editorial board. We had an overwhelming response this year, and we thank the many students who were inspired to become involved.

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Variations in the electrocommunication behaviour of the weakly electric fish *Apteronotus Leptorhynchus*

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Abstract
***Apteronotus leptorhynchus* (Gymnotiformes) is a weakly electric fish which produces wave-like electric organ discharges as well as brief modulations in the frequency of these discharges, termed chirps. Thought to be used for communicating, this study explores variations in previously described chirp types by investigating fish chirping behaviour through recordings of external electric field modulations, and several novel phenomena were characterized. An alternative form of the type 1 chirp was discovered that is preceded by a gradual rise in frequency lasting 8-10 ms. Two sub-categories of type 2 chirps emerged, distinguished by frequency excursions of 23-86 Hz and 86-183 Hz respectively, and individual fish chirp almost exclusively within a single subgroup. Type 3 chirps occupied a much lower range of durations (10-60 ms) than those previously described, and no type 4, 5 or 6 chirps were observed. Finally, a single unusual chirp characterized by an extreme frequency and amplitude modulation raises interesting questions about chirp production mechanisms. While we cannot exclude that the differences observed across chirping studies are a consequence of subtle differences in methodology, we propose that geographical variation in electrocommunication behaviour should be investigated as an alternative explanation with possible implications for speciation.**

Keywords
Electrocommunication: *communication through weak electric fields generated by the specialized cells of an animal which can be perceived by special receptors in the skin*; **chirps**: *a short and sharp rise in the frequency of electrical discharges which create the animal's external electric field*; **Apteronotus leptorhynchus**: *the electric fish used in this study*.

Introduction

Weakly electric fish generate weak electric fields around themselves which they use to monitor their environment and interact with conspecifics. This field is generated by means of electrical discharges produced by a group of specialized cells called electrocytes that make up the electric organ.

The brown ghost knifefish (*Apteronotus leptorhynchus*, **Figure 1)** used in this study is a native to freshwater habitats of Central and South America and is a wave-type electric fish whose electric organ discharges (EODs) are quasi-sinusoidal and extremely regular (Moller 1995). The frequency of EODs in this fish are set by the pacemaker nucleus in the brain and can be driven up by excitatory input from a prepacemaker nucleus to produce short and sharp rises in EOD frequency, called "chirps", which are frequently studied in this and other species of wave-type fish (Dunlap et al. 1998; Engler & Zupanc 2001; Zakon et al. 2002). Chirps are produced primarily by male fish in the presence of conspecifics and behavioural evidence suggests that they play an important role in aggressive and courtship encounters (Hagedorn & Heiligenberg 1985; Dunlap & Larkins-Ford 2003).

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Figure 1

In laboratory studies of electric communication behaviour, an artificial electrical stimulus is typically used to mimic the EOD of a conspecific and elicit chirping (Larimer & MacDonald 1968; Dye 1987; Maler & Ellis 1987; Zupanc & Maler 1993; Dulka & Maler 1994; Dunlap et al. 1998; Engler et al. 2000). At least four main chirp types (named types 1 through 4) have been identified to date based on their duration, frequency excursion, and amplitude reduction characteristics (Engler et al. 2000; Zupanc et al. 2006). However, fish tested by different experimental groups have not performed completely consistently. For example, great variation in the range of frequency excursions is often reported for the same chirp type. Zupanc et al. (2006) describes type 2 chirps as having frequency excursions up to 156 Hz, but in an earlier report Zupanc (2002) placed them in a much lower range around 50 Hz, and Kolodziejski et al. (2005) describe an even lower range around 20 - 40 Hz. Furthermore, different studies often report completely different and sometimes unique chirp types. Beyond the typical chirp types, Kolodziejski et al (2005) describes a long modulation with a frequency excursion of only 10 Hz lasting for up to 500ms, while Zupanc et al. (2006) describes novel modulations (types 5 and 6) characterized by frequency depressions rather than increases.

This investigation began as an exploratory study intended to search for further variants in chirping behaviour in order to compare them to the results of previous investigations, and several novel chirp characteristics emerged, serving as a further testament to the variability of this system. While some researchers have attributed these discrepancies to differences in stimulation procedures, there is no obvious difference in methodology which explains finding different chirp structures. Instead, we propose that this emergent variability between studies might be a product of local geographical variations in chirping behaviour emerging between studies using fish collected at different geographical locations.

Materials & Methods

Animals

Eight brown ghost knifefish (*Apteronotus leptorhynchus; Gymnotiformes, Teleostei*) originating from the Peruvian Amazon were used in this study (DAP Aquatic Haven,

Etobicoke, ON). Fish ranged in length from 12.1 – 18.5 cm, and EOD frequencies at 28 ± 1 ºC ranged from 889 – 1025 Hz **(Table 1)**. Because female fish do not typically respond to external electrical stimulation with chirps (Zupanc 2002), only males were admitted to the experiment by excluding fish that did not respond to a trial stimulus and whose baseline EOD was below the threshold of male EOD frequencies (850Hz).

Between experiments, fish were kept in communal tanks, under a 12 h light / dark cycle, with other males as well as females. These tanks were maintained at approximately 28 ºC, pH 7.0 - 7.5, conductivity 100 - 150 µS cm-1, and aquarium water was continuously filtered and aerated.

EOD Recording

Recordings of chirping behaviour were performed in a test tank (31 x 32 x 61 cm) whose water was conditioned to match that of the respective home tank. To obtain recordings, fish were housed in a cylindrical chirp chamber (19.0 x 5.6 cm) similar to those used in previous studies (e.g. Dye 1987).

EOD fluctuations were recorded using paired carbon-rod electrodes, 6 mm in diameter, placed at the ends of the chirp chamber **(Figure 2)**, and were simultaneously monitored on an oscilloscope. The signal was amplified 500x (bandpass-filter 300 - 3000 Hz) on a differential amplifier (Model 3000, A-M Systems, Sequim, WA) before being digitized at a sampling rate of 20 kHz via a Digidata 1320-A, 16-bit data acquisition device and accompanying Axoscope (v9.0) software (Axon Instruments, Sunnyvale, CA). Recordings were analyzed in MATLAB v7.0 (MathWorks, Natick, MA) using custom-written programs.

Baseline EOD was determined in MATLAB, by calculating the median value of the instantaneous frequency measured during a 2 second sample recording taken prior to each trial.

Electrical Stimulation

The stimuli were sine waves generated by MATLAB and output through the computer's 16-bit soundcard. The signal was fed through an attenuator (SmartStep 8310-1-2-R Attenuator; Aeroflex/Weinschel, Frederick, MD) and an analog stimulus isolator (Model 2200 A-M Systems, Sequim, WA) and was delivered by paired carbon rod electrodes (6 cm long and 6 cm apart) situated orthogonally on either side of the fish **(Figure 2)**. Stimulus amplitude was calibrated with a pair of silver wire electrodes at the location usually occupied by the fish and was set to 1 mV/cm by adjusting the attenuator.

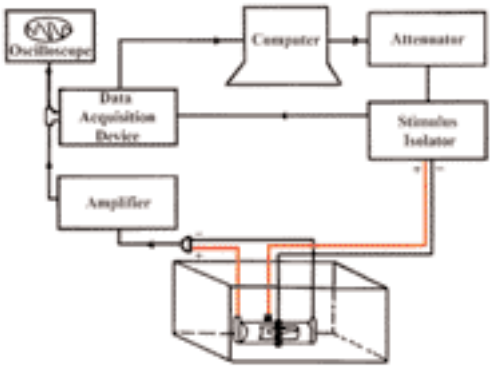

Figure 2. An information flow diagram of key experimental components and their connectivity. White electrodes represent the recording electrodes while black electrodes represent stimulation electrodes

Experimental Design

Fish were tested for chirping response in the presence of the artificial EOD, whose frequency was defined as the fish's baseline EOD frequency immediately prior to each trial, plus the difference frequency (Df) being tested (Df = -300, -200, -100, -50, -20, -10, -4, 0, 4, 10, 20, 50, 100, 200, 300 Hz).

Different stimulation frequencies were ordered randomly and served primarily to mimic the presence of male and female conspecifics to elicit the production of different chirp types.

One fish was tested per day. Each fish was measured and photographed before being placed inside the chirp chamber, and allowed to acclimate for 10 minutes prior to testing. The stimulus regime consisted of 15 trials corresponding to the randomly ordered Df, each lasting 60 seconds, separated by 2 minutes of rest to avoid habituation. Each trial recording was saved to file, and once all trials were completed, fish were returned to a separate compartment of the communal tank.

Data Analysis

Data were analyzed primarily using MATLAB. Peak-to-peak amplitude was calculated using Hilbert Transform, and instantaneous EOD frequency was calculated by determining the periods between successive zero-crossings of the EOD signal. Chirps were detected automatically when the instantaneous frequency rose to ≥ 10 Hz above baseline frequency and did not return below that value for at least 5 ms. When chirps were found, their duration, frequency excursion (defined as the maximum frequency reached minus the baseline EOD), and time of occurrence relative to the start of stimulation were calculated and automatically recorded in a text file upon completion of analysis. In-depth analysis of chirp structure was also performed in MATLAB by expanding and smoothing chirps within a given time interval using a 3-point gliding average function. Chirp frequency excursions, which typically increase with increasing temperature, were normalized to a temperature of 28 ºC by using the slope of a linear fit to a correlation of frequency excursion and temperature that indicated a small rise in frequency excursions of 2.9 Hz per degree (data not shown).

Results

Chirp Types Observed

A total of 3338 chirps produced by 8 fish in 112 individual trials were recorded and examined in the course of this study **(Figure 4a)**. Qualitative analysis revealed at least three chirp types produced in response to electric stimulation, each characterized mainly by frequency excursion from the fish's baseline and by duration. Many chirps observed resemble those described previously as types 1 through 3 (Engler et al. 2000; Zupanc et al. 2006), and so the same nomenclature shall be employed here to facilitate comparisons. Nevertheless, several novel and interesting variations of established chirp types were observed throughout this study and will be described presently. No chirps were found which corresponded to the "classical" type 1 chirp, characterized by a steep EOD frequency increase to a peak of 338 - 537 Hz above baseline followed by an equally steep decline leading to an undershoot of the baseline EOD and having a total duration of 18 - 31ms (Zupanc et al. 2006). Instead, what appears to be a variant of this type consisted of a gradual and approximately linear rise of the EOD followed by a sudden steep rise to the peak frequency and an equally steep decline and undershoot typical of a type 1 chirp (n = 5, **Figure 3a)**. While the total chirp duration of 15 - 25ms is similar to that for type 1 chirps, the frequency excursions observed were 400 - 700 Hz, a higher range than previously observed for chirps of this type. Because of their distinctive gradual rise and their resemblance to type 1 chirps,

these chirps will henceforth be referred to as gradual rise type 1 chirps (GR-1).

Chirps corresponding to type 2 were most common (n = 3313, **Figure 3b**), and conformed well to the classical definition of a chirp lasting 10 – 27 ms and having a relatively small frequency excursion of 50 - 200 Hz without a terminal undershoot (Zupanc et al. 2006). Interestingly, two previously undescribed sub-categories of type 2 chirps emerged consisting of a group concentrated in the 23 - 86 Hz and 86 - 183 Hz ranges respectively, with few chirps occurring at the boundary as defined by the minimum of this clearly bimodal distribution **(Figure 4b)**. Each fish produced chirps almost exclusively within one subgroup. This bias was not influenced by variations in test temperatures, as the chirp distributions were essentially identical after corrected to a standard temperature of 28 ºC **(Table 1, Figure 4c)**.

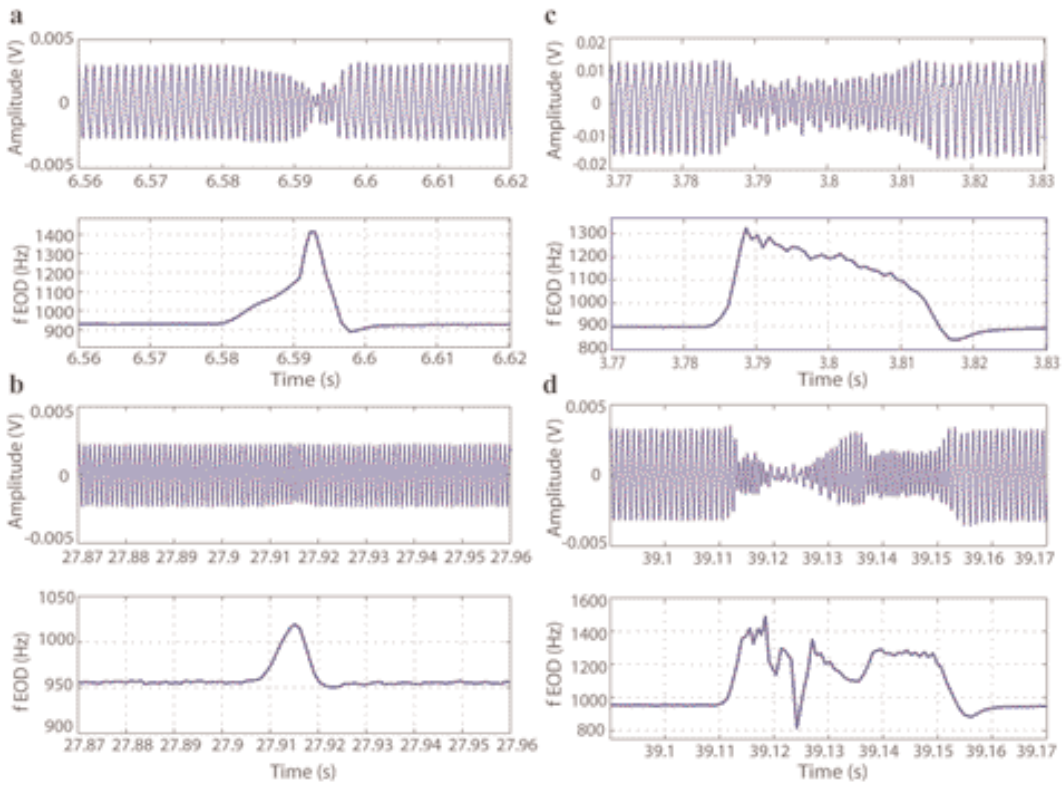

Figure 3. Chirp types observed. Instantaneous frequency plots were smoothed using a 3-point gliding average function (note different scaling). [a] A typical gradual rise type 1 chirp (GR-1). [b] A typical type 2 chirp. [c] A typical type 3 chirp. [d] A chirp with extreme frequency and amplitude modulations. fEOD: frequency of electric organ discharge.

A third group of chirps correspond to those of type 3, as distinguished having a sharp rise in frequency of several hundred Hz sustained with some jitter over 40 – 100 ms followed by a sharp return to baseline accompanied by an undershoot (Engler et al. 2000; Zupanc 2002, Zupanc et al. 2006.) The corresponding chirps observed during this study occupied the lower part of this range, from 10 to 60ms (n = 20, **Figure 3c)**. No chirps corresponding to those of type 4, 5, or 6 were observed.

Because type 2 chirps constitute the bulk of those recorded they will be henceforth referred to as "small chirps". Chirp type 1 and 3 analogues were rarer and will be grouped together under the term "large chirps" for the purposes of quantitative data analysis. However, each chirp type was considered individually in qualitative analysis.

An Unusual Chirp

One unique chirp was recorded which shows some unusual characteristics **(Figure 3d)**. It was the sole chirp produced by fish 4 in response to a stimulation frequency of -300 Hz. While the chirp's duration (41 ms) resembles that of type 3 chirps, its frequency excursion of 1057 Hz exceeds that of commonly produced chirps of any type and puts the fish's EOD at slightly over 2000 Hz at the peak of the chirp. This chirp resembles a type 3 chirp in shape, but the usual plateau is more variable and is punctuated by one brief but dramatic frequency collapse to well below baseline EOD rate accompanied by a simultaneous amplitude collapse nearly to zero.

Effect of Stimulation Frequency Difference (Df)

Stimulation of fish with signals of varying frequency differences yielded responses of varying degrees. The magnitude of the response was gauged by the number of chirps produced during each stimulation period at a particular Df **(Figure 5)**.

The number of small chirps was greatest for small frequency differences between Df = ±10 Hz, and in most cases small chirps were rare or absent outside the range of -100 Hz ≤ Df ≤ +100 Hz. In contrast, large chirps (corresponding to type 1 and type 3 chirps) were usually absent at small absolute Df but became more common for larger absolute Df ≤ -100 Hz or ≥ 100 Hz.

Discussion

The present study examined several aspects of the chirping behaviour of A. leptorhynchus in order to investigate variations in chirping behaviour in comparison to previous studies, which may indicate that these fish have a richer communicatory repertoire than was previously thought.

Chirp Types Observed

Chirps corresponding to those of type 2 were by far the most frequently observed. Although identical in structure to those previously described, two distinct subgroups emerged within this category that have been noted in passing by Zupanc & Maler (1993), but were eventually combined and left uncharacterized. The reappearance of a strong bimodal distribution during this study provides evidence for the existence of real subgroups, and although our sample size was small (8 fish), the larger number of chirps recorded (3338) mitigates this limitation. This phenomenon may even explain the discrepancies in type 2 frequency excursion ranges previously reported (Engler et al. 2000; Zupanc 2001; Zupanc 2002; Kolodziejski et al. 2005; Zupanc et al. 2006). Here, we demonstrate for the first time that these sub-categories occupy the frequency excursion ranges of 50 - 86 Hz and 86 - 120 Hz, where the boundary is defined as the minimum of the bimodal distribution. Interestingly, individual fish produce chirps almost exclusively in a single sub-category. The basis

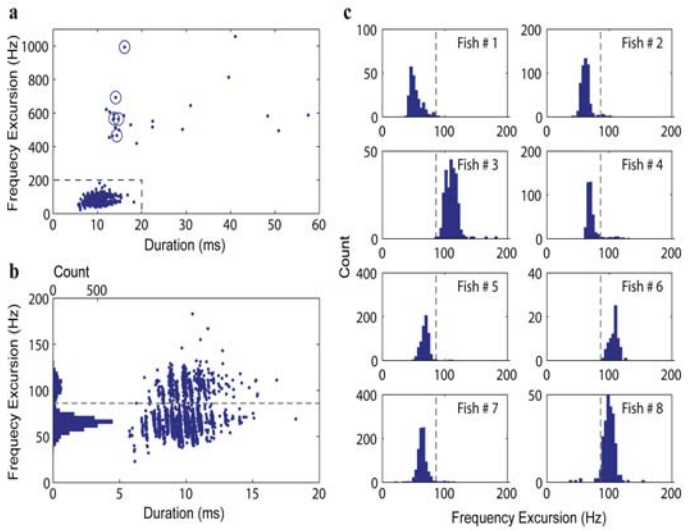

Figure 4. [a/b] Cumulative plots of frequency excursion versus duration for small and large chirps (note different scaling). Mean duration and frequency are plotted as a circle with standard deviation bars radiating outwards. [c] Frequency excursion range of small chirps produced by individuals, demonstrating a bias to produce chirps predominantly either above or below a threshold of approximately 80Hz.

for this selection is still unknown and we speculate that it might reflect social status or perhaps the difference between immature and mature males, in which case it would be interesting to see whether there is a shift from one chosen subgroup to the other as young males mature.

Chirps loosely corresponding to those of type 1 were observed, but much less frequently. The main difference from the traditional type 1 chirp is the presence of a previously undescribed gradual frequency rise preceding the frequency spike. Frequency excursions also ranged slightly higher than has been previously observed for type 1 chirps, most recently defined as 338 - 537 Hz (Zupanc 2002; Zupanc et al. 2006), instead easily reaching 700 Hz. These chirps could be construed as an entirely novel chirp type or as a type 1 chirp to which an additional motif has been added, and whether or not they fulfill the same function as type 1 chirps do in other members of this species remains to be seen.

Fish Number	Total Body Length (cm)	fEOD at 28˚C (Hz)	Temperature During Trials (˚C)
1	18.5	889 - 920	28.0
2	15.5	1025 - 1035	25.5
3	13.8	928 - 935	29.0
4	13.7	950 - 957	28.0
5	12.1	989 - 1011	27.5
6	13.6	942 - 949	28.9
7	12.8	950 - 962	29.0
8	13.6	898 - 903	27.9

Table 1. Key physiological characteristics of each subject fish.

Chirps corresponding to those of type 3 were observed least frequently of all. While these chirps adhere very well to the frequency modulation profile previously described, their 10 – 60 ms durations occupy a much lower range than the 48 – 128 ms chirps of this type that are typically observed (Zupanc 2002; Zupanc et al. 2006). Although this range creates some overlap with type 1 chirps, their distinct shapes distinguish them from one another. Further investigation is required to accumulate enough specimens of these rare chirps for quantitative analysis in order to determine the true bounds of their duration range in this group of fish.

No chirps corresponding to those of types 4, 5 or 6 were observed, which may be an intrinsic trait of this group of fish or may simply reflect their general rarity or even the absence of real conspecifics which might more easily provoke these chirps (Zupanc et al. 2006).

The central findings on the effect of stimulation frequency on the incidence probability of type 1, 2 and 3 chirps agree with those of previous studies, in which small chirps are produced predominantly at small Df ($-10 \leq Df \leq 10$) while large chirps are produced predominantly at large Df ($-50 \geq Df \geq 50$) (Bastian et al. 2001; Engler & Zupanc 2001). This is consistent with the interpretation of the earlier studies that small chirps play a role in intrasexual communication, whereas large chirps are involved in intersexual communication, in which the difference in EOD frequency is larger. Since the relationship between chirp type and stimulation frequency has been well described by these previous studies, and since our results agree with those studies, we did not analyze this interaction in further depth.

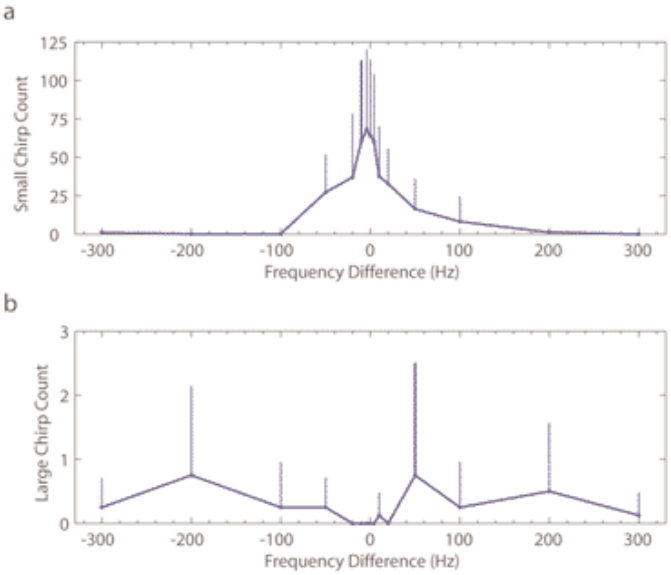

Figure 5. [a/b] Mean chirp frequency distributions for all fish at each Df, with the frequency of small chirps plotted in blue and the frequency of large chirps plotted in red (note different scaling). Positive standard deviation bars (the negative standard deviation bars being symmetrical) are plotted for each mean value.

Extreme frequency and amplitude modulations

The occurrence of a novel and exceptional chirp type with extreme frequency and amplitude modulations beyond those observed in any previously described chirps, although it was singular among all the chirps observed, warrants discussion as to the possible underlying mechanisms because of the very fact that it is physically possible in an apparently healthy fish. While its duration (41 ms) and the presence of an undershoot make it similar to type 3 chirps, its maximal frequency excursion of 1057 Hz, as well as the dramatic frequency and amplitude collapse, distinguish it from any chirps previously described. Zupanc & Maler (1993) briefly mention rare chirps which "displayed very large frequency increases…coupled with a near collapse of the EOD amplitude", but they were left unexamined and likely represent a similar type of modulation which will be considered more carefully here.

While the frequency and amplitude collapse observed in this chirp might be an artifactual response caused by inaccurate measurements of EOD cycles at low values, another possible explanation is a strong momentary desynchronization of the discharges of the individual electrocytes in the electric

organ (EO) resulting in maximally destructive interference. This raises the interesting question of whether or not the A. leptorhynchus EO can fire asynchronously, a question which would be simple to address in future work by recording neural activity from different parts of the EO in an immobilized fish exposed to electrical stimuli. If the EO can indeed fire asynchronously, this would open a dimension for generating communication signals that has not been considered thus far. Alternatively, the collapse could be explained by the refractory period of the electrocytes. If the refractory period of the electrocytes were longer than the refractory period of the relay cells of the pacemaker nucleus driving them (Smith & Zakon 2000), then the electrocytes could miss EOD cycles at the highest driving frequencies. This explanation might identify this chirp as a type 3 chirp which reached such an exceptionally high frequency excursion that just such a collapse took place, and further investigation on a cellular level is required to investigate this hypothesis.

Conclusions

The results of this study provide numerous examples of variations in all properties of chirping behaviour when compared to previous bodies of work on A. leptorhynchus. While it seems increasingly clear that previously defined chirp categories may not be as rigid as it was previously believed, these findings also raise interesting questions about the nature of chirp behaviour variability across studies.

As no obvious methodological reasons have arisen to explain the structural differences in chirps obtained during different studies, the possibility of natural geographical variations in chirping behaviour remains a possibility that warrants investigation. This tentative hypothesis is supported by the fact that the chirp behaviour of the fish used in this study, which to the best of our knowledge originate from Peru, are disparate from the behaviour of fish used in another recent study of chirp behaviour by Zakon (2002) which originated from a geographically distinct river system in Columbia (Zakon personal communication, February 5, 2007).

The phenomenon of unique forms of communication in different populations is well documented in other species of fish, as well as in insects, birds, and even mammals, and may be an indicator of incipient speciation (Martens 1996; Pillay 2000; Boughman 2002; Yamada et al. 2002; Magurran & Ramnarine 2004; Patten et al. 2004). Consequently, the examination of chirping variation in geographically isolated populations of A. leptorhynchus could provide an opportunity to investigate real-time speciation as a result of sensory drive. Future work should aim to compare chirp characteristics between fish from different known geographical locations, and if systematic differences do exist, to determine whether they might form the basis for reproductive isolation between populations which could lead to speciation.

Acknowledgements

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Lipopolysaccharide-induced lung injury does not require production of reactive oxygen species by NAD(P)H oxidase

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Abstract

We examined toll-like receptor 4 (TLR4) protein signaling in the innate immune response to the invasion of the body by gram negative bacteria. When humans are exposed to lipopolysaccharide (LPS or endotoxin), neutrophils attach to the endothelium and infiltrate the lung. This involves activation of TLR4 on the endothelium and on neutrophils and the subsequent activation of cell signaling pathways, such as NF-kB and other transcription factors that stimulate reactive oxygen species (ROS) production, to promote the inflammation process. The activation of endothelial TLR4 produces a generalized inflammatory response that includes activation of adhesion molecules which results in the accumulation of neutrophils on the endothelial side of the inflamed tissue. To test the role of ROS production by NAD(P)H oxidase in this process, wild type (WT) mice and mice deficient in the p47phox component of NAD(P)H oxidase enzyme (KO) were challenged with LPS and the degree of pulmonary injury was assessed. In both WT and KO mice maximal lung injury was observed 4 hours after challenge with LPS. In contrast to WT mice, lung injury in KO mice was more severe and persisted for 8 hours

Keywords

Sepsis: *A generalized inflammatory response produced by invading organisms such as bacteria or their toxins*; **Edema**: *Abnormal accumulation of water-based fluid in the tissues or cavities of the body, often causing visible swelling*; **Endotoxin**: *the lipopolysaccharide coating of gram negative bacteria that is released only upon death of that cell and acts through toll-like 4 receptors to activate an innate immune response*; **Innate immunity**: *The early response of an organism to a foreign agent. It does not require previous exposure to the agent or the production of lymphocytes*; **Neutropenia**: *A hematological condition characterized by an abnormally low number of neutrophils, this makes affected patients more susceptible to bacterial infection;* **Cytokine**: *Proteins and peptides that signal a variety of immunological and inflammatory responses by activating other immune responsive cells*.

Introduction

Sepsis is a systemic response to infection which is characterized by an intense state of widespread inflammation. A prime target of the generalized inflammatory response is the lung, and the inflammatory responses in the lung tissue can eventually lead to pulmonary failure (Hirsh et al. 2004). This severe condition affects thousands of intensive care unit (ICU) patients every year, and there is a great need for efficient therapies that treat this condition. We conducted this research to elucidate the mechanisms underlying the septic shock-related inflammatory response. By understanding how the mediators involved in this pathway interact, researchers may be able to develop effective drugs and/or therapies that regulate the action of these mediators.

Toll-like receptor 4 (TLR4) is one in a family of receptors that provides a critical link between microbial recognition and the induction of immune and inflammatory responses (Medzhitov & Janeway 2000). Lipopolysaccharide (LPS) derived from gram-negative bacteria binds to TLR4 on neutrophils and this initiates an immune response. Bacteria or LPS in the blood can activate the inflammatory process, resulting in multiorgan, dysfunction with the lung being a primary target. Cellular events that occur due to LPS infection include endothelial vascular leak and neutrophil attachment to the endothelium and subsequent infiltration into the lung. These immune responses are mediated by activation of NF-kB as well as other cell signaling pathways. ROS, which are produced in a receptor-mediated fashion, are usually considered as a source of intracellular injury. However, recent studies have shown that NF-kB activation can be controlled by the presence of reactive oxygen species (ROS) such as superoxide (Shah et al. 2001; Magder 2006). Moreover, ROS are also being recognized as important intracellular signaling molecules that can regulate various biological activities including host defense and metabolic conversions. Therefore, the importance of ROS may have been underestimated due to the fact that their detrimental reputation seems to take heed over their role in biological regulation.

The NAD(P)H oxidase enzyme has a signaling role in a variety of cells. In neutrophils, the complex mediates the oxidative burst that occurs during the inflammatory response. Its cytosolic components, $p47^{phox}$, $P67^{phox}$ and RAC interact with membrane components, p22phox and members of the Nox family to generate ROS. Nox proteins work by transferring electrons from NADPH to O2, generating superoxide, a free radical, and reactive oxygen species (Park et al. 2004). $P47^{phox}$ is the cytosolic component of NAD(P)H that helps regulate superoxide production. A loss of function in this component renders the NAD(P)H enzyme incapable of producing ROS (Nobuhisa et al. 2006). We hypothesized that LPS signaling through TLR4 requires ROS from NAD(P)H oxidase to activate an innate immune response by the endothelium.

Materials and Methods

Animal preparation

We were provided with $P47^{phox}$ WT and KO mice ($p47^{phox-/-}$) by

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Dr. S Holland (Laboratory of Host Defense, National Institutes of Health). We bred the mice, and the offspring were genotyped 21 days after birth. For this study, only homozygotes (WT or KO) were used. ROS production was impaired in some mice, though not in others; so the immunological effects of this duality could be determined. WT and KO mice were separated into 3 groups. Animals underwent three different experimental protocols to evaluate lung injury. Mice were injected with a final volume of 200µl of a 0.5mg/kg LPS solution or 200µl of a saline solution. After injections, the mice were kept under observation for 2, 4, or 8 hours and then were subject to a specific procedure that would asses the amount of lung injury they had experienced.

Wet-to-dry weight ratio

Mice were anesthetized with ketamine (200mg/kg) and xylazine (10mg/kg). The left lung was removed at 2, 4, or 8 hours following the LPS challenge, and immediately weighed (wet weight). The lungs were then placed in an oven set at 60°C for 24 hours and then re-weighed to obtain the dry weight. An increase in the wet-to-dry ratio compared to the values obtained with control experiments indicates edema formation in the lung and lung injury.

