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Utilizing Lipid Biomarkers for Animal Holobiont Ecology

By

CHRISTOPHER MULLIGAN
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Earth and Planetary Sciences

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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Dedication

I dedicate this to my family, who have patiently put up with my endless fun facts and sense of wanderlust. Mom, you are one of the bravest and most kind people I've ever met – thank you for your limitless support and mysterious packages of homemade bread. My two siblings, Cain and Alyssa, are my lifelong comrades and I could not have done any of this without our constant banter. Thank you to my wonderful grandparents: Grandma, you taught me to be constantly curious and to cherish the little things, and Grandpa you taught me there's always more to do and how essential a sense of humor is.

I dedicate this to the cherished friends I have met in grad school. Thank you all for the thousands of miles of good company, countless cups of coffee, and too many late-night coworking sessions.

Lastly, I dedicate this to Burger Patch – I told everyone I would thank you in my thesis and here it is – you were the very best vegan fast-food place I have ever known and you are greatly missed.

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I appreciate my thesis committee, Christy Grettenberger and Titus Brown, who helped with my many questions during Chapter 1 and supported my work in the rest of my thesis.

Although the inclusion of new sample data in Chapter 2 was not possible in time to submit this thesis (but will be included in the paper publication), I am thankful to the many people who answered my awkward emails and helped to coordinate and collect stool samples. Utah's Hogle Zoo: Erika Crook, Janice Thompson, Colten Gurney; LA Zoo: Cathleen Cox; Oakland Zoo: Darren Minier, Quincy Gray, and Adam Moreno; Sequoia Park Zoo: Ruth Mock; Savannah River Ecology Lab, UGA: Amanda Hurst; Folsom City Zoo Sanctuary: Nichole Lemcke, Helen Chu, Jessica Donahoo; Saint Louis Zoo: Mary Duncan, Anne Tieber, Emily Wagner, and David Powell; and the Living Coast Discovery Center: Taryn Chiong and Kaitlyn Jones. Additionally, Philip Barruel helped with shipping the primate samples safely, and special thanks to Jose Martín at the National Museum of Natural Sciences in Madrid, Spain for sharing his unpublished lizard fecal data.

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Abstract

Lipids are a fascinating but under-utilized molecular tool - they are taxonomically specific, can be used to reconstruct environmental conditions, and once their functional groups are removed, they are stable over geologic timescales. Lipids are a diverse class of hydrophobic biomolecules, commonly called fats, that have many cell functions including: the bulk of cell membranes, signaling molecules, and energy storage. Different organisms biosynthesize different lipids, these informative biomarkers allow lipids to serve as proxies for which organisms were present in addition to broader environmental conditions. Chemotaxonomy is the study of unique lipids that have very constrained taxonomic sources, for example 2Me-hopanoids are only produced by methanogenic bacteria. Of the many different types of lipids, this thesis focuses primarily on two classes: sterols and fatty acids (FAs). Sterols, such as cholesterol, are a major group of lipids that modulate cell membrane permeability, in addition to providing the building blocks for more complex molecules like hormones and bile acids. FAs are a common lipid class that make up the majority of the phospholipid bilayer in cell membranes, in addition to larger molecules for energy storage. By coordinating lipid biomarker research with bioinformatics and field experiments, we can make robust proxies that can be used for pressing ecological questions, with geologic data providing crucial context.

Studying the ecology of animals often requires direct observations of their diet or signs of their presence, but lipids provide an indirect method to study these interactions. This thesis is founded on two major concepts to disentangle many details of animal ecology using lipids: the 'holobiont,' and trophic transfer of lipids. The holobiont concept unifies a host animal and all of the microbiomes nested on and within it to a single meta-organism. This framework recognizes the independent metabolic importance of microbial symbionts - for example, a cow as a single organism cannot digest fiber from grass but the cow holobiont can, owing to their fermentation-adapted foregut microbiome. The metabolic capacity of holobionts impacts how they digest their lipids, and therefore the recovery of different lipids in stool or environmental samples can have implications for the animal's functional

ecology and conservation. The other significant pattern of lipid ecology is that animals partially take on a lipid signature reflecting their diet, or their prey's diet, allowing researchers to estimate their trophic linkages using lipid tracers. For example, essential omega FAs are not synthesized by most animals but instead are primarily produced by plants and algae and are highly retained trophically, with nearly a 1:1 transfer rate between prey and predators. The unique lipid metabolites found in animals can provide novel proxy insights to presence (e.g. lipids extracted from sediments), taxonomic affinity, diets, and microbial symbionts.

