ON THE COVER

In the recent years, awareness about biodiversity in cities and a renewed effort to improve the sustainability of cities has been established. On Page ___, Jacob Garrah, Katherine Berton, and Sophia Chen propose establishing a relationship between biodiversity preservation and urban planning and design to build a more connected, healthy, and resilient community.

The cover image shows the Montreal Biodome, a facility featuring various ecosystems, alongside an urban city skyline.

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FOREWORD

Dear Reader,

After starting as one of North America's first peer-reviewed undergraduate research journals over a decade ago, the McGill Science Undergraduate Research Journal has come a long way and has continued to foster an ever-changing scientific community of passionate undergraduate researchers. This year MSURJ is proud to present Volume 12. While continuing to uphold our rigorous editorial process and uphold a high standard of scientific acumen and writing quality, we present this year's Journal as an instrument to purvey research excellence and uphold our mission of promoting impactful research at the undergraduate level.

With each new year our journal receives more submissions than the last, coming from various universities and departments around the world. In this edition you will find nine original research papers written by undergraduates with a passion for science reflected well in the works themselves.

Now and in the future, we hope to provide student researchers demonstrating strength and potential with the opportunity to share their work with the scientific community on a platform which continues to garner the respect of its readers.

Alexander Chatron-Michaud & Sapan Patel
Co-Editors-in-Chief

ACKNOWLEDGEMENTS

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We thank Dean Bruce Lennox for his continuing support as we set new goals for the upcoming decade. Without his generosity and expertise, MSURJ would not be the success it currently is. We would also like to acknowledge Mr. Victor Chisholm's dedication to the undergraduate research community and thank him for his guidance. Lastly, we thank the librarians of McGill University for their assistance in bringing MSURJ to a digital medium.

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We are grateful to the many peer reviewers who donated time to review our submissions and the student contributors who offer their work. This journal would not be possible without them.

Gabriel Yahya Haage[1]

Are species largely redundant? Testing the reliability of increasingly complex trait-based classifications in understanding Canadian Arctic ecosystems

Research Article

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Keywords

Functional traits, Species redundancies, arctic aquatic ecosystems

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Abstract

Background: In recent years, some ecologists have advocated the use of functional groups instead of direct species in linking site composition to the environment. They could potentially reveal connections between distant sites and aid in the formation of widely-applicable environmental policies. Several studies have compared the efficiency of using functional groups, in which species are grouped based on functional traits, like feeding method or size, to using species directly. However, few have looked at the effect of varying the complexity of functional groups when compared to species data. This study compares functional group classes of varying complexity, with complexity defined as the number of traits considered, to species data. The hypothesis that more complex functional group classes, compared to less complex classes, tend to approach the results obtained when using taxonomy, is tested.

Methods: In testing this hypothesis, this study uses site composition data from aquatic floor (benthic) ecosystems in the Canadian Arctic. Four functional traits were considered important to describe these species: Bioturbation (sediment disturbance), body size, feeding habit and mobility. These traits were used to segregate species into functional groups of varying complexity, with complexity level determined by the number of traits (out of four) being used. Four environmental characteristics were considered for each site: Chlorophyll a, phaeopigments, depth and salinity. In order to test how similar functional group data is to species data, we sought to determine whether the same environmental variables were important in explaining site composition. This was determined by BIO-ENV analyses and Spearman Rank correlations. Mantel permutation tests then determined whether the correlations were significant.

Results: While all levels of complexity, from one to four functional traits, showed some significant correlations (Spearman Rank ≥ 0.5, $p \leq 0.05$) between site composition and environmental variables, there was no general trend suggesting functional group complexity correlates with greater similarity to taxonomic data. For presence/absence data, all functional results, regardless of complexity, pinpointed only phaeopigments as important, while presence/absence species data also included chlorophyll a and depth. All results with strong and significant correlations ($r \geq 0.5$ $p \leq 0.05$), regardless of data type or complexity, maintained a measure of food supply (Chlorophyll a or phaeopigments), demonstrating its importance in determining ecosystem composition at these sites.

Limitations: Potential improvements include measuring traits directly from the organisms, considering more environmental variables and increasing the number of functional traits considered. Which traits are considered also vary with each study.

Conclusions: The hypothesis was not validated by the results. When pinpointing the most complex functional group class (the most important variable), rather than a less complex class, it was not guaranteed that the chosen variables would be the same as species data. Some classes of less complexity showed greater similarity to full species data. Some outcomes, like the presence/absence results, also imply certain species redundancies in the ecosystem, particularly regarding depth. These results have implications for the concept of functional redundancies in ecosystems, an important point in developing widely applicable environmental policies.

Introduction

Linking species compositions to environmental variables is an important concept in ecology. Some ecologists have pushed the use of functional traits, instead of taxonomic information, in relating organisms to environmental variables. Functional traits, from feeding type to size, can be used to form functional groups, with many ways of defining a functional trait (1). Some researchers define them as the traits which impact the fitness and individual performance of organisms through effects on growth, reproduction and survival (2). Others have focused on ecosystem processes and an organism's response to environmental variables (3). The definition given by Harrington et al. (4) attempts to combine the two views by suggesting that a functional trait can both determine how an organism responds to pressures (response trait) and/or the effects the organism has on ecosystem processes (effect trait). Depending on the type of organism, functional traits can include morphological, biochemical, life-history and behavioral traits (4). Functional groups are formed by grouping species with the same functional traits together (4). For example, if the two functional traits of mobility and feeding are considered, all species that are Hemimobile Carnivores are categorized in the same functional group.

Understanding functional groups is vital in the study of redundancy in ecosystems. There are many theories regarding species redundancy. Species may be seen as unique, each offering a specific and significant effect on the ecosystem. In the "riveting" hypothesis, each species is like a rivet – remove enough and all ecosystem functions fail (6). Alternatively, many species may be redundant. Provided that there exists some species that can fill a functional role, like primary consumers or decomposers, the ecosystem functions (7). One species can replace another with the same function and fitting the same functional group. These are two extreme views, of course. Even if species are partially redundant, having redundant species can make ecosystem function more reliable and offer greater resilience to perturbation (8, 9). Understanding how closely functional information relates to species information is necessary to understand the role of redundancy.

The use of functional groups has several advantages over taxonomy. Its taxon-independent nature can harmonize data from different studies and remove linguistic confusion (10). Collecting functional data is also less costly and requires less taxonomic expertise (30). Trait-based systems can also help compare data from different locations with ecologically similar species (36). They can make ecological similarities between areas clearer than simply relying on species present (10). Functional group information about community composition is particularly helpful if many species are functionally equivalent and substitutable between sites (i.e. there is redundancy) (12).

A potential benefit of using functional groups is in the formation/management of environmental policies. Generally, environmental policies seek to be standardized and to have wide geographic application (27). Policies should also be trustworthy when considering distinct species compositions at different locations (27).

Potentially, if one could understand how species that fit into a functional group react to environmental changes (i.e. "medium size active burrowers"), wide-reaching policies could be formed. Functional traits have been used in several management fields, including in creating protected areas and detecting/predicting anthropogenic impacts. For instance, by considering trait compositions at key sites rather than only taxa, relevant Marine Protected Areas and habitat models can be developed (35, 13). Trait-based systems can also help predict the success of ecological restorations (14).
Similarly, several studies have looked at using functional classifications in gauging anthropogenic stress. Several stressors, including metal leeching and eutrophication can be considered (27, 28, 29, 32). In aquatic systems, benthic invertebrate species are commonly used as ecosystem health bio-indicators and the stability of benthic communities often hinges on the pollution sensitivity of these species (30, 31). Mobility, size and feeding mode can be traits affecting pollution tolerance in benthic communities (30, 32, 25). Functional traits are also beneficial in predicting extinctions. For instance, extinction scenarios can follow clear size patterns, with larger organisms becoming extinct first (28). So, if considering body size is as valid as taxonomy, general extinction patterns could be calculated.
Naturally, considering only one trait, like size or feeding, might not be as specific as taxonomy. Increasing the complexity of functional group information could potentially remedy this, and several approaches exist. For instance, Rawer-Jost et al. (32) discuss potential benefits/drawbacks of increasing levels within one category of trait (i.e. feeding) when identifying anthropogenic stressors. Others suggest combining evolutionary information and biological traits (33).

A common approach is to combine distinct categories of functional traits, including feeding, size and mobility, in segregating species into functional groups (27-29, 34). For instance, several functional traits, including feeding mode, reproductive strategy and spawning season, were combined to segregate fish species into functional groups based on stress sensitivity (29). Similarly, to understand extinction effects in coastal benthic communities, several traits including size, mobility and sediment mixing/bioturbation were used to segregate species into groups according to their impacts on ecological processes (28). In fact, the use of functional groups based on multiple traits has been shown, in certain benthic communities, to be more efficient than species abundances at indicating/identifying anthropogenic impacts (27). As Kenney et al. (30) point out, however, a remaining issue regarding functional traits in policy is the value of considering traits in combination versus individually.

While several studies have sought to compare the use of functional groups to taxonomic data, few have looked at the effects of increasing the complexity of functional groups (15-17). For the purposes of this study, complexity in functional group class is determined by how many traits are used in forming the functional groups within this class (See Table 1 and Table 2 for terminology).

There have been several studies on varying taxonomic resolution, which can be used as a template to understand why varying functional group class complexity is informative. For instance, several researchers tested whether using lower resolution taxonomic data, such as genus, family or order, is as effective as using species information (15-17).

We applied a similar conceptual model. For instance, as taxonomic categories become more detailed and approach the species level, functional group classes can become more complex as they approach the species level data. Conceptually, an order could be analogous to a simple functional group class constructed from one functional trait, whereas a genus might be similar to a more complex functional group class containing groups based on a greater number of traits. Of course, such analogies are imperfect, but help visualize the logic of increasing functional group complexity. Functional group information is more complex to work with, however, because the same level of complexity can result from different functional traits. For instance, a class that considers two traits–mobility and size–is at the same level of complexity as another that considers two traits, feeding and bioturbation, but each class considers different traits.

This study explores how the number of traits used to form functional groups (i.e. the complexity of functional group classes) affects the reliability of trait-based systems. We sought to determine whether results obtained using more complex functional group classes are closer to full species data than using less complex classes. To assess their similarity, we relied on identifying the environmental variables that are important in explaining site composition. We hypothesized that as one moves from simple to more complex functional group classes, the environmental variables pinpointed as essential should become more similar to results obtained using taxonomic data. Greater similarity is predicted in results between complex functional group classes and species data, than simple functional group classes and the same species data. For instance, if species data pinpoints only variables A, B, and C as necessary, and one of the functional group classes also pinpoints only these variables as necessary, it should be the most complex functional group class (i.e. the one that uses the greatest number of traits in grouping species).

Methods

Data Source

To test the hypothesis, a dataset of species composition at various sites, along with environmental measures, was required. Data previously collected by Link et al. (18) from Canadian Arctic marine benthic ecosystems (as part of the Canadian Healthy Oceans Network-NSERC) was used. Samples were collected from nine sites in 2008 and 2009. Several environmental variables, including chlorophyll a, phaeopigments concentrations (µgg-1), depth (m) and salinity (µgg-1) were recorded at each site. Taxonomic information, both diversity and abundance, was also recorded for each site by Link et al. (18). Invertebrate organisms were identified to the lowest possible taxonomic level, usually species, resulting in 311 taxa. Species predominantly fell into the Polychaeta and Malacostraca classes. See Link et al. (18) for a complete list of taxa identified. The identified taxa were also classified using four functional traits commonly considered important in benthic ecosystem processes: Feeding/diet, body size, bioturbation (sediment disturbance), and mobility (18).

We used this dataset, focusing on the year 2009, to experimentally test my hypothesis. We segregated species into functional groups based on different combinations of the four functional traits (body size, mobility, bioturbation, feeding/diet). The number of levels within each functional trait varies. Size and mobility have three levels, while bioturbation has four

(Table 1). The six feeding levels were not mutually exclusive and combinations were possible. For instance, some species were both omnivores and filter feeders. This resulted in 11 possible levels for this trait.

Functional group classes were formed for each level of complexity, ranging from Complexity 1 (considering only one functional trait) to Complexity 4 (considering all four functional traits). A total of 15 classes were formed. For instance, functional group class "Size-Feeding-Bioturbation" is a class of Complexity 3, in which the functional groups within it consider those three functional traits (See Tables 1 and 2). Three types of site composition data were considered for both functional groups and species: Raw abundance, relative abundance and presence/absence data.

Four environmental variables were considered: Chlorophyll a, phaeopigments, salinity and depth. They were chosen due to their key roles in arctic benthic ecosystems. Both chlorophyll a and phaeopigments, as components of algal biomass, are commonly used measures of food supply (24). Benthic organisms rely heavily on this biomass, which descends through the water column to the sediment floor (19). Depth is also important in determining ecosystem composition and has been shown to play a role in determining functional trait compositions in benthic ecosystems (25). Salinity is also important in the benthos, often affecting an organism's ability to survive (26). To reduce the effect of outliers, all environmental measures were log (1+x) transformed.

Category	Level
Feeding/Diet	C=Carnivorous (predator or passive suspension)
	D=Surface deposit feeder
	F=Filter/Suspension feeder
	O=Omnivorous (scavenger)
	P=Parasite
	S=Surface deposit feeder
Size	S< 3 mm
	3 mm<M<10 mm
	L>10 mm
Mobility	M=Mobile
	S=Sessile
	H=Hemimobile
Bioturbation	B=Active burrower (diffusive)
	G=Gallery burrower
	S=Surface dweller
	T=Tube burrower

Table 1. The Functional Traits considered as well as the levels within each trait

Terminology	Example
Functional Trait	eg. Mobility
Functional Trait Level	eg. Sessile
Functional Group	eg. SLD for sessile, large, surface deposit feeders
Functional Group Class	eg. "Mobility-Size-Feeding"

Table 2. The terminology used in the study and examples for each term. A Functional Group Class contains the Functional Groups formed using specific Functional Traits.

Statistical Analysis

We used a procedure similar to Heino (15), who compared results obtained with species data with that of lower taxonomic resolutions, including genus, family and order. He determined, using BIO-ENV analysis, the "best subset of environmental variables" to explain the relationship between site composition and the environmental variables for each case (15). Results were analyzed by comparing which variables were found in all levels of taxonomy and how reliable using lower resolution information can be. A similar procedure was done in this study, with the added aspect that functional group classes can be of the same complexity but be formed using different traits.

We performed similar statistical tests to determine whether more complex functional group classes are closer to the results obtained with species data. The hypothesis predicts that the variables selected as vital in explaining the relationship between site composition and the environment, determined with BIO-ENV, will vary with functional group class complexity. The most complex class should pinpoint the same variables as those selected with species data. For the purposes of this study, the hypothesis does not prohibit a less complex class from also selecting the same variables as species data, providing the most complex functional group class does likewise.
Specifically, the BIO-ENV analysis calculated Spearman Rank correlations for each functional group class to determine the best subset of environmental variables to explain the composition in the nine sites (20). Mantel permutation tests were used to determine the significance of these correlations. Functional group classes that did not give significant results ($p \leq 0.05$) were discarded and only results with correlations ≥ 0.5 were included in interpretation.

Results

The results of the BIO-ENV analysis, which determined the best subset of variables available to explain the relationship between site composition and the environment, were varied in terms of significance and correlation strength (Spearman's Rank correlation rs), although all correlations were positive (Table 3 and Table 4). Results were divided based on type of data used (raw abundance, relative abundance and presence/absence data) and then divided by complexity of functional group class. For example, Complexity 2 refers to the use of two functional traits in forming a functional group class. Individual functional group classes are referred to by the functional traits used to form them. So, a Complexity 3 functional group class based on the traits of mobility, bioturbation and feeding, which used presence/absence data, is referred to as "Mobility-Bioturbation-Feeding Presence/Absence."

Chl a (1), Phaeopigments (2), Depth (3), Salinity (4)
log (1+x)

Raw Abundance Data		spearman	variables	significance
	Full Species Raw Abundance	0.585	1,2,4	0.001
Complexity 4	Mobility-Size-Feeding-Bioturbation Raw Abundance	0.722	1,2,3,4	0.001
Complexity 3	Size-Feeding-Mobility Raw Abundance	0.5071	1,2,4	0.007
Complexity 3	Size-Mobility-Bioturbation Raw Abundance	0.732	1,2,3,4	0.002
Complexity 3	Size-Bioturbation-Feeding Raw Abundance	0.61	1,2,3,4	0.002
Complexity 3	Mobility-Bioturbation-Feeding Raw Abundance	0.552	1,2,3,4	0.007
Complexity 2	Mobility-Bioturbation Raw Abundance	0.65	1,2,3,4	0.002
Complexity 2	Size-Feeding Raw Abundance	0.558	2	0.008
Complexity 2	Size-Mobility Raw Abundance	0,725	1,2,3,4	0.001
Complexity 2	Size-Bioturbation Raw Abundance	0.847	1,3,4	0.001
Complexity 1	Bioturbation Raw Abundance	**0.481**	4	0.048
Complexity 1	Mobility Raw Abundance	0.781	1,3,	0.001
Complexity 1	Size Raw Abundance	0.51	1,3	0.046

Relative Abundance Data		spearman	variables	significance
	Full Species Relative Abundance	0.659	2,4	0.001
Complexity 4	Mobility-Size-Feeding-Bioturbation Relative Abundance	0.65	1,2,3,4	0.002
Complexity 3	Size-Mobility-Bioturbation Relative Abundance	0.634	1,2,3,4	0.003
Complexity 2	Bioturbation-Feeding Relative Abundance	0.501	1,2,3	0.004
Complexity 2	Size-Mobility Relative Abundance	0.558	1,3	0.018
Complexity 2	Size-Bioturbation Relative Abundance	0.681	2,3,4	0.001
Complexity 1	Feeding Relative Abundance	0.502	1,4	0.034
Complexity 1	Motility Relative Abundance	0.71	1,3	0.001
Complexity 1	Size Relative Abundance	**0.499**	1,3	0.045

Presence/Absence Data		spearman	variables	significance
	Full Species Presence/Absence	0.687	1,2,3	0.001
Complexity 4	Mobility-Size-Feeding-Bioturbation Presence/Absence	0.607	2	0.008
Complexity 3	Size-Feeding-Mobility Presence/Absence	0.575	2	0.004
Complexity 3	Size-Mobility-Bioturbation Presence/Absence	**0.487**	2	0.029
Complexity 3	Size-Bioturbation-Feeding Presence/Absence	0.565	2	0.013
Complexity 3	Mobility-Bioturbation-Feeding Presence/Absence	0.583	2	0.015
Complexity 2	Bioturbation-Feeding Presence/Absence	0.575	2	0.013
Complexity 2	Mobility-Feeding Presence/Absence	0.595	2	0.007
Complexity 2	Size-Feeding Presence/Absence	0.558	2	0.003
Complexity 2	Size-Bioturbation Presence/Absence	0.514	2	0.029

Table 3. BIO-ENV results with significant ($p \leq 0.05$) Spearman Rank correlations that exceed the 0.5 correlation threshold. Three correlations that are slightly below the threshold are included in bold.

Presence/Absence Results

For Complexity 1, only "Feeding Presence/Absence" gave a result. It had a significant (p=0.035) but weak correlation (rs=0.374), and pinpointed phaeopigments as the single key variable. The other Presence/Absence results did not isolate any variables. For Complexity 2, all results except two were statistically significant, had correlations greater than 0.5, and put forth phaeopigments as the single key variable. For Complexity 3, all results were statistically significant, with only "Size-Mobility-Bioturbation Presence/Absence" falling short of the correlation threshold (rs=0.487). All pinpointed only one key variable, phaeopigments. For Complexity 4, the use of all traits ("Mobility-Size-Feeding-Bioturbation Presence/Absence"), gave a significant result (p=0.008), with a correlation of 0.607. It pinpointed only phaeopigments as a key variable. For species data, the result was significant (p=0.001), with a correlation of 0.687, and selected chlorophyll a, phaeopigments and depth as key variables. So, there is a clear difference between species and functional data. For the latter, three variables were selected by the statistical test. In contrast, the only variable that was selected for functional data, regardless of complexity, was phaeopigments.

Chl a (1), Phaeopigments (2), Depth (3), Salinity (4) log (1+x)

	Spearman correlation	variables	significance
Full Species Raw Abundance	0.585	1,2,4	0.001
Full species Relative Abundance	0.659	2,4	0.001
Full Species Presence/Absence	0.687	1,2,3	0.001
Mobility-Size-Feeding-Bioturbation Raw Abundance	0.722	1,2,3,4	0.001
Mobility-Size-Feeding-Bioturbation Relative Abundance	0.65	1,2,3,4	0.002
Mobility-Size-Feeding-Bioturbation Presence/Absence	0.607	2	0.008
Size-Feeding-Mobility Raw Abundance	0.5071	1,2,4	0.007
Size-Feeding-Mobility Relative Abundance	0.369	2,4	0.048
Size-Feeding-Mobility Presence/Absence	0.575	2	0.004
Size-Mobility-Bioturbation Raw Abundance	0.732	1,2,3,4	0.002
Size-Mobility-Bioturbation Relative Abundance	0.634	1,2,3,4	0.003
Size-Mobility-Bioturbation Presence/Absence	0.487	2	0.029
Size-Bioturbation-Feeding Raw Abundance	0.61	1,2,3,4	0.002
Size-Bioturbation-Feeding Relative Abundance	0.44	1,2,3,4	0.016
Size-Bioturbation-Feeding Presence/Absence	0.565	2	0.013
Mobility-Bioturbation-Feeding Raw Abundance	0.552	1,2,3,4	0.007
Mobility-Bioturbation-Feeding Relative Abundance	0.452	3	0.013
Mobility-Bioturbation-Feeding Presence/Absence	0.583	2	0.015
Bioturbation-Feeding Raw Abundance	0.409	1,2,3,4	0.073
Bioturbation-Feeding Relative Abundance	0.501	1,2,3	0.004
Bioturbation-Feeding Presence/Absence	0.575	2	0.013
Mobility-Feeding Raw Abundance	0.323	1,2,4	0.08
Mobility-Feeding Relative Abundance	0.352	1,2	0.039
Mobility-Feeding Presence/Absence	0.595	2	0.007
Mobility-Bioturbation Raw Abundance	0.65	1,2,3,4	0.002
Mobility-Bioturbation Relative Abundance	0.469	1,2,3,4	0.019
Mobility-Bioturbation Presence/Absence	0.27	1,2,4	0.144
Size-Feeding Raw Abundance	0.558	2	0.008
Size-Feeding Relative Abundance	0.473	1,2,4	0.009
Size-Feeding Presence/Absence	0.558	2	0.003
Size-Mobility Raw Abundance	0,725	1,2,3,4	0.001
Size-Mobility Relative Abundance	0.558	1,3	0.018
Size-Mobility Presence/Absence	0.282	1,2,3,4	0.968
Size-Bioturbation Raw Abundance	0.847	1,3,4	0.001
Size-Bioturbation Relative Abundance	0.681	2,3,4	0.001
Size-Bioturbation Presence/Absence	0.514	2	0.029
Feeding Raw Abundance	0.445	1,2,3,4	0.059
Feeding Relative Abundance	0.502	1,4	0.034
Feeding Presence/Absence	0.374	2	0.035
Bioturbation Raw Abundance	0.481	4	0.048
Bioturbation Relative	0.45	4	0.04
Bioturbation Presence/Absence	NA	NA	NA
Mobility Raw Abundance	0.781	1,3,	0.001
Mobility Relative Abundance	0.71	1,3	0.001
Mobility Presence/Absence	NA	NA	NA
Size Raw Abundance	0.51	1,3	0.046
Size Relative Abundance	0.499	1,3	0.045
Size Presence/Absence	NA	NA	NA

Table 4. All BIO-ENV results. This includes results that were not statistically significant and results with Spearman Rank correlations less than the 0.5 threshold. In three cases, no results could be obtained at all (labeled "NA").

Relative Abundance Results

For Complexity 1, all functional group classes gave significant results, but only "Feeding Relative Abundance" and "Mobility Relative Abundance" had a correlation greater than 0.5 (rs=0.502 and rs=0.71 respectively). "Size Relative Abundance" and "Bioturbation Relative Abundance" had correlations of 0.499 and 0.45, slightly below the threshold. The variable chlorophyll a was included in three out of four classes. For Complexity 2, all functional group classes gave significant results, with "Size-Bioturbation Relative Abundance," "Size-Mobility Relative Abundance," and "Bioturbation-Feeding Relative Abundance" giving correlations greater than 0.5. Phaeopigment was included as important in five out of six functional group classes. Chlorophyll a was also considered important in five out of six classes. Interestingly, the two variables were not always found together. For Complexity 3, all correlations were significant, but only "Size-Mobility-Bioturbation Relative Abundance" had a correlation exceeding 0.5 (rs=0.634). This functional group class pinpointed all four variables as important. For Complexity 4, "Mobility-Size-Feeding-Bioturbation Relative Abundance," there was a significant (p=0.002) correlation of 0.65 and all variables were found to be important. For species data, the significant (p=0.001) correlation of 0.659 pinpointed only phaeopigments and salinity as important.

Raw Abundance Results

For Complexity 1, all correlations except "Feeding Raw Abundance" were significant (p=0.059). Both "Mobility Raw Abundance" and "Size Raw Abundance" had correlations exceeding 0.5 and both found chlorophyll a and depth to be the only key variables. For Complexity 2, all correlations were significant except "Bioturbation-Feeding Raw Abundance" (p=0.073) and "Mobility-Feeding Raw Abundance" (p=0.073). All significant correlations exceeded 0.5, with the greatest being 0.847 for "Size-Bioturbation Raw Abundance." Chlorophyll a was considered important in all significant results except "Size-Feeding Raw Abundance," which only had phaeopigments as important. In general, phaeopigments were quite important, being present in all but one of the significant results.For Complexity 3, all results had correlation coefficients greater than 0.5 and all were statistically significant. Variables chlorophyll a, phaeopigments and salinity were considered important in all functional group classes. Depth was considered important in all groups, except "Size-Feeding-Mobility Raw Abundance." For Complexity 4, the correlation was significant (p=0.001) and high (rs=0.722). All four variables were considered important. For species data, a significant (p=0.001) correlation of 0.585 was found, with chlorophyll a, phaeopigment, and salinity found to be important.

Discussion

The hypothesis that more complex functional group classes, when compared to less complex classes, are closer to the use of direct species data in determining the important environmental variables was not supported by the results. More complex functional group classes did not necessarily yield better results. Some classes of lower complexity showed greater similarity to full species data. For example, a functional group class of Complexity 3, "Size-Feeding-Mobility Raw Abundance," required only chlorophyll a, phaeopigments and salinity for the best relationship between site composition and the environment, which was also the result obtained for full species raw abundance data. The most complex functional group class, "Mobility-Size-Feeding-Bioturbation Raw Abundance," required all four variables. Contrary to the prediction, results obtained with species data could be equivalent to a class that was not the most complex functional group class.

While establishing concrete relationships between environmental variables and site composition is a difficult task, the results could offer insights into the ecology of this benthic ecosystem. For both raw and relative abundance data, the Complexity 4 class includes bioturbation as a trait and depth as a variable, unlike with species data, suggesting this functional trait may be closely linked to depth while taxonomic composition is not. In fact, all Complexity 2 and Complexity 3 functional group classes that include bioturbation also select depth as important, at least with raw abundance data. This demonstrates why complexity is not always preferable. The functional traits that make up groups are not all equivalent. Adding a trait like bioturbation, even if it increases complexity, can create less similarity between functional and species data if the trait is not particularly important in determining how species composition varies between sites. The results also show there is no steady pattern as data complexity increases and there is no clear additive pattern for the functional traits, suggesting interactions between variables. Although the BIO-ENV results suggest each variable adds new information, the levels of chlorophyll a and phae-

opigments could be linked as both deal with primary productivity and are measures of food supply (24).

Interestingly, all useful presence/absence results for functional group classes, regardless of complexity, selected only phaeopigments as the important variable. While both phaeopigments and chlorophyll a are measures of food supply, they also differ (24). Phaeopigments, which tend to accumulate, can be seen as a measure of overall food supply. Chlorophyll a, which is more short-lived, can be considered a measure of fresh food supply (24). In fact, in Link et al. (18), the retention of chlorophyll a but not phaeopigments in their model suggested fresh food supply, rather than general food supply, was important in benthic processes. Conversely, my results suggest that overall food supply is more important when considering functional groups.