Histological Score for edema

Mice were anesthetized, and the organs of the thorax exposed. We removed all the blood from the lungs by injecting 5ml of Hank's bank salt solution (HBSS) into the right ventricle and used cardiac puncture to allow the blood to escape. The trachea was then cannulated with a PE 20 tube which was attached to a 2ml syringe filled with a polymer of optimal compound temperature (OCT). OCT was injected into the trachea to promote lung expansion. A single lobe of the left lung was excised, harvested and snap frozen. Lungs were sectioned with a microcytometer, and the sections were mounted on glass slides. Sections were stained with Diff-Quik and analyzed using light microscopy. Edema was assessed by a histological score that scaled the thickness of the alveolar walls. Each lung section created was measured at 100 different regions of the lung and each measurement was classified as pertaining to levels 1 to 3 of edema. (0-6.5mm= level 1- no edema, 6.5-11.0= level 2- moderate edema, 11.0 and higher= level 3- maximal edema). A total score was then generated for each mouse. For example, a mouse with 85 measurements in level 3, 14 measurements in level 2 and 1 measurement in level 1 had a total score of (85x3 + 14x2 + 1x1) = 284. Edema was confirmed as being observed in the lung when the total score was >130.

Pulmonary microvascular protein leak

Leakage of protein from the vascular lumen of lung tissue is a good indication that inflammation has occurred. Evans blue (EB) can be used as a sensitive marker of protein leak. For this experiment, all mice were infused with Evans blue dye (20mg/kg), 90 minutes after LPS or saline injection. After anesthesia, we flushed the pulmonary circulation with 10ml of PBS (pH 7.4, 20°C). Lungs were then excised, blotted dry, and snap frozen in liquid nitrogen. We then homogenized the frozen tissues with PBS (600µl) and formamide (1.2ml). After centrifugation (1500rpm, 5 minutes), supernatant absorbances at 620nm and 740nm were recorded, and tissue EB content was calculated as a ratio (µg of EB in plasma/µg of EB in lungs). EB content calculations were achieved by correcting the absorbance at 620nm for the presence of heme pigments and comparing this value to a standard curve of EB in formamide/PBS (Razavi et al. 2004).

Statistical Analysis

All results are expressed as a mean from the data collected. Differences between groups were assessed by a two-way and three-way ANOVA to analyze variance. Pairwise multiple comparisons were performed with a Student-Newman-Keuls t test. Significance was accepted at $p<0.05$.

Results

Lung injury was more dramatic in P47$^{phox-/-}$ mice. Lung sections for all control mice showed no inflammation of the alveolar walls **(Figure 1a, b)**. After 2 hours, we observed minimal alveolar inflammation in both WT and KO mice **(Figure 1c, d)**. After 4 hours, inflammation was present in both WT and KO mice but the injury was more severe in KO mice **(Figure 1e, f)**. After 8 hours there was a decline in inflammation in WT but severe alveolar damage persisted in KO mice **(Figure 1g, h)**. These data suggest that p47phox needs to be present to allow resolution of the inflammatory response induced by LPS. The p47$^{phox-/-}$ mice (KO) possess the other components of NAD(P)H oxidase, but its activity does not seem to increase without the p47phox regulatory component in response to LPS. Therefore the products of its activity (ROS) are never released and the

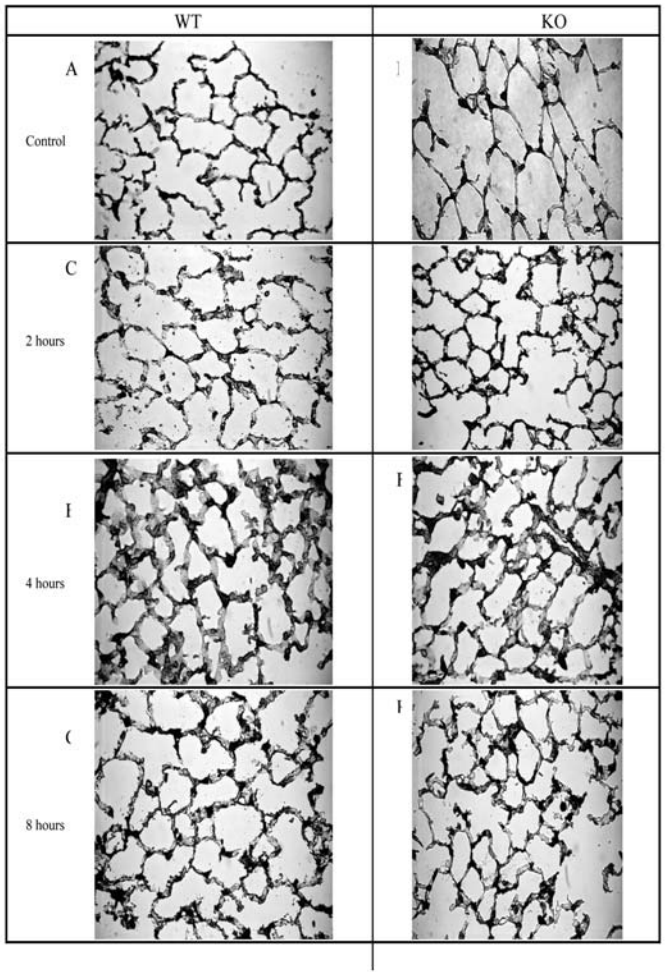

Figure 1. Histological analysis of lung sections, investigating pulmonary edema in WT and KO mice. Thick alveolar walls can be observed at 4 hours in both groups, and at 8 hours in KO mice. All lung sections were visualized via Diff-Quick differential staining; magnification: 40X.

immune response persists instead of resolving. Figure 2 shows a summary of the histological score.

Untreated mice had histological scores that ranged from 116-128 units. The average histological score for KO mice, after 4 hours, was 254.5 units and was significantly greater than WT mice after 4 hours. The difference was even more striking at 8 hours; the average scores were 116 and 254 respectively. These results further exemplify that the resolution of pulmonary edema that is evident in WT mice does not occur in KO mice.

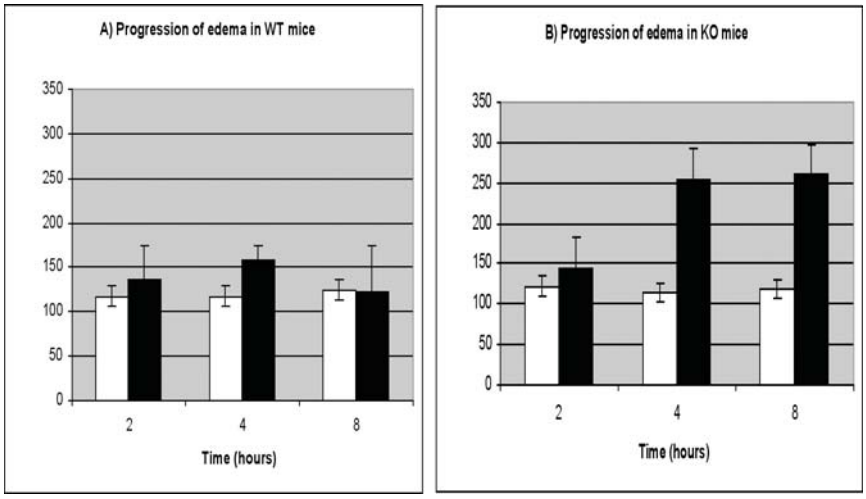

Figure 2. Graphical representation of the histological score for edema for WT (a) and KO (b) mice. The total score decreases after reaching its maximum in WT mice; however this does not occur in KO mice. The open bars represent the control group of mice; the closed bars represent LPS injected mice. Values for histological score were deduced in a blinded manner.

P47$^{phox-/-}$ mice had higher wet-to-dry ratio values.

The ratio of lung weight when wet and lung weight when dry is another measure of lung injury. We tabulated and statistically analyzed the results of this experiment to test their significance **(Table 1)**. The ratio was <4.5 for control mice and these values were similar to those observed by others (Koay et al. 2001).

WT mice had an increase in the wet-to-dry ratio at 4 hours, whereas KO mice had higher ratios after 4 and 8 hours. This assessment further confirms that LPS-induced lung injury occurred in both WT and KO mice, but the injury persisted in KO mice.

P47$^{phox-/-}$ mice had more leakage of Evans blue into the lung vasculature than WT. Capillary protein leak is a significant aspect of syndromes related to lung injury (Green et al. 1988). Evans blue is a common marker used in assessing lung injury because its ability to accurately measure protein leak from the lung vasculature. This experiment was conducted 4 hours after LPS challenge. KO mice had a much higher level of Evans blue, an indication of capillary leak (Figure 3). This indicates that capillary permeability in the alveolar walls increased, and this finding can be linked to the fact that lung inflammation has occurred.

Discussion

Contrary to what we expected, p47$^{phox-/-}$ mice had greater lung injury with time than WT mice. Recent findings suggest that p47phox may interact with TLR4 to downregulate the LPS-induced inflammatory response (Dusting et al. 2004). This could account for the lower degree of lung injury in WT mice compared to KO mice.

A previous study showed that NF-kB activation is significantly attenuated in lungs of p47$^{phox-/-}$ mice. WT mice experience this same attenuation when neutropenia is induced beforehand (Fan et al. 2002). These findings led us to expect that KO mice would show less lung injury than WT mice, because they would not produce ROS to activate NF-kB. A potential difference between their study and ours is that they treated their mice with TNFγ , an inflammatory cytokine that acts though its own receptor and can directly activate the NF-kB pathway, whereas we used LPS, which acts through TLR4. Using TNFγ as the endotoxin may initially promote the inflammatory response, but with time, mediation and correction of the immune response will occur.

There are definite limitations to using mouse models to systematically study biochemical responses. Future experiments should analyze lung injury at a biochemical level to confirm exactly which signaling pathways are activated, and these analyses should also be obtained at different time points. Research with cytokines and signaling peptides could provide more information on what is occurring in the lung at a cellular level. Ultimately, it is the turning off of the inflammatory response that needs to be achieved for any stress-induced injury to ameliorate. Therefore, designating cellular factors that promote anti-inflammatory effects on the endothelium is important for the field to move in the direction of developing efficient therapies. One of these factors, the peroxisome proliferator activated receptor γ (PPARγ), has already been identified as a repressor of pro-inflammatory genes. PPARγ has been shown to be expressed in adipocytes, macrophages and lymphocytes and upon activation by lipophilic ligands; it appears to antagonize NF-kB action in macrophages (Rogler 2005). Future experiments where PPARγ is overexpressed or where ligands specific to this receptor are tested may result in observed amelioration of experimental inflammation, leading to powerful therapeutic agents against the inflammatory response.

During 8 hours of LPS challenge, different mediators are released to activate many signaling pathways. The regulation of each pathway needs to be determined so that the immune response can be understood as a whole. How the mechanism of the immune response proceeds with time is still the main focus of our research, and we hope to soon elucidate this enigma.

Average values for wet/dry ratios		
	WT	KO
Control	4.2±0.09	4.3±0.09
2 hours	4.26±0.1	4.8±0.5
4 hours	4.77±0.07	5.5±0.2
8 hours	4.33±0.45	5.27±0.7

Table 1. Table summarizes the values obtained from the wet/dry ratio experiments. WT mice show lower ratios is general. KO mice have higher wet/dry ratios due to the higher level of pulmonary edema that these mice experience. Statistical significance was assessed using a three-way analysis of variance.

Conclusion

Pulmonary edema, a symptom of septic shock, is characterized by swelling of the lung due to fluid buildup in the lung

tissue and the infiltration of neutrophils in the parenchyma of the lung. LPS can induce this rapid and profound neutrophil infiltration in the lung. When the NAD(P)H enzyme is functioning normally, the neutrophils can produce ROS, which regulates the immune response and decreases the inflammation that occurs. The NF-kB pathway plays an important role in immune responses and inflammation processes and may be activated by the redox status of the cell. Therefore, a regulated concentration of ROS is needed to activate the NF-kB pathways so that the immune response can evolve instead of persisting at the inflammation stage. Our results suggest that when the production of ROS is impaired, the inflammatory response persists and may worsen. Investigators may be skeptical in believing that ROS, which promote oxidative injury, can also promote the regulation of the immune response. However, our data suggests that ROS are necessary factors that promote both the activation of other pathways and the release of key cytokines. In effect, the activated pathways and the key cytokines collectively function to regulate the immune response. This regulation will eventually lead to stabilization of the response and minimize lung injury.

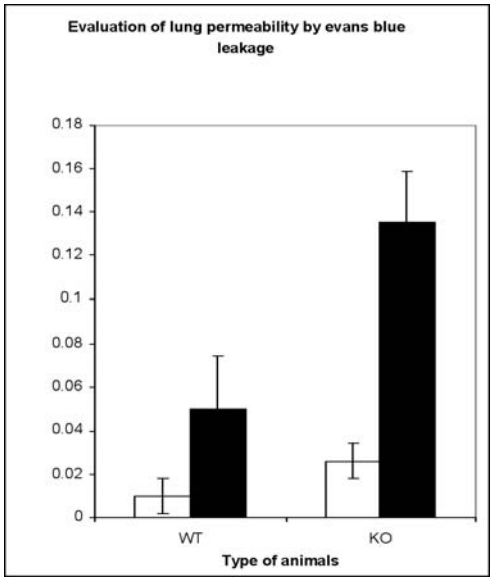

Figure 3. Protein leak was assessed using the Evan's blue marker. The open bars represent the control mice; the closed bars represent LPS injected mice. The KO mice showed a significantly higher level of protein leak due to excessive lung injury. Statistical significance wasassessed using a two-way analysis of variance.

Acknowledgments

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Investigation on stereoconfiguration of azoxybenzene formed via Grignard reaction and further Consequences on Z & E isomerism of diazeniumdiolates

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Abstract

Nitric oxide's importance in biochemistry was recognized in the mid 1980's. Nitric oxide (NO) is critical for the function of neuronal cells, for blood flow and to defend against tumour cells and microorganisms. Diazeniumdiolates, ions of structure $[RN(O)NO]^-$, are being used as probes to study the biological and pharmacological implications of NO because compounds bearing this functional group have been found to release NO. The conformation about the N=N double bond of diazeniumdiolates has been shown through crystallographic studies to be predominantly Z. To study the stereospecificity of diazeniumdiolates, azoxybenzene was synthesized via a "forgotten" synthetic pathway pioneered by Stevens in 1963 which involved a reaction between an organonitrosohydroxylamine tosylate, RN(O)NOTs, and a Grignard reagent. The product from this preparation was directly compared to azoxybenzene made from a coupling reaction.

Key words

Grignard reaction: *an addition reaction involving and organomagnesium halide*; **stereoisomers**: *compounds with the same molecular formula that differ only in the arrangement of their atoms in space*; **crystallography**: *the experimental science of determining the arrangement of atoms in solids*, **diazeniumdiolate**, **nitric oxide, Azoxybenzene**, **RMgX**.

Introduction

Nitric oxide (NO) was named molecule of the year in 1992 by Science (Koshland D. E. Jr. 1992) and a Nobel Prize was awarded in 1998 for establishing its biological importance. However, its roles in critical physiological pathways have only been fully uncovered since the mid 1980's.

NO is a ubiquitous biochemical agent responsible for many critical biochemical processes and is synthesized in the body by a number of enzymes. This small molecule plays a critical role in regulating blood flow; enzymatically released from L-arginine, NO acts upon enzymes in the smooth muscle cells, which surround blood vessels, to cause their relaxation and consequent vasodilation. Moreover, NO is known to inhibit the adhesion, aggregation and recruitment of platelets to a growing thrombus, the plug necessary in wound healing; NO is essential to regulate platelet hyperactivity by reducing the size of the blood clot (Walford G et al. 2003). Furthermore, the cytotoxicity of macrophages against tumor target cells has been linked to the macrophage's ability to secrete NO. As NO is part of the body's innate immune pathway, it also aids in the protection against parasites (notably *Leishamania major* and *Plasmodium* species) and some bacteria (Butler AR et al. 1993).

When NO is produced acutely, it can lead to large blood pressure drops and destruction of tissue leading to inflammatory disease and degeneration of nerve and brain tissue, whereas chronic overproduction of NO is associated with immune-type diabetes, inflammatory bowel disease, rheumatoid arthritis, carcinogenesis, septic shock, multiple sclerosis, transplant rejection and stroke. Insufficient NO production is linked to hypertension, impotence, arteriosclerosis, and susceptibility to infection (Miessler GL et al. 2004).

Ions of structure $[RN(O)NO]^-$ are called diazeniumdiolates and are used as probes to study the biological and pharmacological implications of NO because many chemical agents bearing this functional group have been found to release bioactive NO when dissolved in physiological fluids either spontaneously or after metabolic transformation (Keefer LK et al. 2001). One of the major problems associated with the current NO-donors is the indiscriminate release of NO. Therefore, the development of donors that can deliver NO at specific times and locations to evoke the desired biological function is of great current interest (Hou Y et al. 2000). Furthermore, some naturally occurring diazeniumdiolates are known to have antitumor, antibiotic, antifungal and even herbicidal properties (Hrabie JA et al. 2002).

Diazeniumdiolates and related azoxy-compounds can be prepared via radically-mediated reactions (Stevens TE 1967, Ogata Y et al 1957, Opolonick N 1935, Bigelow HE et al 1931, Pizzolatti at al 1990, Shemyakin MM et al 1957, Hwu JR et al 1997). Radicals are believed to react non-stereospecifically and to readily attack the closest reactive site. Surprisingly however, most radically-mediated reactions generate the Z-conformer of the diazeniumdiolate; this "discriminate" choice in stereospecificity is seen for compounds containing the R'[N(O)NO]R" unit is almost independent of the identity of R' and R" (Keefer LK et al. 2001).

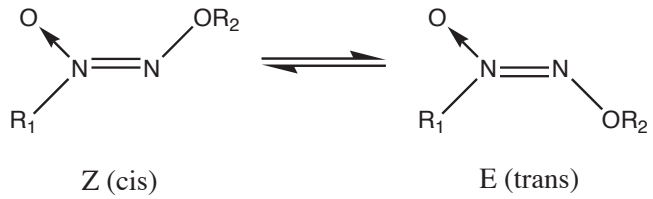

Figure 1. Interconversion of Z/E Isomers of Diazeniumdiolates

This conformational preference of diazeniumdiolates has been under investigation for quite some time. The conformations about the N=N double bond **(Figure 1)** have been studied through ^{13}C and ^{15}N NMR (Schwotzer et al 1977, Simova et al 1983). Crystallographic studies have shown the conformation about the double bond to be Z in all but 5 cases (Bohle DS et al. 2005), where the oxygen atoms are arranged *cis* to each other. If the species with the E stereochemistry could be

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isolated, it could then be compared to its Z analogue to elucidate the relative difference between their respective NO release rates, which greatly affect their pharmacological properties (Wang YN et al. 2005).

Furthermore, an alternate approach to investigate this conformational preference is to probe a related compound, azoxybenzene (III). An alternate method to the synthesis of III was previously examined by Stevens in 1963 (Stevens TE, 1970) (A). An organonitrosohydroxylamine tosylate (II) was prepared from cupferron (I) and followed by a Grignard reaction. The p-toluenesulfonyl derivative of I, an organonitrosohydroxylamine ammonium salt, was prepared in order to have a substituent capable of easy displacement so that the conversion of II to III via a Grignard reaction would take place readily.

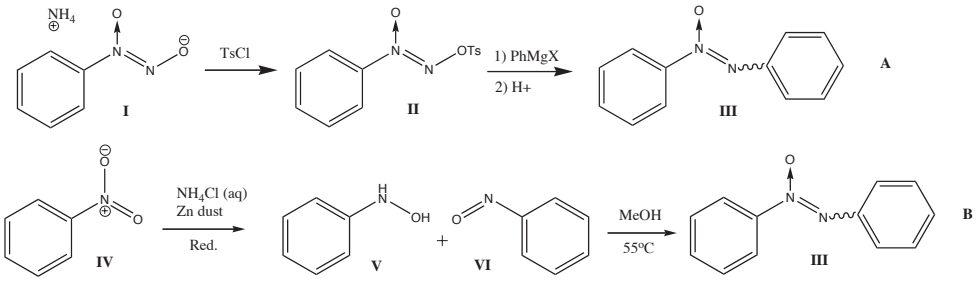

Figure 2. Reaction Pathway A: Formation of N-phenyl-N'-tosyloxydiimide N-oxide **(II)** from cupferron **(I)** and p-toluene sulfonyl chloride followed by the Grignard reaction on the tosylate derivative via phenyl magnesium bromide yielding azoxybenzene **(III)**; Reaction Pathway B: Formation of azoxybenzene **(III)** via the coupling of nitrosobenzene **(VI)** and phenylhydroxylamine **(V)**, synthesized by reducing nitrobenzene **(IV)**

This study focuses on the comparison of both compounds identified as III, the one synthesized from reaction scheme A, a pathway pioneered by Stevens, and the one formed by a conventional radically mediated pathway (B), followed by extensive characterization of all products synthesized. The main focus of this "novel" method of synthesizing diazeniumdiolates precursors is to uncover whether only one configuration (E or Z) is preferred. The method of investigating this resides in proton NMR, IR, UV, and, mainly, crystallographic studies.

Experimental Procedure

Preparation of N-phenyl-N'-tosyloxydiimide N-oxide (Stevens TE, 1970)
A solution of 10.3 g (66.4 mmol) of cupferron in 135 mL of 10% aqueous sodium bicarbonate while 14.2 g (74.5 mmol) of para-toluenesulfonyl chloride was added in one portion without any noticeable heat evolution from the reaction mixture. After 2 hours, an additional 1.5 g (8.0 mmoles) of the para-toluenesulfonyl chloride was added. The mixture was stirred overnight and then extracted with methylene chloride. The dark residue obtained by evaporation of the methylene chloride was treated with 25 mL of methanol to give a colorless precipitate, which was then filtered. The crude white solid obtained was recrystallized by adding chloroform followed by an equal volume of hexane. A total of 11.208 g (38.3 mmoles) of N-phenyl-N'-tosyloxydiimide N-oxide was obtained (58% yield). Crystals suitable for crystallographic characterization **(Figure 3)** were grown from chloroform and hexanes and diffraction data was collected on a Bruker D-8 diffractometer with Mo Kα radiation. ^{1}H NMR (400 MHz, $CDCl_3$, δ) 2.47 (s, 3H), 7.39 (d, J=8.0 Hz, 1H), 7.49 (t, J=8.0 Hz, 2H), 7.59 (t, J=7.2 Hz, 2H), 7.90 (d, J=3.6 Hz, 2H), 7.96 (d, J=8.4 Hz, 2H). IR (KBr, cm^{-1}) 3096 (w, υ_{CH}), 3074 (w, υ_{CH}), 1595 (s, υ_{NO}), 1385 (vs, υ_{SO2}), 1194 (vs, υ_{SO2}), 903 (s, υ_{NOS}), 743.39 (br, υ_{SO}). Raman (cm^{-1}): 1598 (w), 1386 (w), 1318 (w), 1292 (m), 1195 (m), 1178 (m), 1089 (w), 1005 (s), 816 (m), 737 (w), 676 (w), 634 (m), 612 (w), 292 (s).

Preparation of Azoxybenzene via Grignard reaction (Stevens TE, 1970)
A solution of 2.20 g (7.526 mmoles) of N-phenyl-N'-tosyloxydiimide N-oxide in 40 mL of dry tetrahydrofuran was stirred at ambient temperature while 11.0 mL of 1.0 M phenylmagnesium bromide (in THF) was added dropwise. The mixture was stirred at 50-60°C for 2 hours, then cooled to room temperature and poured into an ice-dilute hydrochloric acid mixture. The organic product was isolated by extraction with methylene chloride. After concentration of the organic layer, the residue was chromatographed on silica gel. Elution of the column with 100% hexanes was performed to remove impurities and was followed by further elution with a 3:1 and 2:1 hexanes-methylene chloride solution to recover azoxybenzene in a 64% yield. UV ($CHCl_3$) λmax (ε) 323.0 nm (br). 1H NMR (400 MHz, CDCl3, δ) 7.40 (m, 1H), 7.49 (m, 2H), 7.52 (m, 2H), 7.54 (m, 1H), 8.16 (d, J=7.6 Hz, 2H), 8.31 (d, J=7.2 Hz, 2H). IR (KBr, cm^{-1}) 3066 (w), 1483 (m), 1473 (m), 1451 (m), 1328 (w), 1299 (w), 1093 (br), 1022 (br), 926 (w), 906 (w), 763 (s), 683 (s), 579 (w).

Preparation of Azoxybenzene via coupling reaction (Ogata Y et al. 1957)
A vigorously stirred mixture of 4.2 ml nitrobenzene (0.04 mol), 2.50 g NH4Cl (0.05 mol) and 80 mL water was maintained below 60°C while 5.90 g of zinc dust (0.09 mol) was slowly added (highly exothermic process). After the addition of all the zinc, the mixture was stirred for an additional 20 minutes and filtered. The filter cake was washed with hot water. The filtrate and washings were combined and saturated with 25.0 g of KCl and cooled to 0°C for 30 minutes. The resultinglong, yellow, needle-like crystals of phenylhydroxylamine (3.7 g, 33.91 mmoles) were filtered, dried and stirred in 20.0 mL of methanol. A methanolic solution (15 mL) of 3.5 g of nitrosobenzene (32.67 mmoles) was added and stirred at room temperature. The greenish color of the solution gradually darkened but remained green even after warming to 60°C. A small amount of water (2 mL) was added and the solution mixture was then chilled to 0°C for 10 minutes until a precipitate formed. The solid material was filtered and the mother liquor was evaporated under vacuum yielding 1.072 g of azoxybenzene (15%). UV ($CHCl_3$) λmax (ε) 324.0 nm (br). 1H NMR (400 MHz, $CDCl_3$, δ): 7.46 (m, 1H), 7.48 (m, 2H), 7.49 (m, 2H), 7.50 (m, 1H), 8.16 (d, J=8.4 Hz, 2H), 8.29 (d, J=5.2 Hz, 2H). IR (KBr, cm^{-1}): 3066 (s), 1572 (w), 1483 (m), 1473 (m), 1451 (m), 1438 (s), 1300 (w), 1275 (w), 1165 (w), 1097 (w-br), 1069 (w), 763 (s), 684 (s), 612 (w), 580 (w).

Reaction of Potassium Tert-butoxide and N-phenyl-N'-tosyloxydiimide N-oxide
A solution of 0.32 g (1.095 mmoles) of N-phenyl-N'-tosyloxydiimide N-oxide in 5 mL of methanol was stirred at ambient temperature while 1.64 mL of 1.0 M potassium tert-butoxide (in tert-butanol) was added. The mixture was stirred at ambient temperature overnight. The organic product was filtered from solution. A mixture of tert-butyl 4-methylbenzenesulfonate and cupferron was collected as a white powder (0.3216 g). 1H NMR (400 MHz, $CDCl_3$,δ): 2.48 (s, 3H, t-Bu-SO_3PhCH_3), 3.31, (s, 9H, t -Bu-SO_3PhCH_3), 7.09 (d, J=7.6 Hz, 2H, PhN_2O_2-), 7.17 (t, J=7.2 Hz, 1H, PhN_2O_2-), 7.31 (t, J=7.8 Hz, 2H, PhN_2O_2-), 7.45 (d, J=8.0 Hz, 2H, t-Bu-SO_3PhCH_3), 7.84 (d, J=7.6 Hz, 2H, t -Bu-SO_3PhCH_3).

Discussion

To date, all diazeniumdiolates and closely related analogues such as azoxy-compounds have been found to exist in the Z (*cis*) conformation; however, only 5 examples of the E (*trans*) configuration analogue have been observed. These involve cyclic ring systems forcing the specific conformation (Bohle DS et al. 2005).

There are various known methods for synthesizing azoxybenzene; the main and most frequently used approaches are the oxidation of amines and azo compounds, reduction of nitro (Opolonick N 1935, Bigelow HE et al 1931) and nitroso compounds, acid-catalyzed or base-catalyzed coupling of nitroso compounds with hydroxylamines (Ogata Y et al 1957, Pizzolatti MG et al 1990, Shemyakin MM et al 1957) and by thermal- and photo-induced transformations of N-aryl-N-nitrosohydroxylamine ammonium salts (Hwu JR et al. 1997). Most of these preparations will proceed, in some form or another, as radicals to form azoxybenzene which, when produced by this particular pathway, always generates the Z (*cis*) conformer.

Therefore, a method that deviated from the conventional radical pathway was of interest. The reaction of Grignard reagents with O2-alkyl diazeniumdiolates producing azoxy-compounds was pioneered by Stevens even before the true structures of the starting materials were known. Stevens was also able to perfect reaction pathway A, the preparation of azoxy-compounds from the O2-tosylated acyl diazeniumdiolates (Stevens TE, 1970).

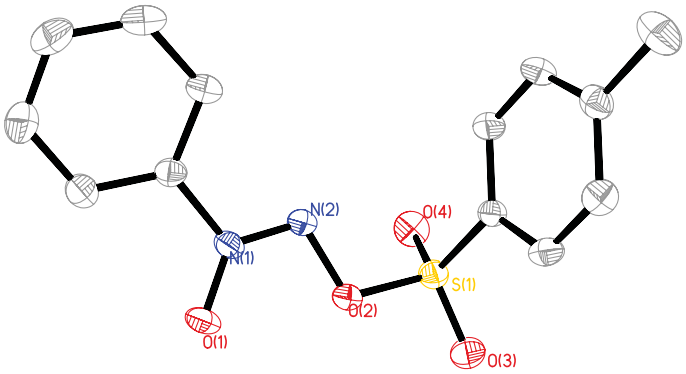

Figure 3. Stereoscopic view of N-phenyl-N'-tosyloxydiimide N-oxide reflects its Z (*cis*) stereoconfiguration

The structure of II was determined by X-ray diffraction techniques and solved by direct methods. The structure obtained clearly reflects the Z configuration about the O(1) atom of II **(Figure 3)**. This structure had also been previously determined but reported only in outline by White and coworkers (White EH et al.), who also synthesized the molecule by tosylating I.

Since II is in the Z configuration, one possible mechanism of this particular Grignard reaction for the generation of azoxybenzene is a back-side attack with respect to the tosyl group by the phenyl anion, leading to an inversion of configuration yielding an E (*trans*) isomer.

To examine this hypothesis, azoxybenzene generated by reaction pathway A was directly compared to azoxybenzene synthesized from B (Ogata Y et al. 1957) (III is formed either when two phenyl nitroxide radicals react or after the formation of the arylnitroxide radical). Radical coupling reactions such as the latter have been previously shown through X-ray crystallography to yield azoxy-compounds in the Z (trans) configuration (Yamamoto J et al. 1987). Proton NMR and IR indicate that azoxybenzene produced via each reaction is relatively similar. Webb and Jaffé have previously published UV-visible values for the E (trans) and Z (cis) conformers of azoxybenzene, 327 nm and 323 nm respectively (Webb DL et al. 1964). Therefore, this points towards the fact that azoxybenzene made from both A and B are in the Z (cis) configuration; however, this would need to be confirmed through crystallographic studies.

Stevens observed that reactions of II with Grignard reagents conducted in THF led to the formation of significant quantities of undesirable side-products (VIII) and thus indicated that the Grignard reaction proceeded via a radical intermediate (VII). He postulated that both III and VIII arise from radical coupling or radical displacement reactions. Although we have found many side products in the Stevens' reaction, none have been identified as compound VIII (Stevens, T. E., 1967). However, the fact that the Grignard reaction of II also proceeds via a radical reaction indicates that the E (trans) isomer would not get generated via this type of reaction.