The three chapters in this thesis each explore a different aspect of lipid biomarker work. The first chapter focuses on a unique sterol metabolite found in a half-billion-year-old animal fossil and searches for the genes responsible for the conversion in living animal gut microbiomes. The second chapter continues working on the same compound but develops new applications for conservation biology and tests if diverse sterols can be used to predict gut fermentation strategies. The last chapter flips the approach to use animals to ask questions about the larger environment, by developing a novel technique to use bird fecal FAs to noninvasively monitor a vulnerable keystone species called seagrass.

Chapter 1: A Reassessment of the Coprostane Biomarker in the Ediacaran

with Implications for *Dickinsonia*

1.1 Abstract

The discovery of cholestane in animal fossils from the Ediacaran (571–541 million years ago) has generated much excitement, but it is not the only interesting biomarker recovered. Coprostane, a geologically stable form of coprostanol, has also been found in Ediacaran rocks. This is surprising, since coprostanol is typically used in modern settings as an environmental biomarker for humans and other mammals, who produce the compound with help from bacteria in their gut. The prevailing hypothesis is that an abundance of coprostane in some Ediacaran fossils - particularly *Dickinsonia* - represents the degradation of the organism's cholesterol by bacteria in the microbial mat, comparable to what is seen in modern vertebrate corpses as they decompose. However, this hypothesis assumes coprostanol-producing bacteria were absent in the guts of Ediacaran organisms, and to date no one has tested whether such bacteria exist in modern invertebrates. In this study, we assembled 115 metagenomes to look for evidence of coprostanol-producing enzymes in invertebrate microbiomes. Ultimately, we did not find any evidence for the enzyme in any invertebrate microbiomes, supporting the hypothesis that coprostane is not a gut biomarker for Ediacaran animals. However, a reassessment of coprostane/cholestane ratios shows *Dickinsonia* was unique in coprostanol enrichment, with ratio levels comparable to waste polluted marine waters and modern vertebrate feces. While we cannot rule out the possibility of contamination, we prefer a novel interpretation of the coprostane signature in dickinsoniomorph fossils, where the elevated level of coprostanol comes from digestion of the microbial mat and concentration of the biologically inert compound. If correct, the elevated coprostanol signal provides new insights into the feeding strategy of these enigmatic animals.

1.2 Background

Coprostanol (or 5β -cholestan- 3β -ol) is an anaerobic metabolic product best characterized from vertebrate guts and feces (Subbiah et al. 1972; Hano et al. 2021; Kemp et al. 2022). Gut bacteria form coprostanol by metabolizing cholesterol (also known as cholest-5-en- 3β -ol) via a dehydrogenase enzyme, ultimately removing the double bond in the B ring (Kenny et al. 2020; Juste and Gérard 2021) (**Figure 1**). Coprostanol is typically passed out as waste from the host and is not thought to play any functional role in cell membrane processes like other sterol lipids, given its non-planar nucleus (Seidel et al. 1986). Human feces are especially enriched in coprostanol, and the compound has therefore been used as an environmental indicator of historic human occupation or wastewater contamination (Bull et al. 2002; Guillemot et al. 2017; Chen et al. 2024). The compound has also been used in forensic and archaeological research, owing to the role of gut microbiomes in carcass decomposition (von der L  he et al. 2018; Ueland et al. 2021). In addition to humans, coprostanol has been recovered in the feces and guts of many modern vertebrates, including diverse mammals and birds, a few reptiles, and a few fish (Subbiah et al. 1972; Hano et al. 2021; Kemp et al. 2022). Diverse coprostanols have often been reported in invertebrates such as sponges, hydrozoans, and tunicates, but these have historically been interpreted as waste accumulation in these filter-feeding animals rather than microbiome-mediated biosynthesis (Popov et al. 1976; Ha et al. 1982; Seidel et al. 1986; Zollo et al. 1986; De Rosa et al. 1999). So, while coprostanol might have a wide environmental distribution, in modern settings it is considered a biomarker of vertebrate waste products and decomposition.