These presence/absence results are also helpful in understanding the general issue of complexity in functional group classes and its relation to taxonomic data. For presence/absence, all classes, regardless of complexity, signaled only phaeopigments. This suggests that complexity is not an important factor when it comes to presence/absence functional data. More research would be necessary to determine why this is the case, but it might be partially due to inherent limitations with using presence/absence data. Resolution is often lost, as rare and abundant species (or functional groups) are given the same weight. Removing rare species beforehand might help reduce this bias (21). The limits of presence/absence data are clearly demonstrated with Complexity 1 functional group classes, as only "Feeding Presence/Absence" gave a result. Perhaps the loss of these low complexity classes made the results appear artificially uniform.

Some information about the relationship between site composition and environmental variables is certainly lost when functional groups are used, but some appears to be retained. For instance, all meaningful results ($r \geq 0.5$, $p \leq 0.05$), regardless of complexity, retained a measure of food supply, whether chlorophyll a or phaeopigments, or both. This suggests food supply is vitally important in such ecosystems.

The results also help address the larger question of ecosystem redundancy, at least for some functional classes. For instance, the presence/absence functional group results pinpointed only phaeopigments as important, while depth was included in species results. This suggests that as depth changes, there is a significant change in species composition but not functional group composition. The species present fall into the same functional groups regardless of depth. In general, however, the results suggest that, while some redundancies exist, one must be cautious in using functional data to develop environmental policies. More research is necessary to determine which functional groups are truly comparable between distant ecosystems.

This study has certain limitations. Only four functional traits were used and more might have helped. No standard number of traits exists, the aim being enough traits to be functionally significant (1). Traits were chosen due to their importance in benthic ecosystems but choosing traits is difficult and always contains an element of subjectivity. As results show, using more traits does not guarantee more meaningful results. The results show complex trait-based systems are not necessarily reliable surrogates for taxonomy. In picking traits, researchers must balance the trait's importance with the ease of obtaining measurements (23). Also, this study used functional traits that were applied after species identification, automatically linking functional groups to taxonomy. Measuring traits beforehand would be preferable (23). Finally, only four environmental variables were used. Including more may have increased result reliability.

While more research is necessary in this field, this study helps clarify the relationship between the use of functional groups and taxonomy. The hypothesis that more complex functional group classes would approach taxonomic data when it comes to identifying key environmental variables was not supported by the BIO-ENV results. The results did offer some insight into benthic community composition. The importance of food supply in these communities was clear and the results suggested overall food supply, rather than fresh food supply, plays a key role in functional group composition, at least when looking at the presence or absence of a group. More generally, the results of this study argue caution should be taken when using functional groups as surrogates for taxonomic data and that the assumption complexity can strengthen the reliability of such methods is unwarranted.

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The Ecosystem Service Benefits of Lab-cultured and Insect Meat

Research Article

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Keywords

Ecosystem services, Lab-cultured meat, Insect meat, Agricultural expansion

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Abstract

Background: Population and income growth are expected to augment meat demand, and consequently, the conversion of natural ecosystems into pasture. Promising alternatives to livestock, particularly lab-cultured and insect meats, use about 1% as much land. Utilizing these technologies could reduce pasture expansion and maintain natural ecosystem service values. This paper investigates: what is the value of the ecosystem services potentially maintained by reducing agricultural expansion through the adoption of cultured and insect meat?

Methods: Total global livestock-associated agricultural expansion by 2050 was predicted using FAO livestock projections (1) multiplied by the average land-use per kilogram of meat (2) yielding 194Mha. This expansion was partitioned among ecosystems according to threat scores derived from past expansion (3). Changes to annual ecosystem service values were calculated using average global values from Costanza et al. (4) multiplied by predicted expansion per ecosystem.

Results & Conclusion: Tropical forests and east-Asia were the most threatened ecosystem and region, respectively, by both area and value. The net loss in annual ecosystem service values in 2050 due to predicted livestock-associated agricultural expansion was calculated to be $732bn/yr, translating to a NPV of $6.62tn to 2050. The potential to save such large ecosystem services value justifies increased research and promotion of these protein production methods.

Limitations: This research does not identify exact ecosystems that are both targeted by agricultural meat expansion and that yield large ecosystem benefits because it is not sufficiently spatially explicit. Thus, it should not be used as a reference for new ecosystem conservation zones.

Introduction

Considering the extent to which current agricultural practices are land-intensive and the risk they pose to natural ecosystems, this research looks at two promising meat production alternatives—lab-grown meat and insect farming—and investigates their potential to mitigate agricultural expansion and loss of ecosystem service value.

Current Protein Production Methods

Current industrial farming activities contribute to many environmental problems including greenhouse gas emissions, the decimation of pollinators, water pollution and waste creation. (5,6,7) These externalities will be exacerbated by the foreseen increase in demand for meat. (8) This increase is due to global population, which is expected to grow from 6.5 billion in 2007 to 9.3 billion by 2050, and per capita consumption of meat across the world, predicted to increase by over 25% in the same period. (1)
In order to respond to the changes in demand, meat production will have to significantly increase, and perhaps double: according to recent predictions, global meat production could reach 524 million tons worldwide by 2050, from 258 million in 2007. (1) With current practices, this translates into more land being used for agriculture as 79% of global agricultural land is used for pasture and cropland for feed. (1) Land use expansion occurs at the cost of natural ecosystems and their ecosystem services.

Added pressure on food systems, and more generally on the environment, prompts questions of sustainability. This creates an interest in alternatives to conventional meat production that could respond to the demand for protein, improve food security and mitigate the externalities of food production.

New Protein Production Methods

Though many meat substitutes exist, we focus on cultured and insect meat as they both have a large potential to reduce the negative impacts associated with livestock farming. In addition, they could respond to global demand as they are each better suited for different relative inputs of land, labor, and capital. Though cultured meat can be a realistic option in developed countries, it is likely to be hard to implement in developing countries as it is currently very capital intensive. Insect farming, on the other hand, may be a more viable solution in those regions where entomophagy (the eating of insects) and vegetarianism are more common culturally.

Producing meat in laboratories from animal stem cells is a novel idea and its potential is being examined with considerable interest. Several studies (9),10,11) and an anticipatory life cycle analysis (12) have been conducted to better assess the benefits and challenges of cultured meat. These have pointed to the promising environmental advantages of such a production method but have also highlighted many uncertainties such as the plausibility of a shift towards the technology. Since creating a market demand for such a product seems to be a primary concern, some consumer surveys have been conducted to further understand and evaluate the future potential of cultured meat. (13) Despite possible challenges with respect to consumer adherence, cultured meat is being looked at as a potential solution with optimism. It will likely be far more resource and pollution efficient: it emits less greenhouse gases and uses significantly less land and water. (11) Insect meat is an older idea but has been regaining attention over the past few years as food system externalities and food security are entering the popular discourse. Like cultured meat, its production is much more energy efficient and requires significantly less land to produce a kilogram of protein. (14) The document prepared by the Food and Agriculture Organization, Edible Insects (14), summarizes the benefits of using insects as a protein source. Other papers, such as Edible insects: Traditional knowledge or western phobia? by Alan L. Yen, (15) highlight the challenges of marketing insects in western societies while emphasising the potential positive impacts.

As we have seen, both alternatives use considerably less land than conventional livestock farming. The interest of this research is to look at cultured meat and insect farming as possible options to diminish the need for land expansion and to value the natural ecosystems that could be maintained.

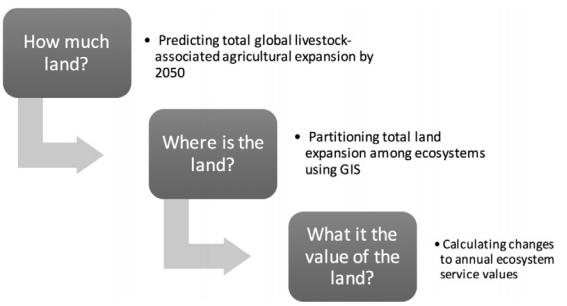
Market Forces

The impacts of cultured meat and insect farming on agricultural expansion will be highly dependent upon the size of the market share they can command. As briefly mentioned above, this depends on various market forces. Primarily, the demand for these alternative meats is highly reliant on sensory expectations, i.e. the ability to mimic conventional meat,16 price, and policy regulations. The supply relies on investment, technological progress, production costs, and policies such as carbon taxes. The evolution of these production methods is dependent on chaotic human systems, justifying the exploration of a variety of scenarios reflecting the myriad of possible futures.

Research Question

The purpose of this research is then to analyse the value of increasing the land efficiency of protein production through technology. The research will look at two alternatives to industrial livestock—cultured meat and insect farming—and the potential environmental benefits that adopting these modes of protein production could lead to, depending on different possible market forces and their interactions.
This study seeks to answer the following question: what is the potential value of the ecosystem services maintained by reducing agricultural land expansion through the adoption of cultured meat and insect farming? This is achieved by identifying the ecosystems at risk due to agricultural expansion and calculating their ecosystem service values. The back-of-the-envelope scenarios help express the long-term value that could be maintained by increasing the market shares of alternatives meat.

Methods



Our methodology consisted of answering three main questions: How much land is predicted to be converted for agricultural purposes? Where is this land expansion predicted to occur? And what is the value of this land in terms of ecosystem services?

The first step of our research involved separating the globe into six geopolitical regions and predicting how much beef, lamb, poultry and pork will be produced in each region by the year 2050. The six geopolitical regions were: East Asia, South Asia, Near East/North Africa, Sub Saharan Africa, Latin America and the Developed Countries.

We used projections from the FAO1 on the number of livestock that are predicted to be produced from 2007 to 2050. This data was provided by geopolitical region. We then multiplied these livestock counts by the predicted edible weight of each animal for each geopolitical region. (1) This yielded the total amount of edible weight that will be produced in 2050 by geopolitical region and species. We then multiplied these total weights by the estimated land use required to produce one kilogram of each type of meat. (2) Cattle and buffaloes use an average of 22 m^2/kg, sheep and goat require 12 m^2/kg, pigs require 10 m^2/kg and poultry require 5 m^2/kg. This yielded the total increase in agricultural land required to match the increase in livestock production in each geopolitical region. We corroborated our estimate with a second calculation and an additional source, which suggest similar values (see Appendix I).

The second step used Geographic Information Systems (GIS) programs to partition the total land expansion calculated in the first step into ecosystems within the geopolitical regions. This requires us to know where agricultural land is expected to expand. This information was found in James Oakleaf and his research team's article A World at Risk: Aggregating Development Trends to Forecast Global Habitat Conversion (3) Oakleaf's research team produced the map shown in Figure 1, which depicts the locations in which agricultural expansion is predicted to occur until the year 2030.

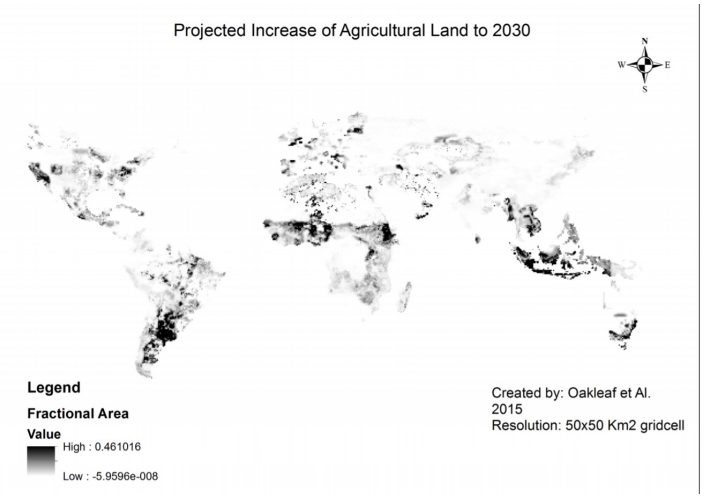


Fig. 1. Projected Increase of Agricultural Land to 2030

The map in Fig. 1 was created using a linear extrapolation from known agricultural land expansion between 2000 and 2011. Each 50x50 km grid cell contains a value between 0 to 0.46, which corresponds to the fractional area of that grid cell that is predicted to be converted to agricultural uses by 2030 after accounting for other trends such as urbanization and mining. (3) This map was used to predict the location of agricultural expansion. However, the specific increase in area calculated from this method is not used directly for two reasons. First, the map only extrapolates until 2030 whereas this research projects until 2050. Secondly, the map includes land used for all types of agriculture, not just livestock and feed production.

The agricultural expansion map was compared with an ecosystem map (17) and a map of country borders. We separated the map of country borders into the six regions defined by the FAO1 in the first step. Next, the ecosystem map was separately clipped to each geopolitical region. This resulted in separate regional maps where each geopolitical region was composed of ecosystems rather than countries. These regional ecosystem maps were then clipped with the agricultural expansion map. GIS software (Arcmap) tools such as "spatial join" and "summary statistics as a table" made it possible to calculate the expansion predicted in each ecosystem and geopolitical region. We then scaled these calculated areas to match the area predictions found in the first step for each geopolitical region.

Costanza et al. Categories	Value (2007$/ha/yr; 2011 values)
Tropical Forest	$5,382.00
Temperate/Boreal Forest	$3,137.00
Grass/Rangelands	$4,166.00
Tidal marsh/Mangroves	$193,843.00
Swamps/Floodplains	$25,681.00
Lakes/Rivers	$12,512.00
Desert	$0
Tundra	$0
Ice/Rock	$0
Cropland (without food production)	$3,244.00

Table 1. Selected average global ecosystem service values, from Costanza et al. (4)

Having calculated predicted land expansion for each ecosystem, the more specific WWF ecoregion categories18 were reclassified into those used by Costanza et al. (4) (See Appendix II.) The predicted change in each (reclassified) ecosystem, was multiplied by the corresponding per-area ecosystem services value from Costanza et al. shown in Table #1. (4) All natural ecosystems lost area and were replaced by an equal amount of cropland. Note that the value of food production was removed from cropland in the analysis. This assumes that, no matter what proportion of future growth in meat demand is satisfied by the proposed alternative meats, the same value of food will be produced as in the baseline prediction of full livestock expansion. This allows us to compare the costs and benefits of protein production scenarios. Also note that the land-use of alternative meats is assumed to be zero in the calculations. In reality, it is about 1% of the land-use of conventional livestock, but that land-use is expected to be in vacant urban areas, where it does not threaten natural ecosystems. (11,14)

Results

Step 1

The total global increase in agricultural land required to meet the increase in livestock production from 2007 to 2050 was calculated to be 194 million hectares. The spatial patterns of predicted land use are shown in Figure 2. The largest increase is predicted to occur in East Asia.

Step 2

The total livestock-associated land expansion from Step 1 was partitioned into nine ecosystems as shown in Fig. 2. Over half of the expansion is predicted to occur in Tropical Forests.

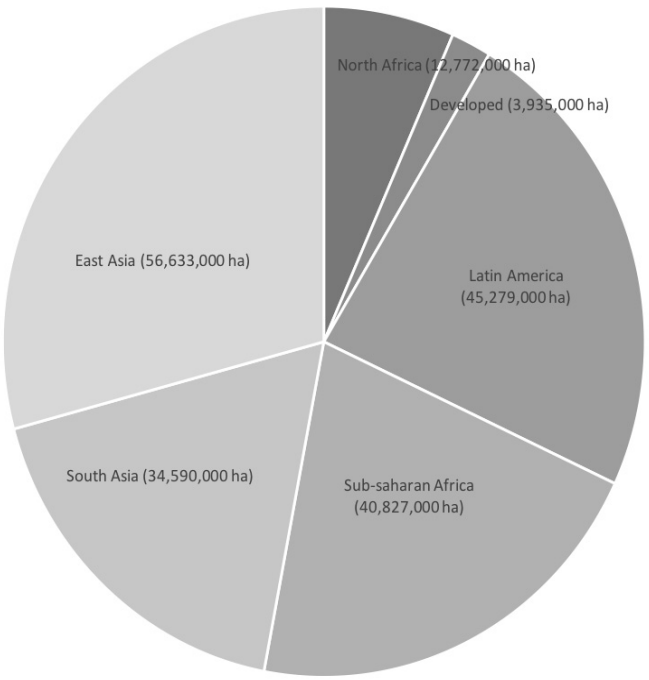


Fig. 2. 2050 Livestock-Associated Agricultural Increase by Geopolitical Region

Step 3

We found the net loss in annual ecosystem service values for 2050, should the predicted livestock increases occur, to be $732 billion dollars each year. In the following tables, this value was divided among geopolitical regions and ecosystems, along with their respective areas.

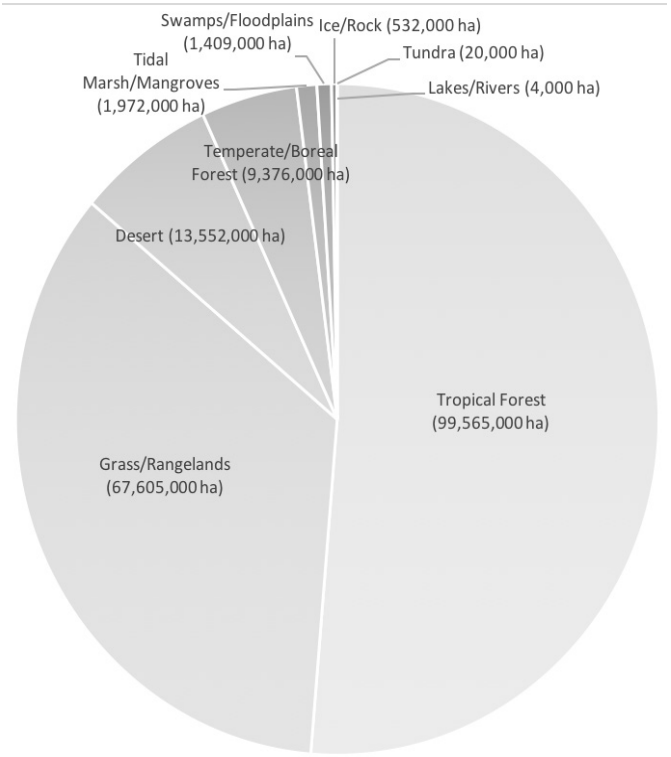


Fig. 3. 2050 Livestock-Associated Agricultural Increase by Natural Ecosystem

Discussion

Table 2 shows how the $732bn/yr and 194Mha are divided among geopolitical regions. Interestingly, North Africa and the Developed Countries have increased ecosystem service values. This is partly because their converted areas are so small, and also because the natural ecosystems that are expected to be expanded upon have lower values than cropland (i.e. deserts and tundra) even without food production values. The region that has the most value and area at risk is East Asia, particularly Indonesia.
In Table 3, the $732bn/yr and 194Mha are divided among ecosystems. While tropical forests are the most threatened in terms of area and value, the next most valuable ecosystem is tidal marsh and mangroves despite the small area under threat. Much of these mangroves are in the east-Asian archipelago.

Net Present Value of Ecosystem Services

Although $732bn/yr is an interesting number, it is only useful for making decisions today if expressed as net present value (NPV). For comparison, global GDP (19) is about $75tn/y and global agricultural subsidies are about $500bn/y (20). As mentioned, there are many human factors that will influence how meat production will evolve, the modeling of which would have been infeasible. Instead, we looked at how ecosystem service values would change depending on how widespread these alternative meats and their production methods become.

Thus, we stated that by 2050, either none of the growth in meat demand will be satisfied by cultured and insect meat with a resulting 194Mha agricultural expansion; all of it will be, resulting in no agricultural expansion; or somewhere in between, resulting in an expansion of some fraction of 194Mha. Using these total land and ecosystem service changes by 2050 we interpolated their change, assuming it to be linear, between now and then. Using a discount rate of 3%, we calculated the net present value of the lost ecosystem service values under the different scenarios. With 0% cultured or insect meat and full pasture expansion, 6.62 trillion dollars are lost in ecosystem service values as net present value to 2050; when tidal marshes and mangroves are conserved, 40% of the 6.62 trillion dollars is

Table 2. Change in total ecosystem value and area by geopolitical region

saved. If cultured and insect meat satisfies half of the growth in demand, 50% of 6.62 trillion dollars is saved. For every 25% of total growth in meat demand that is satisfied with cultured meat, we save a net present value of 1.65 trillion dollars.

Geopolitical Region	Net Ecosystem Service Value (2016$/yr)	Change in Natural Ecosystem Area (ha)
North Africa	+$4,670,768,504.41	(12,772,500)
Developed	+$1,777,353,527.95	(3,935,320)
Latin America	-$85,502,151,066.15	(45,279,080)
Sub-Saharan Africa	-$109,099,358,800.03	(40,827,400)
South Asia	-$162,572,757,215.45	(34,590,280)
East Asia	-$381,731,014,132.53	(56,633,290)
World	-$732,457,159,181.80	(194,037,870)

Table 2. Change in total ecosystem value and area by geopolitical region

Ecosystem Type	Net Natural Ecosystem Service Value (2016$/yr)	Change in Ecosystem Area (ha)
Grass/Rangelands	-$324,352,509,693.33	(67,605,228)
Tidal Marsh/Mangroves	-$440,279,305,089.42	(1,972,258)
Swamps/Floodplains	-$41,683,356,700.56	(1,409,407)
Tropical Forest	-$617,114,831,039.67	(99,565,295)
Temperate/Boreal Forest	-$33,872,824,698.85	(9,376,107)
Lakes/Rivers	-$62,796,196.56	(4,358)
Desert	$0.00	(13,552,285)
Tundra	$0.00	(20,337)
Ice/Rock	$0.00	(532,590)
Total Natural Ecosystems	-$1,457,365,623,418.39	(194,037,870)
Cropland (without food production)	+$724,908,464,236.59	194,037,870

Table 3. Change in total ecosystem value and area by ecosystem type

Financing Investment and Conserving Ecosystems

Expressed as net present value, we should be willing to invest similar sums of money to prevent these ecosystem service value losses. Thus, the upper-bound of socially-optimal investment in alternative meats, if only considering the loss of ecosystem services, is $6.62tn. Such investment would only be socially optimal if it is enough to allow alternative meats to satisfy all growth in the demand for meat, which is unclear. Thus, a fee of around $2 per kilogram of livestock meat would generate a total revenue with NPV of $6.79tn after 10 years to be invested in alternative meats.

According to our analysis, mangroves are the second most threatened ecosystem by value, but fifth by area. As these ecosystems are being converted for aquaculture, mangrove conversion is a plausible trend. (21) However, tidal marshes and mangroves only occur on the coasts: their high value and narrow, precise locations make the results of spatial analyses such as ours very sensitive to imprecision related to our raster resolution. Thus, we need to be cautious when interpreting the mangrove estimates.

Assuming the accuracy of these results, if we could conserve all threatened mangroves we would maintain 30% of threatened ecosystem service value by protecting only 1% of threatened area. This alone would reduce the net loss of annual ecosystem services to $292bn/yr by 2050 and a NPV of $2.64tn, the equivalent of satisfying between 50% and 75% of the growth in meat demand with alternative meats. If we view the threat to mangroves as an error due to our raster resolution we can interpret the $292bn/yr and $2.64tn as the values of the baseline scenario of full livestock expansion.

Limitations of the Data and Further Research

Crucial to our initial calculation of 194Mha were our estimates of the land needed to produce one kilogram of livestock meat, which were quite conservative (2). This was done for several reasons. First, it is predicted that future livestock production will mostly be intensive, thus requiring less land to produce a kilogram of meat.1 Second, there are no robust estimates for how land requirements differ by geopolitical region, thus a standard value had to be chosen for each species. Therefore, our results may be inaccurate where land use per kilogram is significantly different from our selected values. In particular, pasture expansion may be underestimated where ranching is common. This makes our final value of $732bn/yr a conservative estimate of threatened value, ceteris paribus. Further research could improve our results by using different per-kilogram land-use values for specific locations.

The partitioning of total expansion among ecosystems relied on a raster dataset with cell resolution of 50x50km (3). As discussed above, while this is suitable for large tracts of tropical forest and rangelands, ecosystems such as mangroves occur in very precise and narrow locations. If a cell had seen high past expansion, resulting in a high threat-score, our calculations assume that future expansion will occur in all ecosystems present in that cell according to their relative areas. A coastal cell in which there has been much expansion into tropical forests, but none into its mangroves, would nonetheless be predicted to lose mangroves. These errors could be rectified by further research in various ways. Raster resolution could be improved; polygons could be used to display separate ecosystems with more precise boundaries between them; or data on threatened ecosystems could be used directly, eliminating the need to overlay threat score data with ecosystem data.

Finally, it is widely argued that Costanza et al. (4) makes fundamentally flawed assumptions about the nature of total versus marginal value, particularly when asserting that the value of the world's ecosystem services is $145 trillion annually. However, for the purposes of this research, we must assume that the changes to ecosystem services we consider are small enough to be marginal, such that they do not change the scarcity and value of each hectare of a natural ecosystem. Further, as these are global averages, they are appropriate for our global analysis; we do not use them to claim that a particular hectare of land has a specific value. However, assuming that each hectare of, say, mangroves has the same value causes errors even in such a global analysis. While we might be confident in the average ecosystem service values of mangroves from Costanza et al., we don't know how the values of the specific mangroves that we expect to be threatened differ from this average. Further research could improve our results by having spatially explicit data on ecosystem service values, rather than global averages for ecosystem types.

Conclusion

By 2050, 194 million hectares of natural ecosystems may be converted to raise livestock. Tropical forests and east-Asia are the most threatened ecosystem and geopolitical region, respectively, both in terms of area and value. If this expansion occurs and the natural ecosystems are lost, it would

represent a loss of $732bn per year in ecosystem services in 2050, cumulating in a net present value of $6.62tn.

Though this research cannot be used to identify which areas are at risk as it is not sufficiently spatially explicit, other similar studies could be replicated in greater detail, locally, and at finer scales to account for spatial heterogeneity in order to better inform the policy-making process. The human systems that will influence market forces and the uptake of alternative meats can be studied and perhaps modeled, to identify the pressure points within these human systems.

However, the large value of threatened ecosystem services demonstrates the potential of these technologies and can be used to influence market forces that can further advance these alternatives. It can encourage further research and development in the specific science and technology of cultured and insect meat. It can also inform public policy if governments recognize the potential to save ecosystem service value by preventing agricultural expansion through alternative meats. It may even encourage new investment within protein industries as there are yet opportunities for first-mover advantages in seizing market share. This would not only include investment in the technologies themselves, but also marketing campaigns and public awareness. Lastly, the dissemination of the ecological benefits of cultured and insect meat may increase market demand for such commodities, while also spreading awareness of the concept of ecosystem services. Consumers respond more favorably to the alternative meats when they understand their personal and societal benefits.13 Even as individuals we can influence how cultured meat expands its market share. Perhaps the most effective way to promote lab-cultured and insect meat, relative to effort, is to purchase cultured meat when it becomes available, and discuss it with others to reduce the stigma against it.

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Examining the Effects of Covered Landfills on Gas Emissions in Parc Baldwin, Montreal

Research Article

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Keywords

Landfill, Landfill Parks, Biogas, Montreal, Parc Baldwin, Carbon Dioxide, Methane

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Abstract

Background: Within recent years, parks built on top of former landfills have come under scrutiny for their effectiveness at mitigating the effects of the landfill underneath. The purpose of this study is to identify the biogas emissions of converted landfills nearly a century after landfill closure.

Methods: Soil and air emissions for methane and carbon dioxide were collected at 112 sites within the North and South portions of Parc Baldwin in Montreal, Quebec, as well as the presumed boundaries of the former landfill.

Results: Overall, it was found that South Baldwin and the immediate area (previously a landfill) had a higher mean average methane concentration, as well as a greater number of sites with methane present than North Baldwin. Particular raised areas in South Baldwin showed anomalously high carbon dioxide concentrations. There was a large degree of heterogeneity between emissions at different sites.

Limitations: The Eagle 2 machine is limited to measuring only up to 5,000 ppm or 0.5% volume. Another difficulty with the variation in collection of the data is the differences in collection dates.

Conclusions: Ultimately, while South Baldwin did have higher CO2 and methane emissions compared to its counterpart, it is inconclusive whether or not this phenomenon is related to the landfill or other factors. Gas concentrations were significantly below the lower explosive limit in both parks.