Furthermore, the synthesis of the diazeniumdiolate, N-phenyl-N'-tert-butyloxydiimide N-oxide, was attempted from II and potassium tert-butoxide. The O-alkylation of I has been well documented (Hou Y et al. 2000) and leads to the Z (cis) isomer therefore, the O-alkylation of II could potentially lead to a single isomeric product. This would occur through simple nucleophilic attack at nitrogen and displace the tosylate group (N-attack) and generate IX, a diazeniumdiolate. However, an alternate mechanism (S-attack) exists and involves initial nucleophilic attack at sulfur to produce I and an intermediate tosyl ester X. This S-attack mechanism has been known to proceed exclusively and has been confirmed through ^{18}O labeling and incorporation studies (D'Sa RA et al. 2003).

Therefore, to bypass the nucleophilic attack at the sulphur atom, a different leaving group such as a diphenylphosphinoyl (Vankayalapati H et al. 2001) or a triflate (Netscher T et al 2002, Olah GA et al 1986) should be fashioned and coupled to I to allow easy displacement and to prevent the aforementioned S-attack. These reactions with various sodium alkoxides will be examined to determine whether O-alkylation of the new derivative will proceed and yield an isomeric diazeniumdiolate, which could then be characterized by X-ray analysis.

An essential question is whether these reactions are thermodynamically controlled (both E and Z products are formed and the E to Z isomerization ensues) or kinetically controlled (one isomer, Z, is exclusively formed from the mechanism of the reaction). Insight into this is best gained by computational density function calculations. The rotation barriers for even the simplest diazeniumdiolates are very high (approximately 40 kcal/mol). Therefore, the absence of the E (trans) isomer is not likely due to

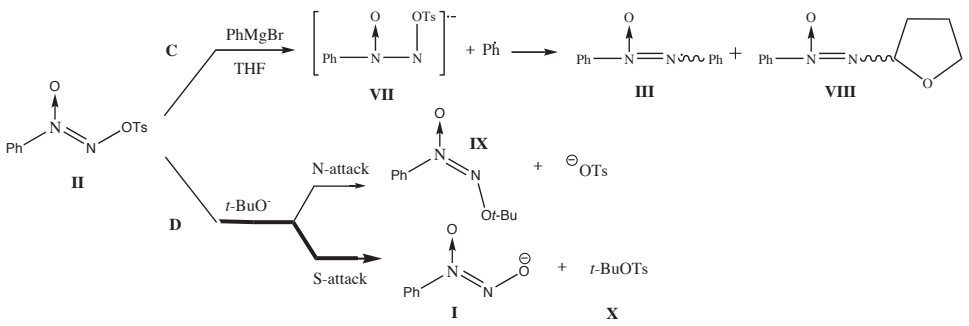

Figure 4. Reaction pathway C: Formation via radical intermediate (VII) of azoxybenzene **(III)** and of side-product **(VIII)** when reaction pathway A is performed in THF as postulated by Stevens; Reaction Pathway D: N-phenyl-N'-tosyloxydiimide N-oxide **(II)** is reacted with tert-butoxide and yields compounds X and I via an S-attack mechanism

isomerization from a product under thermodynamic control, but rather due to product distribution that is Z (*cis*)-preferential and is thus kinetically controlled (Bohle DS et al. 2005). Therefore, the observed products correspond to the isomers initially formed and reflect their mechanism of formation.

Conclusion

X-ray diffraction will be the only way to confirm the stereo-configuration of the azoxybenzene made with the Grignard reaction and from the coupling reaction; it will also verify that that both adopt the Z (*cis*) conformation. Moreover, to produce diazeniumdiolates from O-alkylation, diphenylphosphinoyl chloride ($P(O)Ph_2Cl$) as well as triflic anhydride ($((CF_3SO_2)_2O$) and I will need to be coupled. Finally, to confirm whether the mechanistic pathway of A truly occurs via VII, the reaction can be performed with a radical quencher, such as TEMPO or DMPO. This would allow us to capture and examine the radical intermediate and determine whether azoxybenzene is still formed (and in the same yield) and whether our hypothesized mechanism would occur.

Acknowledgements

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Unwinding the universe: a brief look at String Theory

Michael Dascal

"String Theory" has been a buzz-word in contemporary physics for years. It is at the very edge of theoretical ontology, predicting bizarre qualities of the universe – even claiming we exist in 11-dimensional space-time. These predictions may be strange, but the Theory can account for many of the laws of the universe if we put aside our preconceptions and accept the strange possibilities the Theory suggests.

The basic precepts of String Theory explain that the most elementary particles of the universe are tiny one-dimensional threads often thought to close in upon themselves, forming loops. These strings combine in various ways to form all the other particles that we know about in the universe, such as electrons, photons, quarks, etc. Because these threads are one-dimensional, they can vibrate at different frequencies and wavelengths, each pair of which corresponds to a different energy state. These different energy states, in turn, produce different properties to varying degrees, including mass, electrical charge, etc.

The foundation for the assumption that these strings exist is a simple one. For years physicists have known that Einstein's Theory of Relativity can successfully describe how the macroscopic universe behaves, and that quantum mechanics does the same for the microscopic world. However, when these two theories combine, the results are very problematic. As we try to understand what the universe looks like at the Planck length[1], about 1.6×10^{-35} metres, relativity and quantum theory paint contradictory pictures. The former requires space-time to be flat and smooth (assuming there are no massive bodies in proximity). The latter describes a chaotic place where energy and mass are spontaneously and continuously created and annihilated. Taking strings into account, the Theory essentially tells us that the universe doesn't really ever 'get' as small as the Planck length, as even the smallest particles – strings – are larger than this size.

The Theory has a number of other positive consequences. First, if we assume that the particles we have already observed are composed of these strings, then different possible string vibrations and wavelengths let us account for the different properties these particles exhibit. For example, the Theory may explain why it is that an electron and a proton have the same magnitude of charge while one is almost 2000 times the mass of the other.

Even more impressively, perhaps, the Theory predicts the existence of certain particles that theoretically should exist, but which we have not yet been able to observe. There are four forces in the universe: the electromagnetic force, the gravitational force, the strong force, and the weak force. (Most of us know of the first two. The strong and weak forces account for atomic nuclear cohesion and decay, respectively.) Each force has a corresponding 'messenger' particle that works to 'tell' other particles how to act. For instance, photons play this role in the electromagnetic force, causing charged particles to be attracted or repelled from one another. We have been able to observe the messenger particles of the strong and weak forces as well, but the messenger particle of the gravitational force – the graviton – is as of yet unseen and not proven to exist. One of the first strengths noticed in String Theory was that it not only tells us that all four forces must exist, but goes on to tell us what the graviton must 'look like' as well!

Even with these strong theoretical consequences, there is reason to be wary. There are many different properties the strings must account for, and each one must be represented by a different 'way' in which the string can vibrate. The three spatial dimensions and one time dimension that we experience simply do not allow for enough freedom – we need more than up/down, left/right, forward/backward, and before/after. For this reason, the Theory tells us that the universe must have many more dimensions – current theories estimate 11, including that of time.

As one of the oddest predictions of String Theory, for many this is enough to disprove the whole idea. If the universe contains so many dimensions, why have we only ever observed four? Even if we accept that our observation and the truth can be very distant from each other, where is there any room for more spatial dimensions anyway?

It is pretty much impossible for us to visualize one extra dimension, let alone seven, but even if we can't truly picture it we can try to describe it through analogy in three dimensions. Consider, first, two of the dimensions as a flat plane. If the third dimension is to be "closed", as the extra dimensions described in String Theory are often held to be, not only is it at right angles to the plane, but it also forms a loop. It is important to note that this third dimension appears at every point in the plane. The loop we draw is simply an example of how the dimension appears from a single point on the original plane. Also, when we consider the plane, we are only looking to a small cross-section of the dimensions it represents. These dimensions, too, may be closed and form their own loops. Now at every point the three dimensions are orthogonal, so you have to imagine that as you travel around the loop, the plane of the first two dimensions travels with you. If this seems confusing, then you've probably gotten it right!

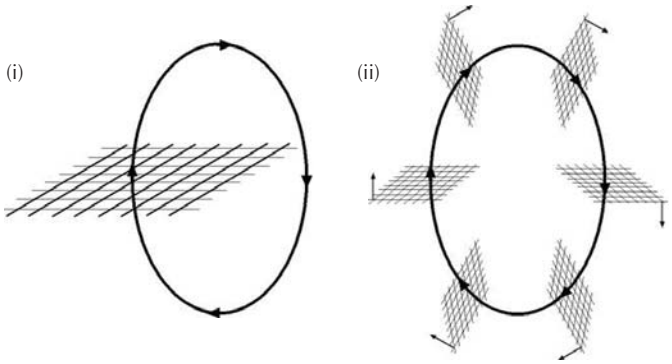
To extend this picture to multiple dimensions, we repeat the procedure. Let's begin with a fourth spatial dimension. Imagine a plane that somehow contains three dimensions. (This is a contradiction of terms, but if we could picture it any better, then extra dimensions wouldn't be a problem to begin with!) We imagine a loop, just as in the three-dimensional case, only this time the first three dimensions all travel along the loop as you follow its path, always orthogonal to the fourth. Repeating this six more times, each times with a plane that represents more dimensions, explains how the 10 spatial dimensions relate to one another.

This isn't the entire picture, as this doesn't explain why we

don't notice the extra dimensions. This has to do with thethe sizes of the extra dimensions. It may seem bizarre for a dimension to have a size at all, but this comes naturally when the dimension forms a loop; all finite loops have a finite radius and circumference. If the three 'everyday' spatial dimensions are closed, they are also very large – their size is that of the universe (which, too, would be closed). However, the extra dimensions proposed in String Theory are essentially too small for us to notice.

Consider, as an analogy, a very fine fishing line hanging taut in the air in front of you. If the wire is thin enough, and close enough to your face, then you can never resolve its image in your eyes, and may even completely disappear from your vision, as if it were invisible. The sizes of the extra dimensions' loops are much smaller than that of the fishing line. Unlike the line though, dimensions aren't solid, and so we carry on in three dimensions without even realizing there may be many more that we pass right through everyday.

Another way to consider the same idea is to imagine traveling around the dimensional loops at a certain speed. Clearly, the amount of time it takes to do so depends on how big the loop is. Now if we make the loop small enough, eventually such a trip would take no time at all. We can even imagine it to be so small that any movement made on the loop involves a number of trips made around it, without the traveler even noticing.



Picturing further dimensions: (i) Adding a closed, third dimension to a plane, we draw a loop. Recognize the same loop occurs at every point on the plan – we only visualize it once. (ii) As we proceed along the loop, the plane rotates with us, remaining always orthogonal to the loop

It should be noted that these analogies offer only a way to think about the consequences of the Theory. No one can properly imagine more than three spatial dimensions – it is simply impossible – and so these results are incredibly difficult to envision, much less accept.

Even if we accept its odd theoretical requirements, String Theory is nowhere near completion. It seems there is a different version of the Theory for every string theorist out there – and not a single prediction has been verified in any way. Because of such difficulties, and the fact that they remain unresolved after 30 years of research, there are more and more physicists who oppose the current expenditure of resources and energy on String Theory research. They feel that perhaps it is a futile exercise, given that we have made no unanimous advances or proven predictions, and that the Theory truly comes out of a desire to explain the universe elegantly and not out of any real experimental data.

Unfortunately, this is an issue that will remain unresolved until a final version of the Theory is developed or until a prediction is proven successful. One thing is certain – the more we find out about the most basic particles that make up the universe, the more we find that they are nothing like what we ever imagined.

Further reading for those interested, who don't necessarily have a physics background:

1. Brian Greene's *The Elegant Universe and The Fabric of the Cosmos* explain String Theory to the layperson from the string theorist's point of view.
2. Lee Smolin explains why he abandoned work in String Theory after years in the field in *The Trouble with Physics: The Rise of String Theory, the Fall of a Science, and What Comes Next*

[1]This is a particular constant that arises naturally from other universal constants, such as the speed of light.

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Shifts in species traits among North American freshwater fish assemblages: ecological homogenization?

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Abstract
This study examines whether the processes of species invasion and extirpation have produced distinct shifts in mean species traits of North American freshwater fish assemblages. An analysis of 54 species (29 invaders, 25 extirpated taxa) in 7 drainages revealed significant differences in maximum length, native latitudinal range size, habitat specificity, and migratory behaviour. Results suggest a pattern in which extirpated species are being replaced by larger, more environmentally tolerant species capable of occupying a broader range of habitats. Freshwater fish assemblages containing introduced generalist species may have a selective advantage over pristine communities as human-dominated landscapes continue to replace natural systems.

Keywords
Freshwater fish: *Actinopterygii*; **biological invasion**: *introduction and spread of non-native species*; **extinction**: *functional or absolute loss of a species*; **extirpation**: *extinction of a species from a region*; **species**: *group of genetically similar, reproducing organisms*; **species/ecological traits**: *characteristic or average values of a trait associated with a species*; **biotic homogenization**, *increasing similarity of species assemblages over time*.

Introduction

Humans have historically exerted novel stresses on ecological communities. One such stress is the introduction of a suite of human commensals and favored species as the human population expands into new geographic regions. Indeed, recent studies have identified human population density and associated development as predictive variables influencing the intensity of invasions in several countries, correcting for the well documented species-area effect whereby greater numbers of species are found at larger spatial scales (Gido and Brown 1999; McKinney 2001; Gido et al. 2004; Olden et al. 2006). One outcome of such anthropogenic influence is biotic homogenization: the increased similarity of assemblages resulting from a combination of the introduction and establishment of non-native species and the loss of native and endemic species (McKinney and Lockwood 1999; Rahel 2000, 2002; McKinney 2001; Olden et al. 2006; Olden and Rooney 2006). Thus far, biotic homogenization has been studied primarily at the species diversity level, without consideration of other ecological factors including life history traits.

Ecological traits of aquatic animals have been examined as predictors of invasion success (Angermeier 1995; Kolar and Lodge 2002; Vila-Gespert et al. 2005; Jeschke and Strayer 2005) and extinction risk (McKinney and Lockwood 1999; Purvis et al. 2000). Certain ecological traits may also influence the impact of introduced species on native communities. For example, larger species physically occupy more space or consume greater amounts of resources than smaller species, while species consuming at different trophic levels depend on different primary food sources. The trophic behaviour of a particular species may cause cascading effects in a community through the magnification of stresses exerted on prey species (Currie et al. 1999).

Changes in the ecological characteristics of communities may be examined through the life history traits of different classes of species, namely those gained (introduced) and lost (extirpated). Threatened species of fishes (Williams et al. 1989) have been compared to taxonomically similar species that are not imperiled in order to determine if threatened species differ from unthreatened species in their life history traits (Angermeier 1995; Reynolds et al. 2005; Vila-Gespert et al. 2005; Alcaraz et al. 2005). The present study seeks to quantify differences in ecological traits between introduced and extirpated fishes within North American drainages. We predict that biases in human preferences and differential susceptibility of aquatic species to extinction are driving a shift in the mean species traits of North American fish assemblages.

Methods and Materials

Data Collection

Information on the distribution of species and their class status (introduced or extirpated) was recorded from Hocutt and Wiley (1986); each drainage in North America that was reported as having 3 extirpated and extinct species was selected for analysis. Data on life history traits of the introduced and extirpated species were collected from FishBase (Froese & Pauly 2006). In cases where life history categories are unreported for a particular subspecies, we substituted data for the species assemblage. A single extirpated subspecies from the Tennessee River drainage which required such data substitution remains in the final data set. Species of indeterminate status (e.g. possibly introduced), or which entirely lack life history information in FishBase, were excluded from the data set. We also excluded one species (Atlantic salmon Salmo salar) that was reported as introduced in two drainages and extirpated from a third.

The final data set consists of 54 species (25 extirpated, 29 introduced) distributed between seven drainages representing four drainage realms, as defined by the contributing authors in Hocutt & Wiley (1986). In order to account for any pseudoreplicative effects stemming from the separate

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evaluation of four of the Great Lakes, a pooled Great Lakes sample was created containing each species examined in the individual lakes. A master list containing species from all drainages was also generated to test cumulative differences across fish assemblages ranging from southern Canada to the Gulf of Mexico. Analyses focused on the most commonly reported traits: maximum recorded length, range of latitudinal distribution, population doubling time, trophic level, habitat preference, typical vertical position in the water column, migratory behaviour and salinity tolerance.

Qualitatively reported life history variables were coded into discrete integer scores after Angermeier (1995) and Alcaraz and coauthors (2005), as averaging the scores over classes (introduced or extirpated) can yield statistically testable class mean values. The scoring convention applied to coded variables is as follows: Habitat preference: lakes 1; rivers 2; lakes and rivers 3; marine and freshwater 4; Migration: nonmigratory 1; potamodromous 2; anadromous 3; Population doubling time: < 15 months 1; 1.4-4.4 years 2; 4.5-14 years 3; > 14 years 4; Salinity tolerance: non-euryhaline 1; euryhaline 2; Vertical preference: demersal 1; benthopelagic 2; pelagic 3. All continuous-trait variables (Maximum reported length, Trophic level, and Latitudinal range) were log-transformed prior to analysis to control for differences of scale.

Statistical Analysis

Data were recorded and transformed in Microsoft Excel. The effects of life history traits on class status were examined through comparative analysis using the XLSTAT program for Excel (Addinsoft 2006). Parametric tests (two sample, two-tailed t-test) were applied to continuous traits, and non-parametric (two-tailed Mann-Whitney) tests were applied to coded traits. Different tests were selected for different categories of variables in order to maximize the statistical power of each comparison (Zar 1999). Analyses were conducted at two levels: between the drainages, and between the two classes of species within a single drainage. The level of significance for all comparative analyses was established at α=0.05. Class means of each variable, from each drainage, were then combined to evaluate overall trends in the dislocation of traits across fish assemblages. To avoid unequal contribution by shared trends in the individual Great Lakes to the overall patterns, the pooled Great Lakes sample was the source of the class means for the between-drainage comparison.

Stepwise discriminant analysis using SAS software (SAS 1996) was used to determine whether the life history traits exerted independent influence on class status within drainages, or if any of the traits were associated in some way with each other. Discriminant analysis is commonly used in analyses of multiple traits (Kolar and Lodge 2002). The probability of falsely rejecting the null hypotheses of no contribution of life history traits to class status was calculated using Wilks' lambda test statistic at a significance level of α=0.15. This significance level is the default value for the SAS stepdisc function, and is in keeping with Angermeier (1995), who observed that establishing a greater level of significance may be beneficial to the elucidation of trends in large scale comparisons.

Results and Discussion

The collaborative nature of the species distribution data (Hocutt and Wiley 1986) leads to ambiguities in the status of certain species in different drainage realms. Some contributing authors in Hocutt and Wiley (1986) list extirpated species and group introduced with natives, while others report introduced species with no extirpations. The selection criteria used to compile the data set for the present analysis excluded several drainages in which introduced or extirpated species were not differentiated from native species, creating a small data set of seven drainages.

Relevance of Traits

Each of the eight life history traits examined was found to contribute significantly to class status (introduced or extirpated) in one or more drainages **(Table 1)**.

Introduced species generally reach greater maximum lengths (LL) (Lakes Huron, Michigan, Ontario), prefer a wider range of habitats (HS) (Lake Ontario, Tennessee River, Galveston Bay, master list), tend to be pelagic or free-swimming as opposed to demersal or bottom-dwelling (HSV) (Lakes Huron, Michigan, Ontario), occupy a wider latitudinal range (LLR) (Galveston Bay), and consume at a lower trophic level (LT) (Lakes Erie and Ontario, Great Lakes). Salinity tolerance (EU) is greater in introduced species in the master list, and greater in extirpated species in Tennessee River. The average population doubling times (DTS) in the master list and Muskingum River are greater for introduced species, and are greater for extirpated species in Lake Ontario.

Variable	Drainage	Direction of Change
Salinity tolerance (EU) – CV	Master list (p=0.0003)	Introduced species are more tolerant
Migratory behaviour (MS) – CV	Lake Erie (p=0.0065)	Introduced species are more migratory
Vertical habitat (HSV) – CV	Lakes Huron (p=0.0013), Michigan (p=0.124), Ontario (p=0.0001); Great Lakes sample (p=0.0005)	Introduced species prefer pelagic habitat
Habitat preference (HS) – CV	Lake Ontario (p=0.0075), Tennessee River (p=0.0383), Galveston Bay (p=0.0353), Master list (p=0.0003)	Introduced species are less specialized in their habitat preferences
Population doubling time (DTS) – CV	Muskingum River (p=0.0401), Master list (p=0.0003)	Introduced species have longer population doubling times
Maximum length (LL)	Lakes Huron (p=0.0004), Michigan (p=0.0004), Ontario (p=0.001); Great Lakes sample (p=0.0002)	Introduced species reach greater maximum lengths
Trophic level (LT)	Lakes Erie (p=0.0164), Ontario (0.0018); Great Lakes sample (p=0.0001)	Introduced species consume at a lower trophic level
Latitudinal range (LLR)	Galveston Bay (p=0.0169)	Introduced species occupy a wider native range

Table 1. Results of stepwise discriminant analysis at level of significance α=0.15. Significant results indicate that variables exert independent influence on class status (introduced or extirpated). Italicized entries denote conflicting directions of change. CV = coded variable.

Significant Changes in Traits

Salinity tolerance (EU) was found to be significantly more frequent in introduced species in all drainage samples

(p<0.0001) except for the Tennessee River, where the trend was significantly reversed (p<0.0001). Groups of introduced species in all drainage samples tended to be migratory; this difference was significant in five out of nine samples. Given that the scoring convention used for migratory behaviour assigned the highest integer score to anadromy (migration between freshwater and marine environments), it is not surprising that more introduced migratory species also display greater salinity tolerance. Habitat preference scored higher in introduced species in all samples and was significant in six out of eight samples. The highest integer scores in this category were assigned to mixed habitat, i.e. lakes, rivers and oceans. Presence in varied habitats is an indication of broad environmental tolerance and little adaptation to particular conditions. Vertical position in the water column tended to be higher for introduced fish in the Great Lakes, significantly so in Lake Erie (p<0.0001) and the combined Great Lakes sample (p<0.0001). Introduced fish in the Muskingum (p<0.0001) and Tennessee Rivers and Galveston Bay (p<0.0001), as well as the master list (p<0.0001), tended to prefer lower positions. The enormous Great Lakes basin likely provides an environment more suited to pelagic fish than the rivers and estuarine bay. Population doubling time varied significantly in seven out of nine samples, although the direction of change varied. The combined Great Lakes sample (p<0.0001), Muskingum River (p<0.0001), Galveston Bay (p<0.0001) and the master list (p<0.0001) contain introduced species with longer population doubling times, while in Lake Ontario and Tennessee River the trend is weakly reversed **(Table 2)**.

There is a distinct trend towards introduced species of greater maximum length, significant in seven out of nine samples. Trophic level is the most underreported life history trait examined, and was only available for three drainages and the two combined (Great Lakes and master) samples. The trophic level of introduced species did not differ significantly from extirpated species, although mean values of trophic level for introduced groups are lower in four out of five samples. Introduced species occupy a wider latitudinal range in all drainages, with the difference being significant in four samples **(Table 2)**.

Between-Drainage Comparison			
Variable	Extirpated	Introduced	P
Salinity Tolerance (CV)	1.06	1.47	0.125
Doubling Time (CV)	2.02	2.25	0.453
Habitat Preference (CV)	1.83	3.39	0.031
Vertical Preference (CV)	1.55	1.61	1.000
Migration (CV)	1.29	2.29	0.016
Maximum Length	1.46	1.92	<0.0001
Trophic Level	0.53	0.51	0.151
Latitudinal Range	1.18	1.43	0.002

Table 3. Comparative analyses of grouped means from all drainages, excluding the master list. The Great Lakes sample is used in place of the individual lakes. CV = coded variable.

Variable				Variable			
	Salinity Tolerance (CV)				Doubling Time (CV)		
	E	I	P		E	I	P
Lake Erie	1	1.5	<0.0001	Lake Erie	2	2.4	<0.0001
Lake Huron	1	1.75	<0.0001	Lake Huron	2.17	2.38	<0.0001
Lake Michigan	1	1.83	<0.0001	Lake Michigan	2	2.17	<0.0001
Lake Ontario	1.17	1.71	<0.0001	Lake Ontario	2.33	2.14	<0.0001
Great Lakes	1.07	1.55	<0.0001	Great Lakes	2.07	2.36	<0.0001
Muskingum R.	1	1.19	<0.0001	Muskingum R.	1.75	2.31	<0.0001
Tennessee R.	1.25	1.2	<0.0001	Tennessee R.	2.25	2.2	<0.0001
Galveston Bay	1	1.13	<0.0001	Galveston Bay	1.67	2.13	0.703
Master List	1.08	1.28	<0.0001	Master List	1.96	2.24	0.021
	Habitat Preference (CV)				Vertical Preference (CV)		
	E	I	P		E	I	P
Lake Erie	-	-	-	Lake Erie	1.43	1.5	<0.0001
Lake Huron	1.17	3.75	0.001	Lake Huron	1.33	1.88	0.839
Lake Michigan	1.333	3.83	0.015	Lake Michigan	1.29	1.83	0.841
Lake Ontario	2	3.71	0.015	Lake Ontario	1.67	1.86	0.58
Great Lakes	1.86	3.55	0	Great Lakes	1.47	1.64	<0.0001
Muskingum R.	2.5	3.06	<0.0001	Muskingum R.	1.5	1.25	<0.0001
Tennessee R.	2	3	0.909	Tennessee R.	2	1.6	0.458
Galveston Bay	2	3	0.485	Galveston Bay	1.67	1.378	<0.0001
Master List	2	3.1	0	Master List	1.6	1.41	<0.0001
	Migratory Score (CV)				Maximum Length		
	E	I	P		E	I	P
Lake Erie	1.29	2.3	0.557	Lake Erie	1.35	1.99	0.007
Lake Huron	1.17	2.63	0.019	Lake Huron	1.69	2.05	0.013
Lake Michigan	1	2.83	0.001	Lake Michigan	1.5	2.07	<0.0001
Lake Ontario	1.5	2.71	0.155	Lake Ontario	1.62	1.99	0.016
Great Lakes	1.27	2.36	0.626	Great Lakes	1.49	1.98	0.001
Muskingum R.	1.25	1.81	<0.0001	Muskingum R.	1.27	1.78	0.113
Tennessee R.	1.5	1.8	0.627	Tennessee R.	1.35	1.79	0.024
Galveston Bay	1.33	2	<0.0001	Galveston Bay	1.46	1.78	0.384
Master List	1.28	1.93	0.042	Master List	1.39	1.78	0.002
	Trophic Level			Latitudinal Range			
	E	I	P		E	I	P
Lake Erie	0.55	0.53	0.488	Lake Erie	1.19	1.42	0.061
Lake Huron	-	-	-	Lake Huron	1.13	1.47	0.001
Lake Michigan	-	-	-	Lake Michigan	1.16	1.52	0.017
Lake Ontario	0.56	0.52	0.177	Lake Ontario	1.25	1.51	0.048
Great Lakes	0.55	0.53	0.449	Great Lakes	1.18	1.43	0.016
Muskingum R.	-	-	-	Muskingum R.	1.36	1.43	0.532
Tennessee R.	-	-	-	Tennessee R.	0.83	1.2	0.288
Galveston Bay	0.49	0.48	0.923	Galveston Bay	1.35	1.43	0.667
Master List	0.52	0.53	0.811	Master List	1.18	1.35	0.096

Table 2. Results of comparative analyses. Parametric tests were applied to continuous variable traits and non-parametric tests to coded variable traits at a level of significance α=0.05. CV = coded variable.

Overall Trends

Comparisons of class means between drainages **(Table 3)** reveal overall significant trends towards introduced species which can occupy a wide variety of habitats (p=0.031), display migratory behaviour (p=0.016), reach greater sizes (p<0.0001), and naturally occur across a broader latitudinal range (p=0.002). A weak trend towards introduced species of lower trophic level (p=0.151) and greater salinity tolerance (p=0.125) is also present. Significant differences between classes at both the group and drainage levels are summarized in **Table 4**.

Most North American freshwater fish introductions and extirpations occurred decades ago (Gido et al. 2004). Almost all introductions were intentional, and the success of the introduced species is most likely the effect of human intervention in the form of stocking, thereby providing suprathreshold propagule pressure (Ruesink 2005). In the southern United States, 44% of introductions were conducted by state agencies specifically for sport and recreation (McKinney 2001).

The interest in introduced species, for aquaculture, sport and nuisance factors, has produced an abundance of ecological and life history data on these species. Extirpated species have not received equal attention, perhaps because most extinctions and extirpations occurred historically; consequently, their life history traits are under-represented in our analysis. Several drainages reported in Hocutt and Wiley (1986) met the selection criteria, but had to be removed from analysis as a result of data deficiency on FishBase. Inferring trait values from related species is unsuitable for comparative analysis because phylogenetic relationships are labile for persistent species (Alcaraz et al. 2005), let alone under-reported species which became extinct prior to the development of genetic analysis.

	Variables							
Drainage	Salinity Tolerance (CV)	Population Doubling Time (CV)	Habitat Preference (CV)	Vertical Preference (CV)	Migration (CV)	Maximum Length	Trophic Level	Latitudinal Range
Lake Erie	+	+		+		+		
Lake Huron	+	+	-		+	+		+
Lake Michigan	+	+	-		+	+		+
Lake Ontario	+	-	-			+		
		+						
Great Lakes	+	+	-	+		+		+
Muskingum River	+	+	-	-	+			
Tennessee River	-	-				+		
Galveston Bay	+			-	+			
Master List	+	+	-	-	+	+		
Between Drainages			-		+	+		+

Table 4. Summary of significant results of parametric and non-parametric comparative analyses. Directions of change are from mean values of extirpated to introduced species. CV = coded variable.

Conclusions

Our results suggest that fish assemblages are undergoing an ecological homogenization characterized by a shift toward species sharing similar traits. The compositions of modern fish assemblages largely reflect human preferences resulting in the stocking of large fishes, particularly migratory species such as salmonids. It may also reflect a selective advantage for generalist, broadly tolerant species to colonize and thrive in increasingly common human-dominated landscapes.

We have expanded upon previous studies of homogenization which have neglected to consider shifts in the ecological traits of species assemblages. In the present data set, diversity increased even as the biotic communities were homogenized. Under climate change scenarios projected for the next century, widely-introduced generalist species will likely become increasingly significant components of species assemblages. Therefore, ecological traits may provide a more informative measure of biotic homogenization than simple measures of diversity.

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Density-dependent succession in Caribbean seagrass communities

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Abstract
It is important to understand the patterns of succession and competition in seagrass beds as a way of explaining recovery processes after disturbances. This project studies macroalgae-seagrass succession dynamics in the Caribbean, and tests the importance of interspecific density-dependence (competition) in predicting the successional sequence of species in a wave-disturbed ecosystem. Competition and gap disturbances seem to be the dominant factors influencing species coexistence in offshore regions whereas habitat partitioning driven by differences in depth, disturbance and wave action creates distinct zones of macroalgae and seagrass inshore. In general, density dependent processes across our study site were influenced by major physical gradients. This study has important consequences for predicting dramatic shifts in large-scale seagrass ecosystems, which act as ecological engineers and provide many ecosystem services.