Like other lipids, coprostanol can be preserved in the geologic record, where it undergoes diagenetic modifications. When lipids are incorporated into the rock record, they are subjected to microbial decomposition and recycling prior to burial, then subsequently heat and pressure alteration. Degraded lipids often lose features such as polar headgroups, double bonds, and alcohol groups. Briefly, cholesterol degradation follows a pathway illustrated in **Figure 1**: first, cholesterol reduces to a stanol with the removal of the double bond in the B ring, leaving a free hydrogen at carbon #5. These can be hydrogenated into geologically stable steranes with the removal of the alcohol group at carbon #3. Compounds in this pathway have different names based on the carbon #5 hydrogen isomers, whether it

goes into the plane (colored yellow in **Figure 1**, called 5α -cholestane or cholestane) or out of the plane (colored green, and called 5β -cholestane or coprostanane) - and likewise for the stanols. All sterols follow similar conversion reactions to stanols then steranes; for example, sitosterol can form both the 5α and 5β isomers of sitostanol and sitosterane. The shorthand notation for sterols records the total number of carbons followed by the type of isomer; for example, C27 5β is coprostanol, and C29 5β is the same isomer for phytosterols (i.e., those produced by plants and algae like sitosterol and stigmasterol). Coprostanol is ultimately preserved in the rock record as coprostanane (5β -cholestane) and has been used as a biomarker for diet in archaeological and paleontological studies (Gill et al. 2009; Gill and Bull 2012; Zocatelli et al. 2017; Umamaheswaran et al. 2019; Tripp et al. 2022).

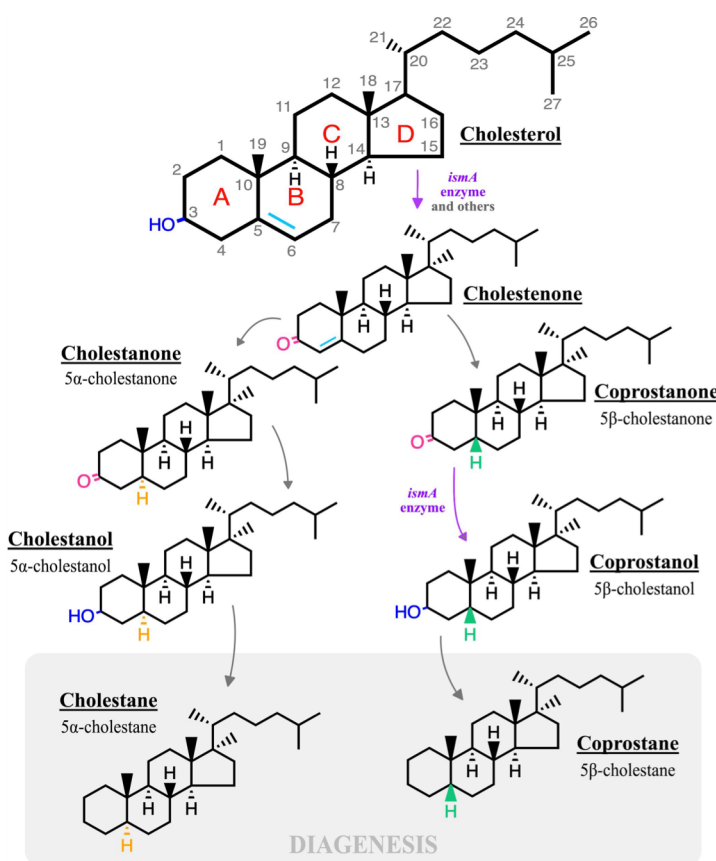


Figure 1.1

Overview of coprostanol biosynthesis. Cholesterol is transformed into cholestanone by moving the double bond from the B ring to the A ring and replacing the alcohol group with a ketone (i.e., oxidation). Next the B ring double bond is removed to form coprostanone (i.e., reduction). Finally, adding a hydrogen to the ketone group forms an alcohol (i.e., another reduction) to create