Introduction

Throughout the 20th century, cities all over the world converted former landfills into parks and residential areas. Many dangers are associated with landfills, including toxicity, dangerous wastes, carcinogenic compounds, and the release of methane and carbon dioxide emissions. (1) Covering landfills was thought to decrease the dangers associated with these exposed areas, however, it is not a perfect solution. (2) Despite conversion, the continued release of methane and carbon dioxide contribute to potential local health problems, risks of explosions, and global climate change. In Montreal alone, there are 62 former landfills and quarries that have been converted to parks or residential areas. (3)

In 1994, the city of Montreal published the results of a four-year study investigating the extent of biogas emissions from multiple converted landfills across the city. The objective of the study was to determine the boundaries and impacts of former landfill sites using methane emissions as a proxy. From this study, one particular park of interest was Parc Baldwin South. Though the landfill under Parc Baldwin south ceased operation in 1924, residents near the park complained about high biogas emissions. Preliminary tests in 1991 showed all locations of Parc Baldwin South to have methane emission levels below the lower explosive limit (LEL) of 50,000 ppm. The survey in 1992 showed no significant changes in methane level from the 1991 results, with the exception of the apartment building at 3480 Fullum where methane concentrations exceeded the LEL. When repeated in 1993 and 1994, the emissions readings showed the same results as the 1992 survey. From these results, the 1994 study concluded that biogas surveillance of the houses along Fullum is crucial. In addition, the study claimed that more research is necessary in order to determine the extent of the biogas in the park itself. (4)

In 2013, the release of data illustrating former landfills through Montreal, as well as the closure of a community garden in South Baldwin due to heavy metal contamination, re-sparked concerns regarding converted landfills. The city of Montreal responded to these concerns in 2016 by conducting additional surveys for methane concentrations in converted landfills through the main districts of Montreal. A major area of concern was the Mont-Royal/Plateau district in which Parc Baldwin is located. In this district, 16 sites were analyzed. Overall, the study concluded that the average methane emissions in the area were 7 ppm with the highest recording at 12 ppm. This contradicts the previous 1994 study in which methane levels in the area exceeded the lower explosive limit of 50,000 ppm. The study has concluded that the Mount-Royal/Plateau district has negligible amounts of methane and the city of Montreal would not be focusing resources towards a threat deemed insignificant.

Due to persistent complaints from residents concerning high methane levels near the Parc Baldwin area, it is unclear how accurate the prior studies were at analyzing biogas emissions. We sought to determine the accuracy of the 1994 and 2016 results produced by the city of Montreal, and analyze the impact a covered landfill can have almost a century after its closure. For our study, gas emissions from both a covered landfill and a non-landfill greenspace were compared at Parc Baldwin North and Parc Baldwin South in the Montreal downtown area. North Baldwin served as the control since it does not cover a landfill, whereas South Baldwin and the surrounding area were observed for anomalous emissions.

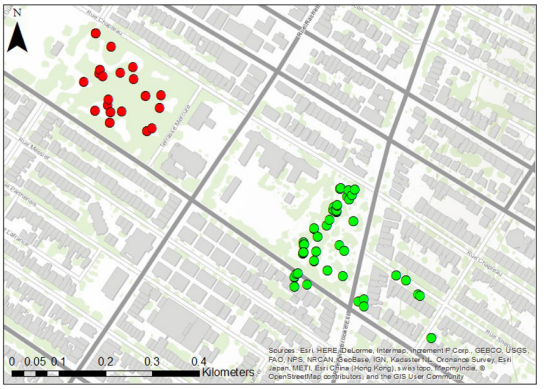
Comparing the gas emissions from these two parks can further facilitate categorizing and identifying key emissions that could indicate the presence of a landfill under a park. The results can also help determine if the construction of parks on covered landfills has an effect on the emissions from the landfill underneath, and if they could possibly reach problematic levels. In addition, the emissions data could potentially be used to create predictive models in order to identify unknown previous landfills covered by parks, as well as be used to identify the boundaries of landfills, if not previously known. Lastly, this study could be used to corroborate or dispute the 1994 and 2016 studies, and help provide a more extensive base for future studies into landfill emissions. It is expected that overall biogas emissions will be higher at Parc Baldwin South compared to Parc Baldwin North due to the presence of the landfill. We expect to see this trend in areas surrounding Baldwin South as well, given that the former landfill extended beyond the park into the surrounding residential areas.

Methods

Emissions data was collected using two machines, the Eagle 2, and the Triple Plus+. Both machines analyze CO2 emissions with differing accuracy; the Eagle 2 detected CO2 up to 10,000 ppm, whereas the Triple Plus+ could measure CO2 up to 50,000 ppm. Only the Eagle 2 was equipped with a methane sensor, which was capable of measuring up to 50,000 ppm CH4. Data was collected over 4 different days between October and November. Care was taken to collect data on days with relatively low wind speeds, to avoid dispersal of any gas emissions in the air. Overall, 112 data points were collected from the four days, consisting of 29 samples from Parc Baldwin North and 83 samples from Parc Baldwin South (including several sites south of Sherbrooke). A more extensive breakdown of the data collection can be seen in Table 1. For the first two data collection days (October 31 and November 7), only the Eagle 2 was used. For the third and fourth data collection days (November 14 and 16), the Triple Plus was also used, and the machines were run in tandem in order to better assess the emissions in both parks. The locations in which samples were taken from Parc Baldwin South and Parc Baldwin North can be seen in Figure 1.

A general assumption was made that Parc Baldwin North was representative of standard background emissions and thus each machine was calibrated to the air in the center of the park. This was done in order to show any relative changes in methane and carbon dioxide when comparing the conditions in Parc Baldwin North and Parc Baldwin South. For the testing of soil emissions, either the Eagle 2 or Triple Plus (or both, in turn) was connected to a metal soil probe using plastic tubing. The probe was then inserted into the ground to a depth of approximately 0.3-0.6m, depending on the soil hardness. Air emissions data was collected in a similar fashion: the tubing for the respective machine was left open (not connected to the soil probe) and held directly over the ground at the site, at a height of approximately 1-2 cm. For both soil and air measurements, adequate time was given for the machine to stabilize before any values were recorded, which was found to be roughly 30 seconds.

Both machines were equipped with alarms that would sound when CO2 concentrations exceeded the threshold of the sensors. The Eagle 2 would sound the alarm at 5,000 ppm, whereas the Triple Plus+ could tolerate concentrations of up to 50,000 ppm. As a result of this, the Triple Plus+ was used first (on days where it was available) to determine if the CO2 emissions exceeded the limits for the Eagle 2 (and therefore no methane or additional CO2 measurements could be collected).



Date of Data Collection	Eagle 2	Triple Plus+	Wind Speed (km/hr)	# of North Baldwin Sites	# of South Baldwin Sites	# of South Sherbrooke Sites	Total
October 31	Yes	No	14	6	9	1	16
November 7	Yes	No	13	3	15	1	19
November 14	Yes	Yes	14	6	25	7	38
November 16	Yes	Yes	6	14	23	2	39

Fig. 1. Location of samples taken from North and South Baldwin over all four days of sampling. North Baldwin sites are darker in colour South Baldwin sites are lighter in colour.
Table 1. Detail about data collection at Parc Baldwin North and South.

Results

As seen in Figure 2A, the correlation between the Triple + and Eagle 2 machines have an R2 value of 0.945 when recording carbon dioxide, indicating that the two machines are quite consistent.

When looking at only soil samples, the correlation between methane and carbon dioxide emissions from the Eagle 2 is weak with a R2 value of 0.005 (Figure 2C), showing that the presence of carbon dioxide emissions are not indicative of the presence of methane emissions for the soil.Comparing methane and carbon emissions for only the air samples from the Eagle 2 shows a weak correlation with a R2 value of 0.016 (Figure 2D). This is

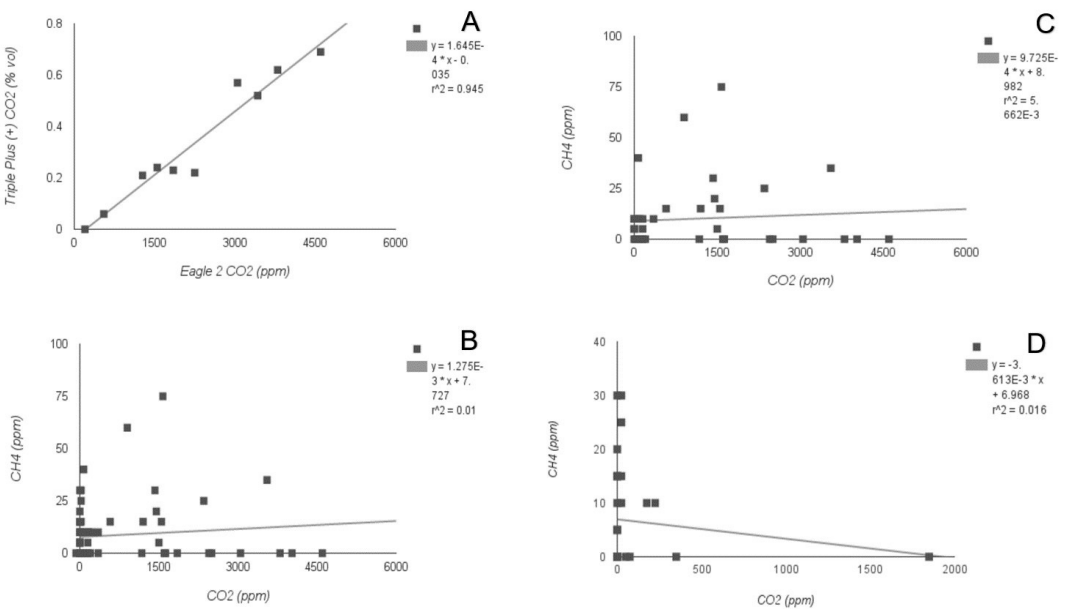


Fig. 2 (A) Correlation Between Triple+ and Eagle 2 Carbon Dioxide Measurements. (B) Correlation Between Methane and Carbon Dioxide Measurements in the Soil and Air using the Eagle 2. (C) Correlation Between Methane and Carbon Dioxide Measurements in the Soil using the Eagle 2. (D) Correlation Between Methane and Carbon Dioxide Measurements in the Air using the Eagle 2.

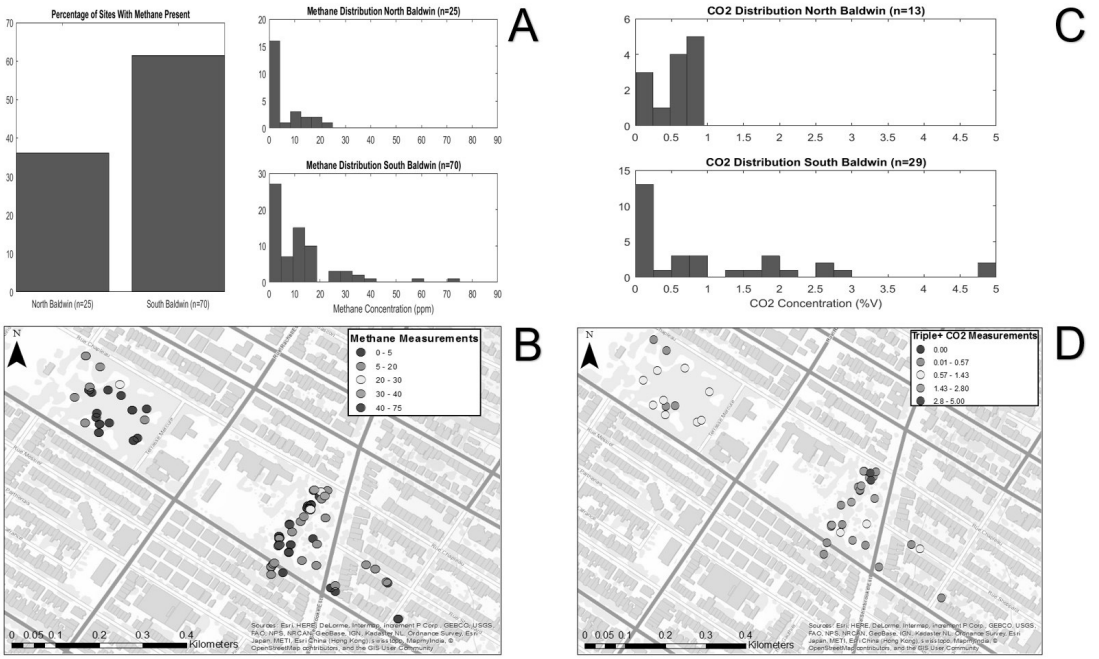


Fig. 3 (A) Statistical distribution of CH4 presence in North and South Baldwin sites, statistical distribution of methane in North Baldwin, statistical distribution of CH4 in South Baldwin. (B) Spatial distribution of CH4 emissions (ppm). (C) Statistical distribution of CO2 in North Baldwin and South Baldwin. (D) Spatial distribution of CO2 emissions (% volume).

consistent with the previously mentioned lack of correlation between soil measurements from the Eagle 2, showing that carbon dioxide emissions are not related to the presence of methane emissions for the air as well.

Overall, Baldwin South had a higher number of sites with methane present than Baldwin North. The highest concentration of methane in North Baldwin was 25 ppm while the highest concentration in South Baldwin was 75 ppm. Figure 3A shows that there were more sites with higher methane concentrations in South Baldwin as compared to North Baldwin, but with a higher standard deviation for South. The spatial distribution of these sites can be seen in Figure 3B. As with methane emissions, Baldwin South had a higher standard deviation for carbon dioxide (Figure 3C). Carbon dioxide concentrations were higher in South Baldwin as well: the highest concentration of CO2 present in South Baldwin was >5% V, compared to 1.43% V in North Baldwin. The spatial distribution of these sites can be seen in Figure 3D.

An interesting trend was observed in South Baldwin, where elevated sections of the park, deemed "lumps", showed extremely high CO2 concentrations. They are clearly visible in Figure 3B as the clusters of darker points in South Baldwin. When CO2 measurements are sorted and grouped, this relationship becomes clearer, as seen in Figure 4.

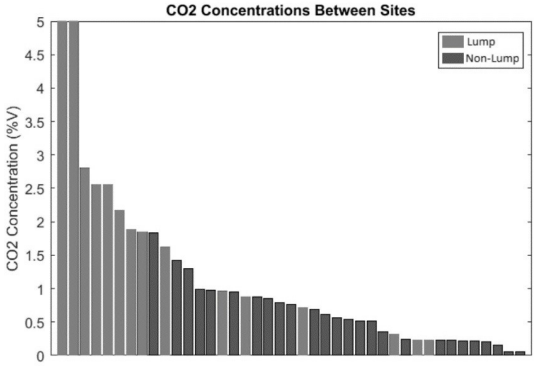


Fig. 4 Sorted Carbon Dioxide emissions grouped by position on or off of "lumps".

Discussion

Comparison to 1994 and 2016 Study

Similar to the 1994 study, our results show negligible amounts of methane in Parc Baldwin itself with respect to the LEL. Furthermore, while the methane emissions near the 3480 Fullum house were higher than ambient levels, they did not come remotely near the LEL, possibly due to successful remediation. Although we did find areas with relatively high methane emissions, the values observed were still extremely low (<0.2% of the LEL). Regarding the 2016 study, our results were far less similar. For the Mont-Royal/Plateau district (where Baldwin Park is located), the city of Montreal found an average methane concentration of 7 ppm, whereas our results suggested an average of 15 ppm. The city also reported that the highest methane concentration was only 12 ppm, whereas the highest concentration that we detected was 75 ppm. While the results of the study did not find methane levels above the LEL, the study did show higher levels of methane than the data recorded in the 2016 study. Ultimately, while we did find higher concentrations of methane than the City of Montreal detected in their 2016 study, the magnitude of emissions is minuscule with respect to the LEL.

Comparing North and South Emissions

Both carbon dioxide and methane concentrations are higher in Parc Baldwin South as compared to North Baldwin, although there is a higher standard deviation in concentrations for South Baldwin. There appears to be no correlation between the presence of CO2 and methane in air and soil samples across both parks. While these overall higher emissions in South Baldwin are potentially due to the presence of the landfill underneath, there are still many unknown factors that would need to be tested in order to draw a more certain conclusion.

Lump Sites

One of the more interesting findings during field data collection was the presence of at least three "lumps" in Parc Baldwin South. The lumps were characterized as being slightly elevated above the surrounding ground and devoid of trees. They ranged in size from a couple meters across to over 10 meters in length (the Eastern Lump). Measurements taken on these lumps showed drastically higher concentrations of methane and carbon dioxide

than the immediate surrounding area off the lump. The lumps consistently had soil CO2 concentrations that were greater than 5,000 ppm, which rendered the Eagle 2 unable to measure soil methane levels. However, air measurements made with the Eagle directly over the ground of the lumps showed higher than normal methane concentrations, sometimes as high as 60 ppm. It was interesting to note that moving off or away from the lump resulted in CO2 and methane levels dropping immediately back down to more ambient levels, even if the distance moved was less than a meter off the lump.

Additionally, the soils group found that the soil type for the lumps was different than for the rest of the park. Clay soil was consistently found at sites near the lumps but not on them, while the soil for the lumps themselves was more broken and less dense. This difference in soil type could possibly explain why the lumps had much higher emissions that the surrounding areas, as the clay soil is less permeable and would block emissions from off the lumps. The less dense soil on the lumps would allow for gas emissions below to more easily escape and cause higher measurements of CO2 and methane emissions at those lump sites.

Connection to Related Study Groups

When analyzing temperature data from the parks, the thermal signature results from the thermal group are not consistent with the emissions results, with the exception of potential elevated temperatures on the eastern lump. However, it is ambiguous whether this is due to the presence of a landfill or other factors such as sunlight exposure, depth of sensor placement, or density of the soil surrounding the sensor when placed into the auger hole. Looking at the results from the soil group regarding the soil chemistry of the park, the soil group has concluded that there is no concrete evidence that the presence of a landfill contributes to higher heavy metal composition. The copper, chromium, and iron samples in the soil were determined to be insignificant and only one site had a large amount of heavy metals. This anomalous site could potentially be caused by the location's proximity to Sherbrooke. While the group was not able to concretely conclude that the presence of the landfill underneath South Baldwin led to increasing heavy metal concentrations, it is important to note that the soil group was unable to test the lump sites. This is important to take into consideration since lump sites are the areas which have the highest emission concentrations and could potentially have the largest concentration of heavy metals as well.

Using the emissions model created by the modelling group, the model has predicted that emissions drastically decline immediately after the closure of landfills, and plateau towards zero after approximately fifty years. Adapting this model to Parc Baldwin South, one can determine that the emissions should be almost zero today, as the landfill closed in 1924. This is consistent with the results of the study since the average methane observed in this area was 15 ppm.

Future Work

Future research on this topic will be able to determine the full effects of landfills on the emissions gas at Parc Baldwin. More analysis regarding the characteristics of lump sites would be necessary in order to determine their importance. These characteristics can include soil properties, which can facilitate this study in understanding if the emissions from these areas are due to soil composition or the presence of a landfill. In addition, having measurements that reach further depths in the ground could lead to more accurate depictions regarding the effects of the landfill. This is because deeper soil would not be affected by any external emissions, which would make the results more accurate. Additionally, historical information regarding the closure of the landfill, the composition of the landfill, and any remediation practices could help determine the cause of these emissions. Analysis of historical information could allow for more conclusive evidence linking the landfill to the emissions. Another important addition to this study would be using this information to create predictive models. These models can help determine the extent of the landfill and predict the boundaries for old landfills now covered by parks. Lastly, expanding this study to other landfill covered parks and not only focusing on Parc Baldwin could determine the extent that parks with landfills below have on biogas emissions.

Limitations

While the data in this study is significant, there are some important caveats to consider. One consideration to recognize is that the Eagle 2 machine is limited to measuring only up to 5,000 ppm or 0.5% volume. Therefore, any concentrations higher than this limit would set off an alarm and the machine would need to reset. This would prevent measuring carbon dioxide as well as methane on any sites with carbon dioxide higher than 5,000 ppm. This means that the Eagle 2 could not be used to measure methane or carbon dioxide on the lumps since these areas had higher than 0.5% volume and the Triple + machine was the sole measurement of carbon dioxide concentrations for the lump areas.

Another difficulty with the collection of the data is the differences in collection dates. The first consideration is the differences in the time of day. Measuring closer to peak rush hour may cause air emissions to be higher due to the high amounts of traffic, which could increase methane and carbon dioxide readings from the influx of combustion engines on the road. In addition, the weather conditions can affect the data. Days with rain could increase microbial decomposition and could increase the carbon dioxide readings on days after rain. Another weather consideration is the amount of wind. Despite the fact that the team attempted to go on days with low wind, even wind speeds of over 10 km/h could drastically decrease and disperse the low levels of methane in the air directly above the ground. Further, there is the change in seasons to consider. The dates where data was collected spanned from October to November, where the leaves were constantly falling from the trees and covering the ground. Therefore, later dates when the leaves were on the ground had higher overall carbon dioxide readings when compared to dates in which the leaves were still on the trees. This could be due to the decomposition of the leaves, as well as the leaves acting as a barrier above the ground that would prevent gas emissions from dispersing into the air.

Conclusion

The results from this study show that there are higher average methane and carbon dioxide concentrations for the South portion of Parc Baldwin as compared to North Baldwin. In addition, a larger percentage of sites in Parc Baldwin South had a presence of methane when compared to North Baldwin. However, it is important to note that South Baldwin had a larger standard deviation in methane concentrations when compared to North Baldwin. While the methane levels reported were all significantly below the lower explosive limit of 5% volume, methane was still present.
While there is evidence that landfill covered Parc South Baldwin has higher emissions compared to its control Parc North Baldwin, the data cannot conclusively determine if the emissions are due to the presence of a landfill or there is some other factor at play. These other factors could be its proximity to Sherbrooke, seasonal or weather factors, or soil composition. More studies and tests as well as access to historical information would be needed in order to confirm that the emissions were due to the landfill below the park.

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Urban Biodiversity Through Sustainable Architecture and Urban Planning

Review Article

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Keywords

urban biodiversity, ecology, built environment, conservation, sustainability

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Abstract

Background: In recent years, ecologists, architects, urban planners and decision makers, and citizens have become more aware of the importance of biodiversity in cities, creating a renewed effort to make cities and new developments better suited towards natural habitats. Sustainable architecture and design practices have offered ground to significant discovery and innovation in the art of city-building.

Methods: A literature review of current practices in the Western world of the last twenty years and two case studies will be used to illustrate current efforts and future directions of biodiversity preservation.

Summary: Integrating building strategies and holistic urban ecosystem development, compounded by encouraging interdisciplinary approaches that promote collaborative and bottom-up urban planning through community activism are the main trends in current sustainable city-building.
The literature review is far from exhaustive and requires a historical perspective to better understand implications of past and present sustainability efforts. The paper serves as introduction to a promising field. Relationships between biodiversity preservation and urban planning and design need to be reinforced in order to build a more connected, healthy, and resilient community.

Introduction

Urban Biodiversity and Motivations to Protect it

The environment is changing – as the world undergoes urbanization, human actions have caused dramatic deterioration of the planet's ecosystems. Continued use of natural resources, agricultural development and growing urban form accentuates the destruction of habitats and leads to an increasingly homogeneous distribution of species on Earth. (1) High extinction rates of local species, accentuated by high introduction rates of foreign species, is estimated to be approximately between 1,000 and 10,000 times higher than the natural extinction rate due mostly to land use change, physical modifications of natural landscape, and anthropogenic climate change and pollution. (1) The above phenomena are all examples of important biodiversity loss that in the short term may benefit the livelihood or the economy of certain regions, but in the long term will result in costly trade-offs. (1) Areas whose economy depends heavily on the quality of the natural environment (for example, regions dependent on the industry of tea) are also the most vulnerable to urbanization and habitat changes.

According to conservationists Dearborn and Kark, (2) not only will natural environments benefit from the protection and restoration of biodiversity, anthropocentric benefits also range from the improvement of human health, including reductions in air pollution, emotional wellbeing from contact with nature, incorporation of the intrinsic value of nature in culture, and preservation of ecosystem services provided by the natural environment. (1) However, due to the multiple dimensions of biodiversity, it is difficult to identify the number one indicator with which to quantify these services. In discussions of urban biodiversity and planning, there is a focus on species diversity – the number, type, and relative dominance of different species, as well as ecosystem diversity – the variety of habitats for animal and plant communities. Large variations in patterns of natural diversity are an indicator of loss of ecosystem function. These indicators account for biotic and abiotic factors within ecosystems to provide an idea of the overall health of these complex networks (3) that count on each link of the chain – every species, niche, and habitat – to ensure it functions as healthily as possible.

Methods

Currently, the goal of urban biodiversity conservation is focused on preserving and reconstructing habitats to minimize species loss and encourage human and wildlife coexistence. (14) Therefore, our literature review will be mainly carried out through focuses on parkland creation, minimization of urban fragmentation, and urban canopy development. (17)

Current ecologists and planners only have direct control over two aspects of urban biodiversity: plant diversity and abundance. The ecosystem is complex and chaotic – it has been shown that introduction of non-plant species rarely works due to difficulty for the population to successfully establish and find its place in the local ecosystem. However, when preserving or reconstructing habitat through plant diversity, natural ecological and evolutionary processes are triggered, increasing overall diversity as well. (14)

A key aspect of this habitat reconstruction is a focus on native plants as opposed to exotic or invasive species not native to the local ecosystem. Historically, cities, parks and green areas have been filled with these ornamental exotics or monoculture trees, which do not act as healthy habitat for local native fauna. For example, in Baltimore County, MD, regulations now stipulate that 80% of county plantings must be local flora, with half being native oaks. The habitat of the oak trees attracts diverse fauna, from the communities of caterpillars that feed songbirds to aquatic invertebrates that feed on the oak detritus, and supports healthy fish populations. (6)

For planners, three basic classifications of urban green spaces exist: formal spaces or parks are highly managed green spaces, vernacular spaces are private green spaces (i.e. yards and gardens), while forgotten spaces (Terrains Vagues) are composed of alleys as well as empty and abandoned lots. Each of these three have a naturalized ecosystem function. Formal spaces are highly managed and have frequently low biodiversity, due to the arrested succession and limited renewal functions of these spaces. (5) Vernacular spaces also face these problems; however, these spaces usually have higher species diversity, resulting in a higher biodiversity, albeit a lower number of native plant species. (5) Even though this high species diversity can increase biodiversity, some is still lost due to the lack of habitat for native species. Forgotten spaces, which are frequently less physically appealing, have high levels of biodiversity. In a study of Berlin's forgotten spaces, there were lower numbers of native species found in other more

formal green spaces within the same area. (29) This may be in part due to a naturalization of ecosystem function in these spaces, as they are not regulated to the same degree as more formal spaces, and natural succession can occur. This allows for habitats to develop at a normal and fully functional forgotten spaces, there were lower numbers of native species found in other more formal green spaces within the same area. (29) This may be in part due to a naturalization of ecosystem function in these spaces, as they are not regulated to the same degree as more formal spaces, and natural succession can occur. This allows for habitats to develop at a normal and fully functional rate. Using these forgotten spaces as examples, we may be able to create urban spaces that mimic natural levels of biodiversity within urban spaces, and perhaps, even create ecosystems with specific dynamics that reflect the unique nature of urban spaces. (28)

Although a green space may be filled with diverse and native flora, an issue arises in the integrity of the landscape due to the heavy fragmentation found in urban environments. This urban environment is known as a "mosaic of patches" (14) with minimal connections, migration routes, and exchanges. Ecologists have analyzed this phenomenon using Island Biogeography Theory, modeling each patch or fragment as an 'ecological island', susceptible to edge effects, small populations, invasive species, and widespread disease. Creating parkland and natural space with native plants is insufficient if they are to be isolated and surrounded by asphalt. This brings rise to the practice of creating green corridors, connecting these habitat spaces with green areas throughout the city. These can take the form of linear parks, restoring habitats along a river or stream, natural greening in the middle of a boulevard, or wildlife crossings that allow animals to safely cross human-made barriers.