Keywords
Succession: *Changes observed in an ecological community following a perturbation that opens up a relatively large space;* **Disturbance**: *Uncommon, irregular events that cause abrupt structural changes in natural communities and create opportunities for new individuals to become established;* **Interspecific density dependence**: *Interactions between individuals of different species that affect population demographic processes;* **Seagrass beds**: *Marine coastal ecosystems formed by various species of angiosperms. Seagrass beds provide an important habitat for an abundance of fish and invertebrate species, as well as many ecosystems services such as water filtration, carbon sequestration and erosion prevention;* **Gaps**: *Vegetation-free depressions within seagrass beds characteristic of regions experiencing moderate to severe wave action. They are typically crescent-shaped and migrate seaward.*

Introduction

Ecologists strive to explain the processes responsible for structuring natural communities in order to better predict how disturbances may alter these groupings of species, and how communities are reassembled following a perturbation. Dynamic disturbance regimes have long been recognized as important mechanisms regulating natural ecosystems (Sousa 1984) and promoting species diversity, especially by allowing subordinate competitors to colonize recently disturbed areas (Paine and Levin 1981). Although these theories have been used to explain the dynamics of seagrass ecosystems (Bell et al. 1999), recent observations suggest that more complicated physical and biotic processes may play important roles in the maintenance of marine communities (Tewfik et al. 2007).

Caribbean seagrass beds are found to alternate between disturbed states which lack vegetation, referred to as gaps, and a variety of successional states which may include both seagrass or macroalgal vegetation (Bell et al. 1999). Constant wave action keeps the area under periodic disturbance and promotes the migration of gaps throughout the beds (Kirkman 1985). The newly exposed sediments are left to be colonized by one of the various marine species, including the grasses Thalassia testudinum and Syringodium filiforme, as well as the rhizophytic algae Avrainvillea longicaulis (Patriquin 1975).

One well-studied seagrass ecosystem at Bath, Barbados was initially described as being dominated by large stands of Thalassia, which was believed to be the competitively dominant species in the seagrass community successional hierarchy (Den Hartog 1971; Patriquin 1975). However, recent studies by Tewfik et al. (2007) at the Bath seagrass beds reported a large-scale shift in species composition from the dominant seagrass cover to monocultures of competitively subordinate macroalgae. More specifically, areas of the seagrass bed that were historically described by Patriquin (1975) as seagrass-dominated zones were found to have transitioned to monocultures of Avrainvillea longicaulis, creating distinct zonation within the community. Recent observations more precisely challenge the competitive dominance of Thalassia (Mavromatis et al. 2006). Rather than observing the expected zones of dominant seagrass in recovered gap areas and subordinate macroalgae in recently disturbed regions, Mavromotis et al. (2006) found that seagrass beds seem to be progressively replaced by large macroalgal beds wherein little to no seagrass is able to grow. However, Mavromatis et al. (2006) did not explicitly test for biotic interactions, such as competition, in the maintenance of the vegetative zones and successional sequences. Although we qualitatively observed that Thalassia does not grow well in the macroalgae zone or in the presence of Avrainvillea, it remains unknown whether habitat effects or density-dependence plays a greater role in maintaining the species assemblages observed. We can address this question by studying the dynamics of species interactions and physical parameters at the boundaries of three described vegetative zones.

The general goal of our study is to test the importance of interspecific density-dependent processes (competition) in explaining successional sequences in wave-disturbed seagrass ecosystems. We will more precisely test the hypotheses that (1) both habitat differences and direct species interactions produce the patterns of species assemblage observed and (2)

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that the relative contributions of these two components differ in the two transition zones. From these hypotheses, we predict that the presence of interspecific competitors of Thalassia in the seagrass bed inhibit the growth and proliferation of Thalassia in areas where species interactions are the strongest. Furthermore, these density dependent processes should be influenced by major physical gradients such as the intensity of water motion and habitat partitioning across our study site. This study will have important consequences for predicting dramatic shifts in large-scale seagrass ecosystems, which act as ecological engineers and provide many ecosystem services.

Methods and Materials

Study site

This study was based on observations and experiments on the seagrass community in Bath, Barbados (N 13° 11′, W 59° 28′) during May and June 2004. The study site covered an area of the seagrass bed 100m wide by 120m long. Three distinct vegetative zones were described in Mavromatis et al. (2006) and named based on the dominant cover of the region. The seagrass zone exists 20 to 40m offshore, and is followed by the macroalgal zone. The macroalgal zone extends from approximately 40 to 90m offshore, making it the largest continuous area of all zones. Finally, the mixed zone extends to 120m offshore **(Figure 1)**.

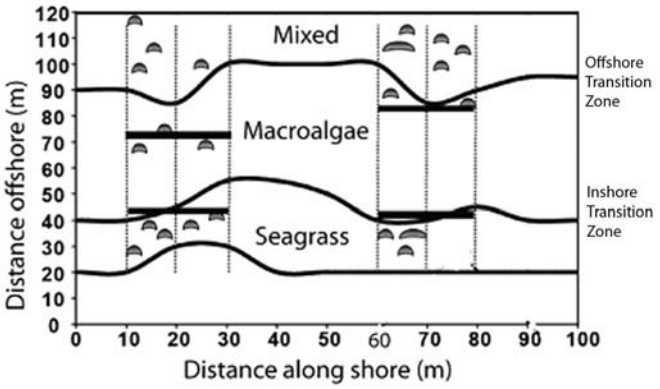

Figure 1. Diagram of the study area depicting 3 vegetation zones, 4 horizontal growth transects, 6 vertical physical gradient transects, and gaps (denoted by grey crescents). Figure modified from Mavromatis et al. 2006.

In order to address our hypothesis that both biotic and abiotic processes affect species assemblages in seagrass beds, we measured a series of physical characteristics of the study site including sediment type, water depth, erosion levels, and disturbance frequency (gap number) to see whether there were strong differences in these parameters between the three vegetation zones that might account for the patterns observed. Since we also wanted to see whether interactions between macroalgae and seagrass species influence the zonation of the study area, we measured blade growth and elongation of Thalassia in 25cm^2 quadrates along transects running lengthwise across the two transition regions between the vegetation zones (approximately 45m and 85m for the inshore and offshore transition zones respectively, 10 quadrates in each transition zone). In quadrates where Thalassia abundances were greater than 30% cover, one of three possible treatments was used: seagrass species only were present (n=6), seagrass and macroalgae species were present (n=7), or macroalgae species were experimentally removed leaving only seagrass in the quadrate (n=7). Thalassia blades in each quadrate (n= 12) were randomly selected, marked and measured after 3-6 days of growth **(Figure 2)**.

Average daily growth rates were calculated for marked blades, and we examined growth rate differences between treatments in the inshore transition zone. Square-root transformations were performed on the growth rate data to improve the normality of residuals. We used a 2-way factorial ANOVA to test for differences between the 2 factors (treatment and transition zone) and Bonferroni-corrected LSD comparisons were used to test for differences between the means.

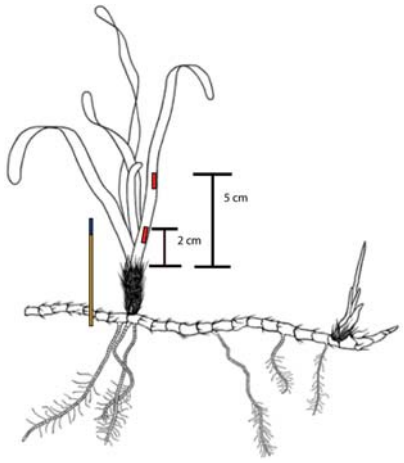

Figure 2. Diagram of blade identification and growth measurement procedure. Coloured toothpicks were inserted into the sediment and used to identify Thalassia blades. Leaves were stapled at 2cm and 5cm above the rhizome base, and after 3-6 days, measurements were taken from rhizome base to each staple to obtain measurements of blade growth and elongation.

Results

Abiotic patterns

Water movement intensity (erosion) was measured overnight using plaster of Paris cylinders (Guichard and Bourget 1998). A one-way ANOVA found that the offshore transition area experiences significantly higher levels of wave action and disturbance than the inshore region (n= 18, $p<0.0003$) **(Figure 3a)**.

Nine measures of water depth associated with each growth transect were analyzed for differences between inshore and offshore transition areas (2 transects per area, n=18). A one-way ANOVA indicated no significant difference in depth at the level of our transects ($p>0.05$). However, water depth was variable across the study site **(Figure 3b)**.

Twenty-three gaps (vegetation-free depressions measuring ≥3m wide, ≥2m long and ≥1m deep) were mapped across the study site **(Figure 1)**: 9 were found in the seagrass zone, 5 in the macroalgae zone and 8 in the mixed zone. Gaps in the seagrass zone occupy an area of approximately 31m^2 representing a total surface area of 3.9%. Gaps in the large macroalgae zone cover only 19.2m^2 or less than 1% of the area. The mixed zone has the highest surface area disturbed by gaps at 56.7m^2, which represents 4.7% of the surface.

Biotic processes

There is a significant effect of treatment on the growth of Thalassia ($p<0.001$) as well as a significant interaction term between transition zone and treatment ($p<0.008$). Bonferroni-corrected LSD comparisons found that inshore areas with no Avrainvillea were significantly different from all other means

(p<0.001), but no other treatment level was found to be significantly different from the others (p>0.0738) **(Figure. 4)**.

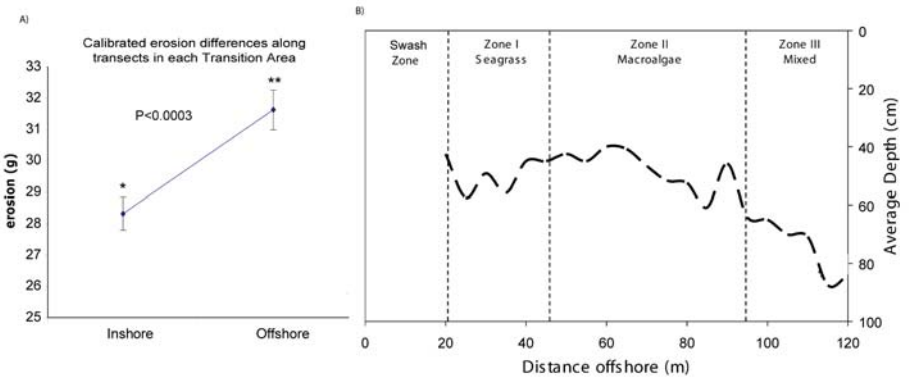

Figure 3. Large-scale environmental gradients. **(A)** Erosion levels between transition zones as measured by differences in plaster cylinder weight **(B)** Calibrated depth differences across the study site.

Discussion

Species interactions in seagrass ecosystems

Studies in community ecology often focus on the fundamental questions of what processes enable species to persist and what processes contribute to extinction. Here, we present a study on a dynamic marine ecosystem currently experiencing global declines for various reasons, many of which are unknown (Duarte 2002). Preliminary observations led us to believe that Thalassia was experiencing negative density-dependent effects of competition from the macroalgae species Avrainvillea, which has recently been established as a late colonizer in this system and is slowly replacing the seagrass at the level of our study site (Mavromatis et al. 2006). However, the effects of direct biotic interactions between these species were not consistent throughout the study site.

Although Thalassia grows better without macroalgae in the inshore transition area, removing the algae experimentally does not significantly increase the growth of seagrass. As a result, biotic processes cannot fully explain the patterns of species assemblage observed in this region; physical parameters must also contribute to the dynamics. The inshore transition zone appears to experience a species sorting effect such that habitat without algae maximizes the growth of Thalassia. Although competition may still be a factor influencing the presence of seagrass, habitat segregation between the species may better explain the distinct zonation observed between the seagrass and macroalgae zones since removal of algae marginally increases Thalassia growth,.

The dynamics change in the offshore transition area. None of the three treatments showed significant differences, suggesting that habitat has less influence on species presence and persistence than biotic interactions such as competition. These results suggest that competitive interactions rather than habitat segregation may enable the persistence of seagrass and macroalgae in mixed assemblages in the offshore zone.

Mechanisms for coexistence in spatial landscapes

Levels of disturbance in a system influence species composition by forcing life-history trade-offs and creating conditions that may enable coexistence (Tilman 1994). It has often been suggested that intermediate levels of disturbance are responsible for the maintenance of diversity in ecological communities (Sousa 1979), and a wide body of literature exists discussing this hypothesis and its implications. However, debate continues about the role of spatial heterogeneity and whether spatially patchy disturbances or simply ecological differences between species are sufficient to promote coexistence (Chesson 1991; Roxburgh et al. 2004).

There is a clear difference in competitive ranks between seagrass and macroalgae established at Bath, Barbados (Mavromatis et al. 2006), although one that is different from previous literature (Davis and Fourqurean 2001). The vegetative zonation observed at the level of our seagrass bed is also associated with gradients of physical parameters including depth, erosion, and disturbance. Depth differences cannot explain the discrepancies in growth patterns observed in our transects. However, water depth is an important physical parameter that may promote habitat segregation. Seagrass is known to suffer desiccation stress rapidly (Birch & Birch, 1984) whereas Avrainvillea longicaulis prefers shallow, open sand areas that may be exposed to the air during low tide (Littler & Littler, 1999). This habitat preference may contribute to the extensive macroalgal zone occupying a relatively shallower part of the bed, since seagrass is at a disadvantage in these habitats.

Wave action differs significantly both between growth

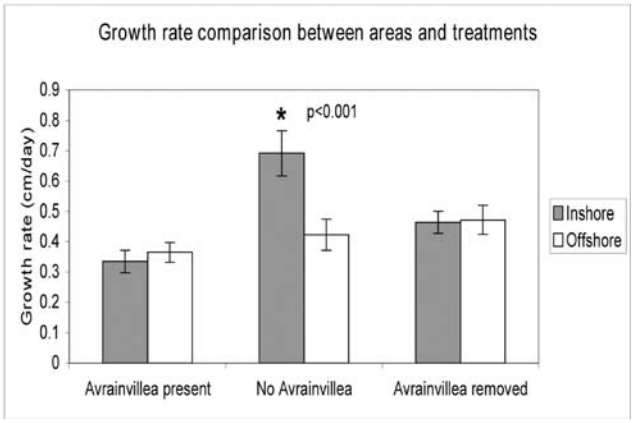

Figure 4. Treatment comparisons between all transects and treatments. Inshore transition zone areas with Avrainvillea naturally absent are significantly different **(a)** from all other means **(b)**.

transects and zones, with the macroalgae and mixed zones experiencing higher levels of water current than the inshore seagrass zone. Since Avrainvillea longicaulis is thought to prefer areas of relatively low current and wave energy (Littler & Littler, 1999), this physical gradient may be a driving factor in producing the patterns observed in the offshore area. Studies have shown that the degree of wave current and action is reported to be one of the main factors determining species composition and the extent of seagrass meadows in comparable sites (Kirkman 1985). High levels of wave action may increase the competitive ability of the seagrass relative to macroalgae, and allow for resource partitioning between these

species in the mixed zone, which Tilman (1994) suggests is a required component for stable coexistence. Although it is unclear whether the seagrass zone is experiencing gradual encroachment by the macroalgae or is temporally stable, the significant differences in physical parameters found across the study zones play an important role in driving the maintenance of diversity. Small-scale disturbances may therefore have a large role in the structuring of seagrass beds since they increase the interactions between biotic and abiotic processes.

Disturbance patterns and species interactions

Competition for space and resources represents a significant challenge in nature. This struggle is especially true for sessile organisms, which are in perpetually close association with both their neighbours and local habitat (Tilman 1994). Space is a limiting resource preventing the proliferation of species in many environments, and disturbance acts as a natural force that renews this resource and allows new individuals to grow (Paine and Levin 1981). Gap phenomena have long been recognized as important processes renewing space and allowing colonization in plant communities (Watt 1947). Even though the macroalgae zone occupies the largest continuous area, it contained the fewest number of gaps and the least amount of disturbed area. The seagrass zone, containing the most gaps in the smallest area, has a much greater percentage of its overall area disturbed. As suggested by Mavromatis et al. (2006), Thalassia is more often found in recently disturbed areas since it is able to colonize newly opened space relatively faster than macroalgae. Differences in disturbance regimes between the macroalgal and seagrass zones may therefore contribute to the habitat segregation observed. Disturbances may play a different role in the mixed zone. Gaps may contribute to the coexistence of seagrass and algae by promoting the rapid turnover of species and preventing the competitive exclusion of Thalassia. In essence, biotic and abiotic processes work in conjunction with one another to enable the persistence of a diverse marine community.

Conclusion

Seagrass beds represent ecologically significant ecosystems that provide a number of vital services to marine coastal communities (Duarte 2002). Unfortunately, many seagrass beds are undergoing rapid changes in their structure and ability to persist as intact systems as a result of both direct and indirect anthropogenic disturbances (Duarte 2002). Even in the thirty years since Patriquin's studies (1972; 1975) at Bath, Barbados, remarkable changes in species assemblages, successional sequences and physical gradients have occurred that are causing a once extensive bed to be overrun with macroalgae. From this study, we were able to elucidate the relative contributions of abiotic and biotic processes in relation to the physical environment. Competition and disturbances seem to be the dominant factors influencing species coexistence in the mixed zone, whereas habitat partitioning driven by differences in depth, disturbance and wave action creates distinct zones of macroalgae and seagrass in inshore regions. Future research should focus on establishing the role of seagrass acting as ecological engineers, the scaling of seagrass recovery from disturbances, and the resistance of beds to physical phenomena such as sedimentation and eutrophication that were not tested during our experimentation. Elucidating the important processes regulating the distribution of seagrass species will be useful in informing managerial decisions regarding the preservation of these endangered systems within larger tropical intertidal communities.

Acknowledgements

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Urban form and climate change

Andrew Salzberg

Cities come in all shapes and sizes. The idea that these different shapes – whether sprawling like Los Angeles or dense like Manhattan – can play a role in determining the environmental impacts of urban areas is an idea that is gaining currency in both popular and scientific circles. This article will attempt to highlight the role that the 'urban form' of a city can play in either attenuating or exacerbating the production of greenhouse gases. 'Urban form' is a term that has been developed to describe the physical composition of a city. It encompasses an urban area's density (inhabitants/hectare), its mix of land uses (divisions between residential, commercial, industrial, etc.), its provision of transportation options (public transit facilities, auto-related infrastructure) as well as the degree to which urban development is contiguous or 'scattered' around the edges.

Although the link between urban form and climate change is still actively debated, research indicates a 'strong but complicated' relationship between higher densities, mixture of residential and commercial uses, and reduced greenhouse gas emissions. Since most of our energy today (and for the foreseeable future) is derived from fossil fuels, a reduction in energy consumption implies a concomitant reduction in greenhouse gas emissions. One study attempted to quantify the connection between urban form and greenhouse gas emissions by plotting a number of cities' private automobile energy consumption versus their gross density. They found a startlingly consistent relationship, as shown in **Figure 1**.

Clearly, increasing density can substantially reduce transportation energy consumption. It is important, however, not to infer too much from this graph. The disparity between automobile usage in places like Houston and Hong Kong involves factors other than density; cultural differences, income distribution, and levels of auto ownership may also play significant roles. Nevertheless, the graph does demonstrate the significance of one measure of urban form in determining energy consumption and, consequently, greenhouse gas emissions.

This density versus greenhouse gases (GHG) emissions relationship can also be demonstrated on a more local level. A study by Feigon et al. (2003) analyzed GHG emissions in several American metropolitan regions and found that Tokyo-like densities are not required for a relationship to become clear. The results of their study of Chicago are presented in **Figure 2**.

On the left, GHG emissions are shown on a per square mile basis. As is expected, the dense urban core produces greater emissions than the periphery. However, as shown on the right, the emissions per household are actually lowest in the central city. The results demonstrated globally by Newman and Kenworthy (2000) are reappearing here at the fine-grained level of urban neighborhoods.

What are the causes of this relationship? How do density and other aspects of urban form exert such a strong influence on urban energy consumption and, consequently, greenhouse gas emissions? The effect can be divided into two main areas: transportation and residential energy consumption.

Urban Form and Transportation Energy Use

Urban Form can have an enormous impact on the way people travel. As one study succinctly put it, 'the physical characteristics of a place, or urban form, influence how often, how far and by what means people travel'. This seems true intuitively:

A person living in a residential subdivision with cul-de-sac streets and few sidewalks has little choice but to drive to the grocery store and to a job. A person living in an area laid out in a grid of interconnecting streets with a mixture of land uses supported by a comprehensive transit system can choose to walk, bicycle, use transit, or drive. Even with the option to drive, the physical layout of the latter community is likely to generate fewer vehicle trips, and shorter trip lengths overall, and will produce fewer CO2 emissions than the former community. (Feigon et al. 2003, p.6)

This description gives one example of how the layout of cities has an impact on the way people choose to get around. It includes the two most essential points: all alternatives to driving require higher densities and a greater variety of uses than exist in most modern, auto-oriented suburbs. This is important since the private automobile has been identified as the most energy-intensive form of transportation. Reducing the frequency and distance of travel as well as allowing a shift from private automobiles to other, less carbon-intensive forms of transportation is one of the primary areas where urban form can play a role in bringing about reductions in GHG emissions. This is particularly true in the case of short trips.

Non-motorized transportation (predominantly walking and bicycling) is an attractive alternative to vehicle travel because, generally, short trips via personal automobile that are of "bikeable or walkable" length tend to be more polluting. They also constitute a significant percentage of all vehicle trips made (Feigon et al, 2003).

In other words, denser urban form allows for more efficient transportation, especially over the short haul.

Density alone, however, is not entirely effective. Another important characteristic of an energy-efficient urban form is that it provides a good mix of uses; in other words, employment and shopping opportunities are mixed in with residential development. This is not a new idea; industrial-era downtowns and pre-automobile neighborhoods often exhibit an effective mixture of primary uses. However, twentieth century automobile-oriented development separated land use by zone according to function: industrial, residential, commercial, etc. By reversing this trend and bringing people closer to their destinations, urban form can bring about reductions in transportation energy consumption. In summary, more people per area closer to their destinations (shopping, work, etc.) reduces the need to travel long distances while enabling cycling, walking and public transit. This reduces GHG emissions and energy consumption.

Quantifying exactly how much energy could be saved is more difficult. A detailed study carried out by the National Round Table on the Environment and the Economy (NRTEE) estimated that urban transportation accounts for about 10% of total Canadian GHG emissions. In the United States, one third of greenhouse gas emissions come from surface transportation. Using this energy breakdown and the fact that American emissions account for 25% of global emissions, one can conclude that 8% of global GHG emissions are created by transportation within the United States, which helps to underline the importance of reducing transportation energy usage in developed countries.

One way of expressing the relationship between urban form and transportation energy use is that a denser, mixed use urban form is a necessary but not necessarily a sufficient condition for reducing energy consumption in cities. Other means, such as congestion pricing or higher fuel taxes, may be necessary to finally push people away from the automobile, but without other options provided by a change in urban form they cannot be effective. As Newman and Kenworthy (2000) write, "Achieving a more sustainable urban form inevitably involves the development of densities that can enable public transport, walking and cycling to be viable options."

Urban Form and Residential Energy Consumption

The residential sector has also been identified as an area where energy efficiency can be improved through physical changes to the urban fabric. The shared walls, floors and ceilings of higher density dwellings inherently increase energy efficiency. Higher densities also allow for more energy efficient technologies. One study estimated that significant energy savings over conventional detached housing could be made by designing houses to make the most of solar energy for heating and cooling, and using shared walls and floors like those in terrace or apartment housing.

Several studies have attempted to quantify the energy savings that are possible in higher density living arrangements. The best of these have incorporated Life-Cycle Analysis (LCA) into their studies. This methodology incorporates all the energy use and greenhouse gas emissions inherent in the construction materials used to construct both low and high-density residential development. LCA allows the development of a more realistic understanding of the environmental impacts of residential densities. Using LCA, two recent studies came up with maximum possible energy reductions on the order of 45% per capita (Norman 2005).

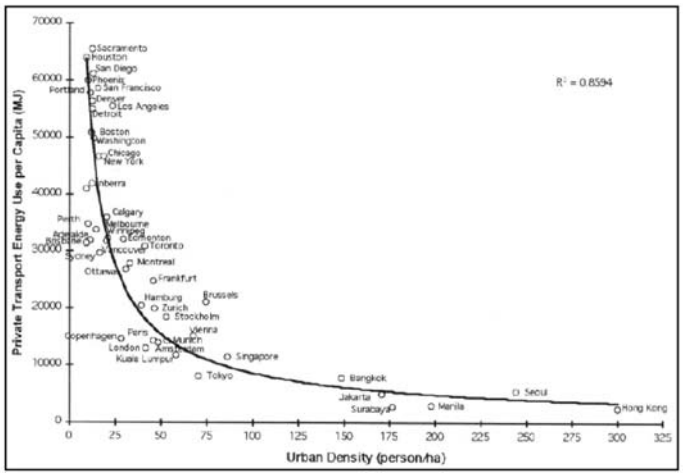

Figure 1. Source: Newman and Kenworthy (2000)

While residential density can help to reduce the greenhouse gas impacts of the residential sector, it is important to note that there are some value judgments implicit in these analyses. For example, reductions in residential energy consumption are often much more significant on a per capita basis than a per square meter of living space basis, which brings up moral and ethical questions about how much living space is really necessary for an individual.

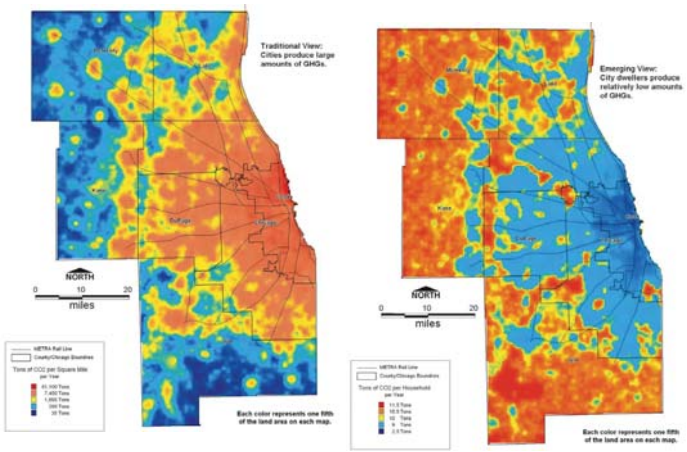

Figure 2. Source: Feigon et al (2003)

åIssues and Conclusion

For those who hope that cities in the developed world can transform to reduce their climate impact, some positive news has emerged in recent years. The process of gentrification is an indication of a renewed desire among affluent people for dense, mixed-use neighborhoods. Other research has demonstrated that American cities are reversing a 50-year trend and becoming denser at the edges. These phenomena demonstrate that we may be moving in the direction of more carbon-friendly cities, at least in the developed world. However, to help achieve reductions commensurate with the scale of the climate problem, practical solutions will need to be found to redesign our cities that are both socially acceptable and environmentally effective. Although a significant body of research has developed around the implications of urban form for energy use and greenhouse gas emissions, the challenge of the 21st century will be to find sensible ways to put this knowledge to good use.

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Stem-loop binding protein localization, expression patterns, and regulation of histone mRNA in wild-type and transgenic mouse oocytes

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Abstract
Histone proteins are essential for the cell cycle and are massively accumulated during S-phase of cell division in somatic cells and during early oogenesis. Histone mRNA differs from the majority of metazoan mRNA by terminating with a highly conserved stem-loop in its 3′-untranslated region. The stem-loop binding protein (SLBP) is the only known protein specifically binding to histone mRNA stem-loops and is believed to be responsible for the regulation of histone translation. SLBP is thought to also control mRNA levels through stabilization and post-transcriptional processing. We studied the localization and expression pattern of SLBP in ovaries of wild-type and transgenic mice using immunohistochemistry and immunofluorescence techniques. Our data show that SLBP translocates from the cytoplasm of developing oocytes to the nucleus shortly after release from meiotic arrest. SLBP subsequently localizes back out into the cytoplasm as the oocyte continues its maturation. Our transgenic mice show a significant decrease in SLBP expression levels. The results present encouraging evidence for the role and localization of SLBP during oogenesis.

Keywords
Histone: *tetrameric proteins responsible for DNA packaging into condensed chromatin and gene regulation;* **Stem-loop**: *a DNA or RNA structure generally involving a single strand folding back onto and binding with itself to form a loop;* **Stem-loop binding protein (SLBP)**: *a protein specifically binding to the stem-loop of histone mRNA;* **Cell cycle**: *the cycle which all somatic and germ cells undergo at some time in their lifespan. Mitosis and meiosis are both parts of the cell cycle;* **Immunofluorescence**: *using an antibody attached to a fluorescent molecule to specifically bind to a target to verify its location and quantify its expression in a cell.*

Introduction

The eukaryotic cell cycle is the result of an accumulation of complex mechanisms employed by the growing cell. The collaboration between these numerous and assorted pathways is crucial to the success of the cell in completing its designated roles. Histone proteins bind to eukaryotic DNA during nearly all phases of the cell cycle and play key roles in the formation of chromatin as a form of DNA packaging, chromatin stability and compaction, as well as gene regulation (Zhao et al., 2004).

During S-phase of the cell cycle, the concentration of histone proteins necessary for DNA condensation is dramatically increased. The abundance of histone proteins is critical to ensure proper DNA packaging of duplicated genetic material in daughter cells (Schumperli, 1988; Marzluff et al., 2002). As such, the transcription of histone mRNA and translation of histone proteins are tightly coupled to cellular proliferation and are amongst the mechanisms essential to cellular division (Whitfield et al., 2000; Zheng et al. 2003).

Whilst increased expression of histone mRNA is important in somatic cells, it is vital in oogenesis, which occurs in several steps. Multiplying primordial germ cells in the embryo and fetus give rise to millions of primary oocytes, which enter meiotic arrest at the first meiotic prophase. At puberty, the arrest is lifted from a small number of primary oocytes, which then continue development and meiotic division (Vanderhyden, 2002). The multiple stages of meiosis and cell division in early oocyte maturation require rapid production of considerable amounts of essential histone proteins. As such, mediation of histone mRNA translation is crucial to ensure proper gene activity.

Translation regulation in metazoan cells involves two classes of cellular mRNA that are differentiated by their 3′-untranslated region. The majority of metazoan protein mRNAs are polyadenylated at their 3′ ends and are regulated by translation initiation factors such as eIF-4E and eIF-4G during protein synthesis. Eukaryotic histone protein mRNAs are not polyadenylated; instead, a highly conserved stem-loop is found at the 3′-untranslated region. The stem-loop is hypothesized to be responsible for the mediation of histone mRNA levels at the posttranscriptional stage through adjustments to mRNA processing and stability. The stem-loop structure is therefore, to a certain degree, functionally homologous to poly (A) tails of other metazoan mRNAs (Muller et al., 1997; Sanchez et al., 2002).

The stem-loop binding protein (SLBP) is a thirty-one kilodalton RNA-binding protein responsible for binding the stem-loop of the histone mRNA and promoting its translation (Muller et al., 1997; Sanchez et al., 2002). The specificity of SLBP binding to the stem-loop of histone mRNAs allows us to monitor the localization and expression level of SLBP as a measure of histone mRNA gene expression. Concurrently, the role of SLBP in translational regulation can be assessed.