coprostanol. During diagenesis, cholestanol and coprostanol lose their alcohol groups to become the geologically stable isomers cholestane and coprostanane, respectively.

As coprostanol is primarily attributed to vertebrate guts, it is surprising that coprostane is also preserved in Ediacaran environments from the Lyamtsa and Zimnie Gory localities of the Ust-Pinega Formation in Russia, where some of the earliest animals are preserved (Bobrovskiy et al. 2018a; Bobrovskiy et al. 2018b; Bobrovskiy et al. 2022). Elevated levels of cholestane are preserved in the dickinsoniomorphs *Dickinsonia* and *Andiva*, which has been used to support the hypothesis that they are animals (cholesterol is the dominant lipid in the cell membranes of animals, although it is also produced by many eukaryotes) (Bobrovskiy et al. 2018a). Dickinsoniomorphs are a classic example of Ediacaran organisms: they were flat, circular, and could get as large as 1 m across. In addition to cholestane, evidence of segmentation-like growth and motility also support an animal-affinity for *Dickinsonia* (Gold et al. 2015; Evans et al. 2019a). Although dickinsoniomorphs lack a discernible mouth or gut, it is generally hypothesized that they fed off the mats of algae and bacteria their fossils are commonly associated with, potentially through external digestion of the mat through their ventral sole (Sperling and Vinther 2010). It is also worth noting that while dickinsoniomorphs share a similar morphology, it is unclear whether the different genera form an evolutionary clade (Xiao and Laflamme 2009; Erwin et al. 2011). Bobrovskiy et al. (2018a) considered whether the coprostane signal in *Dickinsonia* came from a gut microbiome, but given the presence of coprostane in the microbial mat, they ultimately preferred the hypothesis that the signal came from bacteria in the mat breaking down the cholesterol of the dickinsoniomorph carcasses. The authors proposed an “Animal Decomposition Index” (ADI), where $ADI = (C27\ 5\beta/5\alpha)/(C29\ 5\beta/5\alpha)$ to show a ratio between steranes metabolized by anaerobic bacteria versus those present in the environment (Bobrovskiy et al. 2018a). The coprostane signature in *Dickinsonia* generally trends positively with animal size - the authors justified this as more animal biomass and therefore a higher decay signal. In a follow-up paper, Bobrovskiy et al. (2022) expanded their analysis to several more Ediacaran taxa from both locations. In addition to more *Dickinsonia* and *Andiva* samples, this second paper added *Yorgia*, *Kimberella*, and *Calyptrina* - the first three are dickinsoniomorphs and the latter two are interpreted as a putative mollusc and annelid respectively (though it is worth noting there are no morphological features to definitively assign *Calyptrina* to the annelids). In comparison to *Dickinsonia*, the levels of coprostane in *Kimberella* and *Calyptrina* were far lower. Bobrovskiy et al. (2022) hypothesized how each animal developed distinct

sterol and sterane isomer compositions, with the elevated coprostanol in dickinsoniomorphs representing decayed animal tissues while *Kimberella* and *Calyptrina* were overprinted with dietary phytostanol isomers from their guts. While this animal decomposition hypothesis helps to explain part of the data seen in Ediacaran fossils, similar observations have not been verified in other fossil or modern systems. Critically, the interpretation by Bobrovskiy et al. (2018a); Bobrovskiy et al. (2022) assumes coprostanol-producing bacteria come from the mat and not the animal guts, but to date there is no understanding of whether such bacteria exist in marine invertebrates.