There are limitations on the success of green corridors. They are heavily susceptible to ecological edge effects, can act as disease vectors, and are not suitable for every species. Another limitation is the number of corridors needed for a successful, resilient ecological system. Rudd (26) found, by quantitatively analyzing connectivity and ecological metrics, that the best network was found by having over 300 discrete corridor connections based on the parkland and habitat spaces in the urban area of Greater Vancouver. Clearly, from a planning perspective, this is an unreasonable amount, interfering with the built environment and daily happenings of a large city. The solution here is to consider green corridors as integrated into the city itself –roads, boulevards, backyards, alleys, and right of ways being areas for people, native flora and fauna, and paths for ecological connectivity. (26)

It is abundantly clear, that even if a native habitat is reconstructed or preserved in an urban zone with all integrity and connectivity issues thought of, an urban habitat resembling a natural one in form does not mean they entirely resemble each other in function. (14) Hostetler (16) notes that although there is a current heavy focus on 'green infrastructure' of protected space and corridors, the design of surrounding developed areas is overlooked. These surrounding areas have a heavy influence on the protected areas through obvious issues (such as connectivity discussed above), and less obvious but heavily influencing factors such as runoff and temperature regulation, altering the function of the habitat's ecosystem. For example, a heavy rain event over a hardened, developed area results in heavy runoff (since developed areas do not hold much water in their surfaces) into parkland, bringing with it pollutants and particulates not expected to be found in a natural area. To have truly green infrastructure, a more integrated approach to planning habitat and the surrounding areas must be taken, considering the entire city as a habitat for native flora and fauna rather than just protected or reconstructed areas.

As easy as it is to say that we should take a more holistic approach, there is a major barrier to implementing this approach in our cities with current practices: there exists a sharp divide between planners, political decision-makers, and natural scientists. Each group works is in its own 'silo', with minimal knowledge sharing and collaboration. In a 2006 study of Swedish planners, Sandström (23) found that biodiversity was an important consideration to most planners, but self-evaluation showed a distinct lack of knowledge and resources to carry it out appropriately. To achieve the planning of a holistically biodiverse and healthy landscape in our urban zones, there must be more collaboration between these groups than currently stands.

Results

As discussed above, current practices include the creation of formal green spaces within urban areas and the connection of these formal spaces via linkages and corridors. However, this may not be enough. As cities become increasingly dense, vernacular green space decreases. (5) This has led to a limitation of habitat provision and, therefore, biodiversity. (5) To create successful habitats that encourage natural growth, succession, and rich biodiversity, we must consider using the built form, at the local and individual level, to increase spaces for habitat in addition to urban agriculture and green space designed for human use by creating habitats within mixed used development complexes.

Methods for increasing urban biodiversity include adaptive design which builds natural habitat into the urban form in ways such as green roofs, terrace garden infrastructure, and green walls or other vegetative vertical structures. (6) These methods for increasing urban green space have been shown to also increase habitat and overall biodiversity in cities. Catalano et al. (27) show that by replicating specific habitats within green roofs and allowing these green roofs to complete natural cycles of growth, death, and rebirth (which ultimately result in natural succession), these immensely rich and biodiverse communities can compensate for the demolition of green spaces due to urban growth. These green roofs should attempt to use as many native species as possible to promote habitat for local populations of insects and animals as well as to allow for aesthetic change over time. (27) The goal is to maintain these green roof installations with as little human interference as possible to allow a natural progression of habitat formation.

Another important structure for increasing biodiversity is vegetative vertical structures, which seem to be crucial in the identification and the protection of biodiverse and vulnerable avian habitats. In one of the first studies on the matter by Culbert et al., (13) vertical structures such as densely covered tall trees, green walls and vertical gardens complement horizontal structures to enhance avian biodiversity. Vertical structures, especially in cases where they are combined with horizontal structures (such as rooftop gardens) can be extremely useful in increasing both bird and insect biodiversity in urban settings that are near forested areas or near migratory routes. Benefits like the mitigation of air pollutants, noise reduction, lessening of the urban heat island effect, increases in walkability and real estate value, and reduction of stress due to its aesthetic advantages are recognizable. The best example is the proliferation of high-end, high-rise apartment buildings around the world that integrate rooftop gardens and vertical forests (for example, one in Porta Nuova, Milan is home to 730 trees, 5,000 bushes and 11,000 covered balconies). These provide inhabitants with an improved microclimate while also contributing to the implementation of urban agriculture, increases in urban density, and the limiting urban sprawl. (22)

Looking ahead, popularization of methods for incorporating different green spaces into residential developments can significantly enhance the urban ecosystem, creating greater biodiversity within the city and, therefore, creating biophysical communities that are more resilient and significantly healthier. From a sociopolitical perspective, optimistic changes are slowly taking place: as the United Nations declared 2011-2020 to be the Decade on Biodiversity, both governments and citizens are engaging in the dialogue (such as UKGNC Task Group of Association of Local Government Ecologists, UN Major Group of Children and Youth who assisted in drafting the New Urban Agenda). Certifications like LEED (Leadership in Energy & Environmental Design), regulations like municipal bird-friendly guidelines and tools like the Biotope Area Factor (proportion of area that is dedicated to be green spaces in a city's inner area), potential vegetation maps (19) in urban areas, and Singapore's City Biodiversity Index (24) are becoming better known. Their usage strongly encourages future cooperation between different actors in the development of a more holistic approach to the question of urban biodiversity conservation.

Discussion

To further discuss and illustrate practices mentioned above, two distinct case studies will now be presented. The first one speaks more to the physical design and planning perspective and is about how Malmö integrated biodiversity in a holistic manner, blurring the boundaries between nature and city, and questioning the very relative definition of nature itself. The second case study pertains more to the question of balance that must be found between social, local economic, and biophysical research of sustainability. As an initiative powered by local citizens' active involvement, the Bronx River project highlights a successful case of bottom-up urban planning model that should be widely reproduced in the future.

Case Study: Bo01 Malmö, Sweden (Waterfront Brownfield Redevelopment Plan)

Bo01 (named for the year of its inauguration (2001), and the Swedish verb 'Bo' meaning 'to dwell') was built on a former industrial port in the Western harbour of Malmo, Sweden. The land that was revitalized suffered from extreme soil pollution. It is currently supplied by 100% renewable energy and serves as an example of sustainable urban renewal. Across over 54 acres of land, Bo01 offers housing to 2343 people with a density of 26 residential units per gross acre, or 43 people per acre. (4) This density is balanced by the 50% open space dedication on the site. (4) Despite the density of urban fabric that makes up Bo01, it has high levels of diverse green space and urban biodiversity. Trees, creeper plants, ponds and green roofs and walls make up a highly-connected network of green spaces which house at least fifty varieties of plants, and offers food and habitat to a variety of seabirds and other fauna. (4)

According to Austin, (4) the greatest gains in biodiversity could be found in the built form, as opposed to naturalized areas. By putting emphasis on building space that was useable as habitat for a variety of species, Bo01 brings aspects of the biophysical environment into residential developments. This was done by maintaining sufficiently high levels of permeable ground, green space, and integrating hydrological features into the development site. (4) Bo01 increased biodiversity through exceptional urban ecosystem development, including built habitats such as bat boxes, bird houses, and hydrological features which run through the entirety of the site, increasing connectivity at a micro scale. (4) Nine species of seabirds breed at Bo01, salamanders, frogs and three species of bat are residents in the courtyards. The incorporation of green roofs into the site helps with storm water management, which minimizes erosion of ecologically sensitive riparian zones, and provides breeding grounds for seabirds. (4) The saltwater canal, which was incorporated as a water management tool, is proving to be a valuable habitat for species of fish, shellfish, and crustaceans. (4)

In addition to the biophysical environment provided within Bo01 it was acknowledged that any development of this density would displace some wildlife and, therefore, there were offsetting activities performed to compensate for potential habitat loss in other parts of the Western harbor. Kruuse, Bo01's main ecologist and their team established a design that created conditions favorable to species that are tolerant of human activity, intelligently recognizing that certain species in the area are better at coping with human settlement conditions, which resulted in a robust urban ecosystem. (4) Bo01 also incorporated a transferable points system for any future development projects to integrate biodiversity and green infrastructure at an early stage of their development that awarded points for a variety of biodiversity implementations, including the use of native herbs and shrubs for ornamental plantings, the creation of urban agricultural spaces, and reserving spaces for natural succession within the open spaces of the development – an immensely important factor in increasing biodiversity and creating robust and healthy urban ecosystems. (4)

What makes Bo01 such a remarkable case is the successful use of integrated built form and habitat and the cooperation between government and private sector actors that was vital to its success. Architects, planners, and ecologists worked side by side to create a space that was beautiful, sustainable, and ecologically sound.

Case Study: Bronx River, Bronx NY

The Bronx River is a 39km freshwater river rising in the Catskill MounThe Bronx River is a 39km freshwater river rising in the Catskill Mountains north of New York City, and flowing into the saltwater tidal estuary of the East River at Hunt's Point in The Bronx. As the only freshwater river in New York City, the Bronx serves important social, ecological, and ecosystem services roles. It is an important transportation corridor between the suburbs of Westchester County and Manhattan, and is a recreational center in the borough of The Bronx, with parks, gardens, and multi-use pathways along the shore. The mouth of the river is characterized by heavy industrial development, with most of the watershed being covered in impervious surfaces. However, the river still supports wildlife from invertebrates to small mammals and diverse vegetation. The Bronx River is an important tributary to the East River and Long Island Sound estuaries, and provides numerous ecosystem services to New Yorkers including storage and transportation of freshwater as well as storm water drainage.

Industrialization and human interference in the river reaches back to the mid 19th century, starting with the construction of the New York Central Railroad in the valley and by the 1880s it was known as an "open sewer". Into the 20th century, efforts began to restore and reclaim the river – beginning with the creation of the Bronx Park and the Bronx River Parkway, which today is a major commuting corridor surrounded by a narrow ribbon of vegetation. However, unfortunately, the vegetation and riparian areas in these 'preserved' areas are of poor quality and have been degraded by heavy urban development around them. During the Robert Moses era (around 1930s to 1960s), these efforts came to a halt as numerous highways were built across the valley, destroying the river, urging industrial development, and lowering connectivity and quality of life in the low income South Bronx area. In the late 20th century, community groups came together to pour life back into the community and ecosystem: volunteers with the Bronx River Restoration Project began with a focus on debris clean up and started a legacy of community stewardship and activism in the Bronx River Valley, particularly in the neighborhoods of the South Bronx.

Today, ecological restoration and biodiversity improvement in The Bronx are still focused on and led by the community. Founded in 2001, the Bronx River Alliance is a partnership between over 40 organizations from the community, business, the public sector, and the municipal government. Following community feedback and coordinating with stakeholders, the Alliance encourages and promotes ecological restoration of the Bronx River using quantifiable goals and indicators from the ecosystem itself. Physical indicators, such as water quality and channel stability, added to biological ones, such as abundance of macroinvertebrates and migratory birds in the valley, contribute to a scientific understanding of the current state of the river and what needs to be accomplished to improve ecosystem services and quality of life in the South Bronx. (8)

Working with the NYC Department of Parks, the Alliance's efforts are currently focused on habitat restoration, regulation, and policy creation, as well as runoff and storm water controls in the entire watershed. Retrofits in the urban area include plantings of water retaining natural vegetation, 'greenstreets', and green roofs to reduce the amount of raw runoff entering the water system. (8)

Perhaps the most important aspect of the Bronx River Alliance, as well as numerous community organizations doing similar sustainability work in the area, is the role of outreach. Community organizations work together to gather input from members and stakeholders on restoration work – this is a project for and by the community. Sustainability organizations have also had a focus on involving youth volunteers from surrounding neighborhoods and schools, giving them exposure to sustainable living practices, science education, and a strong sense of place and pride in their community. Initiatives such as these foster a sense of nature as an integral part of the urban ecosystem, promoting better understanding and stewardship of natural resources in the future generation. (18)

The Bronx River is in a much better state today than was found in the mid 20th century, acting as a neighborhood center for recreation, education, and active transportation, while continuously improving to be a quality

habitat for a host of diverse native flora and fauna performing crucial ecosystem services for the surrounding communities. In 2007, as "a testament to an increasingly healthy Bronx River", a beaver was spotted living in the river and building a lodge: the first beaver seen in New York City in over 200 years. (21)

Conclusion

The practice of preserving and improving biodiversity within urban regions which aggregate an increasing number of humans must exist within a greater framework of sustainability. The impacts of the urban ecosystem extend well beyond the limits of an individual city as well as beyond the realm of ecology. Urban biodiversity shapes the economics, health, and social and cultural heritage of our communities, which in turn shape the biodiversity of our urban spaces. Current practices in urban biodiversity focus too heavily on the separation of human and natural environments. While preserving formal green space is important to the work of urban ecology, incorporating adaptive design and natural habitats directly into the built form will result in higher levels of biodiversity, a healthier urban ecosystem as well as a healthier, more livable human environment. By working within a holistic framework which values all life and all habitats equally, planners, architects and ecologists can work together to create communities that are biodiverse, and, in turn, sustainable.

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Dirty Turtles: Examining the Effects of Persistent Pollutants on Embryonic Turtle Development

Research Article

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Keywords

turtles, turtle embryonic development, endocrine disruptors, sex reversal, toxicology

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Abstract

Background: In an anthropogenically-altered world, it is common to find pollutants such as plastics, pesticides, fertilizers, and heavy metals in waterways and soil. Many plastics, such as BPAs; organic chemicals that can absorb plastics, such as PCBs; and pesticides, such as atrazine, are known to be endocrine disrupting chemicals (EDCs). Many EDCs act as xenoestrogens and can affect the sexual development of numerous organisms, from mammals, such as humans; to reptiles, such as turtles. Turtles are long-living organisms that often have an omnivorous diet and demonstrate site fidelity, thus they are useful subjects in which to study the effect of EDCs on organismal development.

Methods: In this study, the effects of plastics, pesticides, flame retardants, and heavy metals on developing turtle embryos were examined across a variety of both freshwater and marine turtles. A search of existing relevant literature was carried out in November of 2016 using the database Web Of Science and Google Scholar by looking for the keywords "turtles" AND "sex-reversal" AND "endocrine disrupting chemicals (EDCs)", with no restrictions used on the years in which these studies were published. Web of Science returned 42 articles and Google scholar returned 3870 articles. Of these, 13 studies were deemed relevant and examined, encompassing 35 cases, and consisting of data from eight different species of turtles.

Summary: It was found that plastics and PCBs, even in low doses, had the greatest effects on sex reversal across turtle species, and could affect their behavior later in life as well. Pesticides showed an ability to alter the sex of the turtle, and also caused developmental defects. Flame-retardants and heavy metals were shown to be maternally transferred to offspring, but studies did not find obvious cases of sex reversal or developmental defects at low doses. Many turtle species are endangered, and thus understanding threats to their health and development is critical to their future survival.

Introduction

Habitat degradation, climate change, disease, and unsustainable collection for the pet trade has put significant stress on turtle populations. (1) As a result of these anthropogenically-driven factors, reptiles, including turtles, are some of the most threatened vertebrate taxa across the globe. (1) Another human-caused factor that is impacting turtle populations is the presence of persistent organic pollutants in the environment which, in tandem with other pollutants, are commonly found in the soil sediments where turtles lay their eggs. (2)

Many of these pollutants are endocrine disrupting chemicals (EDCs). (3) Polychlorinated biphenyls (PCBs), Bisphenol A (BPA), pesticides, nitrates and heavy metals have all been reported to be EDCs. (3) Adult turtles can be affected by these pollutants in their environment, but turtles can also be exposed to these chemicals during embryonic development, as these compounds can penetrate the eggshell in both their aqueous and gaseous phases through the soil. (4) Sex determination in many turtle species is temperature dependent (TSD), wherein sex is not determined by chromosomes, but instead by the temperature of the nest during the middle third of development. (5) Reptile and amphibian species where temperature or other environmental factors determine the sex of the embryo are highly susceptible to the effects of EDCs. (3)

EDCs can work either alone or in combination with other pollutants, and can affect the turtle as a developing embryo, hatchling, and later in life. (3) EDCs can affect sex determination, gonadal development, bone mass, immune response, testosterone levels, germ cell numbers, adult sexual behaviors—primarily those driven by testosterone, viability of offspring, fertility, and hatching rates. (2-3) Many EDCs are lipophilic, and can thus bioaccumulate in the turtle's tissues, resulting in important negative consequences for the rest of the food chain. (3)

Furthermore, there may be similarities in the way that turtles and humans respond to these EDCs. (6) The strong site fidelity, longevity, and omnivorous diet of turtles not only makes them important indicators of surrounding environmental health, but may also make them a good analog for how EDCs affect other long-living vertebrates, such as humans. (2,6) Furthermore, an important enzyme involved in sex determination is aromatase, which has been found to be conserved across most vertebrate taxa. (7) Similar pathways and structures in sex determining mechanisms may mean that all vertebrate taxa have a similar susceptibility to EDCs. (6) In humans, EDCs have been linked to reproductive disorders and reproductive cancers. (3) Thus, examining the effect of EDCs on turtles may be important for both environmental and human health.

Between 8 and 15 billion pounds of BPA are produced globally each year, and their high abundance makes it safe to assume it has a high incidence in the environment. (1) PCBs have been banned since 1970 in the United States, but are still present in the soil due to their long half-life. (2) Flame retardants were banned almost forty years ago, but can also still be found in soil today. (2) Many pesticides that are suspected to be EDCs are still in use. (4) Turtles are subject to a wide range of environmental and human-driven stressors, which can act together to bring greater stress to the species. For instance, increased temperature predicted from global climate change may drive TSD species to unsustainable sex ratios, (5) as well as increasing the ability of EDCs to have feminizing effects on the sex of the developing embryo, resulting in further skewing of the population's operational sex ratio. (3) It is critical to understand the full range of effects these environmental stressors produce in order to best conserve the many endangered turtle species.

The purpose of this study is to examine plastics and PCBs, pesticides and fertilizers, flame retardants and heavy metals, and to determine which of these could be considered the most detrimental to turtles based on their

effects on sex determination, embryonic development, and traits and behaviors later in life. It is predicted that the EDCs which are known to be xenoestrogens—synthetic compounds that imitate estrogen in the body, such as PCBs, organic compounds associated with plastic; and atrazine, a pesticide, would have the greatest effect on turtles overall. It is expected that turtles subjected to known xenoestrogens will show particularly high rates of sex reversal, as estrogen is necessary for sex determination in turtles with TSD and can have a feminizing effect. (8)

Methods

13 articles were identified and deemed to be useful for this study, and some of these articles were comprised of multiple cases. As such, the results of 35 different cases of turtles being subjected to EDC exposure were examined. Across these 35 cases, eight species of turtles were examined including *Trachemys scripta elegans*, *Malaclemys terrapin*, *Chrysemys picta*, *Chelydra serpentina*, *Graptemys pseudogeographica*, *Graptemys ouachitensis*, and *Chelonia mydas*. All turtles were freshwater species except Chelonia mydas, a marine species. Studies were grouped into three sections: "Plastics, PCBs and 17β-estradiol", "Pesticides" and "Other", consisting of flame retardants and heavy metals. Plastics and PCBs were grouped together as PCBs are often associated with plastics in the environment. Plastics examined included Bisphenol A, organic chemicals examined included PCB, as well as one hormone, 17β-estradiol. Pesticides and fertilizers examined included atrazine, simazine, metolachlor, azinphos-methyl, dimethoate, chlorpyrifos, carbaryl, endosulfan I, endosulfan II, captan, chlorothalonil, dimethenamid, glyphosate, tefluthrin, ammonia, and DDE. Flame retardants examined included BDE-47, BDE-48, BDE-99, and BDE-100. Heavy metals examined included manganese, copper, zinc, selenium, and arsenic. All data was recorded in a table. The effects of the pollutants on the turtle eggs were separated into three categories: "effect on sex", "effect on development", and "effect later in life". Data for the categories of "effect on sex" and "effect on development" were recorded as "Yes", "No", or "NA" for when no information was given in a paper. The "effect later in life" category had more descriptive entries, such as "maternally transferred" or "reduced fertility". For the "effect later in life" category, any entry of a consequence of the chemical was recorded as a "Yes". The frequency of "No" or "NA" entries was also recorded for the "effect later in life" category.

Results

Plastics and PCBs, and 17β-estradiol

Of the seven studies (1,2,6,7,10,11,14) where the effects of plastics and PCBs on developing turtle embryos were examined, all chemicals examined: PCB, 17β-estradiol, and Bisphenol A had feminizing effects on the sex of the turtle. One study found that PCB caused deformities such as malformed scutes in hatchlings of *Malaclemys terrapin*. (2) The other six studies (1,6,7,10,11,14) either reported that there were no deformities, or did not examine the hatchlings for any gross abnormalities. Five studies (1,2,6,7,10) reported an effect later in life on the turtles. These effects included reduced fertility, altered mating behavior, and the ability of the toxin to be maternally transferred to offspring. PCB, 17β-estradiol, and Bisphenol A were all reported to be capable of producing one or more of these effects.

Pesticides and Fertilizers

The majority of studies involving the effects of pesticides and fertilizers on turtle embryos and hatchlings did not examine the turtles for abnormalities in sexual development. Fourteen of the nineteen cases examined did not look for any evidence of sex reversal in turtles subjected to pesticides or fertilizers. (4,9) Of the cases that did look for an effect on sex, three (9,13) of the five (4,9,13,20) cases reported either full or partial sex reversal of the hatchlings. In the studies, three species across two genera were examined for the effects of atrazine exposure: *Chrysemys picta, Graptemys pseudogeographica*, and *Graptemys ouachitensis*. All cases where atrazine was applied to the eggs reported some degree of sex reversal upon hatching.

All of the studies examined searched for an effect on the development of the turtle embryo after being exposed to pesticides or fertilizers. Of the 19 cases examined, four (9,13) reported gross abnormalities and 15 (4,9,20) reported that there were no gross abnormalities. Two of the reported cases of developmental defects were concluded to have been again caused by atrazine, and were once again reported in *Graptemys pseudogeographica* and *Graptemys ouachitensis*. (13) The other two reported cases were caused by tefluthrin and ammonia and developmental defects were observed in *Chelydra serpentina*. (9)

Fifteen of the nineteen cases did not extend their studies to look for an effect later in life of pesticides and fertilizers on the turtles. Of the four (9,13,20) cases that did, three (13,20) reported either a lower first year survival in the turtle, as for atrazine in *Graptemys pseudogeographica* and *Graptemys ouachitensis*, or reported the ability of the compound to be maternally transferred, as for DDE in *Chelonia mydas*.

Only one pesticide, chlorothalonil, was found to not have an effect on either the sex, development, or had an effect later in life. (9) Chlorothalonil was applied to the eggs of *Chelydra serpentina*.

Other

None of the nine cases that examined the effects of flame retardants or heavy metals on turtles studied the embryos or hatchlings for evidence of sex reversal. Moreover, none of the studies on flame retardants examined the turtle embryos for gross abnormalities. All heavy metals tested were found to have no effect on the development of the turtle. However, all flame-retardants and heavy metals tested were found to be maternally transferrable from the affected mother to her eggs. (15, 17)

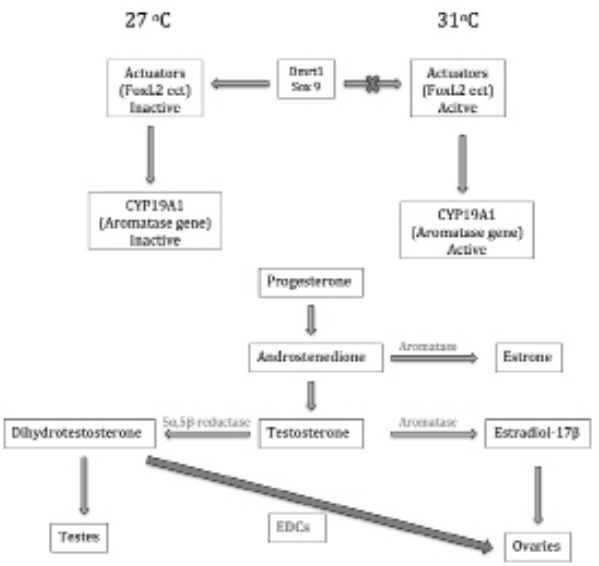

Figure 1: Simplified mechanism of EDCs on the TSD pathway. Modified from Jeyasuria and Place, 1998 and Mizoguchi and Valenzuela, 2016.

Discussion

These results suggest that the most potent EDCs are PCBs and the pesticide atrazine, as both these compounds have the ability to induce complete sex reversal and cause gross abnormalities in the embryos, as well as affect the turtle later in life (Table 1, 2, 3). EDCs are capable of interfering with the sex determination process of animals (Figure 1) and can cause feminization and demasculinization of the animals affected. (1) Furthermore, both PCB and atrazine are known xenoestrogens, so exposure to these compounds during embryonic development can thus cause complete sex reversal during the development process. (3) Additionally, due to their prevalence in soils, these two compounds are also the most like

ly for turtles to encounter while still in the egg—the time when they are most susceptible to the effects of EDCs. (1, 7, 9) These results support the prediction that known xenoestrogens would not only affect the sex of embryos, but other traits throughout the growth and development of turtles, even after hatching.

Pollutant	Species of Turtle	Effect on Sex	Effect on Development	Effect later in life	Source
PCB	*Trachemys scripta elegans*	Yes	N/A	Reduced fertility	(10)
PCB	*Malaclemys terrapin*	Yes	Yes	Yes	(2, 12)
PCB	*Trachemys scripta elegans*	Yes	N/A	N/A	(11)
PCB	*Trachemys scripta elegans*	Yes	No	Malformed mullerian ducts	(7)
17β-estradiol	*Trachemys scripta elegans*	Yes	N/A	Yes (Mating behavioral)	(6)
Bisphenol A	*Trachemys scripta elegans*	More estradiol	N/A	N/A	(14)
Bisphenol A	*Chrysemys picta*	Yes	No	Yes (Maternal transfer)	(1)

Table 1: Summary of the effects of plastics and associated organic pollutants on various turtle species from literature review.

Pollutant	Species of Turtle	Effect on sex	Effect on development	Effect later in life	Source
Atrazine	*Chelydra serpentina*	Yes	No	N/A	(9)
Atrazine	*Chelydra serpentina*	N/A	No	N/A	(9)
Atrazine	*Chrysemys pseudogeographica*	Yes (low chemical exposure, growth in males)	Yes	Yes (Lower 1st year survival)	(13)
Atrazine	*Graptemys ouachitensis*	Yes (Mid chemical exposure, growth in males)	Yes	Yes (Lower 1st year survival)	(13)
Atrazine	*Chelydra serpentina*	N/A	No	N/A	(1)
Nitrates	*Chelydra serpentina*	N/A	No	N/A	(9)
Ammonium nitrate	*Chelydra serpentina*	N/A	No	N/A	(9)
Ammonium perchlorate	*Chelydra serpentina*	N/A	No	N/A	(9)
Cyanazine	*Chelydra serpentina*	N/A	No	N/A	(9)
Chlorothalonil	*Chelydra serpentina*	N/A	No	N/A	(9)
Pentachlorophenol	*Chelydra serpentina*	N/A	No	N/A	(9)
Glyphosate	*Chelydra serpentina*	N/A	No	N/A	(9)
Cadmium	*Chelydra serpentina*	N/A	Yes	N/A	(9)
Ammonia	*Chelydra serpentina*	N/A	Yes	N/A	(9)
EDC	*Chelonia mydas*	No	No	Yes (Maternal Transfer)	(15)

Table 2: Summary of the effects of pesticides on various turtle species from literature review.

Pollutant	Species of Turtle	Effect on Sex	Effect of Development	Effect Later in Life	Source
BDE-47	*Chelydra serpentina*	N/A	N/A	Yes (Maternal Transfer)	(13)
BDE-48	*Trachemys scripta elegans*	N/A	N/A	Yes (Maternal Transfer)	(13)
BDE-99	*Chelydra serpentina*	N/A	N/A	Yes (Maternal Transfer)	(13)
BDE-100	*Trachemys scripta elegans*	N/A	N/A	Yes (Maternal Transfer)	(13)
Mn	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
Cu	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
Zn	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
Se	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
As	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)

Table 3: Summary of the effects of flame retardants and heavy metals on various turtle species from literature review.