Proper SLBP and histone mRNA and protein expression is critical to oogenesis and oocyte survival (Song et al., 2005). During early embryogenesis, control of gene expression is

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dependant on mRNAs that have already been synthesized and stored in the oocyte (Sanchez et al., 2002). Histone mRNAs are therefore accumulated in early oogenesis and translation is activated during oocyte maturation. Thus, as an oocyte moves past meiotic arrest, interacts, and develops alongside its follicle, histone mRNAs should localize from the nucleus to the cytoplasm where translation can take place. The role of SLBP suggests possible localization to the nucleus to bind histone mRNA and subsequent relocation to the cytoplasm. Given that SLBP is the only known protein specifically binding histone mRNA stem-loop, observation of its localization and expressions could prove invaluable to the understanding of replication-dependent histone transcription and translation.

Materials and Methods

The localization and expression of SLBP and underlying processes of mediation of histone mRNA by SLBP were observed using immunofluorescence and immunohistochemistry techniques on sectioned mouse ovaries aged five days, ten days, fifteen days, and twenty days. Immunofluorescence is ideally used to localize proteins within cells, and sections of ovarian tissue serve as excellent templates for antibodies binding specifically to SLBP. Furthermore, the levels of fluorescence can be quantified to reveal the level of expression of a particular protein. All results were controlled for by replacing the primary antibody with PBS (Phosphate Buffered Saline), while the same materials and procedures were applied to the remainder of the experiment.

Transgenic mice

A transgenic mouse line was created in Dr. Hugh Clarke's laboratory to study the effects of SLBP loss. A 700 base pair SLBP dsRNA cassette was inserted into mouse DNA next to the Zona-pellucida-3 gene promoter. The promoter is responsible for gene expression during oocyte growth (Arnold and Clarke, unpublished data). Therefore, the dsRNA is only transcribed during oocyte growth. When it is expressed, the dsRNA specifically inhibits SLBP mRNA by RNA interference, resulting in SLBP knockdown during oogenesis after the first meiotic arrest.

Histology

Ovaries dissected from CD-1 wild-type mice and SLBP-dsRNA-knockout transgenic mice were fixed overnight in 4% paraformaldehyde at 4ºC with agitation. We then dehydrated the ovaries and removed the fixative by washing the tissues in a graded ethanol and xylene series. Tissues were then sent to the McGill Cancer Centre to be embedded in paraffin blocks and stored at -20ºC. We cut 5mm thick sections of tissue from the paraffin block using a microtome and subsequently mounted them on slides, dried them overnight, and adhered them to the slides at 60ºC for 20 minutes the next day. The slides can be stored at either 4ºC or room temperature. Rehydration of the sectioned tissues through the ethanol and xylene series was followed by 20 minutes of antigen recovery using 0.1% sodium citrate antigen recovery buffer at 80ºC to 90ºC.

Immunofluorescence and Immunohistochemistry

Once antigen recovery has been performed, we treated the slides for 30 minutes with a blocking solution composed of 5% goat serum, 5% bovine serum albumin, and 0.1% PBST (Phosphate Buffered Saline and 0.1% Tween). We then incubated the tissues with a 1:100 blocking buffer dilution of anti-SLBP primary antibody, provided by the Clarke laboratory, overnight at 4ºC with agitation. The slides were then washed with blocking buffer and incubated with a 1:100 PBST dilution of horseradish peroxidase-conjugated secondary antibody and YOYO-1 DNA stain for 60 minutes at room temperature. After several PBS washes, the sections were treated with tyramide signal amplification working solution. The slides were then washed and mounted with Mowiol. Storage was at 4ºC and measurements were taken with a confocal microscope.

Image analysis

Image analysis and fluorescence levels were determined using Image J and the colour histogram plug-in. When possible, 20 pixel by 20 pixel selections were made for analysis. Graphs created by the program separate red, green, and blue channels. The program also calculates the mean level of luminescence intensity for each. SLBP fluorescence is shown in the red channel and is of primary interest. DNA fluorescence is shown in the green channel.

Results

Five-day-old ovaries generally had a high number of both dormant and developing primordial and primary follicles. Ten-day-old ovaries retained a considerable amount of primordial follicles; however, a small number of primordial follicles had developed into the primary and secondary stages. Both fifteen and twenty-day-old ovaries had large amounts of secondary follicles and tertiary follicles.

The granulosa cells of primordial follicles have a flat structure. As these primordial follicles develop into primary follicles, the granulosa cells assume a cuboidal configuration. The acquisition of a second granulosa layer marks the development of the follicle into the secondary stage. Maturation continues as the follicle continues to grow, gaining ever more layers of granulosa cells. Tertiary follicles can be identified by the formation of a fluid filled space adjacent to the oocyte.

Immunofluorescence results showed SLBP presence in the cytoplasm of primary oocytes surrounded by flat-structured granulosa cells of primary follicles **(Figure 1a)**. No significant amount of fluorescence was found in the nucleus of these arrested oocytes **(Figure 1b)**. However, in oocytes that have initiated growth and moved past the first meiotic arrest, fluorescence of the antibody is confined to the nucleus, with significantly less staining in the cytoplasm **(Figure 1c)**. Indeed, image analysis shows that fluorescence in the nucleus is double the intensity of staining in the cytoplasm **(Figure 1d)**. The low levels of staining in the cytoplasm indicate that oocytes in primary follicles have high concentrations of SLBP in their nucleus, and relatively low concentrations elsewhere. Furthermore, it is important to note that, in CD-1 wild-type mice, staining is two times stronger in primary follicles than in primordial follicles **(Figure 6)**.

Slides containing transitional follicles caught between primordial and primary stages or primary and secondary stages appear to have equal staining in both the nucleus and the cytoplasm **(Figure 2a; Figure 2b)**. However, as the follicle acquires its second layer of granulosa cells, fluorescence in

the cytoplasm of the oocyte is more than double the intensity of the nuclear staining, indicating relocation of SLBP from the nucleus back into the cytosol **(Figure 2a; Figure 2c)**.

In later stages of oogenesis and folliculogenesis, as seen in the ten-day-old, fifteen-day-old, and twenty-day-old ovary sections, SLBP staining is limited to the cytoplasm of the oocytes, with no staining in the nucleus **(Figure 3)**.

SLBP-knockout mice had strong SLBP staining in the cytoplasm of non-developing oocytes enclosed in primordial follicles **(Figure 4)**. However, only extremely low levels of fluorescence were detected in developing oocytes enclosed in primary, secondary, and tertiary follicles **(Figure 5)**. Wild-type oocytes exhibited more than five times stronger fluorescence than transgenic oocytes **(Figure 5b)**.

Figure 1. Primordial and primary follicles of CD-1 wild-type mouse ovaries. Left: YOYO-1 DNA staining in green. Middle: immunofluorescence SLBP staining in red. Right: combination overlap. (* marks the oocyte nucleus; " marks the cytoplasm)

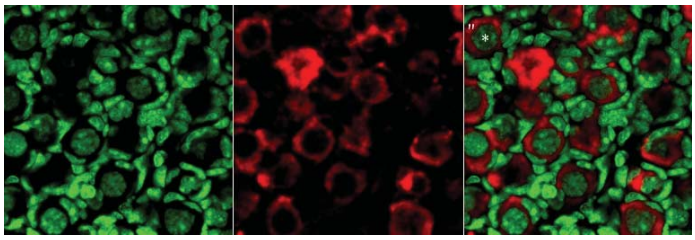

Figure 1a. Primordial follicles in 5-day-old CD-1 wild-type mouse ovaries.

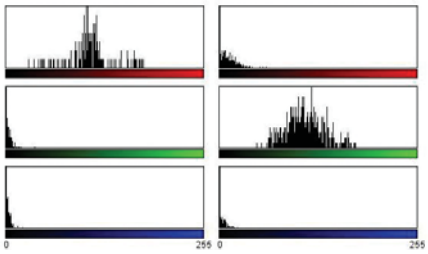

Figure 1b. SLBP fluorescence RGB (colour intensity histogram of primordial follicle and oocyte seen in **Figure 1a**. Left column: cytoplasm. Right column: nucleus.

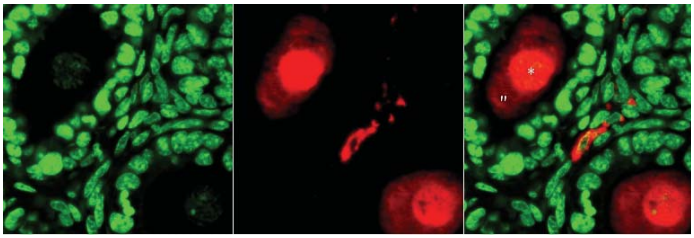

Figure 1c. Primary follicles in 5-day-old CD-1 wild-type mouse ovaries.

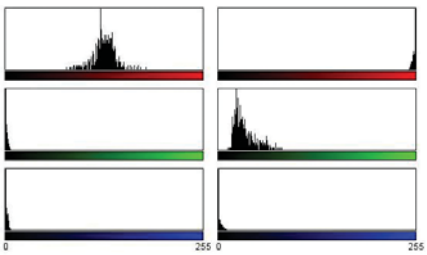

Figure 1d. SLBP fluorescence RGB (colour intensity) histograms of oocyte seen in **Figure 1b**. Left column: cytoplasm marked by " Right column: nucleus marked by *

Discussion

Our data from CD-1 wild-type mice suggests that in the early stages of oogenesis and folliculogenesis, SLBP is translated in and localized in moderate amounts to the cytoplasm of the primary oocyte **(Figure 1)**. Indeed, oocytes in primordial follicles have no SLBP staining the nucleus **(Figure 1b)**, indicating that the protein is first stored in the cytosol after its initial translation. The development of the oocyte is then interrupted. However, when the oocyte is released from meiotic arrest, SLBP production is dramatically increased. In fact, primary follicle oocytes fluoresce with double the intensity of primordial follicle oocytes **(Figure 6)**. As the primordial follicle surrounding the oocyte begins to develop into its primary stage, the increase in SLBP production is accompanied by SLBP localization from the cytoplasm of the oocyte to the nucleus, where it is sequestered until metaphase II **(Figure 1c)**. At this phase, the antibody staining in the nucleus of the oocyte is twice as strong as the fluorescence of the cytoplasm **(Figure 1d)**. Therefore, the data seem to indicate that SLBP expression is greatly enhanced after the commencement of oocyte development and reaches its peak at the primary follicle stage.

Figure 2. Primary follicles in transition to secondary follicle stage in CD-1 wild-type mouse ovaries. YOYO-1 DNA staining (green) and SLBP immunofluorescence staining (red) merged overlap. (* marks the oocyte nucleus; " marks the cytoplasm)

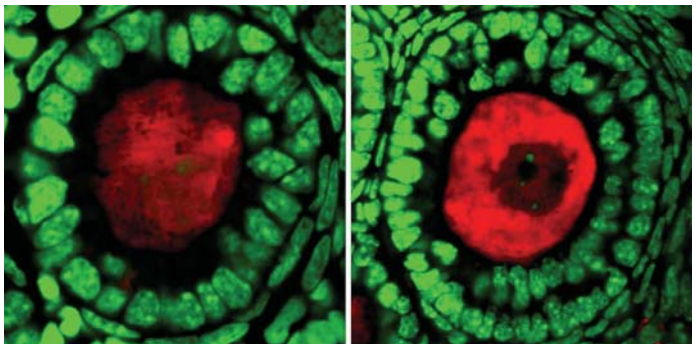

Figure 2a. Follicles transitioning form primary to secondary stage. Left: oocyte at beginning of transition from primary to secondary stage. Right: oocyte at end of transition from primary to secondary stage.

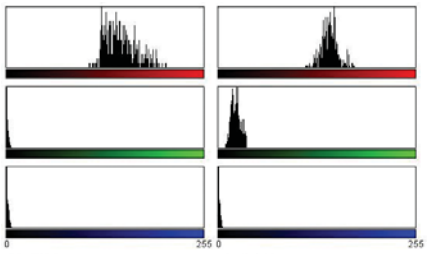

Figure 2b. SLBP fluorescence RGB (colour intensity) histograms of oocyte on left side of **Figure 2a**. Left column: cytoplasm. Right column: nucleus.

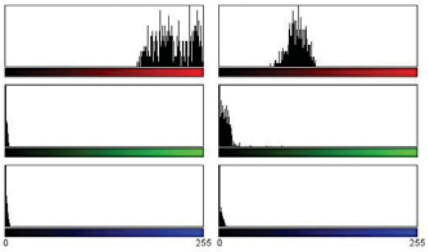

Figure 2c. SLBP fluorescence RGB (colour intensity) histograms of oocyte on right side of **Figure 2a**. Left column: cytoplasm. Right column: nucleus.

The movement of SLBP across the nuclear membrane is thus directly correlated with the growth of the oocyte and development of the follicle. The localization of SLBP into the nucleus is highly significant, as it suggests that histone mRNA stored in the nucleus of the oocyte requires binding with SLBP to exit the nucleus and commence translation.

As the oocyte continues to develop past metaphase II of its first meiotic division, the follicle containing it moves past primary stage and becomes a secondary follicle (Allard et al.,

2002). As the transition takes place, the SLBP concentration in the cytoplasm becomes as high as the concentration in the nucleus and continues to increase until it is more than double the amount **(Figure 2b; Figure 2c)**. Molecularly, it is expected that the translocated SLBP has bound specifically to the stem-loop of the histone mRNA stored in the nucleus. SLBP then appears to return to the cytoplasm as the nucleus is completely emptied of the protein **(Figure 3a; Figure 2)**. As such, we can hypothesize that the translation of histone mRNA within the maturing oocyte occurs at the secondary follicle stage.

Figure 3. Secondary and tertiary follicles of CD-1 mouse ovaries. YOYO-1 DNA staining in green; immunofluorescence SLBP staining in red.

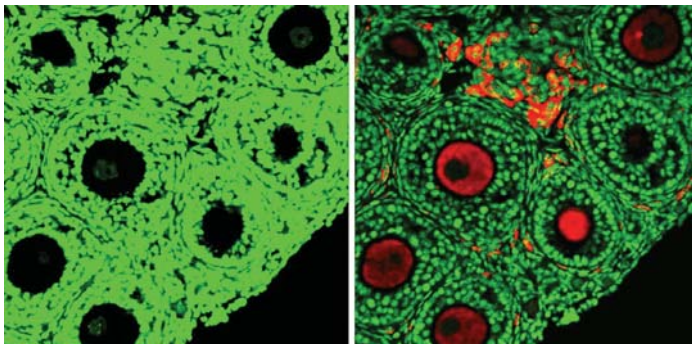

Figure 3a. Secondary follicles in 10-day-old CD-1 wild-type mouse ovaries. Left: YOYO-1 DNA staining. Right: merged overlap of YOYO-1 with immunofluorescence SLBP staining. Note that in oocytes appearing to have uniform staining, the nucleus is not included in the section, and only cytoplasmic staining is visible.

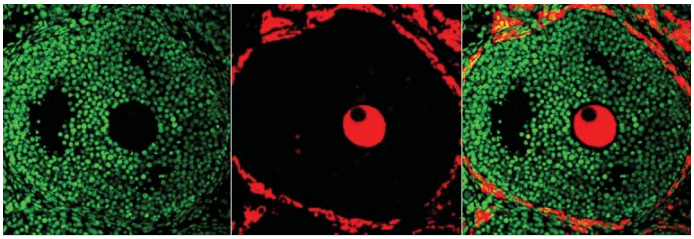

Figure 3b. Early tertiary follicle in 20-day-old CD-1 wild-type mouse ovaries. Left: YOYO-1 DNA staining. Middle: immunofluorescence SLBP staining. Right: merged overlap.

Further analysis shows persistently strong staining in the cytoplasm of maturing oocytes in early and late tertiary follicles **(Figure 3b)**. Indeed, significant quantities of histone proteins may have already been synthesized at this point. Remaining histone mRNA will be regulated by SLBP in the cytoplasm until translation is complete. Thus, SLBP is accordingly kept in the cytoplasm instead of being degraded.

Although our data correlates the localization of SLBP with oocyte development, it is important to note certain limitations of the study. Previous research has determined the target of SLBP to be the stem-loop of the histone mRNA (Muller et al., 1997; Sanchez et al., 2002). However, detection of histone mRNA was not performed. Further research on this subject should focus on the visualization of histone mRNA through in situ hybridization or other mRNA detection techniques.

Figure 4. Primordial follicles in five-day-old transgenic mouse ovaries. Left: YOYO-1 DNA staining in green. Middle: immunofluorescence SLBP staining in red. Right: merged overlap.

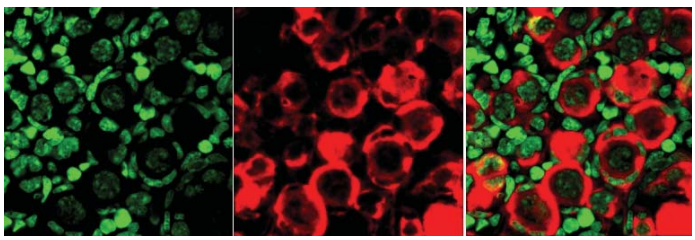

Figure 5. Primary follicles in transition to secondary follicle stage in CD-1 wild-type mouse ovaries. YOYO-1 DNA staining (green) and SLBP immunofluorescence staining (red) merged overlap. (* marks the oocyte nucleus; " marks the cytoplasm)

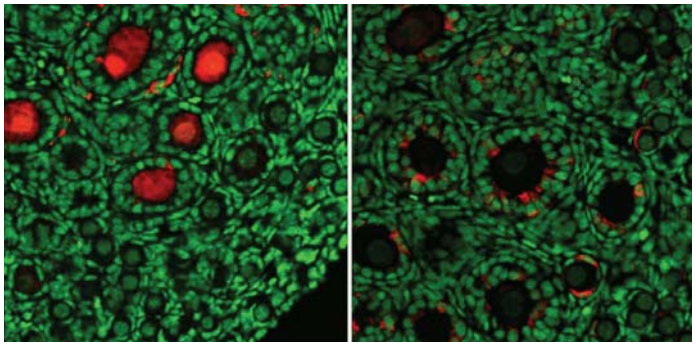

Figure 5a. Primordial and primary follicles in 5-day-old and 10-day-old ovaries. Left: CD-1 wild-type ovary section. Right: transgenic (right) ovary section.

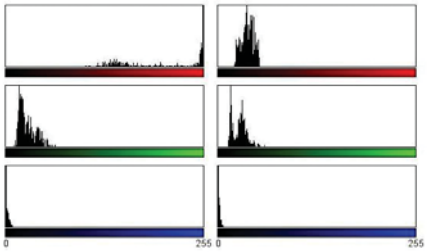

Figure 5b. SLBP fluorescence RGB (colour intensity) histograms of CD-1/transgenic comparison. Left column: CD-1 mouse SLBP immunofluorescence. Right column: transgenic mouse SLBP immunofluorescence.

Figure 6. SLBP fluorescence comparison in primordial and primary follicles of CD-1 mice. YOYO-1 DNA staining in green; immunofluorescence SLBP staining in red.

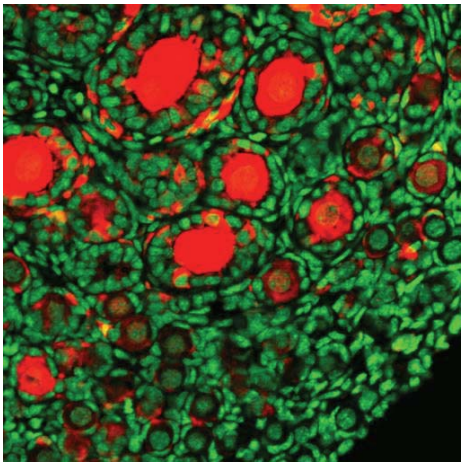

Figure 6a. Primordial and primary follicles in CD-1 mouse ovary.

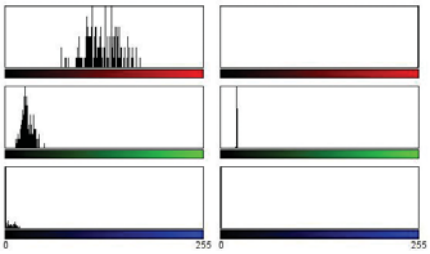

Figure 6b. SLBP fluorescence RGB (colour intensity) histograms of primordial and primary follicles in **Figure 6a**. Left column: Oocyte contained in a primordial follicle. Right column: Oocyte contained in a primary follicle.

Transgenic SLBP-knockdown mice have been created using RNA interference techniques in the Clarke laboratory. The expression of dsRNA in these mutant mice is triggered only in the maturing oocyte. Therefore, oocytes that are in meiotic arrest are not affected by the induced mutation and are indistinguishable from wild-type oocytes. Immunohistochemistry and immunofluorescence performed on ovarian sections from these mutant mice showed identical results for SLBP staining in non-developing oocytes in primordial follicles **(Figure 4)**. Since the expression of mutant phenotype is only present in maturing oocytes and follicles, the moderate levels of fluorescence in primordial follicles is expected. However, as oocytes in older ovaries matured, SLBP signal became very weak throughout all stages of folliculogenesis, implying successful SLBP knockout **(Figure 5)**. Actually, SLBP antibody fluorescence in wild-type mice is five times stronger than fluorescence in transgenic mice. **(Figure 5b)**. Data from other experiments performed by colleagues in the Clarke laboratory indicate that transgenic mouse oocytes abruptly stop development at the two-cell stage. Presumably, this is due to the lack of SLBP regulation of histone mRNA translation, resulting in a shortage of histone proteins necessary for continued meiotic division.

Development in both somatic and germ cells are undeniably critical to the growth of an organism. The exploration of underlying mechanisms is one of many current pursuits to further our knowledge and offers insight on the complexity of the cellular cycle. The regulation of histone mRNA is an important step for cellular replication in all eukaryotes, without which cell division would be difficult if not impossible. By ascertaining patterns of localization of SLBP and its role in the regulation of histone mRNA translation, we have increased our understanding of an important gateway affecting the proliferation of not only individual cells, but of the organism as well. Although still in its early stages, future research involving SLBP and histone mRNA pathways could lead to novel methods of controlling cell growth and development. Such progress has the potential to not only greatly advance our knowledge of the natural world, but may lead to breakthroughs in the treatment of tumours and infertility through inhibition or activation of somatic and germ cell growth.

Acknowlegements

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MSURJ McGill Science Undergraduate Research Journal

A recipe for laboratory-grown crystals

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Abstract
Clinopyroxenes are among the first minerals to crystallize out of a ferromagnesian silicate magma. They commonly exhibit "sector zoning", a phenomenon whereby the crystal incorporates elements in different proportions on non-equivalent crystal faces. By growing clinopyroxene crystals in the laboratory, it is possible to investigate controls on compositional variation, which provides insight on magmatic processes. The goal of this research was to develop an experimental method for growing synthetic clinopyroxene (a silicate mineral) in a carbonate melt rather than in a silicate one. This is advantageous since silicate residue on the clinopyroxene crystal may damage crystal faces, which contain important information on growth features, unlike carbonate residue which is easily dissolved leaving crystal faces intact. The carbonate melt was modeled after the alkali-rich carbonatite lavas erupting at Oldoinyo Lengai, Tanzania by using powdered clinopyroxene, magnetite and alkali carbonates containing up to 5% wt. water as starting materials, and running the experiment at conditions of 800∞C and 10 kbars. Clinopyroxene crystals in a carbonate crystalline matrix were retrieved from the experiment capsules, and cleaned for imaging and analysis with the atomic force microscope (AFM), scanning electron microscope (SEM) and electron microprobe (EMP). This experimental approach provides well-preserved crystal faces whose surfaces can be examined at nanoscale resolution. This technique could be applied to a wide range of synthetic silicate minerals, and the resulting observations help to better understand the relationship between crystal surface structure and trace element uptake during crystal growth.

Keywords
Carbonate/silicate melt: *a synthetic liquid meant to mimic natural magma in which crystals grow; the melt can be silica-dominated (silicate melt) or carbonate-dominated (carbonate melt);* **clinopyroxene**: *a silicate mineral commonly found in nature with a composition of* $Ca(Mg,Fe)Si_2O_6$; **crystal lattice**: *3D geometric arrangement of atoms in a crystal;* **crystalline matrix**: *a fine-grained crystalline matter in which larger crystals are embedded;* **dislocation**: *a linear defect in the crystal structure which promotes growth;* **nucleation**: *the initial step in development of a crystal, where a sufficient number of ions must cluster together to overcome spontaneous separation of ions due to unstable configuration;* **sector zoning**: *phenomenon whereby different crystal faces unequally incorporate trace elements during crystal growth;* **weathering**: *processes by which rocks break down.*

Introduction

Clinopyroxenes are a mineral group described by the chemical formula $Ca(Mg,Fe)Si_2O_6$ and characterized by single chains of silica tetrahedra (SiO_4^{4-}), giving them a prismatic shape elongated along the direction of the c-axis **(Figure 1)**. They are abundant in nature and are one of the first minerals to crystallize from ferromagnesian (iron and magnesium-rich) silicate magma. Like most minerals, they incorporate small amounts of foreign ions during crystal growth, and their crystal chemistry offers insight into mantle and crustal processes (Skulski et al, 1994). A particular feature of clinopyroxene crystallization is the development of sector zoning, the unequal incorporation of trace elements (or "impurities") into the crystal lattice on different crystal faces. Natural and synthetic clinopyroxenes with sector-zoned trace (Sc, Ti, V, Cr, Mn, Co and Zr) and major (Mg, Al, Si, and Fe) elements have been described in the literature (Shimizu, 1981; Kouchi et al., 1983).

Although clinopyroxene crystals are commonly found in nature, it is advantageous to synthesize them in the laboratory for crystal chemical studies since the surfaces of naturally-occurring crystals are commonly damaged by physical and chemical weathering. Laboratory-grown crystals offer control over growth conditions; parameters are chosen to favor development of specific crystal features of interest. In this study, the preservation of intact crystal surfaces was prioritized. This enables observation of growth morphology on crystal faces, which may influence the development of sector zoning (Watson, 1996). Although the studies by Shimizu (1981) and Kouchi et al. (1983) analyzed impurities incorporated in natural and synthetic clinopyroxene crystals, they did not observe in detail the surface structure of freshly-grown crystal faces. The research presented here aims to develop a new and efficient experimental technique for growing clinopyroxene crystals that preserves the pristine crystal faces upon extraction from an encrusting crystalline matrix. Removing the freshly-grown crystals from their encrusting crystalline matrix without damaging the surface features developed during the experiment presents a significant challenge. The present study overcomes this problem by the use of a carbonate melt as the growth medium for clinopyroxene crystals, rather than the conventional silicate melt, since a carbonate matrix is easily dissolved in comparison to a more resistant silicate matrix. Undamaged experimentally grown crystals permit a thorough study of the influence of surface structure on sector zoning.

Methods

An alkali-rich carbonate lava erupted from Oldoinyo Lengai, Tanzania in 1993 (Dawson et al. 1994) was used as a model melt composition because clinopyroxene was reported as one of the minerals crystallized in the lava **(Table 1)**. The lava composition was replicated in the laboratory by using reagent-grade calcium carbonate ($CaCO_3$), magnesium

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carbonate ($MgCO_3$), sodium carbonate (Na_2CO_3), potassium carbonate (K_2CO_3) and iron oxide (Fe_2O_3). For the source of silica, crushed natural clinopyroxene crystals from the Orford nickel mine in Québec, Canada **(Table 1)** were added to the reactant mixture rather than reagent-grade silica, since the presence of natural clinopyroxene in the melt encourages growth, even in its powder form. By using natural clinopyroxene powder, minor amounts of trace elements are added (Co, Cu, Zn, Sr, etc.), but these are insignificant to this study which focuses on sector zoning of the major elements (Ca, Mg, Fe, Al, Si). The powders were thoroughly mixed with ethanol for 30 minutes using a mortar and pestle, and left under a heating lamp overnight to dry. The reactant mixture was then placed in three 2 x 9 mm AuPd capsules to which were added 0, 2.5, and 5 wt% distilled H_2O respectively, with a microliter-scale syringe. The addition of water to the experimental capsules tests the ability of an anhydrous melt (0 wt% H_2O) in comparison to a hydrous melt (2.5 and 5 wt% H_2O) in generating growth. A risk presented by introducing H_2O to the melt is growth of a hydrous mineral rather than clinopyroxene, which is an anhydrous mineral. A 1 mm fragment of natural clinopyroxene, also from the Orford nickel mine, was added to each capsule to seed growth. The capsules were inserted into a piston-cylinder apparatus, which produces the high temperatures and pressures typical of the Earth's crust. The experiment ran for 24 hours at a temperature of 800˚C and a pressure of 10 kbar, approximately equivalent to a depth of 37 km below the Earth's surface, or the base of the crust, and subsequently quenched isobarically. Material recovered from the capsules was submerged overnight in dilute acetic acid, in the proportions four parts distilled water to one part acetic acid, to dissolve the carbonate matrix and retrieve the clinopyroxene crystals.

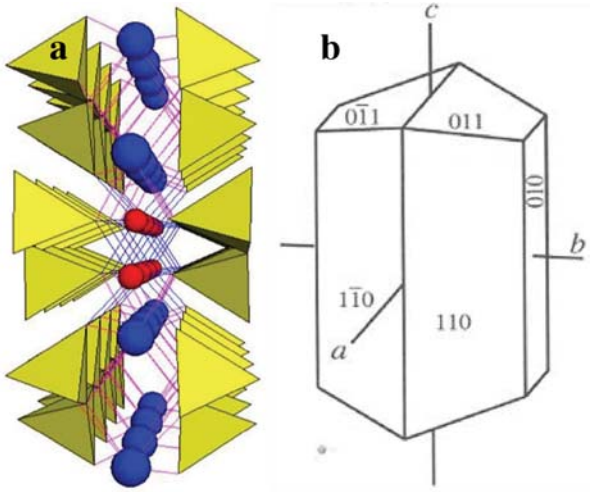

Figure 1. a) Chains forming the atomic structure of clinopyroxene. Yellow pyramids: tetrahedrally-bonded silica (SiO_4^{4-}). Blue spheres: Ca^{2+} cations. Red speheres: Mg^{2+} and Fe^{2+} cations. b) Dominant clinopyroxene crystal faces (110), (011) and (010) and crystal axes a, b and c. Chains of silica tetrahedra are oriented parallel to the c-axis, giving the crystal its elongate shape.

The AFM, SEM and the EMPwere used to identify the presence of crystal overgrowth on the natural clinopyroxene seed by observing growth morphology and analyzing chemical composition. The EMP cannot distinguish between atoms of different valence state, therefore the chemical analyses report all iron as ferrous iron (FeO). The amount of ferric iron (Fe_2O_3) present may be determined by performing a recalculation which satisfies charge balance. This recalculation uses an equation which assumes that iron is the only atom in the melt with variable valency, that oxygen is the only anion and that all cation sites in the mineral formula of clinopyroxene are full (Droop, 1987).

Results

The crystals retrieved ranged in size from 0.1 – 1.0 mm, and displayed a greenish hue typical of Fe-rich clinopyroxene, whereas the seed was 1mm in size and of a yellowish colour. Several analytical tests were performed on the retrieved crystals to identify the presence of crystal overgrowths, their mineralogy and chemical composition.

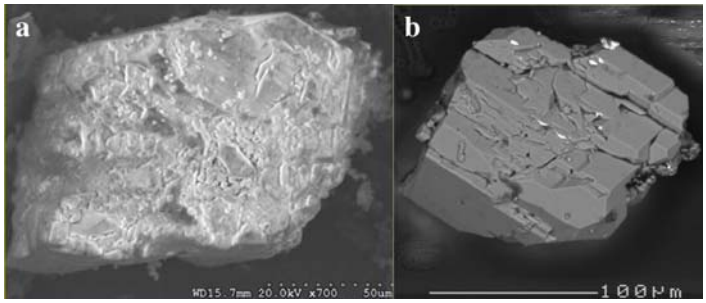

Figure 2. SEM images of clinopyroxene crystals. a) Before acetic acid wash: abundant carbonate residue covers the crystal surface. b) After acetic acid wash: the carbonate residue has dissolved, exposing well-defined crystal faces and edges.