Fortunately, comparative genomics can be leveraged to better interpret biomarkers like coprostane (Summons et al. 2022). In human and mice studies, coprostanol formation has been attributed to the *ismA* gene (short for “Intestinal Sterol Metabolism A”) which has only been described in *Clostridium* cluster IV gut bacteria (Lopetuso et al. 2013; Kenny et al. 2020). Clostridiales are a large group of anaerobic Gram-positive bacteria within the phylum of Bacillota, and have been found as obligate symbionts in 98% of vertebrate gut microbiomes (Youngblut et al. 2019). While the origin and distribution of the *ismA* gene within Clostridiales (and bacteria more generally) is currently unknown, the *Clostridia* genus has been estimated to be at least 1 billion years old (Battistuzzi et al. 2004). The *ismA* gene belongs to the very large and diverse SDR gene family (Short-chain Dehydrogenases and Reductases). These genes are found in all 3 domains of life and help metabolize many compounds, including sugars, alcohols, and steroids (Kallberg et al. 2002). SDR-mediated reactions typically bind to coenzymes like NAD(H) or NADP(H), while the terminal end of the protein typically binds to their specific substrate (Gräff et al. 2019). The IsmA protein is thought to be involved in the first and last steps of the conversion (from cholesterol to cholestenone and from coprostanone to coprostanol), although the full process has yet to be observed in culture (Kenny et al. 2020).

The goal of this project is to better constrain the interpretation of coprostane in Ediacaran-age invertebrates by determining whether any evidence exists for coprostanol biosynthesis in the guts of living invertebrates. Assuming the Ediacaran coprostane signal is endogenous (something we will consider in detail in the Discussion), and that the relevant fossils represent invertebrate animals, there

are two potential sources for coprostanol: either it came from gut-associated bacteria or it was produced by environmental bacteria. While coprostanol has been reported in living invertebrates before, it has generally been dismissed as a contaminant (Popov et al. 1976; Ha et al. 1982; Seidel et al. 1986; Zollo et al. 1986; De Rosa et al. 1999). But if it could be shown that invertebrate guts carry the gene required to make coprostanol, this could support a “gut biomarker” interpretation for coprostanol in Ediacaran taxa.

1.3 Methods

The first aim was to collect all putative IsmA protein sequences from the National Center for Biotechnology Information (NCBI). An IsmA protein from *Eubacterium coprostanoligenes* (NCBI Accession ID: WP 078769004.1) was used as a query to search NCBI's NR and RefSeq databases with default settings except to return the top 500 results. This IsmA protein has been shown to be involved in the conversion of cholesterol into coprostanol in human guts (Kenny et al. 2020). As the matching sequences were primarily from metagenomic bacteria, host information for each sequence was retrieved from the metadata using Entrez when available and appended to sequence names in R v4.0.2 (R Core Team 2021). Additional vetted IsmA protein sequences from human microbiomes were taken from Kenny et al. (2020). Outgroup SDRs were obtained by searching NCBI's RefSeq with the *ismA* queries in bacterial species that do not have *ismAs* according to Kenny et al. (2020) (i.e., using BLAST to get non-*ismA* *Clostridia* SDRs), as well as diverse and well-characterized SDRs from Gräff et al. (2019) and Gabrielli et al. (2022). All sequences were aligned with MAFFT then made into a tree in fastTree v2.1 (Price et al. 2009). We used this starting tree (**Figure S1**) to constrain the IsmA clade.

Most metagenomic data from animal guts is not readily accessible through NCBI BLAST; instead, they are buried in raw sequencing data that must be *de novo* assembled before querying. We looked for a diverse set of invertebrate species and then chose the largest metagenome dataset available. Additionally, sequences from environmental and carcass tissue samples were included. Databases analyzed include the NCBI SRA database (<https://www.ncbi.nlm.nih.gov/sra>), Zenodo (<https://zenodo.org/>), and JGI-IMG (<https://img.jgi.doe.gov/>). The results are summarized in **Table 1**