In reference to TSD, the presence of EDCs can produce females at otherwise male producing temperatures (Figure 1). (6) EDCs are also more likely to affect the development of animals with TSD, such as turtles, which could further explain the cases of complete sex reversal, gross abnormalities, lower first year survival, reduced fertility and maternal transfer found when turtle eggs were subjected to PCB or atrazine. (1,2,10- 13)

Regardless of whether they exhibit genotypic sex determination (GSD) or TSD, all turtles also use hormones to determine sex. TSD pathways differ from GSD pathways in that the sex-determining hormones that are activated depend on environmental cues, not genetic markers. (3) EDCs can infiltrate and disrupt this hormonal pathway. Unlike mammals, reptiles do not have an Sry gene, but do have several downstream genes that are components of the male sex determining pathway, such as Dmrt1 and Sox9. (3) Normally, in turtles, at low temperatures Dmrt1 and Sox9 are active. This results in actuators such as FoxL2 to be inactive, which in turn results in CYP19A1, the gene that codes for aromatase, to be inactive as well (Figure 1). (3) Aromatase is an enzyme important to the female sex determining pathway across vertebrates, and converts testosterone into 17β-estradiol, which causes embryonic ovary development. (3) Without aromatase, testosterone is instead converted to dihydrotestosterone by 5α,5β-reductase (Figure 1). (3) Dihydrotestosterone is a hormone that leads to the development of testes in the embryo. (3)

There are several ways in which EDCs can disrupt the normal pathway of sexual development. EDCs can act as aromatase inhibitors, hormone mimics, hormone antagonists blocking the action of the hormones or alter when a hormone is produced. (3) PCBs, for instance, can mimic estrogen; BPAs can decrease steroid binding affinities; atrazine can inhibit 5α,5β-reductase; nitrates often found in fertilizers can alter steroid levels; and heavy metals can interfere with estrogen action. (3) As the pathways of sexual development in turtles normally operate in a dosage dependent manner, the introduction of an EDC can upset the balance of hormones and alter the sex of the embryo. (3) EDCs can also affect hormones that drive phenotype during development, which may explain the gross abnormalities observed by some studies when the turtle embryos had been subjected to EDCs.

Adult turtles can also be affected by EDCs. Since EDCs have the ability to bioaccumulate, (3) turtles living in contaminated environments may absorb them through their diets. In females, high levels of EDCs in the tissues may lead to maternal transfer. In turtle eggs, the yolk can be maternally loaded with hormones, and these maternally loaded hormones have been found to be able to override the sex determining pathways in the embryo should the concentration be high enough. (3) EDCs that mimic hormones may be loaded into the yolks in the same way through maternal transfer, subjecting the developing turtle embryo to significant EDC exposure even if the environment in which the egg was laid is not contaminated. In males, adult exposure to EDCs may result in "demasculinizing" effects, lowering their testosterone levels and perhaps altering mating behavior. (6) For instance, male painted turtles use their long, sexually dimorphic nails to stroke the face of a potential mate, a behavior that is noted to be driven by testosterone levels. (6) Lower testosterone levels could alter this behavior and result in lower mating success, even without visible abnormalities in the turtle. (6)

Another important anthropogenic source of estrogen is 17β-estradiol (often mentioned in articles alongside BPAs). 17β-estradiol is the main estrogen found in birth control pills. (6) On average, 10μg of 17β-estradiol is excreted daily in the urine of humans who take oral contraceptives, and though there exists technology to completely remove 17β-estradiol in waste treatment plants, it has not been used. (6) Consequently, traces of 17β-estradiol have been found contaminating the water and groundwater in areas where turtles could be exposed, and these areas are often also contaminated by other EDCs. (6) The abundant amounts of feminizing chemicals could cause increasingly more female turtles to hatch, skewing the sex ratio towards unsustainable levels and heavily impacting the future of turtle populations.

A limitation of this study is that many of the studies examined only looked at the effect of EDCs on either the hatchling or the adult, and rarely followed a turtle throughout its life. Some potential and serious effects of

EDCs are high deformity rates in juveniles, increased mortality, and slower growth, (2) but these results are unlikely to be seen in studies looking only at hatchlings and adults. Additionally, many studies euthanized and dissected hatchlings to examine them for signs of sex reversal, making it impossible to see how the sex reversal affected the behavior or reproductive success of those turtles later in life. These premature analyses of the turtles may create an underestimate of the potential effects of EDCs.
Moreover, there were no standard protocols used across all studies. Some studies were conducted in a laboratory setting, (1,2,4,6,7,9,10,11,13,14) while others focused on observations in the field. (15,17,20) Some studies in the lab only applied the contaminant once to the turtle eggs, whereas others applied it continuously throughout the experiment. The dosage of the EDCs applied was also not consistent across the studies. These inconsistencies may have created false positives in my collected results if one technique was more successful at inducing abnormalities than another.
It is also important to note that there may be a publication bias towards papers that have significant or interesting results. This may result in papers focusing on contaminants known to cause abnormalities in developing turtles, and consequently cause an overestimate of the effects of EDCs on turtles.

It is important to examine the effects of EDCs across the life of the turtle, and to look at a wider range of EDCs across varying concentrations in future studies in order to determine the full range and severity of the effects of EDCs on turtle development.

Conclusion

Although many EDCs known to be particularly potent, such as PCBs, are now banned from use, they can still be found in the soil and water. (1,2,4,7) Certain pesticides that are known to be xenoestrogens, however, such as atrazine, are still widely in use. (9) As turtles live in the water and lay their eggs in the soil, they can be exposed to these EDCs at varying stages of embryonic development and throughout their lives. Turtles are subject to a wide range of stressors surrounding habitat loss, climate change, and human activities. These results show that EDCs could cause further population declines in already stressed turtle populations by reducing first year survival, reducing mating success, decreasing immune response, causing gross abnormalities, and skewing the operational sex ratio to unsustainable levels. (2,3) As the hormone levels in the egg yolk can also affect sex determination, EDCs absorbed into the egg could mimic maternally loaded hormones and override genetic factors and/or the temperature-influenced and temperature-activated pathway of sexual development in the embryo. (3) It is thus suspected that EDCs with such characteristics would result in the highest incidence of sex reversal in turtles.

Turtles are already heavily endangered, and in order to properly and thoroughly protect these animals we must understand the full range of stressors that they are subjected to, of which EDCs are a major stressor. It is important for both the health of aquatic ecosystems and the organisms that rely on these ecosystems to understand and mitigate the detrimental effects of EDCs and to determine why and how these substances should be regulated and limited.

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David Scarlata[1]

The CBS Domain: Its Structure, Ligand Binding, and Emerging Role in Regulation

Review Article

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Abstract

Background: Cystathionine β-synthase (CBS) domains are structurally conserved motifs that are present in the proteomes of species from all kingdoms of life. Signifying their importance are the hereditary diseases resulting from mutations within the CBS sequence. They are usually encoded in tandem within a plethora of non-functionally related cytosolic or transmembrane proteins, often intramolecularly dimerizing to afford what is known as a CBS pair or Bateman module. It is also known that these CBS pairs can further multimerize to form higher-order assemblies, which have functions that remain to be elucidated. Moreover, a wide range of adenosyl ligands, divalent cations and nucleic acids have been documented to bind CBS domains and induce conformational changes to the larger protein in which they reside, thus suggesting their involvement in protein regulation in response to intracellular energy status.

Methods: This review was written based on the existing data currently available in the literature and included findings from 44 papers. Selection of papers was based on those that provided up-to-date information on the structural characteristics of CBS domains and their involvement in protein regulation.

Summary: This review aims to conceptualize the architectural characteristics of CBS domains, the structural basis of ligand binding, and its involvement in the regulation of protein function.

Introduction

Cystathionine β-synthase (CBS) domains are small, evolutionarily conserved motifs, consisting of approximately 60 residues associated in tandem repeats. (1) They are known to be widespread throughout all living species (InterPro IPR000644), either as independent proteins or as fusions with cytosolic, nuclear or transmembrane domains. (2) These proteins were initially identified in the archaebacterium *Methanococcus jannaschii* by Alexander Bateman in 1997, through a serendipitous discovery made from his investigation on the homocystinuria-causing enzyme cystathionine β-synthase, from which its name was conceived. (3) CBS domains have since been identified in numerous non-functionally related proteins, with 134,987 protein matches across all species within the EBI registry, as shown in Table 1. (2) They are considered to be adenosine oligophosphate-sensing modules, due to their capacity to regulate protein function in response to fluctuations in cellular energy levels; however, their specific function remains to be elucidated. (4) For instance, CBS domains have been noted to be involved in osmoregulation (5) and in the binding or transport of Mg^{2+} across membranes (6), as seen in several magnesium transporters, a few of which are the mammalian CNNMs (7), and the bacterial MgtE and CorC. (8) They are also known to be involved in the intracellular modulation of chloride channel trafficking (9), in nitrate transport, and as internal inhibitors of inorganic pyrophosphatases. (10)

To date, there are upwards of 120 solved crystal structures for the CBS domain, which all demonstrate an association between contiguous CBS motifs to form what is often referred to as a CBS pair or Bateman module. (11) Furthermore, CBS domains have been shown to bind adenosyl ligands and their derivatives with widely varying affinities and stoichiometries, often inducing conformational rearrangements depending on the construct being studied. (12) CBS modules are attributed with oligomerization into higher-order assemblies, an additional mode of regulation. This mode of regulation confers increased stability and solvent accessibility of certain interstices to ligands. (13) Interestingly, several hereditary diseases have been associated with mutations in the CBS sequence. For instance, mutations in inosine-5'-monophosphate dehydrogenase (IMPDH) lead to retinitis pigmentosa (14); chloride channel (ClC) mutations cause hypercalciuric nephrolithiasis, among other deleterious conditions (15); mutations within the γ-2 subunit of AMP-activated kinase (AMPK) lead to familial hypertrophic cardiomyopathy with Wolff-Parkinson-White syndrome; (16) homocystinuria is caused by mutations within the CBS enzyme; (17) and Bartter syndrome results from mutations within ClC-Kb, (18) osteopetrosis from mutations in ClC-7 (19), and Dent's disease from mutations within ClC-5. (20) This list of diseases emphasizes the (patho)physiological importance of CBS domains as a target for rational drug design. In this review, we aim to discuss the structural and functional characteristics of the CBS domain to better conceptualize their ligand binding and regulatory activity, which may provide useful insight into developing compounds of medicinal interest.

Table 1: Widespread Distribution of CBS Domains Across Various Phylae

Species	No. of Proteins With CBS Domains
Viruses	1
Prokarya	118,813
Archaea	6,654
Eubacteria	112,159
Escherichia coli (Strain K12)	9
Eukarya	16,174
Plantae	
Oryza savita (Asian rice)	99
Arabidopsis thaliana	76
Chordata	
Homo sapiens	87
Mus musculus (Mouse)	67
Danio rerio (Zebrafish)	57
Arthropoda	
Drosophila melanogaster	25
Nematoda	
Caenorhabditis elegans	39
Fungi	
Saccharomyces cerevisiae	10

*Data were obtained from http://www.ebi.ac.uk/interpro/entry/IPR000644/taxonomy.

Conserved Structural Characteristics of CBS Domain

Despite their low level of sequence homology, all CBS domains maintain oligomeric folds consisting of three-stranded β-sheets, where β1 and β2 run parallel to each other and antiparallel to β3, with two α-helices arranged in a β1-α1-β2-β3-α2 topology. (21) Its overall folding is somewhat irregular pyramidal, whereby the loop connecting β1-β2 defines the apex, and helices α1 and α2 make up its base. (22) Additionally, there is always a flexible linker that precedes β1, and most often contains at its N-terminus one turn of a helix, denoted by α0. (22) Tandem CBS motifs preferentially associate into a dimeric state forming what is referred to as a CBS pair or Bateman module, with pseudo-C_2 symmetry running parallel to the central β-sheets. (23) There are numerous examples of these CBS pairs undergoing further multimerization to form higher-order structures, thus emphasizing the many possible combinations that can be achieved and the consequent diversity of functions that arise from this variability. The flexible linkers containing the α0 helical turn are integrated well into the adjacent CBS motif due to the tight antiparallel association between the α-helices and β-sheets lining the dimerization interface. (22)

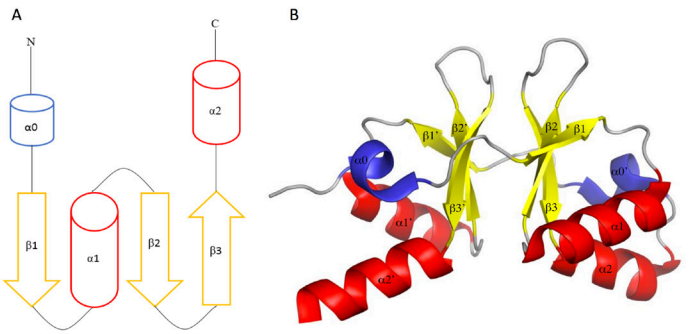

Fig. 1: The Structural Features of CBS Domains. (A) The topology of the CBS motif consists of an α0 helix (blue), helices α1 and α2 (red) and β sheets 1, 2 and 3 (yellow). (B) The CBS pair is represented with an irregular pyramid fold, where helices α1 and α2 make up the base and the loop connecting β1-2 denote the apex. PDB ID: 3KPC.

Crystallographic studies have revealed that CBS domains are not part of the catalytic core. Rather, CBS domains reside on the periphery of enzyme complexes, suggesting that they do not participate in enzymatic catalysis but rather in regulation. (24) Truncation experiments validated this assertion, whereby enzymes missing the CBS domain retained their catalytic activity, but lost their regulatory capacity. (25) Such examples include *Streptococcus pyogenes* IMPDH, which catalyzes the rate-limiting step in de novo guanine nucleotide biosynthesis, and consists of both a TIM barrel that embodies the catalytic framework and a CBS pair at its periphery. (26) The latter acts as an accessory domain that confers to the enzyme the ability to be *trans*-regulated by adenosyl ligands, thus coupling GTP/dGTP production to cellular energy status. (27) As shown in mutagenesis studies, deletion of the CBS domain does not impair the *in vitro* catalytic activity; however, it does result in loss of sensitivity to nucleotides. (22) The capacity of CBS domains to bind a wide variety of nucleotides may contribute to the number of effector signals that it is capable of transducing, emphasizing the importance in characterizing its ligands.

Ligans and Their Binding Site

CBS motifs have attracted considerable attention in recent years due to their regulatory role in enzyme complexes of pathological significance. Perhaps even more interestingly, CBS motifs have been documented to bind a myriad of adenosyl ligands in conserved locations, allosterically regulating the activity of the catalytic core. (28) Solved Bateman module crystal structures reveal a cleft at the CBS pair dimerization interface, which has been found to bear two ligand binding sites, S1 and S2, respectively. (29) Therefore, the number of binding sites is equal to the number of CBS motifs within the protein. (29)

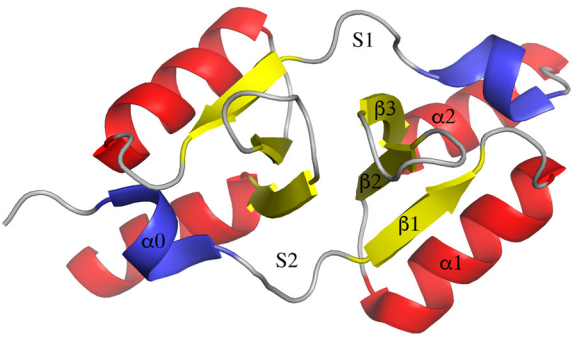

Fig. 2: An Apical View of the S1 and S2 Ligand Binding Sites within the CBS Pair. An apical view of the CBS pair is represented, indicating the positions of the two canonical ligand binding sites, S1 and S2. Shown in blue are α0, in red α1-2 and in yellow β1-3 for each CBS motif in the pair. PDB ID: 3KPC.

Each of these binding sites consists of three subsections, which confer ligand binding selectivity (i.e. preference for one base over another), sensitivity to ligand energy-charge (i.e. the number of appending phosphate substituents) and the orientation of the ligand within the cavity. The first section comprises the residues of the flexible linker region preceding β1, which has been found to show the most sequence diversity and inconsistency in terms of length across CBS-containing proteins. (22) The N-terminal α0 helix and the residues immediately preceding β1 within this flexible linker are well nested into the adjacent CBS motif and serve to maintain the hydrophobic contacts between residues at the dimerization interface. (22) Between these two points are several key residues. The first residue is a conserved serine or threonine that hydrogen bonds with the hydroxyl substituents of the ribose moiety. The second is centered in the middle of the linker and confers the selectivity of adenosyl derivatives over its guanine counterparts by orienting its main-chain carbonyl oxygen towards C2 of the adenine ring as to sterically hinder the recruitment of guanine, which contains an amino group at that position. (22) The third residue is positioned two residues further down the chain from the second key residue and also confers adenine selectivity through hydrogen bonding between its carbonyl oxygen and the N6-amino group. (22) The second section contains residues found within the loop between α1 and β2. This region bears hydrophobic residues that accommodate the purine ring through exclusion of water. (29) In some cases, the carbonyls of polar residues can interact with the exocyclic N6-amino group on the adenine ring. (29) The last section, making up the remainder of the interstice contact surface, includes the first two turns of α2 with the entirety of β3. (22) This section contains a G-h-x-S/T-x-S/T-D motif that functions in ribose-phosphate recognition, where h denotes a hydrophobic residue, x is any residue and G, S, T and D are their corresponding amino acids, respectively. (29) The highly conserved aspartate has been shown in some instances to be substituted by asparagine and instead functions in hydrogen bonding to the hydroxyl substituents of the ribofuranose moiety. (29)

Numerous research groups have documented that these cavities interact with a plethora of adenosyl derivatives with both varying affinities and stoichiometries. These derivatives include ligands such as AMP, ADP, ATP, diadenosine polyphosphate (ApnA), nicotinamide adenine dinucleotide (NAD), S-adenosyl methionine (SAM), 5'-deoxy-5'-methylthioadenosine (MTA), adenosine 5'-(β,γ-imido)triphosphate (AMP-PNP) and 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR). (10,29,30) The structures of these ligands are provided in Fig. 3.

Additionally, CBS motifs have also been shown to bind divalent cations and nucleic acids. (31) In vitro studies reveal that Mg^{2+}, Mn^{2+} and Zn^{2+} are all found to accompany ATP within the binding cavity, serving to stabilize the anionic electrostatic repulsion between phosphate groups. (32,33) These cations have also been found to bind single-stranded DNA and RNA, and

in some cases have even been shown to associate with double-stranded DNA. (34) There is clearly much that has yet to be understood, presenting a new avenue of research in determining the many possible ligands and their modes of binding.

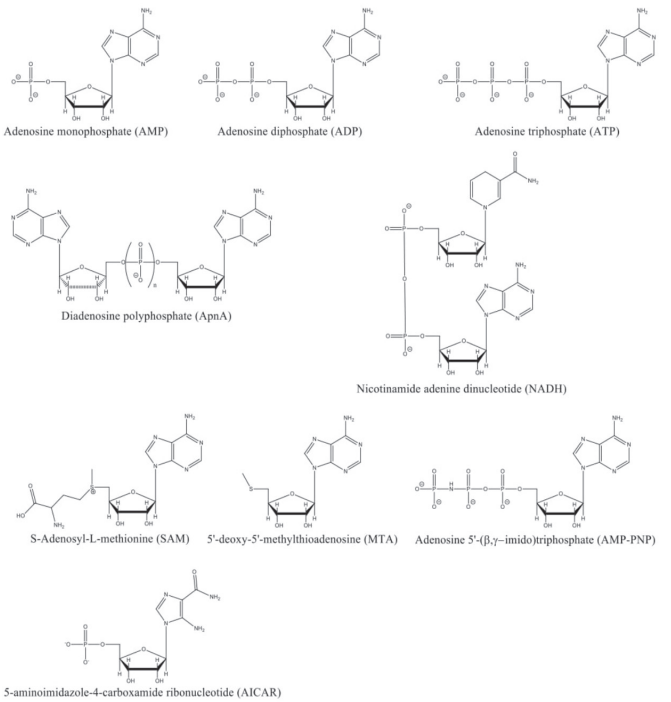

Fig. 3: The Structures of Adenosyl Derivatives known to bind CBS Motifs. The structures provided illustrate the wide variability of possible adenosyl-derived ligands that can bind CBS domains.

Dimerization/oligomerization modes of CBS pairs

Approximately 50-70% of all known protein structures self-assemble to form homomeric species, emphasizing its importance in functioning biological systems. (35) From an evolutionary standpoint, selection of the oligomerization property of proteins has allowed for increased stability, control over the solvent accessibility of certain sites, specificity of certain sites for ligands, and restriction of enzyme activity when necessary. In recent years, CBS pairs have also been found to associate with other such modules, forming higher-order assemblies. Studies conducted on the hyperthermophilic *Methanocaldococcus jannaschii* CBS-containing protein MJ0729 have proposed that its oligomerization state is pH-dependent. (36) MJ0729 is a 124 residue protein of 14.3 kDa that is thought to be involved in the regulation of the electron transport chain, although its specific function remains to be elucidated. (36) At a pH less than 2.5 and with the use of hydrodynamic and spectroscopic techniques in, the protein has been demonstrated to exist in a high oligomeric molten globular state. (36) At pHs within the range of 4.5-5.3, the species undergoes dissociation into an oblong tetrameric state. (36) This dissociation has been postulated to be caused by the titration of key aspartic acid or glutamic acid residues which provoke the switching between α-helix and β-sheet secondary structures, resulting in gain or loss of multimerization contact surfaces. At physiological pH, and even up to pH 9, the elongated dimeric species predominates. These findings are consistent with the observation that most proteins known to harbour a CBS domain exist predominantly in a dimeric state at physiological pH.

Certain eukaryotic proteins, including the γ-subunit of AMP activated protein kinase (AMPK), contain four contiguous CBS motifs within their primary sequence that associate to form two Bateman modules joined together by a linker of variable length. (37) This arrangement consequently only allows for the formation of parallel CBS modules, also referred to as a head-to-head orientation. (22) In 95% of cases, the linker is too short to accommodate an anti-parallel, or head-to-tail, orientation due to the physical strain which limits the potential reorientation of the Bateman modules, thus significantly favouring the abundance of the parallel species. (22)

Bacterial magnesium transporter MgtE is a notable example of how CBS pair oligomerization states regulate the transmission of an effector signal to the protein core. Under conditions of low Mg^{2+} concentration, the two CBS pairs associate to form a disc-like, flat-parallel module that suffers less compaction at its dimerization interface compared with the Mg^{2+} bound form. (38) This association is due to the repulsive electrostatic forces imparted by the α-helical acidic clusters within the ligand binding interstice that would otherwise be stabilized by a Mg^{2+} ion. Conversely, under conditions of high Mg^{2+} concentrations, the metal ion binds these clusters, effectively constricting the crevice, resulting in a transition from a flat-open to flat-closed conformation. This rearrangement causes movement of a long α-helix bridging the CBS and transmembrane domains which results in opening of the ion channel. The prevalence of these architectural features in which CBS domains confer regulation to larger proteins by forming higher-order assemblies emphasizes their versatility and indispensability in biological systems.

Cystathionine β-synthase regulation by CBS modulatory domains

To better conceptualize the molecular mechanisms involved in the regulation of enzymes by CBS domains, we turn to the example of Cystathionine β-synthase. Human CBS (*h*CBS) is a 61 kDa pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyzes the condensation of L-serine with L-homocysteine to form cystathionine. (2) CBS therefore plays a pivotal role in mammalian sulfur metabolism, lying at the junction of the transsulfuration pathway whereby homocysteine is either converted to methionine or used in the production of cysteine. This positioning of the CBS domain proves to be a critical determinant in how sulfur is distributed endogenously. (39)

The *h*CBS enzyme consists of three structurally distinct domains: an N-terminal domain bearing a heme moiety which is thought to act in redox sensing; a central domain that confers PLP dependent modulation; and a C-terminal domain containing a Bateman module which allows for CBS pair homotetramerization *in vivo*. (40) The CBS pair domain has also been postulated to regulate enzyme function intrasterically and allosterically through the binding of S-adenosyl-methionine (SAM). (2) SAM was verified to bind with a dissociation constant (K_D) of 34 μM and to induce a conformational change that abates this subunit's association with the catalytic core, thus facilitating substrate binding and increasing enzyme activity approximately three-fold. (41) As depicted in Fig. 4, the binding of SAM brings both CBS pairs within close proximity at the internal axis of symmetry, consequently resulting in breakage of the hydrogen bonds formed between key residues T460, N463, S466, and Y484 with loop L191-202 residues R196, D198, S199, P200, and E201 on the catalytic core.

This conformational change relieves the occlusion at the entrance of the catalytic pocket and releases the autoinhibitory clamp on the protein core allowing for binding of substrate, as shown in Fig. 5. These crystallographic findings have been confirmed by mutagenesis studies on some of the key residues lining the pocket. For example, S466L, as reported by *Janosik et al.*, has been shown to compromise SAM sensitivity. (42) The overall fold of the protein core does not undergo any major structural rearrangements. This enzymatic reaction proves to be critical in the biosynthesis of cysteine by providing a regulatory control point for SAM. (42) At low concentrations of SAM, CBS pair activity is diminished, resulting in an accumulation of homocysteine which funnels toward the production of SAM. (43) Conversely, at high concentrations, CBS pair activity increases resulting in the clearance of homocysteine and the increase of cysteine production. (43)

In humans, there are currently 150 known loss-of-function mutations within the CBS gene that lead to homocystinuria, a marked accumulation of homocysteine manifesting itself in connective tissue defects, skeletal deformities, vascular thrombosis, and mental retardation. (44) Some of these CBS point mutations, including I435T, D444N, and S466L, can oc-

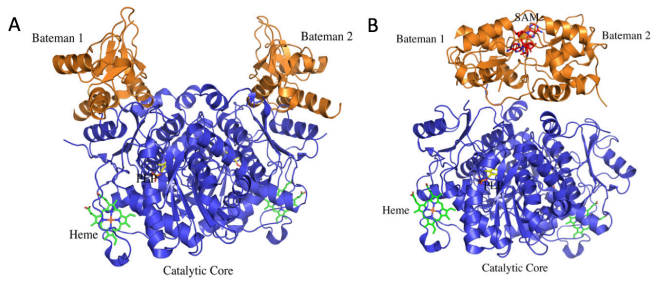

Fig. 4: The Structural Rearrangement of Human Cystathionine β-synthase upon Binding of SAM. The two CBS pair domains are featured in orange, and the catalytic core in blue. (A) In its unbound state, the two CBS pairs are distal from each other. PDB ID 4L0D. (B) When bound to SAM at its CBS module, the CBS pairs join at the central axis. The overall conformation of the catalytic core does not change. Shown in yellow is PLP, and in green are the heme moieties. PDB ID 4PCU.

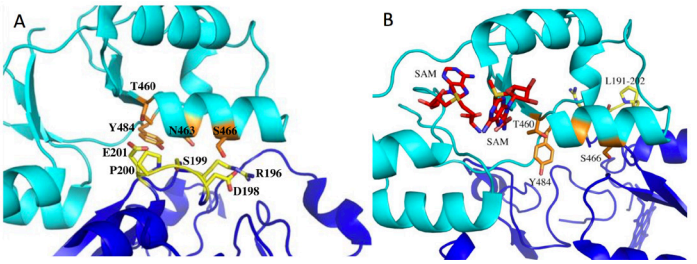

Fig. 5: Interaction between the CBS domain and the catalytic core within *h*CBS in the SAM bound state. (A) In the unbound state, key residues T460, Y484, N463 and S466 (orange) from the CBS domain interact with residues from the L191-202 loop (yellow), thus occluding the binding of SAM. (B) The binding of SAM causes a conformational change that disrupts the interactions between the residues on the CBS domain and L191-202, thus relieving the autoinhibitory clamp on the catalytic core.

cur in both domains and lead to either a dramatic decrease or complete loss of SAM activation. (42) Intriguingly, there are many other mutations that do not interfere with binding of SAM and still manage to impinge on enzymatic activation, suggesting that further studies are required to better understand this regulatory mechanism (42).

Conclusion

The current available information on the CBS domain reveals that its best-known feature is its tertiary structure. Indeed, these small motifs share poor sequence identity, yet have consistently shown through numerous examples of proteins from all kingdoms of life, to maintain a conserved three-dimensional structure. These motifs are often found in tandem, and associate to form what is known as a CBS pair or Bateman module. Furthermore, higher-order assemblies of CBS domains have been documented, and are thought to be peripheral units that relay signals from the intracellular environment to the protein core. Interestingly, careful studies of these domains reveal a cleft harbouring two ligand binding sites, S1 and S2. These canonical sites have been found to accommodate a wide variety of adenosyl derivatives as a means of sensing intracellular energy status, and in some particular cases have even been shown to interact with numerous divalent cations and nucleic acids. Indeed, there are many deleterious mutations within these domains that interfere with their ligand binding activity, consequently resulting in disease. Even though a relationship between the individual domains relaying signals and their larger protein structure has increased our understanding of the CBS domains, there is much that is still not understood. More investigation is required to decipher the rules governing the different modes of association of the Bateman modules, the identification and characterization of the chemical properties of their binding sites, and the structural effects induced by ligand binding. Once thought to be simple and obscure, CBS motifs have unveiled their involvement in certain pathologies, making them promising targets for therapeutic intervention.