Scanning Electron Microscope (SEM)

The SEM produces high resolution images with well-defined three-dimensional features by firing an electron beam at an area and recording the ensuing emission of electrons from the surface. A SEM images shows the general morphology of the crystal, and permits a preliminary identification of visible crystal faces, as depicted in **Figure 1b**. SEM imaging also reveals any matrix residue still encrusted on crystal surfaces. The crystal cleaning protocol may then be modified accordingly, as a longer soak or a stronger acid may be necessary to successfully dissolve all crystalline matrix. **Figures 2a** and **2b** are images taken with the SEM. **Figure 2a** shows a seed fragment after a 12-hour soak in distilled water. The crystal is still considerably encrusted with its crystalline matrix. Subsequently, the crystals were submerged in dilute

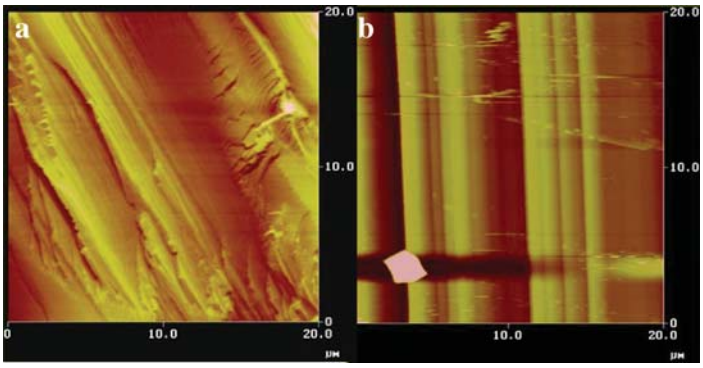

Figure 3. AFM images of synthetic crystal surface topography. a) Fractured surface. b) Parallel growth steps; cubic form at bottom left is likely an iron oxide crystal.

acetic acid for 12 hours which successfully dissolved the crystalline matrix, as shown by the clean crystal in **Figure 2b**. **Figure 2b** also reveals some well-developed crystal faces.

If the faces are large enough, the crystals may be mounted with such faces facing upwards for scanning with the atomic force microscope (AFM).

Atomic Force Microscope (AFM)

The AFM is an instrument equipped with a sharp tip mounted on a cantilever which scans the surface of crystals and detects surface topography down to the near-atomic scale. This tool thus enables visualization of growth structure, if present. Although this method permits the qualitative analysis of growth features, it cannot be determined whether the features observed belong to the natural seed fragment or were generated over the course of the experiment and indicate new growth. Imaging of the experimental crystals must therefore take into account the possibility that surface topography observed predates the experiment. Nonetheless, this analysis is a crucial step in thoroughly characterizing experimental results. **Figures 3a** and **3b** were taken with the AFM. The splintery texture and jagged edges observed in **Figure 3a** strongly suggest a freshly cleaved surface. In contrast, **Figure 3b** reveals parallel, elongate growth steps which either reflect fresh overgrowth from the experiment, or a growth surface present on the original clinopyroxene seed.

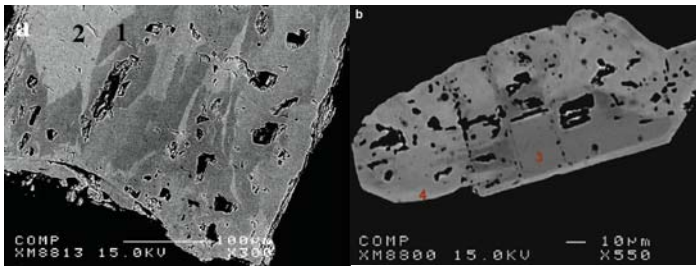

Figure 4. BSE EMP images of synthetized clinopyroxene crystals. Dark and light zones indicate areas of heavier and lighter average mass respectively. Black areas on the crystal surfaces are holes due to polishing. Chemical analyses for numbered areas are given in **Table 1**, with special care given to sampling both dark and light zones.

Electron Microprobe (EMP)

The EMP determines the chemical composition of a solid surface by bombarding the mineral surface with electrons and recording the energy of the emitted x-rays. Minerals and their composition may be identified, as each constituent element emits a characteristic reflected x-ray photon energy. The EMP is performed last in the series of analytical techniques since it requires a polished surface for probing, hence destroying all surface growth features. For this experiment, the reactant mixture departed significantly from the original Orford nickel mine clinopyroxene composition by the addition of alkalis (Na_2O, K_2O) and ferric iron (Fe_2O_3) to the melt. The seed, which was submitted for EMP chemical analysis, contained only trace amounts of Na_2O and K_2O, and mostly ferrous iron (FeO) **(Table 1)**. This contrast in composition between the seed and the melt facilitates monitoring of element exchange between the melt and growing crystal. **Figures 4a** and **4b** are images taken by back-scattered electron imaging with the EMP, and corresponding quantitative chemical analyses for the numbered spots are reported in **Table 1**. The lighter-shade zones numbered 2 and 4 reflect lighter average atomic mass. The darker-shade areas numbered 1 and 3 have heavier average atomic mass, as they have higher concentrations of FeO, CaO and MgO than the lighter areas, which in turn have higher concentrations of Na_2O, K_2O and Fe_2O_3. These lighter patches therefore indicate incorporation of melt elements into the crystal structure.

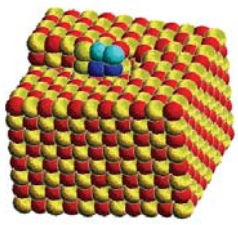

Figure 5. Dislocation–linear defect in the crystal lattice which promotes growth by creating nucleation sites.

Discussion

A 1mm natural clinopyroxene fragment was used to seed growth, yet several smaller crystals were retrieved from each capsule after the experiment. This suggests that the 1mm seed fragmented into several smaller pieces during the experiment, onto which growth may or may not have occurred. Another possible explanation is that the seed was partially to completely dissolved over the course of the experiment, and nucleation gave rise to several small crystals. The latter possibility is unlikely, as nucleation is a difficult process to initiate and requires a considerable amount of energy. If the first explanation is accepted, the fragmented seed could considerably enhance chances of new growth by increasing available crystal surface area.

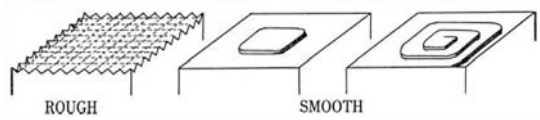

Figure 6. The stages of dislocation-controlled growth: initially rough surface, formation of a growth hillock and growth layers around the hillock.

The initial appearance of the retrieved crystals suggests the experiment was successful in generating element exchange between the melt and seed: the synthetic crystals were a darker shade of green than the seed, which was closer to yellow. Darker coloring indicates higher iron content; as the melt was enriched in ferric iron, the fragmented seed likely incorporated iron from the surrounding melt. This greenish hue may only signify re-equilibration of the crystal with its growing medium, and not necessarily the occurrence of new growth layers. Further evidence for growth is provided by AFM and EMP analyses.

	SiO2	TiO2	Al2O3	Fe2O3	FeO	MnO	MgO	CaO	Na2O	K2O	Cr2O3	Total
1	54.00	-	0.26	0.94	4.11	0.22	15.16	25.48	0.05	0.01	0.01	100.24
2	54.00	0.02	0.24	8.90	-	0.08	13.93	19.89	3.19	0.01	0.04	100.3
3	53.44	-	0.39	0.86	5.45	0.21	14.33	25.16	0.08	-	-	99.92
4	52.48	-	0.80	17.63	-	0.01	7.69	11.41	7.43	0.13	0.13	97.71
Seed (Orford nickel mind)	53.28	-	0.73	1.12	4.87	0.35	14.26	25.18	0.17	-	-	99.96
1993 lava	3.12	0.12	1.02	1.49	-	0.37	0.30	15.93	29.09	5.87	-	-

Table 1. EMP spot chemical analyses corresponding to numbered areas on crystals in **Figure 4** and of the original Orford clinopyroxene used as a seed in the experiments. Composition of the model 1993 lava from Oldoinyo Lengai. Values are reported in weight %.

Imaging with the AFM revealed the presence of parallel growth steps, a feature associated with dislocation-controlled growth. Dislocations result in the formation of a ramp on an otherwise flat surface **(Figure 5)**. This ramp encourages growth as nucleation sites are created along the leading edge of the ramp. Initial stages of growth resemble hillocks (**Figure 6)**. The structure then follows a helical path around the hillock, continuously adding growth layers. On a smaller scale, these resemble steps, as those observed in **Figure 3b**. The parallel steps strongly suggest a freshly-grown surface. As discussed previously, the possibility exists that growth steps were present on the original seed before the experiment. Conversely, the parallel steps are not likely due to growth on the seed predating the experiment since the seed was heavily fragmented during the experiment; consequently, we would expect fractured surfaces as in **Figure 2a** rather than growth surfaces. Further evidence from the EMP chemical analyses is necessary to conclude the presence of new growth.

The EMP images and chemical analyses offer many possible interpretations. At first glance, **Figure 4a** and especially **Figure 4b** have darker-colored patches at the center of the imaged crystal. From **Table 1**, the numbered spots 1 and 3 in these darker areas have a composition very close to that of the seed. This observation leads to the interpretation that the central dark area is seed material, surrounded by a significant lighter-colored rim of overgrowth, which is high in melt constituents Na_2O, K_2O and Fe_2O_3. Another possible interpretation is that both dark and light areas represent new growth, and the uneven distribution of elements is the manifestation of sector zoning. Accordingly, the dark areas are enriched in Ca^{2+}, Mg^{2}+ and Fe^{2}+, and the light areas in Na^{+}, K^{+} and Fe^{3+}. In sector zoning, charge balance must be maintained, and coupled substitution of ions satisfies this condition. In this case, the coupled substitution reaction could be expressed as (Ca^{2+}, Mg^{2+}) + Fe^{2}+ ‡ (Na^{+}, K^{+}) + Fe^{3+}. This clarifies why the light domains are enriched in Na^{+} (Na_2O) but depleted in Fe^{2+} (FeO): the incorporation of Na^{+} into the crystal lattice is paired with Fe^{3+} to maintain charge balance. A third interpretation is that no new growth occurred. Although the dark centers with light rims are highly suggestive of overgrowth, the Fe_2O_3 and Na_2O enriched rims could simply reflect the re-equilibration of the seed fragment with the surrounding melt of differing composition by diffusion of ions through the crystal lattice until equilibrium is achieved. However, this third possibility is quite unlikely, due to the compelling evidence from the AFM images that growth steps are present on several crystal surfaces.

Conclusion

The major innovation of this research project was the choice of a carbonate melt as a growth medium for crystallizing a silicate mineral, clinopyroxene. Although clinopyroxene has been the subject of numerous crystal chemical studies, none have attempted this technique. The reference to a modern carbonate analogue, the Tanzanian volcano Oldoinyo Lengai, thus proves useful in modeling the experiment. The importance of developing an effective method for clinopyroxene crystallization lies in their common incorporation of trace elements from their surrounding magma. A better understanding of the mechanisms influencing this trace element uptake may lead to considerable advances regarding mantle and crustal studies. One such mechanism is the surface morphology of a growing crystal face, which may cause preferential elemental enrichment. To explore this concept, detailed observations of freshly grown crystal faces are imperative. The carbonate melt offers the advantage of an easily dissolvable crystalline matrix, from which clinopyroxene crystals may be freed while conserving undamaged surfaces. The results presented here confirm the efficacy of this technique, as the AFM imaged remarkably well-preserved growth steps, and the EMP confirmed the ability of a silicate mineral to incorporate elements from its carbonate environment. The method could therefore potentially be extended to a wide range of silicate minerals whose surface features one wishes to investigate.

The unconventional use of a carbonate melt as a growth medium for a silicate mineral does present potential limitations in applicability. Little information on the diffusivity of carbonate melts is available in the scientific literature, which makes a comparison with silicate melt diffusivity impossible. The diffusivity of the melt can play a major role in facilitating the migration of ions towards growth surfaces, and subsequently, may influence the ability of a crystal to incorporate trace elements. Carbonate magmas represent only a small fraction of all magmas on Earth, the majority being silicate in composition. The experiment would need to be replicated using a silicate melt to assess the degree of compositional and/or morphological difference in the results, but such a study is made difficult by the resistant encrusting matrix formed by silicates which destroys morphological features. Silicate environments also require higher running temperatures, which makes the experiments technically riskier to perform. A thorough comparative study of carbonate and silicate melt characteristics must be undertaken to justly assess the applicability of the experimental results presented in this study.

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The effect of density stratification and a cape in a baroclinic western boundary current separation experiment

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Abstract

Western boundary current separation has long been a mystery. For the Gulf Stream, different factors such as the coastal shape, inflow and outflow location, wind stresses, continental shelf slope, shallow underwater plateaus, and interaction with the deep circulation potentially play unique and important roles in separating the Gulf Stream from the coast. To study these effects, a model consisting of a circular tank of water rotating on a spinning table was set up. Sloping planes form the upper and lower boundaries of the enclosed tank, approximating the Coriolis force. Then, water was pumped through gaps in the tank, producing a western boundary current and an artificial cape having the geometry of Cape Hatteras at the Gulf Stream's point of separation was introduced into the system. Finally, to study the effects of stratification on the point of separation, a 2-layer system was used. The results of varying different parameters, such as flow rate or density difference between layers, were compared with observations of the Gulf Stream and output from a numerical model. Ultimately, the experimental results showed that density differences alone do not affect the separation point to a meaningful degree, but rather that it is the position of inflow and outflow gaps that are much more significant. Density differences alone do not significantly affect the separation point. The relationship between high flow rates tending to create more modes of oscillation and a moving separation point was also observed. More so than any other setup, a cape at high density difference and low flow rate deflects the western boundary current flow. The results suggest that the interplay between the 1-layer and 2-layer modes is relevant to the oceanic case.

Keywords

Western boundary currents: *Warm, deep, narrow, and fast flowing currents that occur on the west side of an ocean basin*; **ocean circulation**: *Any permanent or continuous, directed movement of ocean water that flows in one of the Earth's oceans*; **rotating table experiments**: *Experiments involving a rotating table to study the effects of planetary rotation on fluid flow*; **geophysical fluid dynamics**: *The study of the naturally occurring, large-scale flows in the atmosphere and oceans, such as in weather patterns, atmospheric fronts, ocean currents, coastal upwelling, and the El Niño phenomenon*; **β-effect**: *A planar approximation for the latitudinal dependence of the Coriolis frequency. Normally, the Coriolis force exhibits a sinusoidal dependence on latitude. After Taylor expansion and elimination of higher order terms, a linear approximation is obtained, with β as the coefficient*; **barotropic**: *A flow characterized by pressure varying as a function of density only. This essentially describes a one-layer experimental setup*; **baroclinic**: *A flow whose pressure varies as a function of both density and temperature. It basically gives a measure of the stratification of the fluid. In the case of this experiment, it describes the vertical variation of density in a 2-layer experimental setup*.

Introduction

Western boundary currents are intensified jets found on western edges of major ocean basins. They are the result of the variation of the Coriolis parameter with changing latitude. Eastern boundary currents do exist, but are significantly lower in strength. A large portion of a western boundary current's length follows continental boundaries, transporting heat and nutrients. The separation point between the western boundary current and the coast has been a hot spot in oceanographic research, as it is important to ocean and current predictions.

The Gulf Stream, found in the Atlantic Ocean basin, flows along the North American continent from the Gulf of Mexico to Cape Hatteras, North Carolina. It is then deflected seaward, crosses over the Mid-Atlantic ridge and heads toward Europe. The core of the Gulf Stream current is about 90 km wide and has peak velocities of greater than 2 m/s, or 5 knots. The mechanisms involved in Gulf Stream separation have long been a mystery to physical oceanographers.

Henry Stommel pioneered general ocean circulation modeling in the 1940s. Since then, many experiments involving rotating tables have been performed to examine a spectrum of different effects ranging from mixing of turbulent eddies, wind stresses, gap leaping, and many others. The rotating table setup allows a small-scale modeling of an entire ocean basin in a rotating frame in which the Coriolis force can be easily approximated. For Gulf Stream separation experiments in particular, many different setups have been examined. There have been many one-layer rotating table experiments (Baines et. al., 1996) as well as flow analysis around cylindrical barriers. There have been few experiments done with more than one layer.

A two-layer model of the ocean basin is used in our study of Gulf Stream separation. This idealization of the ocean's density stratification allows one to experiment with physics involving a free, parabolic interface. The bathymetry under the northern portion of the Gulf Stream is thought to act as a sort of streamlining or pumping mechanism that could direct the current's flow. Inflow and outflow locations are used to simulate this effect and provide a way to control the

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separation point of our artificial western boundary current. Experiments with a cape are used to determine the impact of geographical coastal features. Results obtained from various setups are then compared to an output of a preliminary numerical model made to mimic the parameters of our tank. This model essentially solves the stream function given the boundary conditions of the tank until a steady state is reached. A comparison between the model and experimental results can be made to verify the correctness of the model. The model is intended to be used in situations which are difficult to achieve in experiment.

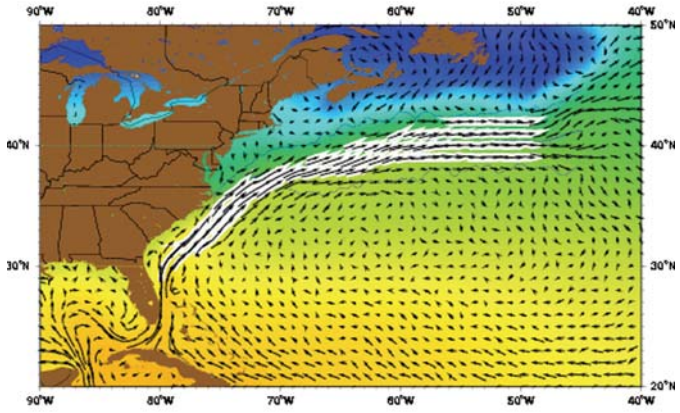

Figure 1. The Gulf Stream as represented by the Mariano Global Surface Velocity Analysis (MGSVA). The Gulf Stream is the western boundary current of the N. Atlantic subtropical gyre. The Gulf Stream transports significant amount of warm water (heat) poleward.

Methods

Tank Setup

A 1 m diameter cylindrical tank is used to create the western boundary current needed for our experiment. The tank is centered on a rotating table and has three compartments: an active area, a northern outflow collection region, and a southern inflow region. An aerial view of the tank is shown in **Figure 1a**. The outer tank walls rise to 45 cm in height. For all experiments, the rotation rate of the tank was set at 1 rad/s. A north-south gradient in the thickness of the water column is created by the sloping bottom and top lids in the tank's active area. These slopes create the β-effect, which approximately simulates the effect of latitudinal dependence of the Coriolis force. This approximation can be made because fast rotating fluids tend to flow with rigid columns aligned parallel to the vertical rotational axis. Since ocean depth increases from high to low latitude measured parallel to the rotational axis, the tank equivalent is to make the depth vary in a similar manner. The bottom lid begins at the southern point of the active region, sloping at +0.05. The top lid is added to prevent a parabolic surface from forming in a freely rotating non-lid experiment, and allows for the β-effect to act upon the top layer of a baroclinic experiment. The top lid also begins at the southern point, and slopes at -0.05, so that the height of the water column in the active region ranges from 14 cm at minimum (north end) to 24 cm at maximum (south end). Lines of constant depth run east-west. The sloped bottom is marked with radial lines every 15 degrees arc and with concentric lines from the midpoint at 5 cm increments.

Both the inflow and outflow gaps of the active tank region are 8 cm wide and are cut out from the first 10 cm layer under the top lid. The inflow and outflow compartments are lined carefully with sponges, taking care to not disturb the flow in or around the gap regions. These sponges serve to diffuse the flow. A digital pump takes water from the northern outflow area, and forces it into the southern inflow compartment, thus creating a pressure difference and causing water to be pushed into the active tank region.

Neutrally buoyant dye was used to trace time-averaged currents in the active region of the tank. A series of small holes was drilled into the top lid of the tank along radial lines at 180, 225, and 270 degrees, concentrated toward the outer rim of the tank. Needles were inserted about 5 cm down from the top lid, and were connected to micro-pumps to inject the dye at a slow rate of 20 mL/hr. A remote-controlled camera was mounted above the active region of the tank, allowing photos to be taken in the rotating tank's frame. Its output was sent by wireless transmitter to a television monitor as well as to a memory card within the camera.

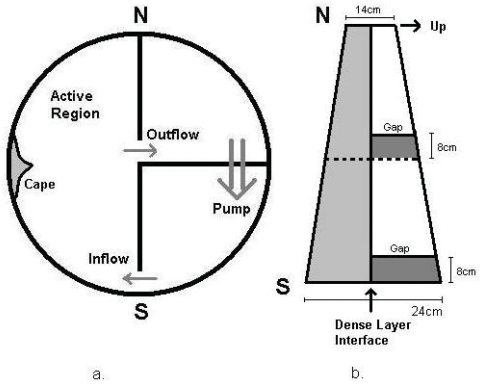

Figure 2. Diagram 2a is a plane view of the tank setup. 2b shows a vertical cross section through the tank from north to south.

Baroclinic System and Cape

To create a stable baroclinic system in the tank, a seawater solution was mixed to our desired density. This layer is allowed to equilibrate to a uniform distribution of temperature. To avoid any currents produced by a vertical temperature gradient, the temperature of all the water used is kept constant at room temperature. The densities tested ranged from 1025 kg/m^3 (pure seawater from Narragansett Bay) to 1002.5 kg/m^3 (diluted seawater). In order to determine the density difference, one defines $\delta=\Delta\rho/\rho$, where $\Delta\rho$ is the difference in densities between the bottom and top layers, ρ is the density of the top layer, and ρ being that of fresh water, 1000 kg/m^3. For example, a setup with a bottom layer density of ρ=1025 kg/m^3 gives δ=0.025. A "high density difference" setup refers to a two-layer system with high δ (δ=0.025 for pure seawater), whereas a "low density difference" refers to a setup with low δ (δ=0.0025 for seawater diluted to 1/10 the amount of seawater compared to pure seawater for a given volume). Testing the extremes of the density range allows us to gauge to what extent the density profile has an effect on separation point. The denser solution is attached to an inflow tube that rotates with the table. Fresh water is put in the tank while the table is stationary – the quantity is measured such that when the tank is completely full with the two-layer system, the denser liquid will rise to the bottom of the inflow and outflow gaps, shown in the vertical section in **Figure 1b**.

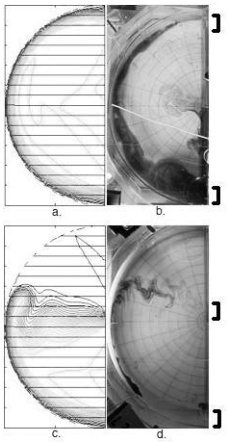

Figure 3. Black brackets designate gaps. Image a shows a barotropic model output for a gap at the north end of the active region. Image b shows the results of the experiment in this setup. Image c is the barotropic model output for a gap in the middle region of the active tank. Image d shows the experimental result of this setup.

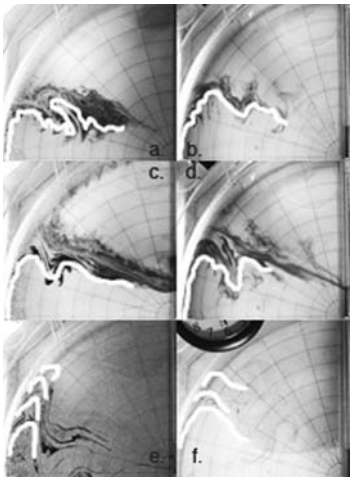

Figure 4. Image a shows results from an experiment at Q=10cm^3/s and δ=0.0025. Image b shows results at Q=10cm^3/s and δ=0.025. Image c shows results at Q=20cm^3/s and δ=0.0025. Image d shows results at Q=20cm^3/s and δ=0.025. Image e shows results at Q=40cm^3/s and δ=0.0025. Image f shows results at Q=40cm^3/s and δ=0.025. White lines are digitally enhanced traces of the dye path for easy viewing.

Once the fresh water is filled to the marked height, the tank is spun up for roughly 10 to 15 minutes until solid body rotation is achieved. The denser seawater solution is then very slowly pumped into the lowest part of the active tank region: the southern point. Neutrally buoyant dye matching the top layer density was injected into the bottom of the tank. This dye would rise through the denser fluid until it reached the interface between layers, allowing us to visually monitor the boundary height. In less dense solutions, dye was used to color the denser solution so that the interface between the bottom and top layer could be monitored more easily. Once the dense water layer is at the correct height, the water pump begins to push the flow, creating the western boundary current.

An artificial cape was made to imitate the presence of Cape Hatteras. It was fit so that its protruding point would line up vertically with the due-west (270 degrees) line of the tank at all heights of the active region. Both sides of the cape were smooth and vertical.

Numerical Model

The numerical model was made to mimic the parameters of the tank setup, including a new addition of a baroclinic, two-layer fluid having a parabolic interface. It finds a numerical solution to the Shallow Water Equations in the cylindrical coordinates of our rotating tank system. The progress of the stream function with trapezoidal time stepping until a steady state is reached can then be tracked. In the case of the baroclinic system, the bottom layer is assumed to be at rest with respect to the rotating table. Contours of the stream function are plotted in the tank's coordinate system. It should be noted that the results of the numerical model depend on the resolution of the model and viscous parameters.

Results and Discussion

Gap Placement

The inflow gap was kept at the southern-most point. Outflow gaps varied from the northern edge to the middle of the tank. Lines of constant depth run east-west. Usually, the western boundary current flows perpendicular to these lines. The global geostrophic contour at which bathymetric features are located is hypothesized to affect the Gulf Stream separation point. Our experimental results show that the point of separation is determined by the placement of the gaps. **Figure 2** shows model output for two different gap placements compared to experimental results at given flow rates.

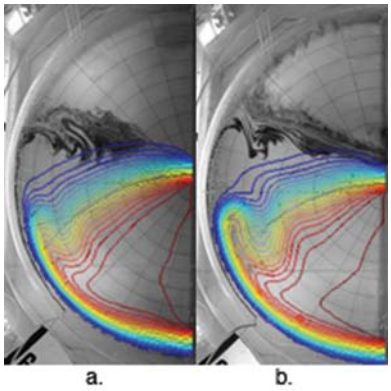

Figure 5. Image a shows the numerical model (colored lines) superimposed over data from an experimental run at Q=10cm^3/s and δ=0.0025. Image b shows the same for Q=20cm^3/s and δ=0.025.

Density Stratification and Flow Rate

By adjusting the flow rate, Q, the amount of water moved through the pump per unit time (measured in cm^3/s), one can compare the effects of density differences between layers at different flow rates. **Figure 3** shows results from both high and low density difference runs at two different flow rates. The dye lines have been digitally enhanced to emphasize the path and separation point of each run. Dye experiments at varying densities exhibit little variation in separation point. This same observation can be made at different flow rates.

At low flow rates, the separation point is easily visible with dye experiments. When the flow rate is increased, dramatic shifts in the point of separation occur, as if the high flow rate is causing the separation point to drift. Many different modes of oscillation make the ink path difficult to map. This observation can be made for different densities. Therefore, flow rate has rather ambiguous effects on the separation point, and further study is needed by more accurate methods. An assessment between instantaneous velocity fields at a number of different times may be more useful in the case of high-oscillation flow rates.

Discrepancies between the model output and the experi-

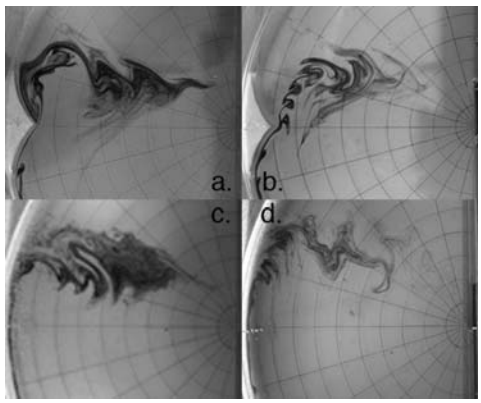

Figure 6. Image a shows results from an experiment at Q=10cm^3/s and δ=0.0025 with the cape insert. Image b shows results at Q=10cm^3/s and δ=0.025 with the cape insert. Image c shows results of the same setup as in a, without the cape. Image d shows results of the same setup as in b, without the cape.

mental setup are shown in **Figure 4**. The model predicts a separation point much further south than what is observed in the lab, even at different flow rates. The experimental setup more closely mimics model outputs of a barotropic system, where there is no velocity change with respect to height of the water column. This can be explained by the baroclinic model's assumption that the lower, denser layer rotates with the tank with a zero relative velocity. Experimental tests with neutrally buoyant dye matching the lower level density show that at sufficiently high flow rates, there is a considerable amount of flow exhibited by the bottom layer, which was previously assumed stationary with respect to the tank. A hypothesis to explain this anomaly is that the bottom layer flow generated enough energy to flow into the outflow gap. At this gap, water from the lower layer was being sucked up and into the outflow compartment. This generated a low-level flow that was less energetic than its upper-layer counterpart. However, the effect was enough to make the system substantially more barotropic, pushing the separation point more northward to match flow patterns predicted and observed in a barotropic equivalent of the setup.

Cape

The cape was tested at multiple density differences and flow rates. **Figure 5** shows the results of runs with the cape compared to those without the cape. At the low density difference extreme, the cape deflects very little water, and the western boundary current literally curls along the cape, back to the side of the tank, and separates at the same point it would have had there not been a cape. The cape's ability to deflect the western boundary current seems greatest at a high density difference, low flow rate situation. This effect can be contributed to the balance between the Rossby waves traveling westward from the outflow gap along the geostrophic contour and the western boundary current's originally intended separation path. In a more baroclinic, or high density difference, system, the upper layer Rossby waves tend to be weaker, thus pushing the current less westward. This allows the current to be deflected away from the tank side more easily.

Conclusions

The Gulf Stream was modeled as a two-layer baroclinic system to examine the impact of different density stratifications on the artificial boundary current. In creating a baroclinic setup, different parameters were adjusted. These included density differences between layers, gap inflow and outflow restriction, flow rate, and presence of a cape. The separation point of the western boundary current from the tank edge was closely examined under each of the different tank setup circumstances.

As seen by the result shown in **Figure 3**, the separation point of an artificial western boundary current in a barotropic, one-layer system is easily controlled by moving the location of the outflow gap in the active area of the tank. Disturbances propagating along geostrophic contours tend to affect the western boundary current's movement. This effect simulates the Gulf Stream flow around Grand Banks, Newfoundland.

The introduction of the two-layer system is a better approximation of the density stratification found in the ocean. It allows for examination of the effects of extreme density differences in the layer and of a parabolic free surface at the interface. At both extremes of very low and very high density differences between layers, results shown in **Figure 4** suggest that the baroclinicity of a two-layer system alone do not affect the separation point in a meaningful way. At high flow rates, this effect is even less obvious, because the flow develops oscillations in the separation point. The baroclinic setup used in this experiment assumed a zero-velocity heavy layer, which was not the case; the bottom layer showed movement, which may explain the discrepancies between the model output and the experimental outcome. A more barotropic system tends to push the separation point further north than a baroclinic system. This result suggests that deep ocean currents may affect western boundary current flow.