and the full dataset can be found in **Table S1**. These files were passed to fastqc v0.11.9 (Andrews 2010) to identify the quality of the reads and flag any adapter content to be removed, and trimmed with Trimmomatic v0.39. Reads were then assembled with Megahit v1.1.4 (Li et al. 2015). All sequences were concatenated together, then made into a BLAST database with BLAST v2.13.0 (Camacho et al. 2009). A tblastn search was used on the total-read database with three diverse *ismA* reads from Kenny et al. 2020 and four from the above NCBI biodiversity search, with an e-value of 10⁻⁵. Transdecoder v5.7.1 was used to extract the reading frames (Haas 2024). This output was made into a BLAST database again to extract the best translation matches. Cd-Hit v4.8.1 (Li and Godzik 2006) was used to remove duplicate sequences, with a similarity cut off at 90%. PfamScan v36.0+ (Finn et al. 2014) was used to identify the protein domains, which were then extracted again with Samtool v1.16.1 (Danecek et al. 2021). The same file from above with known *ismAs* and known non-*ismA* sequences was appended to the file. The sequences were aligned with MAFFT v7.505 (Kato et al. 2002), then passed to trimAl v1.5.0 (Capella-Gutiérrez et al. 2009) to remove any poorly aligned sequences. Finally, the file was passed to IqTree v2.3.6 (von Haeseler et al. 2014) to generate a phylogeny using 1000 bootstraps and the default parameters, and visualized using FigTree v1.4.4 (Rambaut 2010) (**Figure S2**).

Select protein sequences were chosen for additional modeling and structural analysis. Candidate protein sequences were chosen based on their distribution on the gene tree and uploaded to ColabFold v1.5.5 AlphaFold2 using MMseqs2 (Jumper et al. 2021) and run with the default settings. Proteins were visualized using Pymol v3.0.0 (Schrödinger and DeLano 2020) and aligned to the known human symbiont *E. coprostanoligenes* IsmA (sequence WP_078769004.1). Root mean square deviation (RMSD) scores were calculated for each alignment, which is the average distance between two atoms in overlaid structures (**see Table 1.2**). R was used to identify the conserved SDR motifs, including the active binding sites in the catalytic triad (Gräff et al. 2019). The Pymol plugin Dockingpie v1.2.1 was used to confirm both the location and binding affinity of these sequences via the RxDock program (Rosignoli and Paiardini 2022). The .sdf model files of cholesterol and coprostanone were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The ligand binding site cavity grid was determined via the two-spheres option based on cholesterol in the *E. coprostanoligenes* model for

ingroup sequences (given the very low RMSD values) and per model+ligand pair for the outgroups. Each sequence was run on each ligand for 10 poses, and the binding score for the top match was recorded only in the correct position guided by the color-coded catalytic triad.

Clade	No. species	Total BP	Avg contig BP
Annelida	5	590947617	2455
Arthropoda	18	331758289	3987
Chordata	51	3662305864	4368
Cnidaria	9	418741599	4261
Ctenophora	1	26319	2393
Echinodermata	1	79368154	4218
Mollusca	7	1167355529	3927
Placozoa	1	96195390	5730
Platyhelminthes	1	24978233	2374
Porifera	8	993772594	3630
Carcasses	3	76875838	3054
Environment	10	2052141817	3341

Table 1.1

Summary of *de novo* assembled metagenomic samples, including the cumulative total base pairs (BP) for that clade and the average BP per contig.

A literature review (see **Table S2**) was conducted for published sterol and sterane data that included levels for both cholesterol and coprostanol (or cholestane and copropane). Papers that did not report any null coprostanol amounts were omitted. All modern water samples, both those reported polluted and unpolluted, were reevaluated at the field standard of 0.2 C27 5 β /5 α cutoff value (Mudge and Ball 1964). Only the ratio of 5 β /5 α is provided by Bobrovskiy et al. (2018a) and Bobrovskiy et al. (2022) as opposed to individual absolute values - therefore only this ratio was used for direct comparison. In R, ANOVA tests were used to determine the statistical correlation between the Ediacaran data types, and boxplots were plotted as a figure using ggplot2 v3.5.1 (Wickham 2016).