Acknowledgements

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Melatonin and its Receptors

Review Article

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Abstract

Background: Melatonin (5-methoxy-N-acetyltryptamine) is a hormone that has numerous physiological functions. Synthesized and released during nighttime, melatonin exerts its physiological effects in a circadian manner. Melatonin acts by binding to its different types of receptors. The purpose of this systematic review is to summarize recent findings about melatonin, its receptors, and the differential functionalities of the most characterized melatonin receptors MT1 and MT2.

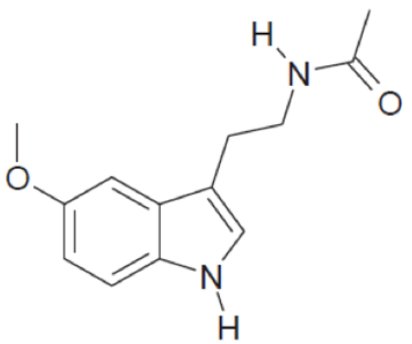
Methods: We searched PubMed and Google Scholar for studies that reported melatonin receptor subtypes, their differential functionalities, biochemical structures, signal transductions, and various functions of melatonin such as pain, sleep, temperature, and antioxidative effects. We chose seventy articles for this systematic review.

Summary: These studies highlight melatonin's range of physiological functions and the differential functionalities of the melatonin receptors MT1 and MT2; they characterize the receptors' signal transduction cascades and their biochemical structures. More studies assessing melatonin receptors' functions would help patients with disorders in sleep, pain, and circadian rhythm.

Introduction

"Think in the morning. Act in the noon. Eat in the evening. Sleep in the night." (William Blake, The Marriage of Heaven and Hell). (1)
Long before our investigation into chronobiology, the daily light/dark cycle dictated our lives. The human physiological system has synchronized to when the sun rises and sets and to when the sky is clear blue or pitch black. The neurohormone melatonin plays a pivotal role in this process.
Melatonin (5-methoxy-N-acetyltryptamine) (Fig. 1) is a hormone that regulates a wide range of physiological functions including circadian rhythms, (2) mood regulation, (3) anxiety, (4) sleep, (4) pain, (3) immune responses, (2) and cell cycle. (2)

Enzymes drive the synthesis of melatonin. Tryptophan hydroxylase (TPH) converts tryptophan, a precursor to melatonin, into 5-hydroxytryptophan. (5) Subsequently, aromatic amino acid decarboxylase (AAD) converts 5-hydroxytryptophan into serotonin. Arylalkylamine-N-acetyl transferase (AANAT) then converts the serotonin into N-acetylserotonin. Finally, hydroxyindole-O-methyltransferase (HIOMT) converts N-acetylserotonin into melatonin (Fig. 2). (6) Melatonin travels through the circulatory system to the capillaries and affects various organs. (6) Its amphiphilic biochemical structure facilitates its transportation throughout the body. (7) where it exerts different physiological effects on different organs.



Melatonin (5-methoxy-N-acetyltryptamine)

Fig. 1: Chemical Structure of Melatonin

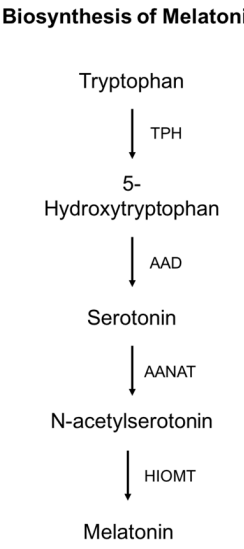


Figure 2. Biosynthesis of Melatonin. (2) Tryptophan hydroxylase (TPH) converts tryptophan, a precursor to melatonin, into 5-hydroxytrytophan. Subsequently, aromatic amino acid decarboxylase (AAD) converts 5-hydroxytrptophan into serotonin. Arylalkylamine-N-acetyl transferase (AANAT) then converts the serotonin into N-acetylserotonin. Finally, hydroxyindole-O-methyltransferase (HIOMT) converts N-acetylserotonin into melatonin.

Melatonin has a short half-life. It shows a biexponential decay with a first distribution half-life of 2 minutes and a second metabolic half-life of 20 minutes. (8) Therefore, melatonin's clearance is rapid upon its release in the bloodstream. Hence, the physiological concentration of circulating melatonin delivers the message of environmental darkness throughout the body; for this reason melatonin is known as the "chemical expression of darkness". (9) The concentration of melatonin peaks during nighttime

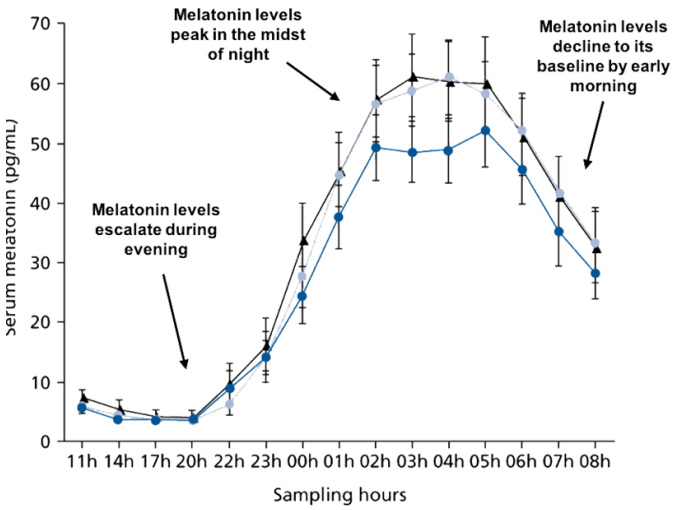


Figure 3. Concentration of melatonin during night and day phase in three different 24-h sessions. The concentration varies depending on the time block due to the circadian regulation of suprachiasmatic nucleus affecting the synthesis and release of melatonin. (13) Its concentration is maximal at 2-4 AM and minimal throughout the daytime. (13) Reproduced from (14) Selmaoui B, Touitou Y. Reproducibility of the circadian rhythms of serum cortisol and melatonin in healthy subjects. A study of three different 24-h cycles over six weeks. Life Sci. 2003;73:3339-49.

(between 2:00 AM and 4:00 AM) and declines during daytime. (10, 11) Melatonin is mostly synthesized by the pineal gland and is secreted during the nighttime. (8) Light exposure suppresses the release and synthesis of melatonin in a dose-response manner. The intensity of environmental light determines the amplitude of the synthesis and the release of melatonin (Fig. 3).

Melatonin also regulates a wide range of physiological functions including circadian rhythmicity. It interacts with a brain region in the hypothalamus known as the suprachiasmatic nucleus (SCN), the so called "master clock." The SCN regulates the synthesis and secretion of melatonin in a circadian pattern that corresponds to the environmental daily light/dark cycle (Fig. 3). Although melatonin has diverse physiological functions, its primary role is to provide photoperiod information about the environmental light/dark phase so that the organism can adjust its physiology to the 24-hour cycle.

If the SCN is lesioned, the rhythm of melatonin release is abolished; (12) this experiment shows the importance of the SCN in generating a circadian pattern of melatonin release. However, melatonin can also send feedback to the SCN. (13) Therefore, melatonin performs a dual role as (a) a possible feedback signal to the SCN by binding to melatonin receptors MT1 and MT2 within the SCN and (b) as an SCN-dependent output signal that influences cells in the central and peripheral nervous system. (2) Communications between the SCN and the pineal gland proceed via a multi-synaptic pathway that allows for the synthesis and the release of melatonin according to the environmental light/dark phase. (14) A limited subset of retinal ganglion cells in the eyes detect the intensity of environmental light. (15) This information from the retina is then transferred to the SCN via the retinohypothalamic tract. (16) This tract enables not only the information to be transmitted from the retina to the SCN, but also allows the SCN to entrain the physiological rhythm to the 24-hour cycle. Pinealectomy (the removal of the pineal gland) abolishes most of the nocturnal rhythm in mammals, (17) demonstrating the importance of the pineal gland and melatonin in the maintenance of biological rhythms.

Though the versatility of melatonin in its physiological functions has been well-elucidated, this review will focus more on the known melatonin receptor subtypes. The purpose of this review is to describe recent findings on melatonin receptor subtypes such as MT1, MT2, GPR50, MT3, and nuclear receptors and to report recent findings about signal transduction and the differential functionalities of the two most characterized melatonin receptor subtypes, MT1 and MT2, with respect to pain sleep and circadian rhythms.

The Melatonin Receptors

Melatonin receptors were first cloned using *Xenopus laevis* melanophore (18) using a method to unbiasedly isolate proteins known as expression cloning strategy. (19) This sparked interest in melatonin and led to research that characterized mammalian high-affinity melatonin receptors. (20)

The melatonin receptors were later characterized in the early-1990s using the high-affinity radioligand 2-[125I] iodomelatonin. (21) This radioligand served as a visual tracer to localize melatonin receptors that are expressed in native tissues. Visualization of 2-[125I] iodomelatonin has been made possible by autoradiography, thus enabling anatomical localization of melatonin receptors.

Initially, melatonin receptors were classified based on their affinity for the 2-[125I]iodomelatonin radioligand as the high-affinity ML1 (dissociation constant Kd <300 pM) and the low-affinity ML2 (Kd 0.9-10 nM). (22) Later, two subtypes of ML1 receptor were cloned (20, 23) and they were initially referred to as Mel1a and Mel1b. Moreover, the nomenclature of ML2 was later modified, and the receptor is now named MT3, a third subtype of the melatonin receptor which, unlike MT1 and MT2, displays low affinity to melatonin. (23) Before, another cloned melatonin receptor from Xenopus laevis melanophores was identified and named Mel1c, but this subtype has only been found in non-mammals. (18) Development of diverse partial agonists and antagonists that show differential affinity to one of the melatonin receptor subtypes (24) has enabled investigators to characterize the differential functionalities of the melatonin receptors. (25)

MT1 and MT2

The human MT1 (formerly named Mel1a) receptor spans 350 amino acids whereas the human MT2 (formerly named Mel1b) receptor spans 362 amino acids. (26) Their molecular weights are 39-40 kDa and they display homology in 55% of their amino acids overall and 70% within the transmembrane domain. (26) Melatonin receptors MT1 and MT2 are both membrane-bound G-protein coupled receptors (GCPRs) with 7 transmembrane alpha-helical domains. (27) The amino terminus is on the extracellular side and the carboxyl terminus is on the intracellular side. (13) Features unique to melatonin receptors among the GCPRs are (a) a NRY motif downstream from the third transmembrane domain and (b) a NAXIY motif in the seventh transmembrane domain. (13, 20, 23, 28)

Two melatonin receptors display different chromosomal localizations. The MT1 melatonin receptor gene was localized in human chromosome 4q35.1 and mouse chromosome 8. (29) Conversely, the MT2 melatonin receptor was localized in human chromosome 11a21-22 and mouse chromosome 9. (23)

Melatonin MT1 and MT2 receptors have been localized through several laboratory techniques including *in situ* hybridization and immunohistochemistry. (13) The peripheral tissues where MT1 is expressed include the cardiovascular system, immune cells, testes, ovaries, skin, liver, kidneys, adrenal cortex, placenta, breasts, retina, pancreas, and spleen. (5) MT2 is distributed in the immune cells, retina, pituitary gland, blood vessels, testes, kidneys, gastrointestinal tract, mammary glands, adipose tissue, and skin. (5)

A recent study examined the distribution of MT1 and MT2 in an adult rat's brain using polyclonal anti-MT1 and anti-MT2 antibodies (30) visualized under light, confocal, and electron microscopes. (31) This study thoroughly mapped the anatomical localization of MT1, finding abundant MT1 receptors in the retrosplenial cerebral cortex, basal forebrain, hippocampus, medial habenula, anterodorsal nucleus of thalamus, dorsal mesencephalon, substantia nigra (pars compacta), and pars tuberalis of the pineal

gland. (31) On the other hand, MT2 receptors were widely expressed in the reticular thalamus, substantia nigra (pars reticulata), supraoptic nucleus, red nucleus, CA2 and CA3 areas of the hippocampus, (31, 32) and on the glutamatergic neurons in the ventral lateral periaqueductal grey matter (vlPAG), which is involved in the descending pain-control pathway (vlPAG-rostral ventral medulla). (33) As opposed to radioligand studies that crudely determined the anatomical localizations of melatonin receptors, this study attempted for the first time to use antibodies to specifically localize different subtypes of melatonin receptors.

Signal Transduction via MT1 and MT2

Since both MT1 and MT2 melatonin receptors are G-protein coupled receptors, this review will briefly touch upon the G-proteins.

G-proteins:

Signal transduction is pivotal to an organism's survival. For an organism to adapt to an ever-changing environment, it must immediately respond to changes in the environment. This detection of changes happens through signal transductions (signal processes), many of which are mediated by G-proteins. Ligands such as hormones, neurotransmitters, and chemokines exert their effects on their target cells by binding to heptahelical transmembrane receptors (G-protein coupled receptors) coupled to heterotrimeric G-proteins. (34) After a ligand binds to a receptor, it induces a conformational change that activates a heterotrimeric G-protein.

Heterotrimeric G-proteins are composed of alpha, beta and gamma subunits; they are located on the intracellular surface of a cell. (35) G-proteins are classically divided into four different families based on their alpha subunits: $G_{i/o}$, G_s, $G_{q/11}$, $G_{12/13}$. If the G-protein is in an inactive state, guanosine diphosphate (GDP) is bound to the alpha-subunit of the G-protein. Once the G-protein is activated, GDP gets released from the alpha subunit and guanosine triphosphate (GTP) associates with the alpha subunit. This association causes a dissociation between the G-beta-gamma and the G-alpha-GTP subunits. The G-beta-gamma and the G-alpha-GTP subunits can activate many intracellular effectors to mediate further signalling. To study the cellular signaling mediated by G-proteins, researchers have used Pertussis Toxin (PTX; a virulence factor synthesized by *B. pertussis*) as a reagent in mammalian cells in signaling studies. PTX has an inhibitory effect on G-protein coupled receptor (36) and PTX sensitivity also displays an involvement of G-proteins in the $G_{i/o}$ family. (37)

Melatonin receptors are seven transmembrane-spanning proteins belonging to the GPCR superfamily. In mammals, two melatonin receptor subtypes have been cloned, the MT1 and MT2 which are encoded by the MTNR1A and MTNR1B genes respectively.

MT1:

The MT1 melatonin receptor is coupled to both PTX-sensitive (G_{i2} and G_{i3}) and insensitive ($G_{q/11}$) G-proteins. (38) Activation of the MT1 melatonin receptor inhibits forskolin-stimulated cyclic adenosine monophosphate (cAMP), (39, 20) protein kinase A signaling, and cAMP-responsive element binding protein (CREB) phosphorylation. (40) The inhibitory effect on forskolin-stimulated cAMP formation was abolished when the non-selective MT1/MT2 antagonist luzindole is pre-administered. (41) Moreover, activation of the MT1 melatonin receptor increases the phosphorylation of mitogen-activated protein kinase 1 and 2 (MEK1 and MEK2), extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), (42) and potassium conductance through K_{ir} inwardly rectifying channels. (43)

MT2:

The MT2 melatonin receptor is also coupled to the inhibition of forskolin-stimulated cAMP formation. (23) Petit and colleagues (44) also examined a potential modulation of cyclic guanosine 3'-5'-monophosphate (cGMP) by expressing them in human embryonic kidney cells. While MT2 modulated cGMP level in a dose-dependent level, MT1 did not show any modulation of the cGMP level. (44) MT2 signaling also increases the level of protein kinase C (PKC) activity in the SCN; this effect is blocked by an administration of the MT2 selective antagonist 4P-PDOT. (41) Like with MT1, MT2's inhibitory effect on forskolin-stimulated cAMP formation is also abolished when luzindole is pre-administered. (41) Moreover, MT2 activation reduces calcium-dependent release from the rabbit retina. (26, 45)

GPR50:

Besides MT1 and MT2, another mammalian melatonin receptor-related receptor known as GPR50 has been isolated. (27) However, unlike MT1 and MT2, melatonin is unable to bind to GPR50 as a ligand. This receptor remains an orphan because its natural ligand is unknown. (46) GPR50 also belongs to the GPCR family. GPR50 is now believed to be a mammalian orthologue of Mel1c melatonin receptor subtype based on an in silico approach and an examination of the synteny between the two genes. (47)

MT3:

As mentioned above, melatonin displays a low affinity for MT3. Originally, it was widely believed that MT3 belonged to the GPCR family. However, MT3 turned out to be a human homologue of cytosolic enzyme quinone reductase II. (48) The existence of MT3 was initially hypothesized after researchers observed its different binding and kinetics profile from those of MT1 and MT2. (48) The existence of MT3 was confirmed when it was purified via affinity chromatography; purified MT3 showed a 95% homology to quinone reductase II, which has detoxifying properties. The pharmacological profile of MT1 and MT2 is 2-iodomelatonin>melatonin>>N-acetylserotonin, whereas that of MT3 is 2-iodomelatonin>melatonin=N-acetylserotonin. (13) More studies should explore the relationship between melatonin's antioxidative effects and the functionality of the MT3 receptor.

Nuclear Receptors:

Melatonin also mediates its physiological actions by binding to nuclear receptors. Nuclear receptors are ligand-inducible transcription factors that can affect gene expression and thereby regulate development, the maintenance of homeostasis, cellular proliferation and differentiation, and apoptosis. (49) There are about 200 members of the nuclear receptor superfamily, a vast number of which are orphans. (50) Melatonin is a natural ligand of nuclear receptors that belong to the subfamily of retinoid Z receptors or retinoid orphan receptors (RZR/ROR). (51) There are three members of the RZR/ROR subfamily: RZR/ROR(alpha), RZR(beta) and ROR(gamma). (52) It has been reported that melatonergic signalling via nuclear receptors mediates aspects of the immune system. Indeed, RZR/ROR(alpha) activation by melatonin increases the level of interleukin (IL) 2 and IL-6 production by human mononuclear immune cells. (53) The RZR(alpha) nuclear receptor is also able to repress the expression of the gene 5-lipoxygenase, an enzyme responsible for the biosynthesis of allergic and inflammatory mediators, in human B lymphocytes. (54) On the other hand, RZR(beta) melatonin receptor mRNA has been found using *in situ* hybridization in sensory regions such as the cortical areas of the somatosensory, visual and auditory systems, the thalamic nuclei for each of the sensory pathway, and the dorsal horn of the spinal cord. (55) These results suggest that RZR(beta) plays a selective role as a transcription factor in the sensory system. (55) After the initial identification of the nuclear receptor subfamily, research in this area has become dormant. More studies are needed to unravel the selective roles of RZR/ROR(alpha), RZR(beta) and ROR(gamma).

To recapitulate, melatonin exerts its physiological effects by binding to the membrane-bound proteins MT1 and MT2 (Kd=10–200 pM) and, with lower affinity, to MT3 (Kd=3–9 nM). (48) It also likely exerts its effects by binding to the nuclear receptors subfamily ROR/RZR.

Functions of MT1 and MT2

Selective Ligands:

Due to a paucity of available selective partial agonists for melatonin receptor subtypes, the functional characterization of MT1 and MT2 melatonin receptors remains incomplete. Pharmacological studies have been carried out using antagonists such as luzindole (MT1/MT2 nonselective)

and 4-phenyl-2-propionamidotetralin (4P-PDOT) (MT2 selective). Recently, there were also studies published that used selective partial agonists including N-{2-([3-bromophenyl]-4-fluorophenylamino)ethyl}acetamide UCM924 (MT2 selective). (24, 25, 33, 32) Characterization of MT1 and MT2 function has also been carried out via genetic deletion of either the MT1 and/or the MT2 receptor. (4)

Complementarity of MT1 and MT2:

MT1 and MT2 fulfill complementary or opposing roles (4) as demonstrated by several studies. For example, melatonin binds to two receptor subtypes in vascular smooth muscle: MT1 mediates vasoconstriction, whereas MT2 mediates vasodilation. (56) Moreover, it has been reported that MT1 and MT2 melatonin receptor subtypes mediate opposing modulations of the type-A gamma-aminobutyric acid (GABAA) receptor. (57) Potentiation of the GABA receptor-mediated current occurred via MT1 in the rat SCN. However, repression of the current occurred via MT2 binding in the hippocampus. GABA is the primary neurotransmitter responsible for synaptic inhibition in the central nervous system. (57) Moreover, MT1 and MT2 melatonin receptor subtypes mediate the regulation of temperature in an opposing manner, contributing to the circadian rhythm of body temperature. (*Gobbi et al.*, unpublished) These results suggest the selective roles of the MT1 and MT2 melatonin receptor subtypes.

Regulation of Sleep and Circadian System:
:
The SCN plays a pivotal role in regulating the wake/sleep cycle. Therefore, this review will first discuss the role of melatonin receptors MT1 and MT2 in the SCN, and then discuss their respective contributions to sleep.

There have been conflicting results on the differential functionalities of the melatonin MT1 and MT2 receptors in the SCN between *in vitro* and in vivo studies. Activation of the MT1 receptor in vitro in rodent SCN slices inhibits the neuronal firing of the SCN in a concentration dependent manner. (58, 59) While in vivo, the inhibition of neuronal firing in the SCN was not observed in mice with disrupted MT1 but was demonstrated in mice with disrupted MT2. (59,60) Activation of MT2 receptor in vitro shifts the phases in the SCN's electrical activity. (59) The melatonin-mediated phase shifts were abolished when the rat SCN slice was incubated with the selective MT2 antagonist 4P-PDOT. (58) However, these studies have never been applied in in vivo models and studies in MT2 knockout (KO) mice and with the selective MT2 agonists have not observed any phase shift. (32, 61) Furthermore, MT2 receptors are scarcely present in the SCN. (31) Dubocovich and her colleagues (62) used wheel running activity as a measure of assessing circadian behaviour (63) and measured the effects of melatonin administration on circadian phase shifts and on re-entrainment after a change in the timing of dark onset on wild type (WT) and MT1 KO mice. Melatonin was given to both groups 2 hours before the onset of the circadian phase shift. MT1 KO mice did not display the phase shifting that was observed in WT mice after the administration of melatonin. This study demonstrates that the MT1 receptor is responsible for mediating phase shifts in vivo.

Altogether, these results suggest that the MT1 receptor, and not the MT2 receptor, is primarily involved in phase shifting. However, it will be crucial to perform further in vivo studies with selective full and/or partial agonists and antagonists to further elucidate the differential functionalities of MT1 and MT2 melatonin receptors.

The architecture of normal sleep involves a progression from wakefulness to non-rapid eye movement sleep (NREMS) and then to rapid eye movement sleep (REMS). The state of wakefulness, REMS, and NREMS show different electrical signatures in electroencephalogram testing (EEG). EEG is paired with electromyogram (EMG) and is the gold standard technique for sleep research at both preclinical and clinical levels. (39)

Melatonin plays an important role in generating the sleep architecture. Recently, Ochoa-Sanchez et al. examined the effects of the partial MT2 receptor agonist UCM765 on the sleep–wake cycle of rats and that of mice lacking MT1 or MT2 receptors. (32) The administration of the novel MT2 receptor partial agonist UCM765 promoted NREMS in WT mice, whereas this administration did not result in these effects in MT2 KO mice. (32) MT2 KO mice showed a reduction in NREMS compared to the WT mice, but the MT1 KO mice did not show reduction in NREMS (Fig. 4). (32) This study demonstrates that the MT2 melatonin receptor is responsible for mediating NREMS. (25, 32) In another study, the same group showed that MT1 KO mice displayed a 37.3% decrease in REMS duration, whereas MT2 KO mice showed only a 17.3% decrease in NREMS duration in a different study. (61). Altogether, these findings illustrate that MT1 and MT2 play selective roles in sleep: MT1 is responsible for mediating the effects of melatonin on REMS whereas MT2 is responsible for mediating the effects of melatonin on NREMS.

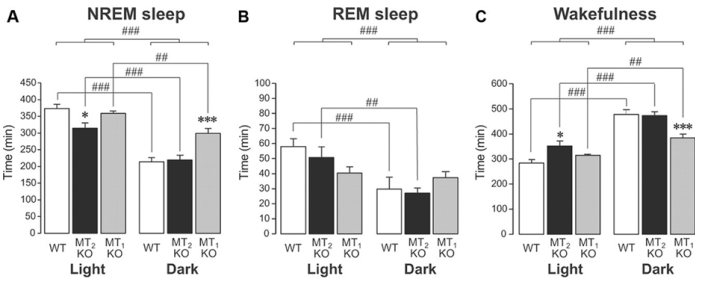


Fig. 5. The distribution of cosmic string loop radii. Note that there is a large drop-off after R_{c1}.

In summary, the melatonin receptors MT1 and MT2 exhibit opposing roles in sleep. The MT1 receptor activation increases REMS whereas the activation of MT2 receptor in reticular thalamus induces NREMS. Since melatonin has similar affinities to both MT1 (pKi=9.85) and MT2 (pKi=9.62), (64) this lack of selectivity can explain why a non-selective agonist such as melatonin is a less valid hypnotic promoter (65). However, a selective MT2 receptor agonist could cause potent hypnotic effects (Fig. 4).

Pain:

Several studies have analyzed the potential role of melatonin to modulate pain. (3, 66) Melatonin reduces nociception to different levels of pain-inducing stimuli. (3) For example, intraperitoneal (i.p.) administration of melatonin demonstrated analgesic effects in mice. (67) Golombek et al evaluated the pain threshold by measuring the latency of the response to the stimulus through the hot plate test. The hot plate evokes spinally processed pain-related behaviors and thus measuring nociception in rodents. (68) Since the synthesis of melatonin occurs in a circadian pattern, (10) a time-dependent melatonin-induced analgesia was observed. (67) The maximal melatonin-induced analgesic effect has always been expected to be at nighttime as melatonin synthesis peaks during the dark phase. (10) The mice displayed a maximal analgesic effect (longest latency in the hot plate test) at 8:00 PM, which supports the claim that melatonin mediates analgesic effects. Moreover, melatonin-induced analgesic mechanisms seem to involve the opioid receptors as melatonin-induced analgesia is blocked by naloxone, a non-selective antagonist of opioid receptors. (3)

Yu and colleagues (69) suggested that the MT2 melatonin receptor subtype is responsible for melatonin-induced antinociception in a study employing the antagonist luzindole, which has a 25-fold greater affinity for MT2 compared to MT1. (70) By using the novel MT2 selective partial agonist UCM924 at the doses of 20-40 mg/kg, Lopez-Canul and colleagues found that UCM924 mediates a superior antinociceptive effect than a larger dose (150mg/kg) of melatonin does. (33) This analgesia was nullified when the rat was pre-injected with MT2 receptor selective antagonist 4P-PDOT, a finding which further supports that MT2 receptors mediate melatonin-induced analgesia (Fig. 5). (33) The authors also demonstrated that this analgesic effect occurs by deactivating pronociceptive (ON) cells and activating antinociceptive (OFF) cells in the descending pain-control pathway (vlPAG-rostral ventral medulla) (Fig. 6). (33) A recent study suggests a novel epigenetic mechanism through which melatonin decreases neuropathic allodynia (pain that occurs from harmless stimuli) via its activity at the MT2 receptor; this mechanism involves a decrease in the expres-

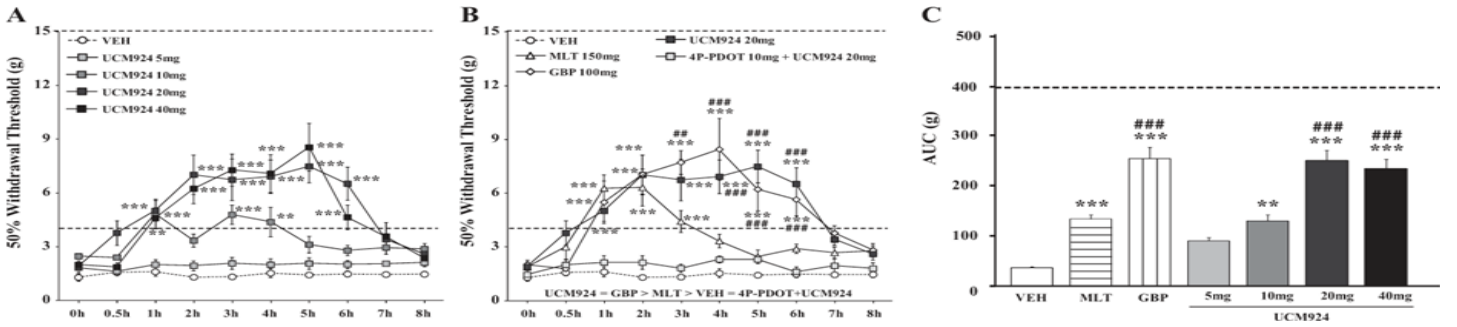


Figure 5. UCM924 reduces tactile allodynia in neuropathic rat model. Time course and dose-response (A). Comparison amongst UCM924, Melatonin and Gabapentin (analgesic drug) effects (B). Area under the curve (AUC) of antiallodynic effect of UCM924, Melatonin and Gabapentin (C). Reprinted from Lopez-Canul et al. Selective melatonin MT2 receptor ligands relieve neuropathic pain through modulation of brainstem descending antinociceptive pathways. Pain. 2015;156(2):305-17. Reprinted with permission.