A cape insertion is placed due west in the active region to mimic its presence in Cape Hatteras, North Carolina. As seen in **Figure 6**, the cape has little or no effect on the separation point at low density difference setups. However, at a high density difference, there seems to be a clear deflection of the western boundary current from its originally intended path. This suggests that under certain circumstances, the cape does indeed play a role in diverting the western boundary current from the continent.

Further experimental setups should be used in examining bathymetric effects such as constructing underwater plateaus. As this was a preliminary exercise in creating a feasible experimental setup, future tests can expand on these methods with instantaneous velocity field tracking of the flow, as opposed to the time-averaged dye tracing method used, in order to mathematically correlate the experimental results with the numerical model. Effort should be put in to experiment with movable capes and physical barriers in order to examine different flow patterns. Lastly, the effects of deep water circulation should be examined by controlling different lower-layer flows and examining the effects.

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The effects of implementation intentions on relationship maintenance responses

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Abstract
Participants in committed relationships may frequently encounter interpersonal adversity. Research has shown that committed partners use relationship maintenance responses to reduce relationship threat in these situations while strengthening relationship commitment. A study was conducted to determine the effects of a relationship-defending implementation intention on three computerized tasks measuring relationship maintenance responses; gender, commitment level, and demographic information were analyzed as covariates. It was found that male and female participants differed in relationship maintenance responses, and that the formation of an implementation intention may cause individuals (particularly men) to defend their current relationship in implicitly relationship-threatening situations. Practical and academic implications of these results, including clinical possibilities and directions for subsequent studies, are discussed.

Introduction
Interpersonal relationships are an integral part of our social lives. In actuality, the perpetuation of our species as a whole is moderated by relationships between compatible human adults. As inevitable as these relationships seem to be, however, adversity within them seems equally prevalent. By extension, any analysis of romantically committed dyads in general cannot be considered comprehensive unless it examines the factors and effects of adversity within them.

Although these relationships have defined our collective existence, objective scientific analysis of the subject is recent, and a working understanding of how our relationships function is elusive. Current research has shown that the success of interpersonal relationships is moderated by commitment between relationship partners, relationship maintenance strategies employed, reactions to others outside of the relationship, and plans and goals regarding the relationship's future (Johnson & Rusbult, 1989; Johnson, 1991; Rusbult, Wieselquist, Foster, & Witcher, 1999; Gagne & Lydon, 2001).

According to Johnson (1991), commitment is composed of three aspects: personal, moral and structural. The externally based structural component represents the influence of one's relationship status on one's commitment to that relationship. In contrast, personal and moral aspects of commitment are internally based. Personal commitment results from one's desire to remain in a relationship due to the satisfaction experienced as part of it. Finally, moral commitment is the perception of an obligation to remain in a relationship for moral reasons.

Individuals in a committed dyad act to defend their relationship by using relationship maintenance strategies in response to adversity (Rusbult et al., 1999; Brehm, Miller, Perlman, & Campbell, 2002). One important relationship maintenance response involves devaluation of relationship alternatives. Johnson and Rusbult (1989) established that there exists a negative linear correlation between commitment to a romantic relationship and the evaluation of attractive alternatives. They found that in couples whose commitment increased over time, ratings of attractive alternatives decreased, whereas in couples that became less committed over time, ratings of alternatives increased. Johnson and Rusbult also found that alternatives were more strongly devalued if they were extremely attractive, representing a "high threat" condition.

According to the commitment calibration hypothesis, the effects of adversity within a relationship are contingent upon the level of commitment present in that relationship (Lydon, Meana, Sepinwall, Richards, and Mayman, 1999; Pearson, 2004). If the adversity level surpasses the level of commitment, the relationship cannot survive, and if the adversity level is lower than the commitment level, the relationship does not experience a threat. However, if the levels of relationship commitment and adversity are comparable, the relationship will resist the threat and become stronger. Lydon et al. tested this theory with a study in which participants in committed relationships were exposed to varying degrees of relationship "threat". This threat was presented in the form of attractive alternatives, following the paradigm developed by Johnson and Rusbult (1989). Participants who were moderately committed and were presented with a moderate threat (evaluating an attractive alternative who ostensibly did not rate the participant) rated the alternative lower in attractiveness than participants who were either less or more committed. Highly committed participants who were presented with a highly threatening situation (evaluating an attractive alternative who was ostensibly attracted to the participant) evaluated the alternative as less attractive than participants who were less committed to their romantic relationships. The commitment calibration hypothesis explains Simpson, Gangestad, and Lerma's (1990) findings that romantically involved individuals gave lower attractiveness ratings to photographs of attractive alternatives in comparison to the ratings of single individuals.

Relationship maintenance responses have recently been linked with the concept of implementation intentions (Lydon & Miners, 2001; Lydon & Nguyen, 2004). According to Gollwitzer (1999), implementation intentions are plans formed in order to associate upcoming goal-related situations with goal-related actions that must be performed. Gollwitzer and Brandstatter (1997; Gollwitzer, 1999) have suggested that implementation intentions reduce or eliminate obstacles preventing goal completion, and that the initiation of goal-directed action will then occur automatically when the goal-

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related situation is presented. Webb and Sheeran (2004) confirmed this suggestion, showing that the formation of an implementation intention led to quicker and more accurate responses to goal-related cues. These studies suggest that forming an implementation intention increases the probability of an individual detecting the correct time and action necessary to act toward their goal.

Lydon and Miners (2001) were among the first to investigate the connection between implementation intentions and relationship maintenance responses. This study bore three relevant findings. First, the commitment level of males was found to be lower than that of females, which was unusual. Second, in a computerized image-distancing task, male participants moved a picture of an attractive female closer to themselves than female participants (who moved a picture of an attractive male). Finally, participants who had formed an implementation intention to defend their current relationship kept an image of an attractive alternative closer to neutral images (but further from themselves) in the image distancing task, suggesting reduced cognitive awareness of the threat, whereas other participants placed the image of the attractive alternative further from neutral images (but closer to themselves). However, this study was limited by a relatively small sample size and methodological issues.

The present study analyzes how forming an implementation intention regarding one's own romantic relationship affects relationship maintenance responses, and is intended primarily as a replication of the Lydon and Miners (2001) study, with the aforementioned limitations corrected. Participants in committed relationships formed an implementation intention to either augment their study habits (the control condition) or defend their relationship (the experimental condition). It was hypothesized that participants in the experimental condition would react more quickly and accurately than control participants to stimuli related to relationship commitment in a lexical decision task, as would women in comparison to men, and highly committed participants in comparison to those less committed. Further, it was believed that experimental condition participants would show decreased preference for a virtual space associated with an attractive alternative compared to control participants, as would highly committed individuals in comparison to those less committed. Finally, it was hypothesized that experimental condition participants would place the image of an attractive alternative further away (but closer to neutral images) compared to control participants in an image distancing task, that highly committed participants would push the attractive alternative further away than other participants, and that men would bring the attractive alternative closer than the neutral images, whereas women would push the attractive alternative further away.

Method

Participants

Participants were males (N=20) and females (N=20) between the ages of 17 and 26 (M=19.38, SD=1.31) who had been dating their current partner for between 1 and 72 months (M=17.98, SD=15.87). Participants were McGill University students (with the exception of one male Concordia University student) who were currently in committed heterosexual relationships, and were fluent in English (self-reported English proficiency on a scale from 1=poor to 7=excellent: M=6.35, SD=0.80). Participants were recruited by phone and email from the McGill psychology subject pool.

Materials

At least two days before testing in the lab, participants completed two questionnaires hosted online at www.surveymonkey.com; these measures included a modified version of the Commitment Evaluation Questionnaire (CEQ) created by Lydon and Miners (2001). The modified CEQ contained the original 15 items (assessing commitment, investment, devotion, loyalty, and dedication towards one's academic, romantic and social life), and added three new items regarding attachment and obligation.

Participants were given a consent form to be signed, which stated that they were aware that all responses were confidential, and that they were free to leave at any time or refuse to answer any questions. Participants were then presented with the implementation intention materials. Four scenarios were prepared; each scenario involved the participant imagining themselves in a given situation, and being asked to form an implementation intention in response to this situation. Each scenario also contained a series of questions assessing the ease and vividness of mental simulation, as well as affect during simulation.

In the male and female experimental condition scenarios, the participant was asked to imagine that their significant other is away visiting family, while the participant is at a bar with friends. In these scenarios, the participant's friends tease him or her about being "single for the weekend", and mention that one of their significant other's attractive friends is very interested in meeting the participant. The participant is then asked to imagine this attractive alternative flirting with them at the bar, consider the situation and write how they will show the attractive alternative that they are not interested in them. In the male and female control scenarios, participants are asked to imagine that their significant other is away visiting family, while the participant is attending a movie with friends.

During the scenario, the friends tease the participant about their recent poor performance on an exam. The participant is then asked to imagine that he or she is in the library studying for an upcoming exam, and to write down how he or she would deal with becoming distracted while studying. All scenarios differed in word count by less than 1%.

For the computerized tasks, the software used for the lexical decision task was e-PRIME, while the balloon placement and image distancing tasks used World Tool Kit 2.0; both programs were designed for the Windows operating system. To perform these tasks, participants used a PC terminal running the Windows OS, including a monitor and keyboard. All of the images used were 225 pixels in length and width.

Following the tasks, participants were given a two-part "funnel debriefing" intended to ascertain the participant's perceptions during the experiment; an example question is, "Did you have any specific strategies or reasons for arranging the images the way you did?" The final questionnaire asked the participant for demographic information such as age, gender, sexual orientation, relationship length, first language, and proficiency in English. The final material distributed was a written debriefing outlining the purpose and experimental manipulation of the study, and also contained the experimenter's contact information.

Procedure

Participants were randomly assigned to either the experimental or control condition prior to testing. Participants were first asked to read and sign the consent form, and then given five

envelopes, ostensibly containing five different scenarios, but in fact all containing the scenario assigned to that participant's condition. The five hypothetical scenarios supposedly were studying for an exam, shopping for clothes, athletic performance, an evening at a bar, and going on a vacation. Participants were given five minutes to complete this portion of the study.

Participants were then asked to complete three computerized tasks. In the first task, 64 strings of letters appeared, half of which were actual words and half of which were non-words (actual words with one letter replaced, forming a nonsensical string). There was a 2500 ms delay between strings, during which participants focused on an asterisk which appeared on the monitor. Participants were asked to determine if each string of letters was either a word or a non-word as fast as possible by pressing designated "word" and "non-word" keys, and were also asked to keep their index fingers over the two keys at all times to ensure that responses were as quick as possible. Participants were told that response time and accuracy would be recorded. Four words previously found to be prototypical of commitment (Fehr, 1988; Rosch, 1973) (such as "dedication" and "devotion") were randomly interspersed among the strings of letters. The remaining strings consisted of four neutral non-interpersonal words, two negative non-interpersonal words, two positive non-interpersonal words, four synonyms for the word "defend", and sixteen non-words (Lydon & Miners, 2001; Lydon & Nguyen, 2004). All words were selected from Anderson's (1968) list of 555 personality-trait words.

During this lexical decision task, participants were subliminally presented with an image of an attractive alternative, which remained on the computer monitor for 10 milliseconds. These images were followed immediately by the presentation of a mask (composed of gray curved lines) for 20 ms, in order to disrupt conscious perception of the attractive alternative. There were 16 images of attractive alternatives in total (eight males and eight females), which had been rated on a seven-point scale that ranged from not at all attractive (1) to extremely attractive (7) by 20 impartial judges prior to testing. Female judges rated the attractiveness of the male images (M=5.88), and male judges rated the attractiveness of female images (M=5.92) (Lydon & Miners, 2001). The first block of trials presented 32 images of males in a random sequence and the second block, 32 images of females; transition between the two blocks was seamless. Presentation of opposite-sex images was intended to prime the concept of attractive alternatives within the participant, whereas same-sex images were considered neutral.

Following the lexical decision task, participants entered a virtual environment program containing one large hall with four small identical rooms. Participants were asked to thoroughly explore each of the four rooms, using the arrow keys on the keyboard, before returning to a small table in the center of the hall. In two of these rooms (randomly selected by the software), the image of an attractive alternative (of the opposite sex of the participant) was automatically presented for 10 ms upon advancing past a certain point in the room, followed immediately for 20 ms by a "retinal disruption" mask. The remaining two rooms presented only the mask. The mask (an improved version of the mask used by Lydon and Miners (2001)) appeared as four horizontal lines interrupted by assorted geometric shapes, and was of identical size to the attractive alternative image. The purpose of this mask was to minimize recollection of the subsequent image at a conscious level, by disrupting the image on the retina. After participants had explored each room and returned to the table, a robotic arm appeared in front of the participant, and a balloon appeared over the table. Participants used the robotic arm to grab and place the image of the balloon in one of the rooms, using the "space" key. The prevalence of balloon placement within the "target" rooms that had presented the image of the attractive alternative was recorded.

Lydon and Miners (2001) encountered a computer error in which participants did not adequately explore each room, which prevented images from appearing. This was corrected in the current study by updating the instructions presented to participants, who were specifically asked to enter each room completely before moving on. Also, the image of the male attractive alternative was replaced (with a new male image selected by impartial raters) for the balloon placement and image distancing tasks, as it was believed (from the results of a pilot study) that the previous image would not be considered sufficiently attractive to female participants.

In the final computer task, eight images were positioned in a circle around the participant's position in virtual space. Participants were able to move each image closer (by pressing the "down" arrow key) or further away (by pressing the "up" key) in order to create an arrangement they were content with. One of these images was a picture of an attractive alternative (the "target" image) of the opposite sex of the participant, whereas the other images were of animals, fruit, and other neutral objects. The distance to which each image was moved in relation to a participant's virtual position was recorded by the software.

Following the image distancing task, participants were given the funnel debriefing and the demographic questionnaire, and were given a written and oral debriefing explaining the true nature of the experiment. Participants were then thanked for their participation and compensated with a movie pass valued at approximately $10.

Results

Contrary to the results of the Lydon and Miners (2001) study, commitment levels of male participants (M=4.03) and female participants (M=4.69) obtained from the modified CEQ did not differ significantly ($t(35) < 1$). Responses to the scenarios revealed that all participants in both experimental groups successfully formed the desired implementation intention, as all participants outlined an implementation intention to deal with their given scenario.

Balloon placement task

25 of the 40 participants placed the balloon in a room in which an attractive alternative was presented, in comparison to the 20 predicted by chance. Men (50% of whom chose a target room) did not differ significantly from women (75%) ($X^2(1)=2.67$, $p>.1$). Women placed the balloon in a target room more than chance ($X^2(1)=5.0$, $p<.05$), while men were exactly at chance. There was no difference in the frequency of target room selection of participants in the experimental condition (65%) compared to of control participants (60%) ($X^2(1)<1$). In analyzing the results for a gender by condition interaction, 30% of male experimental participants, 70% of the control males, 90% of experimental females, and 60% of control females placed a balloon in a target room. When aggregated with the data of Lydon and

Miners (2001) to increase sample size, experimental participants (43%) were not significantly less likely than controls (60%) to choose a target room ($X^2(1)=2.67$, $p>.05$). However, when broken down by gender, males in the experimental condition (25%) were less likely than males in the control condition (63%) to choose a target room ($X^2(1)=4.571$, $p<.05$), whereas women's behavior was random across conditions ($X^2(1)=0$). All participants entered each room far enough to trigger presentation of the intended image(s), as witnessed by the experimenter.

Image distancing task

Males in the experimental condition kept the image of the attractive alternative at approximately the same distance as the neutral images ($t(18)<1$), as did males in the control condition ($t(18)<1$). Women in the experimental condition did not move the target image further away than women in the control condition ($t(18)=1.136$, $p>.1$), although women in general moved the target image significantly further away from themselves than the neutral images ($t(36)=1.768$, $p<.05$). Overall, the target image was moved further away than neutral images, although the result was only marginally significant ($t(36)=1.344$, $p<.1$). However, when results were aggregated with those of Lydon and Miners (2001), there was an interaction effect for image and gender ($F(1,66)=5.820$, $p<.025$); specifically, women placed the target image further away than men ($t(66)=2.534$, $p<.01$).

Debriefing

Funnel debriefing measures revealed that none of the participants correctly guessed the purpose of the experiment or what the experiment was attempting to study. Also, none of the participants were able to correctly identify the images that appeared during the lexical decision task, and many stated that they saw no images at all during this task. Similarly, very few participants were able to identify the content of images in the balloon placement task, and none could describe them accurately. When asked how they arranged the images in the distancing task, the most common strategy described was to move closest those images that were "liked" the most. No participants had prior knowledge of the tasks or methods used in this study.

Discussion

The current study explored whether the formation of an implementation intention affected the relationship maintenance response of devaluing attractive alternatives. The effects of gender and commitment level were similarly examined. It was hypothesized that participants who formed an implementation intention to defend their current relationship would respond more quickly to prototypical commitment words, would be less likely to place a balloon in a target room, and would move an image of an attractive alternative further away from themselves in an image distancing task.

Although the balloon placement task data indicated that men in the experimental condition were less likely to place the balloon in a target room than control males, the effect was not significant. This is likely due to the small sample size. When the data are aggregated with Lydon and Miners' (2001) results of the same task, however, the effect is in fact significant. This indicates that men were influenced by the formation of a relationship-defensive implementation intention to avoid those rooms associated with an attractive alternative. It seems that this behavior is indicative of these men augmenting their relationship maintenance responses to adversity as a result of the experimental manipulation.

The results of the image distancing task suggest that the formation of an implementation intention did not significantly affect relationship maintenance responses for this explicit task. Although women in general moved the target image further away from themselves than neutral images, women in the experimental condition did not move the attractive alternative further away than women in the control condition, and men in both conditions kept the target and neutral images at relatively the same distance.

The current study replicated the finding of Lydon and Miners (2001) that men and women do not respond equally to relationship threat (shown by the gender differences in task results). Lydon and Miners suggest that this difference occurs because men and women differ in their self-concepts, in that women have a more interdependent self-concept, whereas men have a more independent self-concept. This difference could cause the aforementioned difference in response to relationship threat, as women would be more likely to defend a relationship that they believe contributes to their interdependent self-concept. Particularly interesting is the fact that the experimental manipulation did not affect performance for the *explicit* distancing task, but had a significant effect on men in the *implicit* placement task. These results support the hypothesis of Lydon (submitted) that committed women already have a chronic internal contingency plan for dealing with situations that are threatening to their relationships, whereas committed men do not; in the current study, it seems that the formation of a relationship-defending implementation intention in men served the same purpose as the pre-existing contingency plan of women.

Limitations

The scope of the current study is limited by several factors. All participants were relatively young (18-23 years of age), reducing the generalizability of these results to older individuals. In addition, participants were undergraduate students, predominately attending McGill University. Results of the current study might not be characteristic of individuals with a different educational or socioeconomic background than the average undergraduate. Because the experimenter was male, it is possible that male and female participants' responses differed as a result. Finally, the current study was limited to individuals in heterosexual relationships, limiting the generalizability to gay, lesbian and bisexual relationships.

Of the individuals indicating an interest in participating, 60 completed the online survey, and only 40 of these respondents were tested in the lab. This may have resulted in a recruitment bias in the results, as less interested individuals may have been less likely to complete the survey, and the most interested subjects may have responded earlier.

In attempting to replicate the results of Lydon and Miners (2001), it was necessary to maintain several methodological issues limiting the predictive power of the results. As all participants experienced the computerized tasks in the same sequence, it is possible that order effects exist. For example, the commitment words in the lexical decision task could prime for commitment in participants, affecting the results of the subsequent tasks. Similarly, since the blocks of images presented in the lexical decision task always occur with male images preceding female images, order effects may exist within this task.

The results of the balloon placement task were aggregated with those of Lydon and Miners (2001) during analysis; as some of their results for this task may have been affected by the aforementioned computer error, the reliability of these data may be reduced. The background of the attractive alternative images was not quite identical, with the male background being slightly lighter in color, potentially making the male image slightly more detectable. This extra salience may have affected the distancing task as well, by reducing the need to bring the male image closer in order to see it clearly. Similarly, although all images were the same size, it is possible that image resolution was not quite identical across all images, potentially causing participants to move lower resolution images closer in order to discern finer detail. Finally, since opinions of what qualifies as "attractive" vary between individuals, it is difficult to find an image that serves as an attractive alternative for all members of a gender.

Implications and future directions

The current study found that the formation of implementation intentions affects relationship maintenance mechanisms in response to relationship threat, particularly in men. It is possible that implementation intentions affect other relationship maintenance responses beside the devaluation of alternatives; if so, members of a committed relationship might deal with other relationship obstacles using a similar strategy.

There are both practical and academic applications for this information. From a research standpoint, the effects of implementation intentions on relationship maintenance responses have only recently been studied, and future research in this area could reveal much about the true nature of interpersonal relationships. Although the current study addresses methodological issues present in previous studies, the design of the current study could still be augmented in future replications. Primarily, increasing the sample size could reveal useful findings. The presentation of images during the lexical decision task could be improved so as to present only male images to female participants and vice versa. The virtual space created for the balloon placement task could also be updated with a more modern interface. Finally, the resolution of the images presented during the experiment could be increased and standardized. Future research could potentially examine the effects of implementation intentions on relationship maintenance responses in gay, lesbian and bisexual relationships as well. The effectiveness of relationship implementation intentions has yet to be fully assessed, as well as their effects on other aspects of relationships.

The practical implications are equally interesting. Relationship therapists could introduce the formation of implementation intentions as a strategy in clinical sessions. Couples in therapy could form implementation intentions to defend their relationship, and potentially limit effects of relationship obstacles in their future. Ideally, this strategy could strengthen existing relationships, reducing the prevalence of divorce. Committed individuals (particularly men) who are tempted by an attractive alternative (or suffer from a "wandering eye") could also benefit from forming implementation intentions to defend their relationship, as the current study suggests they would have reduced cognitive recognition of attractive alternatives in social settings.

Although this area of research has only recently emerged, it shows promise in uncovering new information regarding relationships that comprise a significant part of our daily lives. Adversity is as inevitable in relationships as relationships are inevitable in our social lives, and as such it should be addressed directly and honestly. As the current study attempts to imply, this can be done on a global level, as researchers attempt to address the causes and results of this adversity, or at an individual level, between members of a committed dyad desiring to protect their relationship.

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While the molecular basis receives attention, development of a molecular-based diagnosis is still in a deficit: understanding Attention Deficit Hyperactivity Disorder

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Abstract

Attention deficit hyperactivity disorder is the most prevalent childhood-onset behavioral disorder, affecting approximately 8% of the population, where a disproportionate amount of males are afflicted. Common symptoms of the disorder include inattentiveness, hyperactivity, and impulsivity. Genomewide linkage analyses have demonstrated that the disorder is likely due to several genetic factors, whereby the dopaminergic, serotonergic, and noradrenergic neurotransmitter systems are highly implicated through various observations. Genetic screens of afflicted individuals have implicated the presence of specific genetic polymorphisms with ADHD, examples being the 10-repeat-40-base-pair allele of the dopamine transporter, DAT-1, and the silent-G861C-substitution allele of the serotonin receptor, 5-HT1B. Evidence is emerging that proteins involved in the release of neurotransmitters from synaptic vesicles, like SNAP-25, may also be involved in the pathology of ADHD. The most common method of treatment is the administration of psychostimulants, like amphetamine derivatives and methylphenidate (Ritalin®), drugs which target the dopaminergic system. New therapies that target other neurotransmitter systems, like the selective noradrenaline transport inhibitor, atomoxetine, are gaining recognition as effective treatments. Common methods to diagnose ADHD reside in psychological assessments. As more insight is gained into the genetic basis for the disorder, it appears likely that a clinical diagnostic test based on genetic screening for these factors, such as specific genetic polymorphisms, could serve as an additional means of diagnosis.

Keywords

ADHD, genomewide linkage scan, genetic loci, genetic polymorphisms, psychostimulants, neurotransmitters.

List of abbreviations

ADHD: attention deficit hyperactivity disorder
DA: dopamine
5-HT: serotonin
MCP: meta-chlorophenylpiperazine
NE: norepinephrine
AMP: dextroamphetamine
MET: methylphenidate (Ritalin®)

Attention deficit hyperactivity disorder (ADHD) is a disorder with a strong genetic component and is observed to have many symptoms that often vary amongst individuals. The few commonalities of the disorder, as stated in the DSM-IV definition (American Psychiatric Association 1994), are that it generally becomes apparent in early to mid-childhood where the symptoms of inattentiveness, hyperactivity, and impulsivity are present. ADHD symptoms have now been sub grouped into three categories: inattentive individuals, those who are hyperactive, and those who possess a combination of both (combined ADHD). More specifically, ADHD individuals are described as constantly fidgeting, impatient, incessantly talkative, interruptive, distractible, engaging in physically dangerous activities, and impatient. Often, a combination of some but not all of these behavioral attributes are observed in ADHD individuals.

Many populations of school-age children from around the world have been assessed for the prevalence of the disorder. The results show that approximately 5-10% of children and adolescents are affected, making this the most common childhood-onset behavioural disorder (Wolraich, Hannah et al. 1996). It has been observed that out of the population diagnosed with ADHD, young males were apparently 4 to 8 times more abundant than young females (Anderson, Williams et al. 1987; Zametkin, Nordahl et al. 1990).

ADHD has been documented since the early nineteen hundreds, when a successful treatment for the disorder was first observed in 1937 by Charles Bradley upon administering amphetamine derivatives to hyperactive children (Bradley 1937). Today, the most commonly employed method of treatment is the administration of psychostimulants (Solanto 1998), such as methylphenidate (Ritalin®), where such treatments have been shown to be effective for the long-term without demonstrating long-term adverse effects (Stevenson and Wolraich 1989; Wilens and Biederman 1992; Gillberg, Melander et al. 1997). Interestingly, since 1990 the use of psychostimulants for the treatment of ADHD has been observed to have more than tripled (Seeman and Madras 1998).

Numerous studies have had this psychological disorder as their focal point but despite modern genetic, psychological, and neurophysiological studies, relatively little is understood concerning the exact molecular mechanisms of ADHD and the specific mechanisms of action of several therapeutic drugs used as treatment (Solanto 1998). The purpose of this review is to provide a general summary of the neuropathology of the disease with focus placed on abnormalities at the biochemical level and review current knowledge on therapeutic drugs for the disorder. The initial section will provide a summary of the genes and gene products associated with ADHD, while the subsequent section will focus on pharmacological

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treatments for the disorder and what is known about their modes of action. The general conclusion will address areas of interest for future research and critical suggestions for a method to develop a diagnostic test for ADHD that is based on biochemical markers for the disorder.

	Dopamine receptor			Dopamine transporter	Serotonin receptor		Neurotransmitter release (SNARE Proteins)		
Biochemical Factor Implicated in ADHD Pathology	DRD-1	DRD-4	DRD-5	DAT-1	5-HT1	5-HT2	SNAP-25	Syntaxin-8	RIM1
Corresponding Chromosomal Loci	5q35.1	11p15.5	4p16.1 -p15.3	5p15.3	6q13	13q14	20p11.2 -q21	17p12	6q12 -q13
Similar Loci Previously Implicated?	Yes Chr.5	No	No	Yes 5p13	Yes 6q14	No	No	Yes 17p11	Yes 6q14
List of Other Previously Implicated Loci	7p, 9q, 11q25, 15q, 16p13, 20q13								

Table 1. Summary of genetic loci and biochemical factors implicated in ADHD. Listed above are biochemical factors, grouped by their general function, implicated in the pathology of ADHD and their known genetic loci. Gray boxes denote genetic loci previously implicated through genome linkage analysis, some of which roughly superimpose to the genetic loci of the aforementioned biochemical factors. It is noted that the SNARE proteins involved in neurotransmitter release, Syntaxin-8 and RIM1, have not been formally implicated in ADHD. 'Chr.5': chromosome 5.

Isolation of genetic factors for ADHD

Numerous studies have demonstrated that ADHD is often familial, just like many other psychological disorders (Smalley 1997). One notable group of studies demonstrating the strong genetic link for the disorder were concordance studies between twins. Two large studies (Goodman and Stevenson 1989; Sherman, McGue et al. 1997) found that monozygotic twins had a concordance rate for ADHD of 51 and 58 % while dizygotic twins had a concordance rate for ADHD of only 33 and 31%, respectively. These studies reported a heritability estimate of 64 and 79%, respectively. Several adoption studies have also demonstrated that ADHD is determined far more by one's heredity rather than the environment in which children were raised. For example, ADHD children who were adopted and raised in separate homes from their biological siblings had higher rates of hyperactivity similar to their biological siblings, but unlike their adoptive siblings (Safer 1973).

The mode of inheritance of ADHD is complex and non-Mendelian since it appears to be a polygenetic disorder that shows incomplete penetrance **(Table 1)**. For instance, a systematic genomewide linkage scan on affected siblings that was performed by Fisher et al. (2002) implicated several genetic loci. One focus of this study was a meticulous screen of the X chromosomes of affected pairs of brothers. From this scan they concluded that the apparent excess of affected males with the disorder was not due to an X-linked recessive factor. A second genome linkage scan performed by Bakker et al. (2003) on siblings implicated five loci, all on different chromosomes. Each region was assessed by a multipoint maximum likelihood score (MLS) where a 15q locus had the maximum MLS value of 3.54, which was obtained from a sample of sibling pairs that met a standard, broad phenotype definition for ADHD. Loci located on the chromosome regions 7p and 9q also had notable MLS values, where these values were obtained from a sample of sibling pairs that met a narrower definition for the ADHD phenotype. Both Fisher et al. (2002) and Bakker et al. (2003) implicated chromosome 5 for possessing a possible factor for ADHD. A third analysis of affected sibling pairs performed by Smalley et al. (2002) found that a 12 centiMorgan region on chromosome 16p13 was a major locus for ADHD. A fourth linkage analysis of affected sibling pairs was performed by Ogdie et al. (2003) where five new regions, each on a separate chromosome, were found to have significant MLS values. These regions were 20q13, 17p11, 11q25, 6q14, and 5p13. The regions on chromosome 17 had the

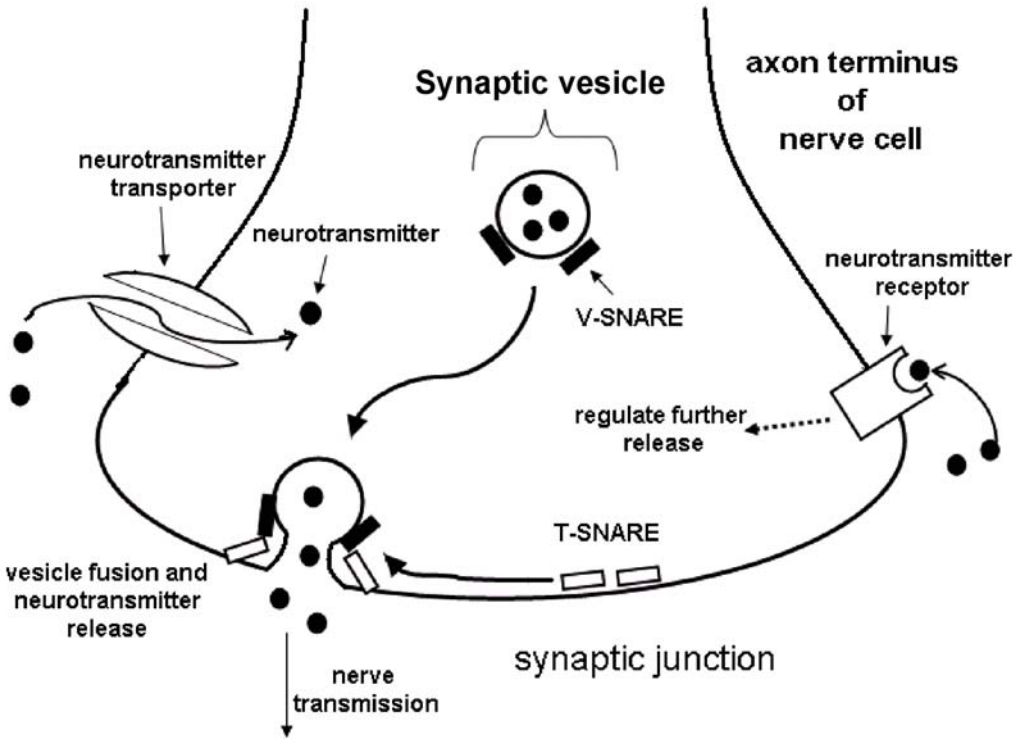

Figure 1. Basic overview of biochemical factors involved in neurotransmitter release that will be pertinent to our discussion of ADHD pathology. Within nerve cells, neurotransmitters that mediate nerve transmission are packaged into vesicle bodies known as synaptic vesicles. Synaptic vesicles are targeted to the presynaptic membrane through SNARE proteins, v-SNARE members being associated with the vesicle and t-SNARE members with the presynaptic membrane. Upon stimulus, membrane fusion occurs and the release of the vesicle cargo into the synaptic space results in nerve transmission. Once nerve transmission is conveyed, neurotransmitter concentration dissipates in the synaptic space by passive diffusion or by being returned into the axon terminus of the presynaptic nerve cell through a specific transporter. Additionally, neurotransmitters in the synaptic space may bind to specific receptors that serve to regulate subsequent neurotransmitter release.

highest MLS score (2.98) of the set. In conclusion, the aforementioned linkage analyses strongly implicate chromosome regions 17p11, 16p13, and 15q in ADHD and these should be the focus of further study. Although regions from chromosome 5 had less significant MLS scores, the fact that this chromosome was identified in three separate analyses is quite noteworthy. Since the disorder is most likely due to a combination of genes, the effect of an individual locus on the appearance of the disorder is likely to be small (Hawi, Dring et al. 2002).