Host Species	Sequence	IqTree Clade	RMSD aligned to <i>E.cop.</i>	Cholesterol score	Coprostanone score
Human <i>E.cop.</i>	WP 078769004.1	Known ingroup	-	37.2317	29.6722
Human	k00196	Known ingroup	0.353	50.6601	68.7007
Human	k00562	Known ingroup	0.38	80.5901	62.0439
Human	k00645	Known ingroup	0.219	52.8085	32.0164
Human	k00741	Known ingroup	0.319	41.1711	48.28
Human	k00764	Known ingroup	0.123	39.3537	83.161
Koala	NR MCC8016609.1	High confidence	0.241	68.0113	64.8497
Chicken	NR HIS05287.1	High confidence	0.340	93.9521	36.2189
Salamander	NR MEG0546937.1	High confidence	0.367	48.7398	20.8018
Buffalo	1387.p14	High confidence	0.510	49.3428	66.3982
Pig	91312.p318	Low confidence	0.563	124.676	163.675
Termite	NR MDR0849881.1	Low confidence	0.643	61.2519	NA
Hydra	685.p116:3-192	Outgroup	0.945	67.6886	7.78951
Sea Hare	SDR7-1C3	Known outgroup	0.723	NA	NA
Fruit Fly	O18404	Known outgroup	0.951	NA	NA
Human	Q9BPW9	Known outgroup	1.046	116.278	40.3697
Yeast	P40580	Known outgroup	1.089	NA	32.4255
Sea Urchin	SDR7-1C3	Known outgroup	1.166	44.7589	NA

Table 1.2

Pymol RMSD values aligned against the known *E. coprostanoligenes* Human ismA gene (top row, WP_078769004.1), and their ligand binding scores modelled with RxDock in Dockingpie.

1.4 Results

IsmA proteins are part of a large and diverse gene family, and constraining IsmAs within the tree proved difficult. We ultimately distinguished between a “high confidence” clade - which constituted the smallest clade containing all functionally verified IsmA enzymes - and a “low confidence” clade, which includes similar sequences whose enzymatic activity is unclear. The high confidence IsmA clade only contains vertebrate hosts - primarily mammals but also a few birds and a giant salamander (*Andrias davidianus*) (**Figure 2A**). The lower confidence IsmA clade contains similar vertebrate species in addition to a dampwood termite (*Hodotermopsis sjostedti*) microbiome. IqTree reports both SH-aLRT and UFBoot support values for nodes: the ingroup (high confidence + low

confidence clades) had values of 98.6/98; and the high confidence clade had values of 94.8/98. All sequences from our assembled invertebrate metagenomes are restricted to the outgroups - none were included in the high nor low confidence clades. These results suggest that no SDRs from our invertebrate microbiomes are IsmA proteins.