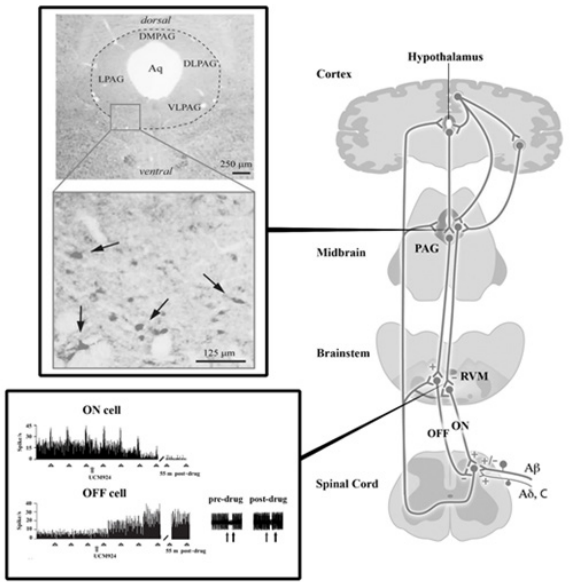


Fig 6. Descending pain-control pathway (vlPAG-rostral ventral medulla) mechanism. MT2 receptors localized in vlPAG activate antinociceptive OFF cells and deactivate pronociceptive ON cell projection in rostral ventral medulla. Reprinted from Posa et al. Targeting melatonin MT2 receptors: A novel pharmacological avenue for inflammatory and neuropathic pain. Curr Med Chem. 2017 Feb 8. doi: 10.2174/0929867324666170209104926.

sion of phosphatase 2A's catalytic subunit. (71) The decrease in allodynia was blocked by the selective MT2 antagonist 4P-PDOT. (71) Additional studies are required to better characterize the biochemical mechanisms of melatonin-mediated pain modulation.

Conclusion

This review summarizes some recent findings about melatonin, its receptor subtypes MT1 and MT2, their differential physiological roles, and their differential molecular pathways. Studies of the melatonin receptors have been made possible by the use of selective partial agonists and antagonists for specific melatonin receptor subtypes, and genetic deletion of each subtype.

Melatonin is a pleiotropic hormone that mediates various physiological functions in different organs of the body. Due to its implication in so many health-related issues, further studies are required to fully characterize the functionalities of each melatonin receptor subtype. Such characterization could help countless patients who suffer from circadian rhythm-, pain-, and sleep-related health issues.

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Si Jia Wang[1]

The Role of Ubiquitin in the Survival of Legionella pneumophila in Eukaryotic Host Cells

Review Article

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Abstract

Background: Eukaryotic cells use essential ubiquitin-mediated pathways in their defense against pathogenic bacteria, such as Legionella pneumophila, the intracellular pathogen of Legionnaire's disease. Despite the protective role of these pathways, L. pneumophila virulence has evolved to secrete numerous effector proteins involved in co-opting host ubiquitin-mediated processes to facilitate their survival. Many of these effector proteins are of great research interest in the quest to demystify the molecular mechanisms underlying L. pneumophila pathogenesis as the bacterium has a vast repertoire of effector proteins.

Methods: Articles were obtained from scientific literature databases such as PubMed and the McGill library. Selected articles provided an overview of the ubiquitination pathway, eukaryotic autophagy, L. pneumophila pathogenesis, and structural and functional analysis of L. pneumophila and other bacterial effectors involved in subverting host ubiquitin systems.

Summary: This review discusses the current structural and functional characterization of L. pneumophila protein effectors involved in exploiting host ubiquitin machinery to facilitate intracellular bacterial survival. These protein effectors include those with E3 ubiquitin ligase activity, LubX, AnkB, and SidC, which respectively mediate bacterial nutrient acquisition, temporal regulation of other effectors, and remodelling of the L. pneumophila replicative niche; the SidE family of effectors, which mediates the first novel, single-enzyme ubiquitination pathway and deubiquitination; and ravZ, a protease promoting evasion of host autophagy. However, the exact molecular functions and biological consequences of these effectors as well as the full repertoire of L. pneumophila effectors facilitating ubiquitin-mediated survival still require further investigation.

Introduction

Legionella pneumophila

Legionella pneumophila is a gram-negative bacterium typically found in aquatic environments that is a facultative intracellular pathogen. (1) Its natural hosts are protozoans, such as the amoeba Acanthamoeba castellanii, but *L. pneumophila* also infects mammalian alveolar macrophages, causing an atypical form of pneumonia known as Legionnaires' disease. Legionnaires' disease has a fatality rate of 8-12% in healthy individuals and up to 34% in nosocomial cases. (2, 3) While person-to-person transmission of *L. pneumophila* infections has not been reported, humans often are known to contract *L. pneumophila* infections by inhaling water droplets contaminated with the bacteria spread through aerosolized systems such as cooling towers or air condition systems. (4) The increased presence of man-made systems is thought to have facilitated the evolution of *L. pneumophila* infection in humans, hence *L. pneumophila* is often referred to as an accidental human pathogen. (4)

Following uptake into host cells by phagocytosis, *L. pneumophila* uses a Dot/Icm type IV secretion system which translocates over 300 bacterial proteins, known as termed effectors, into the host cytosol. (5) Some of these effectors are known to modulate eukaryotic pathways to establish a replicative niche, the *Legionella*-containing vacuole (LCV), which evades the endosomal-lysosomal degradation pathway activated by the host cell's immune response. (1) Other effectors are likely involved in triggering apoptosis of macrophages and alveolar epithelial cells during early infection, bacterial replication and growth, and finally, a pore-formation mechanism that induces lysis of the host cell during late infection. (6) Eukaryotic cells modulate numerous host processes to support *L. pneumophila* proliferation, which makes these effectors potential targets for drug development. (7) While antibiotic resistance is not a current issue for *L. pneumophila*, many effectors interfere with host cell immune signalling pathways and characterizing these effectors may also facilitate the future adaption of bacterial effectors to treat human diseases such as autoimmune disorders. (8) However, functional elucidation of *L. pneumophila*'s effectors has proven challenging given their highly redundant nature and lack of homology to currently characterized proteins. (7)

Legionella pneumophila Growth Requires Ubiquitin

Upon microbial infection, eukaryotic cells activate ubiquitin-mediated processes, such as proteasomal degradation of ubiquitinated pathogenic proteins, as part of their defense response. (9) Despite this protective role in host cells, studies delineate a paradoxical importance of ubiquitin in *L. pneumophila* infection. (10-12) LCVs are enriched in polyubiquitinated conjugates, and this vacuolar membrane remodelling is credited to the functions of effector proteins, although the exact mechanisms are not well established. (10) A proteomic study revealed that the majority of these ubiquitinated proteins are involved in host immune response, signaling, regulation, intracellular trafficking, and amino acid transport pathways. (11) Furthermore, inhibition of ubiquitin-mediated proteasome function using dsRNA-mediated knockdown of the proteasomal subunit Rpn11 or the proteasomal inhibitor Mg-132 resulted in a significantly decreased intracellular bacterial replication in *Drosophila* cells. (10) SiRNA depletion of the host cdc48/p57 complex, an AAA ATPase required for proteasomal degradation of polyubiquitinated proteins, also diminished *L. pneumophila* proliferation and produced an accumulation of ubiquitinated proteins on the LCV surface. (10) In accordance to this dependence on host ubiquitin systems, *L. pneumophila* has been shown to employ several effectors to co-opt these processes and facilitate its survival. (12) This review will explore the effectors involved and the current understanding of how they manipulate ubiquitin-mediated processes in *L. pneumophila* infections.

Overview of the Canonical Ubiquitin System

Ubiquitination is a highly conserved and regulated eukaryotic post-translation modification that targets proteins for degradation or modifies their function. (9, 13) Specifically, ubiquitination is the addition of the 8.5 kDa eukaryotic protein ubiquitin on amino groups of residues, frequently lysine, in protein substrates through covalent linkages. The seven lysine residues within ubiquitin can be used to conjugate subsequent ubiquitin

moieties, forming polyubiquitin chains. (14) This molecular modification regulates a myriad of intracellular processes such as endocytosis, signal transduction, and transmembrane protein trafficking. (15, 16) Substrates may be monoubiquitinated or multiubiquitinated, where multiple lysine residues are monoubiquinated. (17) In addition, Lys63-linked chains mark substrates involved in lysosomal degradation, DNA damage repair, cellular signaling, intracellular trafficking, and ribosomal biogenesis. (18, 19) Among these forms of ubiquitination, substrates with polyubiquitin chains linked through the Lys48 side chains of ubiquitin are destined for proteasomal degradation.

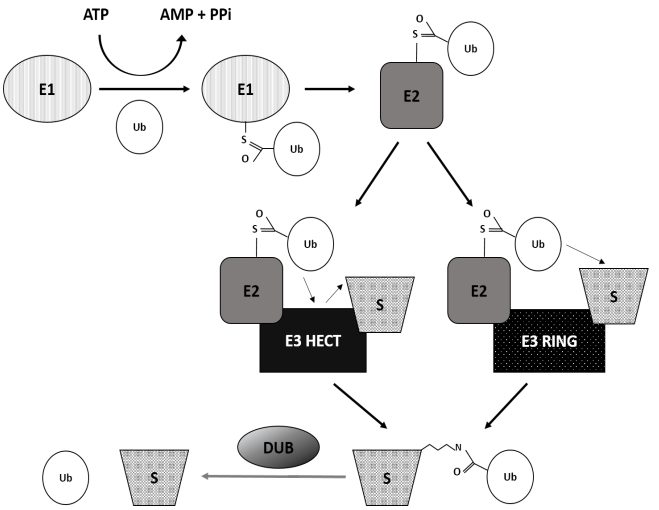

Figure 1: Eukaryotic Ubiquitination Pathway. E1 binds activates ubiquitin (Ub) using ATP before transferring it to E216. E3 binds the E2-Ub complex, catalyzing ubiquitination of the substrate (S) on a lysine residue through a HECT domain, which covalently bind ubiquitin before transferring it to substrates, or a RING domain, which bring ubiquitin and substrates close together16. DUBs remove ubiquitin16.

The aforementioned forms of ubiquitination are catalyzed by the same sequential, three-step enzymatic cascade (Fig. 1). (17) This process begins with the E1 ubiquitin activating enzyme, which catalyzes the formation of a thioester bond between its catalytic cysteine residue and the C-terminal glycine of ubiquitin in an ATP and Mg2+-dependent manner. (17) Following the transfer of the activated ubiquitin to E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases coordinate the final transfer of ubiquitin onto substrates. (17) Finally, this process is reversible through the action of substrate-specific deubiquitinases (DUBs), which hydrolyses linkages between the substrate and ubiquitin or between ubiquitin moieties. (13)

E3 ligases mediate substrate selectivity, allowing cells accordingly to encode numerous E3 enzymes: human cells, for example, have two E1 enzymes, 37 E2 enzymes, and over 600 E3 ligases. (17) E3 ligases are classified according to four particular domains: HECT (homologous to the E6-AP C-terminus) domain, RING (really interesting new gene) finger domain, a U-box domain, or an RBR (Ring Between Ring) domain. (20, 21) HECT domains bind ubiquitinated E2 and catalyzes the formation of a thioester linkage between its cysteine residue and ubiquitin prior to transferring ubiquitin to the substrate. (20) In contrast, RING finger domains function as adaptors, forming protein binding motifs stabilized by the coordination of Zn2+ with their cysteine and histidine residues, which serve as scaffolds that bring E2 and the substrate close together to catalyze ubiquitin transfer. (20) Skp1-Cullin-F-box (SCF) complex is a major RING finger-containing E3 ubiquitin ligase family: the F-box binds target substrates, Cullin is a scaffold protein, and Skp1 acts as an adaptor protein. (22) U-box domains are classified as modified RING domains that function as adaptors. While they structurally resemble RING domains, U-box domains lack the key residues involved in Zn2+ chelation. (23) Lastly, the RBR E3 ligases are multi-domain proteins comprising of an IBR (InBetweenRING) domain and two domains whose sequences bear resemblances to the RING1 and RING2 domains. (21) While all three domains contain several cysteine residues that co-ordinate Zn2+, the IBR lacks the catalytic cysteine required for ubiquitination. (21) Furthermore, the RING2-like domain does not structurally conform to canonical RING2 domains, but it contains the essential catalytic cysteine which mediates ubiquitin transfer from an E2 enzyme to the substrate via a thioester linkage. (21) Given the diversity and function of E3 ubiquitin ligases, *L. pneumophila* have unsurprisingly developed several effectors mimicking E3 ubiquitin ligases. (24)

Discovery of Noncanonical Ubiquitination in Legionelle pneumophila

The SidE effectors represent the first examples of an all-in-one ubiquitination machineries. (25) Recently, Qiu et al. discovered that the SidE effector family of *L. pneumophila* mediates ubiquitination independent of E1 and E2 enzymes. (26) Previously, ubiquitination has been reported to occur with E2 enzymes directly ubiquitinating proteins containing a ubiquitin-binding domain. (27) SidE ubiquitination proceeds in the absence of not only E1 and E2 enzymes but also of cofactors ATP and Mg2+. (26) Furthermore, the C-terminal glycine and lysine residues of ubiquitin were non-essential. (26)

The SidE family consists of SdeA, SdeB, SdeC, and SidE which all reside on the cytosolic face of the LCV. (28) Through sequence analysis, all four proteins were found to contain a mono-ADP-ribosyltransferase (mART) motif, R-S-ExE, which catalyzes the transfer of ADP-ribose groups from nicotinamide adenine dinucleotide (NAD) to arginine residues of substrates. (28) This noncanonical ubiquitination (Fig. 2) begins with the transfer of ADP-ribose onto R42 of ubiquitin by the mART motif followed by the transfer of the activated ubiquitin to its substrate. (26, 28) Currently, known substrates of the SidE effectors are the eukaryotic Rab GTPases Rab1, Rab6A, and Rab33b. (25, 26) However, the biochemical consequences of the SidE effector family, substrate selectivity, the mechanisms of activated ubiquitin transfer to the substrate, and the nature of the linkage between ubiquitin and Rab remain to be investigated. (28)

Compared to wild-type *L. pneumophila*, strains lacking all SidE genes were observed to have reduced virulence in the natural host D. discoideum. (29) However, this effect was not observed in the infection of alveolar macrophages and the exact downstream effects of the SidE effectors in eukaryotic hosts are still unclear. (29) Since SidE proteins are expressed early in host cell infection and interact with Rab proteins, which are involved in membrane trafficking and phagosome formation, they are hypothesized to play a role in the evasion of the endocytic pathway and/or LCV maturation. (26, 30)

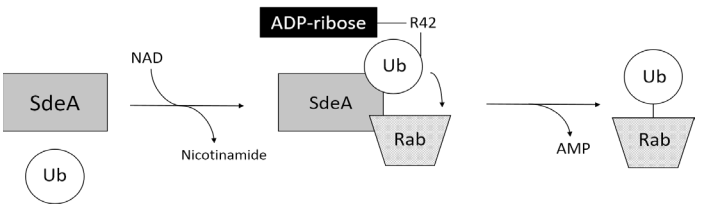

Figure 2: Schematic of Noncanonical Ubiquitination Mediated by SdeA. SdeA catalyzes ADP-ribosylation of R42 on ubiquitin (Ub) using NAD. SdeA then ubiquitinates its substrate, Rab proteins24. Currently, the mechanism of ubiquitin transfer and the nature of the substrate-ubiquitin linkage remain to be elucidated24.

Deubiquitinating Activity in *L. pneumophila*

Numerous bacterial DUBs have been characterized, such as ChlaDub1, YoP, and YopJ from Chlamydia trachomatis, Yersinia enterocolitica, and Yersinia pseudotuberculosis respectively, which all function to inhib-

it host cell NF-κB activation. (31-33) DUBs are postulated to exist in *L. pneumophila*, although few have been discovered or fully characterized. (24, 34) Currently, effectors of the SidE family are known to possess an N-terminal DUB domain. (35) Through structural studies, this particular DUB domain was found to contain a canonical ubiquitin-like protease domain, which cleaves ubiquitin from substrates. (35, 36) Mechanistically, the DUBs of the SidE family mediate deubiquitination of Lys11, Lys48, and Lys63-linked polyubiquitinated proteins on the LCV using its conserved Cys-His-Asp catalytic triad. (35) Infection of mouse bone marrow macrophages with *L. pneumophila* lacking all members of the SidE family exhibited decreased proliferation and a 90% increase in ubiquitinated species surrounding the LCV. (35) However, while the addition of an inactivated catalytic cysteine to alanine mutant of SdeA with a DUB domain mutation rescued the growth defect, the accumulation of ubiquitin species was not restored to wild-type levels. (35) This DUB domain was shown to be non-essential to the novel ubiquitination mechanism, but is believed to play a role in polyubiquitination of the LCV. (35)

E3 Ubiquitin Ligases in *Legionella pneumophila*

Although bacteria lack the proteins involved in a canonical eukaryotic ubiquitin system, numerous studies indicate that bacteria have developed an array of compatible ubiquitin ligase-like effectors. (37) These effectors enable bacteria to hijack host ubiquitin systems and modulate a variety of signalling cascades to secure their survival. (37) For example, *Salmonella typhimurium* contains SspH2 and SlrP, E3 ubiquitin ligase-like effectors involved in inducing IL8 secretion and host cell death respectively. (38, 39) In *L. pneumophila*, several secreted effectors were found to mimic eukaryotic E3 ubiquitin ligases through their possession of F-box or U-box domains, which facilitate *L. pneumophila* co-option of its host ubiquitin system. (40) Bioinformatic analyses hypothesize that acquisition of these eukaryotic-like effectors occurred through an inter-domain horizontal gene transfer, the process by which pathogens acquire and incorporate foreign eukaryotic genetic material into their genome. (41) This review will focus on the currently identified and characterized E3 effectors in *L. pneumophila*, summarized in Table 1, with their observed functions.

LubX

LubX (*Legionella* U-box protein) is a 215 amino acid long effector that contains two U-box domains. (42) In vitro reactions indicate that LubX mediates auto-ubiquitination and polyubiquitination. (43) This process is E1 and E2 dependent in which LubX interacts with the currently defined subset of E2 enzymes: UBE2D1, UBE2D3, UBE2D2, UBE2D4, UBE2E2, UBE2E3, and UBE2W1. (42, 43) Of note, U-box 1, the motif with the most N-terminals, retains canonical E3 ubiquitin ligase activity while U-box 2, located after U-box 1, binds target substrates, a non-canonical function

Effector	Lpg No.	E3-like Domain	Function(s)	Substrate(s)	References
LubX	2830	U-box	Promote bacterial growth in macrophages; regulate function/activity of other effectors	CLK1, SidH	31, 32, 33
AnkB	2144	F-box (RING domain)	Increase protein turnover, generating amino acids to support proliferation	ParvB, TRIM21	34, 35, 36
SidC	2511	Noncanonical 1	Recruit ER proteins and polyubiquitinated conjugates to the LCV	Unknown	37, 38, 39

Table 1: Summary of E3 Ubiquitin Ligases Secreted by L. pneumophila and Their Functions

previously not observed in eukaryotic U-box domains. (43) This functional difference is explained in part by the conservation of hydrophobic residues critical for E2 binding in U-boxes which have been lost in U-box 2. (42) Furthermore, sequence alignment indicates that an invariant proline residue found in active U-box domains was missing from loop 2 of U-box 2. (42, 44) While both U-box domains are highly similar to eukaryotic versions, these amino acid differences are postulated to account for their divergent functions. (42)

LuxB substrates have been found to be similar to Cdc2 kinase 1 (Clk1) through yeast two-hybrid and co-immunoprecipitation assay and SidH through bioinformatics assessment. (43, 45) Clk1, a eukaryotic protein whose expression is essential to *L. pneumophila* virulence, is involved in regulating alternative mRNA splicing by phosphorylating members of a family of serine and arginine-rich splicing factors expression. (43, 46) While LubX mediates Clk1 polyubiquitination, Clk1 is not degraded and the biological consequences which are likely linked to modulation of gene expression are unclear. (43) However, splicing regulation has been observed to be involved in disarming host-induced antimicrobial responses. A recent study showed that *L. pneumophila* secretes effectors lgt1 and lgt2 which inhibit splicing of the XBP1 mRNA, suppressing the host unfolded protein response that arises following *L. pneumophila* infection. (46) In contrast, LubX, whose expression is elevated in late phases of infection, regulates the function of SidH, an effector expressed early during infection. (45) This activity in *L. pneumophila* makes LubX the first identified metaeffector, an effector that regulates the function of other effectors. (45) Unlike Clk1, LubX-mediated polyubiquitination of SidH leads to proteasome degradation in late stages of host-cell infection. (45) This temporal downregulation is necessary for *L. pneumophila* proliferation. Infection of *Drosophila melanogaster* with LubX mutants led to hyper-lethality in the flies and also decreased intracellular viable bacterial counts relative to wild-type *L. pneumophila*. (45) These phenotypes were rescued through introducing SidH mutants with LubX mutants. (45) Prolonged SidH expression is toxic to both host and bacteria, necessitating the ubiquitination activity of LubX. (45, 47)

AnkB

AnkB is essential for *L. pneumophila* virulence as AnkB mutants exhibit severe defects in proliferation in both human and amoeba hosts. While the LCV of these mutants retained proper vacuolar remodelling, and evaded lysosomal fusion, the loss of AnkB activity was associated with a decreased level of ubiquitinated proteins at the LCV. (48, 49) However, supplementing AnkB null mutants with amino acids, especially cysteine, serine, and pyruvate, rescued the growth defect and indicated that AnkB activity caters to the nutritional needs of *L. pneumophila*. (48)

Through bioinformatics and structural analysis, AnkB was confirmed to possess an N-terminal F-box domain which interacts with the host SCF E3 ubiquitin ligase complex. (50, 51) Furthermore, in vitro ubiquitination assays confirmed that AnkB mediates robust ubiquitination in the presence of E2 enzymes UBCH4A and UBCH5C. (52) AnkB is anchored to the LCV membrane through host-mediated farnesylation at the C-terminal CaaX farnesylation motif. (11) Here, the effector recruits Lys48-linked polyubiquitinated proteins to the LCV, which are subsequently degraded by the proteasome to release free amino acids. (11, 48) These amino acids represent a major carbon-rich source *L. pneumophila* use to produce energy via the tricarboxylic acid cycle to power bacterial growth and replication. (48)

AnkB substrates are not well established, but two interaction partners have been identified: eukaryotic parvinB (ParvB) and Trim21 proteins. (49) ParvB is involved in focal adhesion, cellular motility, and pro-apoptotic pathways. (53) Its deficiency decreases *L. pneumophila* proliferation but does not affect normal host viability. (49) Interestingly, AnkB null strains results in increased ParvB ubiquitination. (49) Furthermore, wild-type *L. pneumophila* was associated with increased caspase-3 activation and DNA fragmentation during infection compared to AnkB null strains. (49) This observation suggests that AnkB protect ParvB from ubiquitination by competing with endogenous ubiquitin ligases for ParvB binding, leading to apoptotic processes stimulation. (49) However, the biological importance of these effects to *L. pneumophila* survival is not clearly established. (49) In addition, Trim21 is a host E3 ubiquitin ligase which was recently

found to mediate Lys11-linked polyubiquitination on AnkB. (54) Host proteins ubiquitinated by Trim21 are typically degraded by the proteasome, but this does not occur for AnkB. (54) Although this phenomenon is the first example of an interaction between Trim21 and a bacterial effector protein, the biological significance remains to be elucidated. (54)

SidC

Unlike AnkB and LubX, SidC exhibits E3 ubiquitin ligase activity mediating polyubiquitination through several lysine linkages while having no structural homology to canonical E3 domains. (55) SidC contains a catalytic triad in the N-terminal domain, termed the SNL domain, that is typical of cysteine-based proteases and DUBs. The triad comprises of amino acids cysteine (C46), histidine (H444), and aspartic acid (D446). (55) While the crystallized SNL domain differs structurally from HECT domains, the cysteine residue of the triad is postulated to function similar to a nucleophile and SidC is thought to define a unique family of ubiquitin ligases. (55)

SidC binds to phosphoinositide lipid PI(4)P, which is abundant on mature LCV surfaces, through its C-terminal PI(4)P binding domain, P4C. (56) Binding of PI(4)P was associated with an increase in the ubiquitin ligase activity of SidC, presumably due to a conformational change in SidC which increased accessibility of the catalytic site. (56) Crystal structures of near full length SidC indicated that hydrophobic interactions mediate interactions between P4C and SNL domains. (56) Accordingly, mutation of a leucine residue in the P4C domain involved in this interaction to an arginine residue (L629R) resulted in a mutant SidC that preferably adopted an open conformation of the catalytic site and exhibited increased E3 ubiquitin ligase activity. (56) This result suggests a model of SidC regulation where SidC is inactive upon secretion and active when it is attached to the LCV and interacts with the host ubiquitin system. (56)

SidC functions as a tethering factor that recruits host ER vesicles, polyubiquitin conjugates, and arf-1 to the LCV via its SNL domain. (56, 57) *L. pneumophila* defective for SidC and its paralog SdcA showed delayed establishment of replicative vacuoles due to decreased recruitment of host ER proteins and polyubiquitin conjugates, necessitating E3 ubiquitin ligase activity in proper tethering. (55, 57) Interestingly, SidC is required in the monoubiquitination of Rab1, but this modification is not a result of direct ubiquitination by SidC. (55) Rather, SidC is thought to tether Rab1 and bring it proximal to its ubiquitin ligase. (55) Based on SidC's tethering functions, SidC ubiquitinated substrates ubiquitinated are currently unknown but are hypothesized to be host proteins involved in trafficking between the ER and membranes. (55)

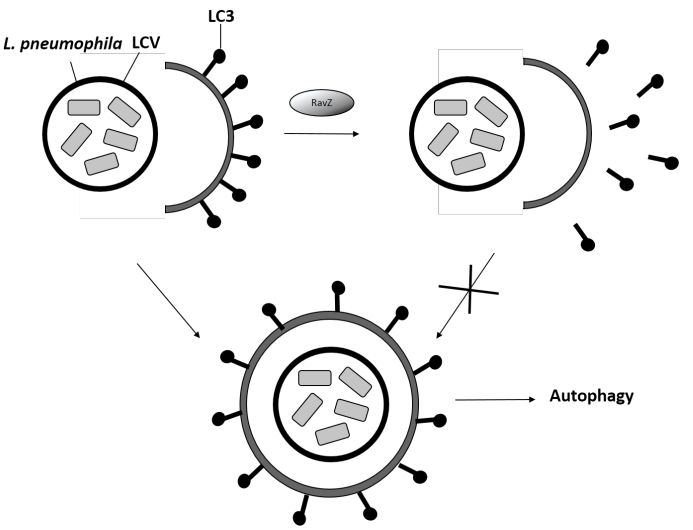

Figure 3: Evasion of Autophagy of L. pneumophila Mediated by RavZ. L. pneumophila secretes RavZ which decouples LC3 conjugated to PE on the membranes of autophagosomes. This blocks LC3-mediated extension of the autophagosome membrane, which prevents degradation of L. pneumophila by host autophagy pathways49.