Biochemical and molecular genetic aspects of ADHD

From a biochemical perspective, the best known causative factors of ADHD mainly involve the dopaminergic, noradrenergic, and the serotonergic neurotransmitter systems (Figure 1). The association of the dopaminergic system with ADHD has been, by far, the most intensively studied (Solanto 1998). The concentration of brain dopamine (DA) shows biphasic action concerning locomotion, where an overabundance of DA release during nerve transmission stimulates abnormal movement (Hornykiewicz 1966), as in patients afflicted with Parkinson's disease, and decreased DA release during nerve transmission reduces movement (Stromberg and Svensson 1975). Certain studies that have monitored DA levels in the brain have concluded that elevated DA release can be correlated with the severity of ADHD symptoms (Castellanos, Elia et al. 1994; Ernst, Zametkin et al. 1997). Two types of dopaminergic system proteins have been associated to ADHD pathology: the DA receptors and DA transporters, where both function as key regulators of DA concentration in the synaptic space (Seeman and Madras 1998). Certain receptors can regulate levels of DA by moderating its release during future nerve impulses while the transporters deplete its concentration through reuptake of DA into the nerve cell.

Five DA receptors, termed DRD-1 to DRD-5, have been identified, and genetic polymorphisms of specific receptors have been implicated in ADHD. A 148-base pair (bp) allele of the DRD-5 receptor containing a dinucleotide repeat was identified as a possible susceptibility locus (Daly, Hawi et al. 1999), and one study found a correlation between the density of DRD-1 receptors and ADHD in primates (Goldman-Rakic 1992). The DRD-4 receptor has been found to be highly polymorphic, where a 48-bp segment in its third exon may be repeated 2 to 11 times. The number of the repeat is known to be highly variable between different ethnicities, where the 7-repeat allele shows a high frequency in American populations, but a low frequency in Asian populations (Chang, Kidd et al. 1996). Numerous studies have implicated the 7-repeat polymorphism in ADHD (Swanson, Flodman et al. 2000), but no information was found on whether Asian populations show a lower incidence of the disorder. Additionally, the location of the DRD-4 gene was found to be at the 11p15.5 locus (Van Tol, Bunzow et al. 1991), which was not implicated in the previously mentioned linkage analyses.

The exact mechanism for the induction of ADHD symptoms by these polymorphisms is unknown, but a possible explanation has been found for DRD-4. DA receptors belong to a group of G-protein coupled receptors that have 7-transmembrane domains. The polymorphic region of DRD-4 corresponds to the third intracellular loop of the protein (Lichter, Barr et al. 1993) that is involved in G-protein coupling. The 7-repeat form of the gene probably has altered coupling capacities that would alter its ability in regulating DA concentrations. This form of the receptor has been found to respond differently to DA antagonists and agonists such that its response has been described as being "blunted" (Asghari, Schoots et al. 1994; Asghari, Sanyal et al. 1995) and has also been suspected of being less sensitive to DA (Seeman and Madras 1998).

The specific DA transporter, DAT-1, also has different polymorphic forms where a 40-bp repeat exists in 3 to 13 copies within the 3′ untranslated region of the gene (Vandenbergh, Persico et al. 1992; Sano, Kondoh et al. 1993). The 10-repeat allelic form has been implicated in ADHD and also corresponds to the most prevalent allelic form of the gene (Swanson, Flodman et al. 2000). It has been proposed that this allele encodes an overactive transporter that would over reuptake DA from the synaptic space (Swanson, Flodman et al. 2000). One particular study (Fuke, Suo et al. 2001) found that the 10-repeat allele significantly increased the expression of the gene in a manner that was not observed with the other allelic forms. This observation indicates that an overabundance of the transporter may be a factor in ADHD, one which would also have the effect of depleting DA in the synaptic space. The genomic locus for the transporter was found to be 5p15.3 (Vandenbergh, Persico et al. 1992), which roughly corresponds to the 5p13 locus implicated in ADHD by Ogdie et al. (2003).

The norepinephrine (NE) system exerts widespread regulatory effects since its terminals are found throughout the brain (Solanto 1998). The NE system is known to be important in attentional processes such as selective attention and vigilance (Aston-Jones, Chiang et al. 1991), where evidence has suggested the possibility that overactivity of the NE system is responsible for ADHD symptoms which may be related to its association with the dopaminergic system (Solanto 1998). Hyperactivity was also found to be induced in animal models through chemically induced legions in the brain which lead to the depletion of cerebral NE (Shaywitz, Cohen et al. 1977). To summarize, it appears that, like DA, NE may display biphasic action where either too little or too much of the neurotransmitter in the synaptic junction may produce symptoms that resemble traits seen with ADHD. The relevance of NE to ADHD will be expanded in a subsequent section that describes the NE transport inhibitor, atomoxetine.

Serotonin (5-hydroxytryptamine; 5-HT) has numerous physiologic functions that range from appetite to sexual behaviour. Upon release into the synaptic junction, 5-HT binds to numerous specific receptors to exert its effects. 5-HT receptors fall into four main groups: 5-HT1, 5-HT2, 5-HT3, and 5-HT4, where each can be subdivided into numerous subgroups. DNA variations in genes of the serotonin system and the abnormal functioning of serotonergic system proteins in relation to their influence on the doaminergic system have been implicated with the pathophysiology of ADHD (Hawi, Dring et al. 2002), where it has been found that both neurotransmitter systems exert regulatory control over one another (Kelland and Chiodo 1996). Much of this evidence has been acquired from studies involving animal models. One such model was of mutant mice that possessed a deletion in the 5-HT1B receptor. They were noted to be far more impulsive and displayed more aggressive behaviour than wild-type controls (Saudou, Amara et al. 1994). This receptor also showed associations with hyperactivity, where controlled stimulation of the

receptor by a specific agonist induced hyperactive tendencies in wild-type mice (Hawi, Dring et al. 2002). This same agonist had no effect on mice possessing a knock out mutation for the receptor. Additional observations specifically pointed to the 5-HT1B receptor as the causative agent for induced hyperactivity (Heisler and Tecott 2000). Hyperactivity was induced in mice containing a knock-out mutation in the 5-HT2C gene upon administration of the non-specific serotonin receptor agonist, meta-chlorophenylpiperazine (MCP). When the same mice were pre-treated with a specific antagonist for the 5-HT1B receptor, no hyperactivity was observed following administration of MCP. Studies on mice involving the 5-HT2A receptor showed similar results where induced hyperactivity was found to be attenuated by 5-HT2A antagonists (O'Neill, Heron-Maxwell et al. 1999).

These observations were extrapolated to humans whereby the orthologous 5-HT1B gene was also shown to be linked with ADHD. The function of this autoreceptor is to adjust the release of 5-HT from the presynaptic serotonergic neurons and has been implicated in the control of movement such that it is predominantly expressed in regions of the brain involved in motor control (Demchyshyn, Sunahara et al. 1992; Quist and Kennedy 2001). This receptor, through the action of 5-HT, has also demonstrated an inhibitory (Sarhan, Cloez-Tayarani et al. 1999) as well as stimulatory (Ng, Lee et al. 1999) role in the release of DA in the brain. Over four polymorphisms of the receptor have been isolated and studies have implicated specific alleles with ADHD. The allele containing the silent G861C substitution was observed to be preferentially transmitted in a sample of ADHD individuals (Quist, Barr et al. 2000; Hawi, Dring et al. 2002). Why a silent mutation would be linked to the disorder is unknown but it has been suggested that a disease-causing-genetic variation may be found close to the location of this silent mutation and would thus also show preferential transmission with the pathologic genetic variation (Hawi, Dring et al. 2002). Additional evidence linking the 5-HT1B gene with the disorder originates from its chromosomal location, 6q13 (Jin, Oksenberg et al. 1992; Lappalainen, Dean et al. 1995). This region roughly coincides with 6q14, a previously implicated locus found through linkage analysis (Ogdie, Macphie et al. 2003).

The human orthologue of 5-HT2A has also been linked to ADHD. Hawi et al. (2002) stated that serotonergic agonists inhibit neuronal firing, possibly due to a decrease in synaptic DA resulting from lack of synthesis or release of the neurotransmitter. They further stated that this effect may be mediated by the 5-HT2A receptor. Several polymorphisms have also been identified with this serotonergic receptor. One allele that encodes an amino acid substitution of histidine for tyrosine shows preferential transmission in ADHD individuals (Quist, Barr et al. 2000; Hawi, Dring et al. 2002). This allelic variation appears to produce a desensitized receptor that may alter the balance of serotonergic transmission (Ozaki, Manji et al. 1997). The 5-HT2 receptor has been mapped to chromosome 13 (Hsieh, Bowcock et al. 1990), a locus not previously implicated with the disorder.

To conclude, the controlled release of DA through the action of 5-HT on its associated receptors is probably the relevant cause of dopamine-mediated symptoms that are seen in ADHD. Interestingly, genes encoding serotonergic receptors may be the reason why there is a disproportion of males with the disorder. There is evidence of genomic imprinting of the 5-HT2 gene where the gene was found to be expressed only from the maternal allele (Kato, Shimizu et al. 1996). Hawi et al. (2002) also implicated genomic imprinting from noting preferential transmission of the 5-HT1B G861C allele from fathers to their offspring. Evidence of genomic imprinting has been found for the 6q27 locus (Xu, Goodyer et al. 1993), one that is proximal to the 6q13 locus of the receptor.

Aside from the observations that neurotransmitter receptors and transporters are factors in ADHD, a different type of protein, SNAP-25, has been proposed as a candidate. Recent evidence has found that SNAP-25 plays a crucial role in the release of classical neurotransmitters (Raber, Mehta et al. 1997). More specifically, SNAP-25, along with the protein Syntaxin, are two major t-SNARE proteins located at the plasma membrane of the axon terminus. The complex of these proteins serves as a docking complex for synaptic vesicles through interactions with the vesicle membrane protein VAMP (a v-SNARE protein). Upon localization of the vesicle at the presynaptic membrane, the two membranes fuse, leading to the release of the vesicle's cargo into the synaptic cleft.

SNAP-25 became implicated in ADHD as a result of the development of the Coloboma mutant (Cm) mouse containing a deletion on chromosome 2. This region is known to contain several genes, including that encoding SNAP-25 (Hess, Collins et al. 1994; Hess, Collins et al. 1996). The heterozygous mouse (containing one Cm mutated chromosome 2 and a wild-type chromosome 2) displays a semidominant effect on SNAP-25 with a 50% reduction of the protein throughout the brain and no change in the expression pattern of the remaining gene (Hess, Jinnah et al. 1992). This mutant mouse displayed spontaneous hyperactivity and now serves as an animal model for ADHD, since its hyperactivity could be successfully treated with amphetamines, and genetic complementation of the deficiency with a SNAP-25 transgene produced wild-type behaviour (Hess, Collins et al. 1996). These observations suggest that the behavioral problems are due to a reduction of functional SNAP-25, which causes a reduced release of neurotransmitters at presynaptic terminals (Raber, Mehta et al. 1997). Studies by Raber et al. (1997) have confirmed that this mutant mouse does show abnormalities in the release of neurotransmitters, where, for example, induced depolarization of the synaptic region was found to no longer release DA and the release of 5-HT was significantly lower.

These observations, although preliminary and based on a mouse model, raise the question of whether biochemical pathways involved in the general release of neurotransmitters are key factors in the etiology of ADHD. An interesting link between human SNAP-25 and ADHD can be made from its chromosomal location, 20p11.2 (Maglott, Feldblyum et al. 1996). This locus roughly coincides with the previously stated 20q13 locus found by linkage analysis (Ogdie, Macphie et al. 2003). Furthermore, I note that the human genes encoding syntaxin-8 and RIM1, SNARE proteins involved in the docking and fusion process of synaptic vesicles, have been mapped to 17p12 (Thoreau, Berges et al. 1999) and chromosome 6 (Nagase, Ishikawa et al. 1997), respectively. Both regions roughly coincide with previously implicated loci found through linkage analysis where the 17p11 locus had the highest MLS value (Ogdie, Macphie et al. 2003). In conclusion, it appears that further evidence asserting the associations of

human SNARE proteins in relation to ADHD may uncover new causative agents and drug targets for the disorder. An interesting avenue for investigation would be to confirm if syntaxin-8 and RIM1 are factors in this disorder, as suggested through previous chromosomal linkage analyses **(Table 1)**s.

Pharmacological treatments for ADHD and their modes of action

Intensive research on the pathology of ADHD has led to the development of numerous pharmacological treatments, of which most exert their activities on specific neurotransmitter systems. Since the dopaminergic system has been the most intensely studied in relation to ADHD, the mechanisms of drugs affecting this system are most understood. The most widely used drugs for the treatment of ADHD are the psychostimulants dextroamphetamine (AMP), methylphenidate (Ritalin®, MET), and pemoline. These drugs both promote the release of DA and block its reuptake into the neuron via the DA transporter, where MET plays a greater role in blocking the transporter (Volkow, Ding et al. 1995) and AMP plays a greater role in enhancing the release of DA (Seeman and Madras 1998). In brief, both drugs increase the DA concentration in synaptic junction. These drugs show molecular homology with DA, which allows them to enter the transporter much like DA but are molecularly structured so that they block, rather than pass through it, as would DA **(Figure 2**). Like DA and suggested for NE, MET and AMP also display a biphasic action where low doses of the drugs promote a calming effect and elevated doses produce stimulation (Seeman and Madras 1998).

Ironically, increased amounts of DA release during nerve transmission are known to increase impulsive movements (as in Parkinson's disease), thus the effects of psychostimulants on increasing DA concentrations in synaptic junctions must have a more complicated mechanism in order to produce a calming effect. A potential mechanism will now be presented.

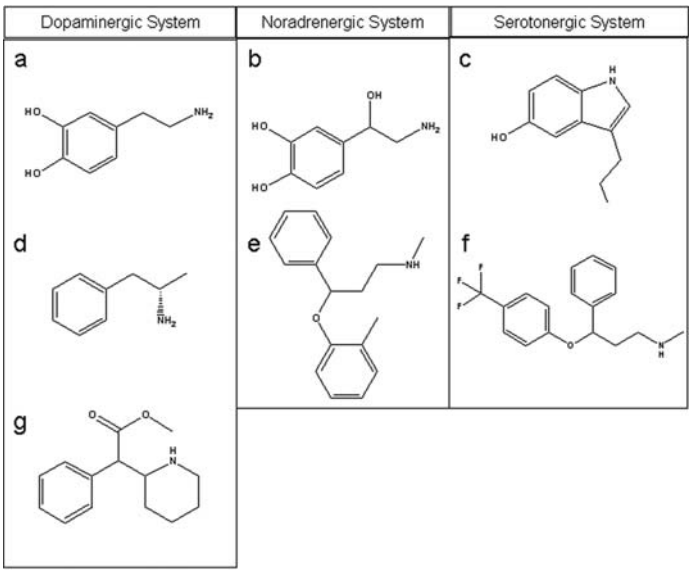

Figure 2. Molecular structures of neurotransmitters and pharmacological therapeutics for ADHD. The therapeutics display structural similarities to the neurotransmitter of the neurotransmitter system they act upon. a) dopamine and the psychostimulants d) dextroamphetamine and g) methylphenidate (Ritalin®). b) norepinephrine and NE transport inhibitor e) atomoxetine. c) serotonin and the 5-HT transporter inhibitor f) fluoxetine (Prozac®).

During a normal nerve impulse, the extracellular level of DA in the synaptic junction may increase to over 60 times the basal pre-stimulatory level (Kawagoe, Garris et al. 1992). Return to the basal level occurs by diffusion and reuptake of DA by DA transporters **(Figure 1)** (Seeman and Madras 1998). Low doses of psychostimulants have been found to increase both basal levels of DA within the synaptic junction and the amount of DA released upon nerve transmission. More specifically, the increase in the basal level of DA in the synaptic junction is several times that of the increase in the amount of DA released upon nerve transmission(Seeman and Madras 1998). Thus, the calming effect of low doses of psychostimulants may reside in the fact that the relative increase of DA in the synaptic junction before and after nerve transmission is reduced compared to the amount obtained in the absence of psychostimulants. Simply put, this appears to mimic a situation where less DA is released during nerve transmission. Additionally, the elevated basal amount of DA in the synaptic junction can mediate subsequent DA release by saturating DA receptors that regulate the release of this neurotransmitter, thus resulting in a decrease of DA release during future nerve impulses. This lowering of pulsatile DA concentrations will result in less activation of DA receptors that mediate the initiation of psychomotor activity (Silvia, King et al. 1994; Seeman and Madras 1998). Furthermore, it has been documented that DA receptors that mediate the initiation of psychomotor activity can lower their affinity for DA and become desensitized through elevated associations with DA (Seeman, Watanabe et al. 1985). It has been suggested that the elevated basal level of DA in the synaptic junction may also cause such DA receptors compensate by becoming desensitized (Seeman and Madras 1998), which would also decrease their ability to initiate psychomotor activity. On the contrary, sudden administration of elevated concentrations of psychostimulants (as is seen with recreational drug use) do not produce the same effect since the basal level of DA within the synaptic junction under this situation becomes extreme and thus produces general stimulation (Seeman and Madras 1998).

Another therapeutic drug that has been found to attenuate ADHD symptoms is fluoxetine (Prozac®), which is a selective inhibitor of 5-HT reuptake. The drug blocks 5-HT transporters, causing an increase in extracellular 5-HT (Gainetdinov, Wetsel et al. 1999). Fluoxetine was noted to diminish ADHD symptoms in DAT-1 gene knockout mice, whereas the drug MET showed no effect (Hawi, Dring et al. 2002).

A recent study has shown that a selective NE transport inhibitor, atomoxetine, was able to alleviate ADHD symptoms (Bymaster, Katner et al. 2002). This study investigated the mechanism of action of the drug on DA, 5-HT, and NE transporters and its effect on the extracellular concentrations of these neurotransmitters. Atomoxetine displayed specificity for NE transporters, causing increased extracellular levels of NE in the brain but having no effect on 5-HT concentrations. Interestingly, extracellular levels of DA were found to have also increased in certain regions of the brain. The extent of this increase was found to be comparable to that produced with the administration of MET. Thus, the therapeutic effects of atomoxetine were concluded to be due to the induced increase in DA and NE. The action of blocking NE transporters by atomoxetine is mimicked by certain tricyclic anti-depressants like desipramine (Delgado, Miller et al. 1993), which have also

been proposed as treatments for ADHD (Spencer, Biederman et al. 1996) but are considered to be less effective than psychostimulants (Pliszka 1987). To conclude, evidence is emerging that atomoxetine may be a favourable alternative to psychostimulant treatments in certain conditions and has the added benefit of not increasing extracellular levels of DA in regions of the brain that are associated with addiction, which is the case with other psychostimulants (Kuczenski and Segal 1997; Bymaster, Katner et al. 2002).

Concluding statements

ADHD is a complex disorder that is linked to multiple neurotransmitter systems. Associations of the disorder with the dopaminergic, noradrenergic, and serotonergic systems are relatively well known. Conversely, associations between the disorder and SNARE proteins, notably SNAP-25, and neurotransmitter release are in their infancy and probably hold much research potential, especially since many SNARE genes appear to have been previously implicated with the disorder through linkage analyses. For example, it is noted in this review that the SNARE proteins RIM1 and syntaxin-8 are of interest for the aforementioned reason. How exactly SNARE proteins may be linked to the disorder remains nebulous and should be the focus of investigation. It is interesting to note that hyperactivity in the Coloboma mouse was due to the 50% reduction in SNAP-25 protein expression. This suggests that depletion or loss-of-function of certain SNARE proteins could be a general trait of ADHD whereby knowledge into the specifics as to how this might occur, such as through genetic polymorphisms as seen with DAT-1, would be invaluable to our understanding of this condition. The genetics of ADHD also show several ambiguities and require focussed investigation; especially why the disorder appears to be predominantly observed in males and whether genomic imprinting is indeed a factor in this discrepancy. Further understanding as to why ADHD shows prominence in males may divulge very specific factors that cause the disorder and their mechanisms in pathology. For example, if genomic imprinting results in the silencing of certain genomic loci, depletion of certain genetic products may induce the disorder whereby therapies to correct this condition may favour alternatives to pharmaceuticals. Increasing knowledge into the specific molecular mechanisms of the disorder is broadening options for successful treatments and is exposing new potential drug targets. It appears that after using psychostimulants for decades, new therapies are providing other options to these compounds as seen with atomoxetine. Research concerning specific factors of ADHD that are not directly related with the dopaminergic system and DA, namely serotonergic receptors and possible factors within the noradrenergic system, appear to be ones that will provide the revolutionary discoveries into this disorder in the years to come. Since several drugs have been observed to be effective (psychostimulants, atomoxetine, fluoxetine), or somewhat effective (tricyclic anti-depressants) in the treatment of ADHD, future investigations into the effectiveness of administering combinations of these drugs might prove valuable. Identification of effective combinations of drug therapies may result in lowering the necessary doses of any one particular therapy, thus reducing the possibility of adverse drug responses, especially since these treatments are often administered well into adulthood with ADHD sufferers.

It has been noted that many studies have focused on the themes of drug development and the identification of causative biological factors associated with ADHD, yet have not focused on the development of a diagnostic test for the disorder that is based on such biological factors. Most ADHD individuals are diagnosed through psychological assessments, while it appears that few are diagnosed through methods concerning genetic or biochemical factors, such as genetic screening. The lack of such tests is probably due to the complexity of the disorder where a successful clinical diagnosis would probably involve numerous biochemical and/or physiological variables, many of which have yet to be confirmed as agents in ADHD pathology. The analysis of specific genomic loci appears to be the most promising approach for the development of a genetics-based clinical test. Several genes implicated in ADHD, their polymorphisms, and their genomic locations are known. Therefore genetic screening of specific genomic locations could be used to identify such polymorphisms or allelic variations, such as through Southern blotting or DNA microarray analysis. Such an approach for diagnosis of the disorder has its flaws, since some polymorphisms are known to be more predominant in certain populations and absent in others. Since ADHD is a polygenetic disorder, several loci would have to be analyzed in order to produce a diagnosis with an appreciable level of certainty. While it is not to be construed that diagnosing ADHD via psychological tests is inefficient or inferior, additional means to diagnose the disorder have their merit. For example, if ambiguities are obtained from a psychological assessment, a subsequent analysis through genetic testing may help to confirm the accurate diagnosis of an individual. Plus, since ADHD is the most prevalent childhood-onset behavioral disorder, developing tests that employ routine procedures requiring a relatively short time per analysis (i.e.: Southern blot analysis to identify specific genetic polymorphisms) may increase the efficiency with which ADHD is diagnosed in large patient populations.

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New Course Announcement

Science Writing
REDM 399

Linda Cooper
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Redpath Museum, Room 203
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Science Writing focuses on writing and editing techniques that enable undergraduate Science students in Research Project courses to write clearly about their research. The course meets on Tuesday from 2:00-3:00 and is structured as follows:

Week 1: Introduction. Features of clear science writing.
Why specialized terminology, abbreviations, and obscure writing alienate interdisciplinary audiences.
The advantages and disadvantages of metaphors in science.

Week 2-6: Editing techniques to make texts more precise and clearer.
The benefits of the active voice.
Limiting the use of weak linking verbs.
Limiting the use of prepositions.
Limiting the use of nominalizations.
Where to put important information.
Using modifiers effectively.

Week 7-8: The Abstract IMRAD format and why it works.
What to put in each section of the Abstract and what tense to use.
How to write an effective title.

Week 10 - 12: Applying the principles of clear writing to students' research projects
How to cite other people's work.

Grading Scheme: 70 % final research project, 15% class participation, 15% class assignment.

Please note: REDM 399 is designed for Science students involved in courses that require a research project. Permission of the Instructor is required. Please contact Linda.Cooper@mcgill.ca

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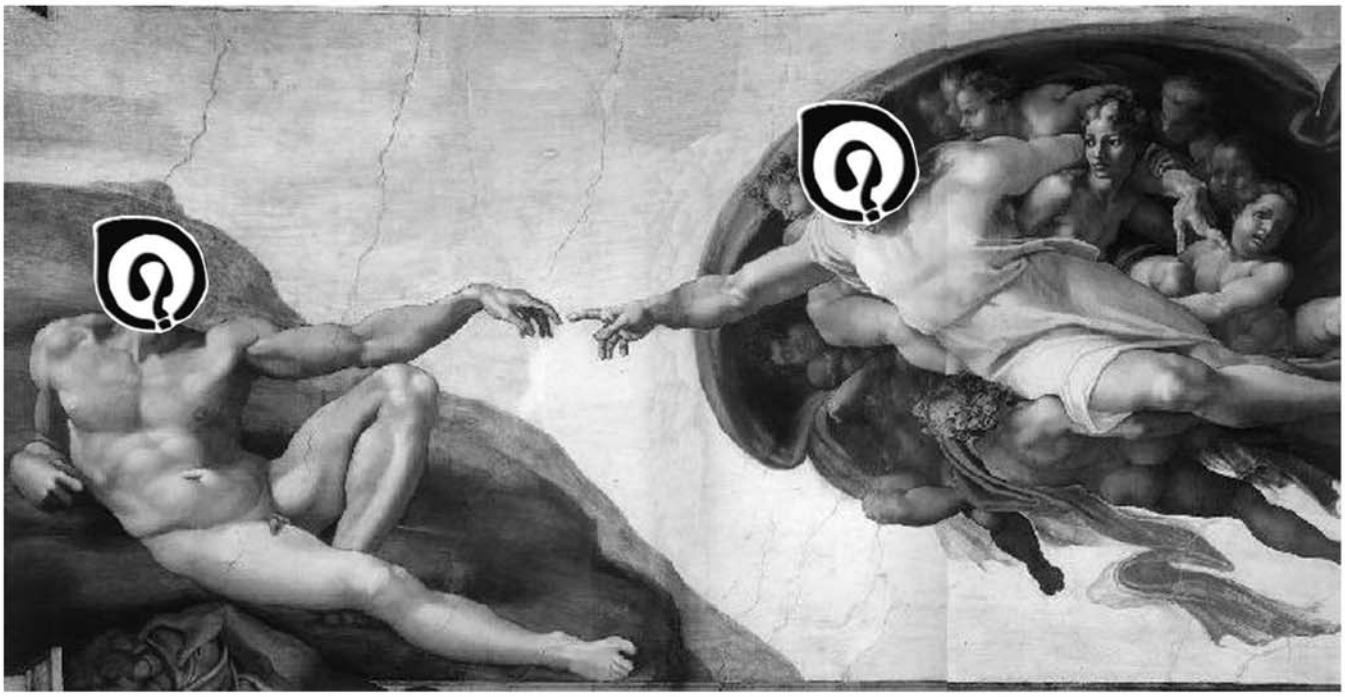

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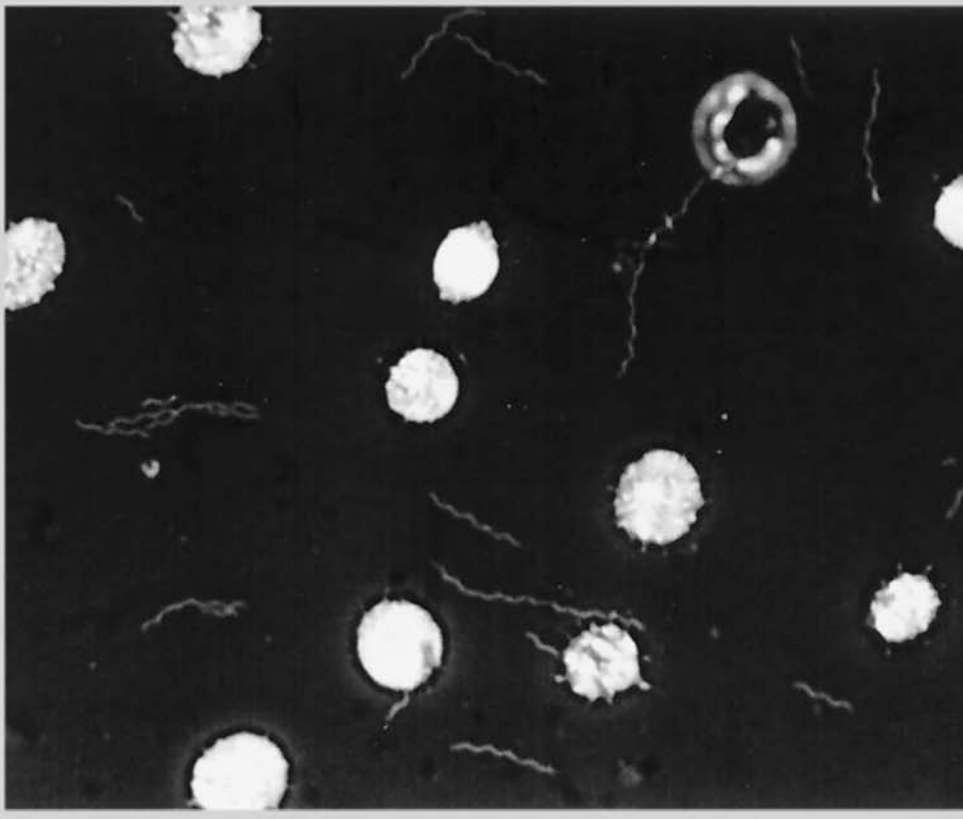

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