Evasion of Autophagy by *Legionella pneumophila*

Eukaryotic cells engage in autophagy to selectively remove protein aggregates and damaged/surplus organelles as well as to non-specifically degrade proteins and organelles during cellular starvation to generate amino acids used to preserve essential processes such as protein synthesis. (58, 59) However, in response to bacterial infections, host cells can activate a form of autophagy termed xenophagy: the elimination of invading microorganisms by engulfing them in autophagosomes followed by fusion with lysosomes for degradation. (58) Selective autophagy of pathogens involves their ubiquitination, and in this case, ubiquitination of the phagosomal surface of the LCV. (60) Adaptor proteins, such as p62, bind to the LCV via the ubiquitin and also bind autophagosome-associated LC3-II which targets the ubiquitinated LCV to the autophagosome for degradation. (60) LC3 is the mammalian homolog of yeast autophagy related (ATG) 8 protein and is a ubiquitin-like protein that is cleaved by ATG4 to form LC3-II, which is conjugated through its C-terminal glycine to phosphatidylethanolamine (PE). (61) Functionally, LC3-II has been observed to facilitate autophagosomal membrane expansion. (61)

Despite facing elimination by the host cell's autophagy pathway, *L. pneumophila* has evolved mechanisms to circumvent ubiquitin-dependent xenophagy as evinced by the fact that *L. pneumophila* replicates in ubiquitinated LCVs evade the autophagy pathway. (62) Furthermore, *L. pneumophila* replication in its natural host, Dictyostelium discoideum, is increased when ATG9 is knocked out. (62) Following these observations, *L. pneumophila* was formally confirmed to interfere with xenophagy at the stage of autophagosome maturation via the secreted effector ravZ, a cysteine protease which functions similarly to ATG4 (Fig. 3). (62) However, unlike ATG4, ravZ is a deconjugating enzyme that specifically cleaves the amide bond between tyrosine and the PE-conjugated glycine of lipidated LC3, producing an LC3 product that cannot be re-conjugated to PE due to loss of the C-terminal glycine. (62) Hence, the loss of membrane-bound LC3 prevents p62 from delivering the ubiquitinated LCVs to the autophagy pathway. (61, 62) Intriguingly, macrophages infected with *L. pneumophila* lacking the ravZ gene were observed to retain the ability to prevent LC3 recruitment to LCVs, suggesting that multiple effectors are involved in disrupting the autophagy pathway. (62)

The ATG8/LC3 protein also plays an important role in susceptibility to bacterial infections such as in Parkinson's disease, a neurodegenerative disorder caused by mutations in the PARK2 gene that can result in decreased parkin expression and impaired protein function. (63, 64) In *Mycobacterium tuberculosis* infections, the ubiquitin ligase parkin mediates K63-linked polyubiquitination of the bacteria-containing phagosomes and was found to be essential for macrophages to impede *M. tuberculosis* replication, supported by the fact that PARK2-/- mice are more sensitive to infection. (63) *D. melanogaster* flies deficient for parkin were also defective in ATG8 processing when infected with *L. pneumophila* monocytogenes, indicating a potential role for ubiquitin ligases in mediating proper autophagic immunity. (63) Furthermore, numerous studies have noted that genetic knockouts of specific ATG genes correlated to increased susceptibility to various bacterial strains. (65-69) Together, the role of ATG8/LC3 in parkin deficient cells and *L. pneumophila* infection along with ATG gene deletion assays suggests the existence of other bacterial mechanisms that interact with ATG proteins to dictate bacterial resistance and susceptibility. (70, 71)

Conclusion

Ubiquitin-mediated processes play important roles in defending host eukaryotic cells against bacterial invasion, yet these processes have been proven indispensable to *L. pneumophila* virulence. (9, 12) As described in this review, *L. pneumophila* secretes several effectors that modulate the host ubiquitin system to bolster their own survival, as summarized in Fig. 4. (12) These results include the SidE family of effectors, which mediate deubiquitination and the first and only E1 and E2 independent ubiquitina-

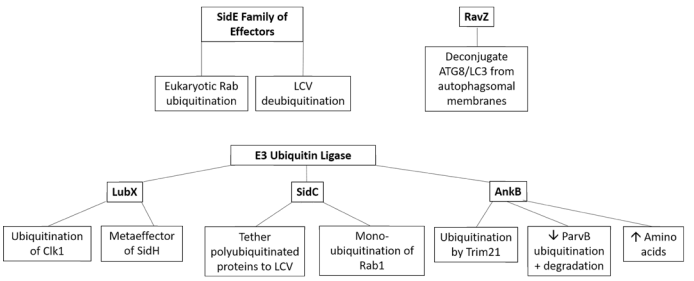

Fig. 4: Summary of L. pneumophila Effectors Involved in Ubiquitin-Mediated Survival Pathways in Eukaryotic Host Cells.

tion discovered to date; structural mimics of eukaryotic F-box and U-box type E3 ubiquitin ligases, which commandeer the canonical host ubiquitination machinery to mediate degradation and regulation of host proteins and bacterial effectors; and RavZ, which actively disrupts the ubiquitin-mediated autophagy pathway. (26, 35, 40, 62) Currently, these effectors are thought to affect various pathways such as LCV maturation, host gene expression, and bacterial nutrient acquisition. (6, 48) However, researchers still have much to elucidate regarding the largely unknown substrate spectrums and biological consequences of these effectors. As exemplified by parkin mediated bacterial resistance via ATG8/LC3, there may exist other undiscovered host protein-effector interactions causing differential susceptibility or fatality of *L. pneumophila* infections in infected individuals with other human diseases compared to healthy individuals. (3) Furthermore, research on numerous other pathogens indicate that bacteria secrete a wide variety of effectors that function as DUBS, post-translationally modify proteins in the host ubiquitin system, or interfere with pathogen ubiquitination in xenophagy. (24) Presently, these effectors are currently unobserved or not well characterized in *L. pneumophila*. (7) Part of the difficulty in identifying functions of effectors stem from the extensive redundancy of effectors in *L. pneumophila*. This challenge could potentially be circumvented by adopting new genetic screening methods, notably the insertional mutagenesis and depletion technique, continuing structural analysis, and developing novel robust assays, such as fluorescence resonance energy transfer, to elucidate novel effector-substrate relationships. (24, 37) Such efforts will facilitate the expansion of the current understanding and identified repertoire of effectors and substrates involved in the ubiquitin-mediated survival of *L. pneumophila*. In the future, this knowledge may contribute towards development of targeted antibacterial drugs and adoption of pathogenic molecules in treating human diseases. (7, 8, 24)

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George Sung[1]

Similar but Different: RBR E3 Ligases and their Domains that are Crucial for Function

Review Article

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Abstract

Background: The E3 ubiquitin ligases can be subdivided into four distinct types (RING, HECT, U-box, and RBR type) based on their domain architecture and ubiquitin transfer mechanism. Recent structures of different RBR E3 ligases have been solved showing enzymes in their autoinhibited state. The only exception is HOIP/HOIL-1L which was recently solved in its "active" conformation. This review discusses the structural and functional characteristics of three different members of the RBR E3 ubiquitin ligase family: Parkin, HOIP/HOIL-1L, and HHARI.

Methods: Searches were performed using PubMed. Search term includes "RBR E3 Ligase", "Parkin", "HOIP/HOIL-1L", "HHARI", "UbcH7", and "E2". In the end, 25 journal articles were selected as the foundation of this review. The structural coordinates of Parkin, HOIP, and HHARI were accessed from the PDB (www.rcsb.org) with the PDB IDs 4ZYN, 5EDV, and 4KBL, respectively.

Summary: Currently, most solved RBR E3 ligase structures are only in their inactive forms, except for HOIP/HOIL-1L, and these inactive forms provide valuable information on how these proteins are regulated in vivo. All the RBR E3 ligases have common domains, but their structures and functions are heavily dependent on their accessory domains, which serve as regulators that orchestrate certain ubiquitin chain syntheses and play a role in the autoinhibition of RBR E3 ligases. Although these domains are structurally different, they use distinct molecular interactions to achieve the same goal. While the regulation of most RBR E3 ligases has been extensively studied, more structural studies are required to further characterize the mechanism that these enzymes use to build different ubiquitin chains. Understanding the mechanisms underlying the formation of each type of ubiquitin chain could help elucidate their functions and related pathways.

Introduction

Protein turnover is an essential cellular process that allows non-functional or nonessential proteins to be degraded and new ones to be made. (1) One of the most prevalent degradation pathways is the protein ubiquitination pathway. (1,2) Protein ubiquitination is a key post-translational modification involved in the marking of cellular proteins for 26S proteosomal degradation.(1) The ubiquitin degradation pathway occurs via three steps: ubiquitin activation, conjugation, and the actual transfer of ubiquitin to the substrate. (1) The ubiquitin is activated by an E1 activation enzyme (E1) in an ATP-dependent reaction. (3) Ubiquitin is then transferred to an E2 conjugating enzyme (E2). (3) The E2 enzyme catalyzes the transfer of ubiquitin to either the E3 ligases (E3) or the substrate, depending on the type of E3 ligases used in the reaction. (4) These enzymes, particularly the RBR E3 ligases, accomplish the transfer of ubiquitin through the formation of thioester bonds. During the last step of the ubiquitination pathway, a stable isopeptide bond is formed between the substrate and the C-terminus of ubiquitin, as shown in Fig. 1. (3)

Ubiquitination can also occur multiple times on the same substrate, generating multiple ubiquitination sites. (1) Ubiquitination usually occurs on a lysine residue of the substrate, but can also occur on the N-terminus or more rarely on serine and threonine residues. Ubiquitination can also happen to ubiquitin to generate ubiquitin chains. (1) Different types of polyubiquitination chains lead to different signalling pathways, but often lead to protein degradation (Fig. 2). (1) For example, linear chains, which are generated by the HOIP/HOIL-1L complex or Linear Ubiquitin Assembly Complex (LUBAC), signal for the NF-kB pathway during an immune response. (1) In addition, K63 polyubiquitination often signals for DNA repair and lysosomal degradation, (1) whereas the K48 polyubiquitination signals for proteasomal or lysosomal degradation. (1, 5)

Since the human proteome is quite large, it is reasonable for there to be many kinds of E3 ligases to accommodate the different substrates. Four different categories of E3s have been characterized based on their domains and ubiquitin transfer mechanism. (7) The first type is the RING type ligase, which allows the direct transfer of ubiquitin from the E2 enzyme to the substrate. (7) The second type of E3 is the HECT type ligase, in which the ubiquitin is first transferred to a cysteine in the E3 ligase and then to the substrate. (7) The third type of ligase is the U-box ligases, in which the transfer mechanism resembles RING type ligases using their U-Box

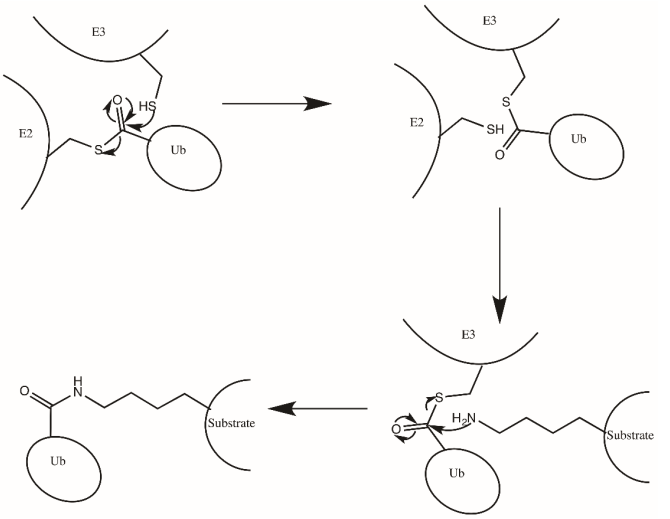

Figure 1: General ubiquitin transfer mechanism for RBR E3 ubiquitin ligases. Ubiquitin is transferred from E2 is transferred to the E3 via a conserved catalytic cysteine residue on the RING2 domain. Upon the binding the thiol from the E3 mediates a nucleophilic attack and facilitates trans-thiolation from the E2. The amine of the lysine on a substrate acts as a nucleophile to displace the E3 thiol and covalently attach the ubiquitin via an isopeptide bond.

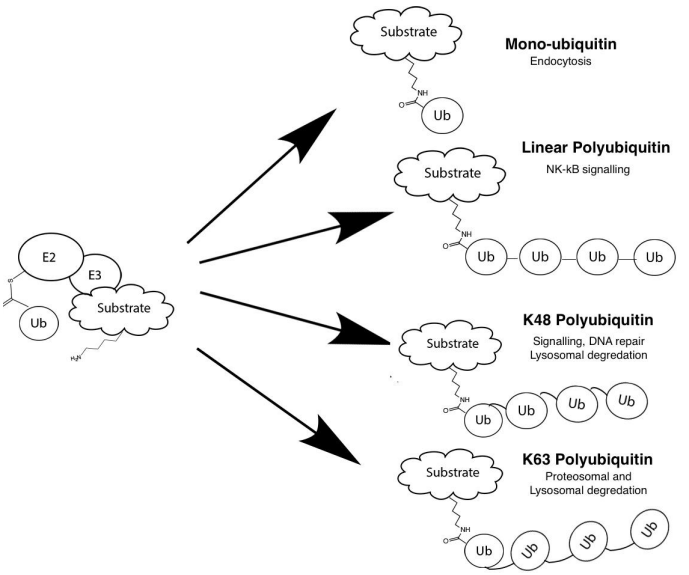

Figure 2: Different ubiquitin chains generated by various RBR E3 ligases. (1, 5, 6) The different ubiquitin chains shown have many different signalling pathways. Linear Chains could be generated by the LUBAC system by HOIP/HOIL-1L, K63 and K48 chains could be generated by either Parkin or the Ariadne Family.

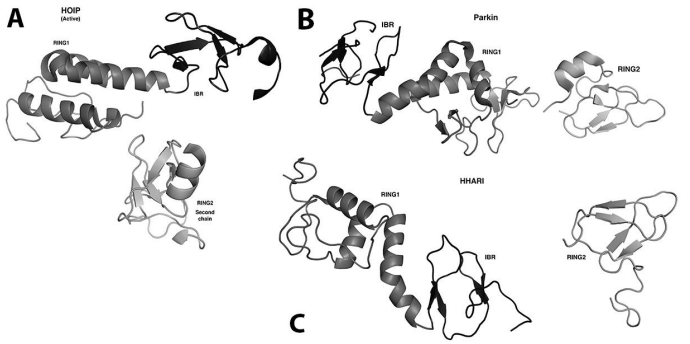

Figure 3: Comparison of the common domains of RBR E3 ligases. (9-11) The structural difference of the RING1, IBR, and RING2 domains among these proteins are shown. A) In the active form, two HOIP molecules interacts with two UbcH5B (an E2). The RING1 and IBR domain interacts with the E2 with another HOIP's RING2 domain. The different arrangement of these domains give rise to different regulation and activation mechanisms. (Structure coordinate from Lechtenberg et al. Nature 2016, Trempe et al. Science 2013, and Duda et al. Structure 2013)

domains rather than the RING domain. (8) The fourth type of ligase, and the focus of this review, is the RBR E3 ligase which uses a unique mechanism incorporating elements from the RING and HECT E3 ligases. (7) The different adjacent domains and ligands of RBR E3 ligases dictate their different functions in the cell. In this paper, the structural and functional characteristics of RBR E3 ligases will be discussed to analyze the common and distinct features between Parkin and other RBR counterparts, with particular focus on the Ariadne family and HOIP/HOIL-1L complex.

Structural Characterists of the RBR E3 Ligase Family

The RBR E3 ubiquitin ligases have three characteristic Zn2+-binding domains known as the RING1, in-between RING (IBR), and RING2 domains (Fig 3). In all of these domains, the coordination with Zn2+ ions occurs through seven cysteines and one histidine. (10) The RING1 domain is structurally similar and exhibits the same functional role for binding an E2 enzyme as typically observed for the RING domains of RING-type E3 ligases. (10) The RING1 domain, which is highly conserved among the RBR E3 ligases in sequence and structure, is important for the binding of an E2 enzyme. (10) For example, the RING1 domain displays the characteristic cross-brace motif structure observed in RING ligases (Fig 4). (10) The RING2 domain in the RBR ligases, despite bearing the same name, is not a true RING domain. Indeed, it is not able to bind to E2 conjugating enzymes and has a different structure. (10) In the RBR E3 ligases, the transfer of ubiquitin is done between the E2 enzyme and the RING2 domain through trans-thiolation (Fig 1) (3) The role of the IBR domain remains unclear, but the IBR domain is required for function and displays flexibility in known structures. (10, 12)

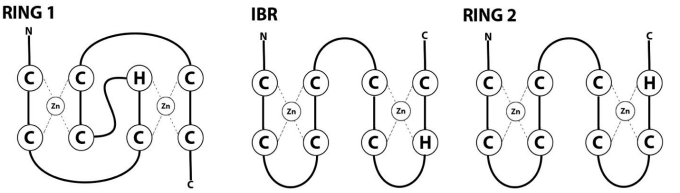

Figure 4: The topology of zinc fingers of each RING and RING-like domain in RBR E3 Ligases. The residues that coordinate structural zinc ions are shown in blue circles labeled as C, cysteine, and H, Histidine. The RING1 domain displays a topology that is characteristic of the RING domain in RING-type E3 ligases. The IBR and RING2 domains show similar topology. Upon studying the structure coordinate for various E3 ligases, the fold observed for the RING1 domain of Parkin is similar across the RBR E3 ligases.

Interaction between E2 and RBR E3 ligases

The RBR E3 ligases all contain a RING1 domain, which is involved in the binding of the E2 conjugating enzyme. (3) There are many E2 conjugating enzymes that have the ability to bind different E3s, and some E2s have the ability to either use the RING-type transfer mechanism (ubiquitin transferred directly onto the lysine residue of a substrate) or HECT-type mechanism (where ubiquitin is first transferred to the cysteine on the HECT E3, then to the lysine on substrate). (4) Moreover, the discovery of UbcH7 (E2) that could not transfer ubiquitin directly to a lysine residue indicated that the RBR E3 ligases behaved like HECT-type ligases. (13) However, since RBR E3s behave like the HECT-type and use the RING1 domain to bind to E2s, there are E2s that can interact with both types. (4) Some of the most common types of E2s that have been shown to interact with most RBR E3s are UbcH7 and UBCH5b. (13) These E2s have been shown to have some preference for the types of ubiquitin chains they make, but it has been demonstrated that E2s might not be as specific as we previously thought. For example, it has been documented that E2s such as Ube2K, which normally produce Lys 48 chains, produce a linear chain in conjunction with the HOIP/HOIL-1L complex. (14-16) Generally, researchers have suggested that the E2 might influence the type of ubiquitin chains it makes, but the HECT and RBR E3 ligases should presumably dictate the types of ubiquitin chains made.

What sets these RBR E3 ligases apart?

In addition to their three core domains, each RBR E3 ligase has other different domains that have similar functions. Parkin activity is modulated by its Ubl (ubiquitin-like) domain, a unique RING0 domain, and the Repressed Element of Parkin (REP) linker. Indeed, the Ubl domain and the REP linker of Parkin affect its binding to E2. (10) The Ubl domain is crucial for Parkin as it contains a site for phosphorylation by PINK1, a kinase localized at the mitochondria, during the mitophagy pathway. (17) PINK1 phosphorylation of Parkin is required for the activation of Parkin ubiquitination activity when mitochondria are depolarized. (17,18) Parkin will then ubiquitinate mitochondrial protein to promote mitophagy. (17) In addition, Parkin's unique RING0 domain contributes to the maintainencemaintenance of the Parkin autoinhibited conformation in the context of healthy mitochondria. (19)

HOIP and HOIL-1L are other types of RBR E3 ligases. Unlike other RBR E3 ligases, they both ligases form a complex to carry out polyubiquiti-

nation reactions. (20) Moreover, HOIP also includes a Ubiquitin Associated (UBA) domain, which is involved in autoinhibition; however,, but the binding site for the UBA domain on HOIP is still unknown. (9) Like Parkin, HOIL-1L contains a Ubl domain, but ; however, it acts as an trans-activating agent for the HOIP ligase instead of an autoinhibiting domain. (21) In addition, both HOIP and HOIL-1L contain NZF domains that are involved in the stabilization of linear ubiquitin chains. (21) The linear ubiquitin chain formed by the HOIP/HOIL-1L complex could then activate the NF-kB pathway by conjugating the ubiquitin chain to NF-κB essential modulator (NEMO). (22)

Another class of RBR E3 ligases is the Ariadne family, which has a signature Ariadne domain at their C-terminus. (23) The Ariadne domain in the HHARI ligase has been shown to interact with its RING2 domain, similarly to the RING0 domain of Parkin, to produce its own autoinhibition. (11). Despite having completely different structures, both Ariadne and RING0 domains restrict the access to the catalytic cysteine. (10, 11) HHARI have been shown to be activated by binding to neddylated Cullin-RING Ligases (CRL) complex, potentially allowing the regulation ofe the activity of HHARI activity. (24) HHARI activation leads to cellular proliferation due to the formation of nuclear bodies. (25)

How do cells regulate the ubiquitination activity of Parkin, A?

Although all the RBR E3 ligases have the three core domains, they are regulated very differently. Generally, the E3 ligases are autoinhibited due to the other domains within the ligase. (23) Different RBR E3 ligases have been found to inhibit themselves via various mechanisms involving different accessory domains. While these accessory domains may have similar functions, they have different structures as isn the case with the of Parkin and the Ariadne Family.

The autoinhibition of Parkin

Parkin has two structural features, the Ubl domain and REP linker, which regulate its activity by modulating the access of its E2 binding site. It has been shown that the Ubl domain interacts with the RING1 domain, acting as a switch between inactive and active Parkin. (26, 27) Lysine 48 (K48) is required for the Ubl to interact with Parkin, with any mutations to K48 resulting in the loss of Ubl-dependent autoinhibition. (10). Furthermore, binding of phospho-ubiquitin and the phosphorylated Ubl domain induce a structural change in Parkin which increases Parkin activity. (12, 27-29) The phosphorylation of Parkin on serine 65 (S65) is performed by PINK1. (17) Phosphorylation of the Ubl domain decreases its affinity to RING1 which results in the activation of the protein. (10, 17)

In addition to the Ubl domain, the REP linker has been shown to occlude the E2 binding site. However, after the phosphorylation of the Ubl domain, a change in its conformation makes the E2 binding site accessible. (26) In the REP linker, tryptophan 403 (W403) is involved in the binding of RING1 domain. Mutations on W403 have been associated with higher Parkin activity because the hydrophobic residue fits in the RING1 domain. (10) Despite the the crucial the tryptophan residue being crucial , a hydrophobic residue, is crucial for the interaction between the REP linker and the RING1 domain, not all hydrophobic residues have the same effect. (10) A mutation to alanine, also a hydrophobic residue, has been shown to increase Parkin's activity, possibly because alanine is not long enough to bind to the groove that would otherwise be occupied by tryptophan. (10)
Aside from the E2 binding site, Parkin has another regulation site located at the RING2 -RING0 interface. (10) The active cysteine (C431) required for the trans-thiolation is buried between the hydrophobic interfaces of the RING2 and RING0 domain. (10) Mutations in the hydrophobic residues involved in the interaction between RING2 and RING0, such as phenylalanine 146, have been shown to increase Parkin's activity. (10) Without this hydrophobic interaction, the RING2 domain can easily dissociate from the RING0 domain, thus exposing the active cysteine to the surrounding environment.

HOIP/HOIL-1L regulation mechanism

Similar to Parkin, an interaction between the third ubiquitin binding region (UBR3) and ubiquitin is important for HOIP's activity. (9) However, the difference between Parkin and HOIP is that Parkin is activated upon the binding by phosphorylated ubiquitin, whereas HOIP is activated by non-phosphorylated ubiquitin. (9) In HOIP, the autoinhibitory function is carried out by its UBA domain, however, upon interaction with HOIL-1L, HOIP becomes activated. (9) Furthermore, linear di-ubiquitin could also remove HOIP-UBA autoinhibition. (9) It has been shown that residues such as Ile807 and Glu809 in the Ubiquitin-Binding Region 3 (UBR3) of HOIP are important in order to bind to ubiquitin or ubiquitin chains. (9) These interaction between the UBR3 and ubiquitin allows HOIP/HOIL-1L to form linear ubiquitin chains. (9)

Ariadne family is also autoinhibited like Parkin

Similar to Parkin, the Ariadne family also has accessory domains that aid in its autoinhibition. These domains are structurally different as Ariadne domains exist as four alpha helices in HHARI, while Parkin's RING0 contains two beta sheets and one alpha helix, as shown in Fig 5. (10, 11) The Ariadne domain behaves functionally like the RING0 domain in Parkin; however, the way that these interactions occur is completely different. The Ariadne domain interacts with its RING2 domain via hydrogen bonds. (11) Consequently, the active cysteine in HHARI (C357) is occluded from interacting with the E2-bound ubiquitin. Not only is the Ariadne domain occluding the RING2 active site, but it also intercalates between the IBR and RING2 domain. (11) The intercalation between IBR and the RING2 domain separates the active site from the RING1-bound E2. (11)

The RING2 domain of HHARI contains 14% aromatic residues. (30) Trends between HHARI and other RBR E3 ligases were compared, and a sequence alignment suggests that the tryptophan and histidine observed in both HHARI and Parkin might play a role in maintaining their protein fold. (30, 31) In Parkin, these aromatics, such as His 433, are involved in the transfer of ubiquitin to its target. (10) Therefore, iIt is possible that these aromatics on HHARI also play an important role in the transfer of ubiquitin.

Types of ubiquitin chains formed by RBR E3 ligases

The ubiquitination signaling pathway is vital in the elimination of misfolded or unwanted proteins. (1) The only way for these different chains to form is through E3 ligases. These chains could be linear, K48, K63, or branched polyubiquitin. (1) Ubiquitin has seven lysine residues and the amino group of its N-terminus can be used to form isopeptide bonds. It is suggested that the type of E2 utilized will affect the types of chains that are made. However, in some cases, the rules might not be as rigid.

It has been previously suggested that Parkin is able to form branched ubiquitin chains such as the formation of Lys 63, Lys 48, and Lys 27 branched polyubiquitin chains. (32) Recently, it was discovered that Parkin has a preference to form Lys 6 chains, although its signalling pathway is poorly understood. (32) Structurally, the crucial residue that has been implicated to aid the transfer of ubiquitin is the histidine residue that is two residues away from the catalytic cysteine. (10) Although the preference for a certain type of chain has been discovered, the mechanisms in which they are transferred are still unclear. It should be expected that the structural difference between these RBR E3 ligases will contribute more to the formation of different ubiquitin chains. Since these E3s use different E2s, it is most likely that the structural difference between these E2s and the structural difference between the E3s contributes to the different chain formations.

In contrast to other RBR ligases, HOIP/HOIL-1L are complexed in the LUBAC (linear ubiquitin chain assembly complex) pathway. (15) It has been shown that HOIL-1L contains a UBL domain, however, in contrast to Parkin, this UBL domain does not participate in the autoinhibitory mechanism, but is used to activate the HOIP once in complex with HOIL-1L and SHARPIN. Furthermore, both HOIL-1L and SHARPIN have the

NZF domain that coordinates one zinc ion with four conserved cysteine residues. (21) These NZF domains facilitate the binding to ubiquitin via a conserved TF/Φ motif. (21) Furthermore, HOIL-1L has been shown to bind linear chains via hydrophobic interaction between the NZF domains and certain ubiquitin residues. (21) It has been shown that Phe4 and Ile44 have been shown to interact with the NZF core and its tail. (21) It is possible that the NZF domain of HOIL-1L or SHARPIN is used to stabilize the di-ubiquitin that could facilitate the linear conjugation of further linear polymerization. (21) Although both proteins have an NZF domain, the NZF domain of SHARPIN is also involved in programmed cell death. (21) Although the mechanism for the formation of different ubiquitin chains is not clear, using the model of the HOIP/HOIL-1L system might shed some light onto the structural variance that can give rise to different types of chains made by the LUBAC complex.

Conclusion

Due to the recentness of its discovery, a lot of ambiguity still surrounds the Hect-type mechanism used by RBR ED ligases. Although all RBR E3 ligases have common domains, their functions are heavily dependent on the accessory domains. In the case of HOIP/HOIL-1L, the NZF domain from HOIL-1L serves as an aid to form linear ubiquitin chains. For the Parkin and Ariadne family, there are accessory domains dedicated to autoinhibition. Their structural differences provide different interactions between these accessory domains and the RING2 domain. In contrast to the Ariadne family and Parkin, the HOIP/HOIL-1L system activation requires another RBR E3 ligase (HOIL-1L). Since the regulation of most RBR E3 ligases has been extensively studied, more structural studies are required to characterize the mechanism in which different ubiquitin chains are formed. Understanding the mechanisms underlying the formation of each type of ubiquitin chain could help to elucidate their functions and related signaling pathways.

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