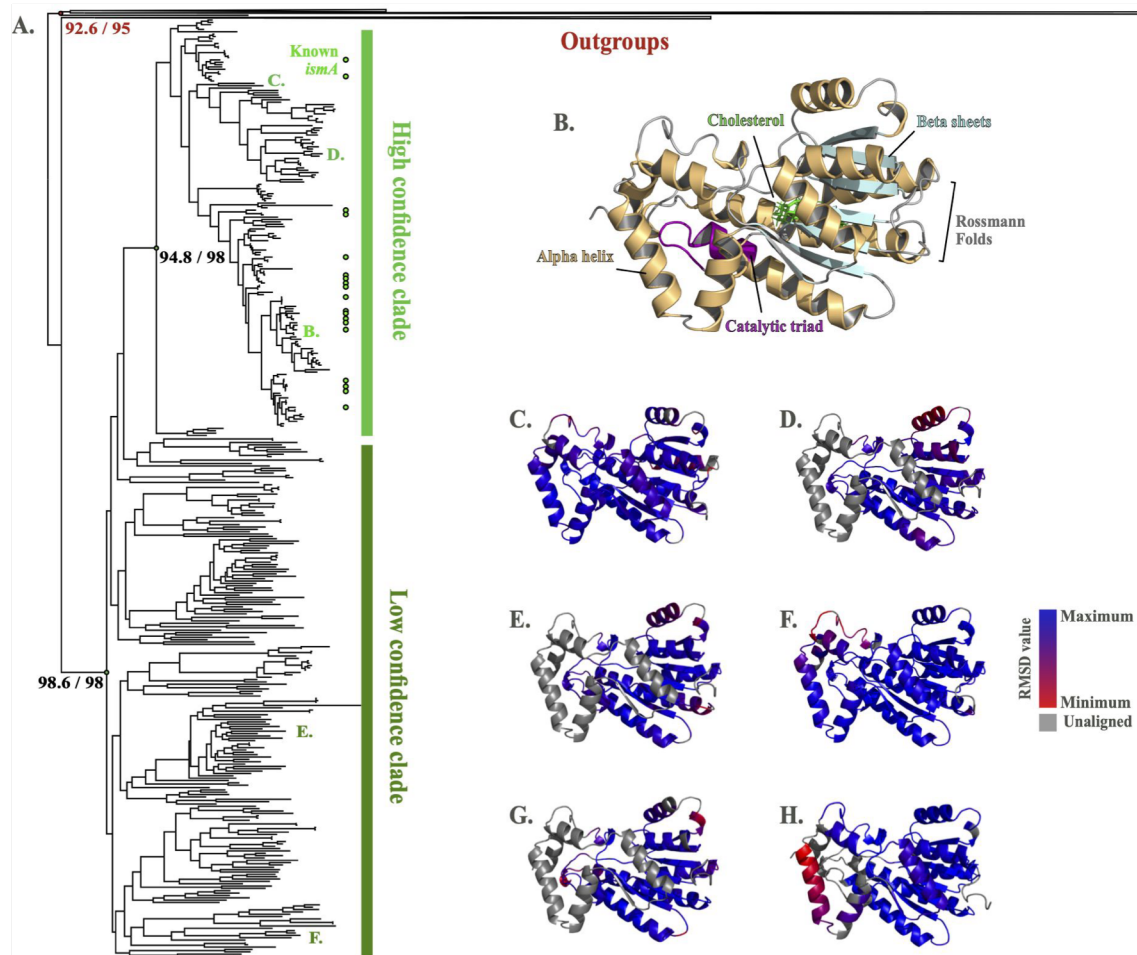


Figure 1.2

Phylogenetics and protein modeling for IsmA. (A) Gene tree showing metagenome results, with the outgroups collapsed and both high-and low- confidence clades and their bootstraps labeled. The letters associated with the tree show where each modeled protein sits in the phylogeny. (B) Human symbiont IsmA protein model, with the cholesterol ligand bound. (C–H) Modeled sequences aligned to the Human symbiont gene above and colored by RMSD values. (C) Chinese giant salamander (high conf.), (D) African buffalo (high conf.), (E) pig (low conf.), (F) dampwood termite (low conf.), (G) hydra (outgroup), and (H) California sea hare (known non-IsmA SDR).

The protein models from AlphaFold2 revealed secondary and tertiary structures consistent with other SDR genes (**Figure 2B–H**). The root-mean-squared deviation, or RMSD, values were all $< 1 \text{ \AA}$, in agreement with published work that all SDR genes have very similar structures (Gräff et al. 2019; Da Costa et al. 2021, see Table 2). SDR proteins all consist of two blocks of α -helices with six β -sheets between them, together forming Rossmann folds. Based on wet lab experimentation, Kenny et al. (2020) identified amino acid sites S-138, Y-151, and K-155 in their sequence alignments as the required active sites in the conversion of cholesterol to coprostanol. These amino acids represent the “catalytic triad” found in all SDRs - this conserved motif in classical SDRs like IsmA is identified as [ST] x_n Y x_3 K (Gräff et al. 2019; Da Costa et al. 2021). Based on X-ray crystallography, cholesterol as a ligand needs a particular protein structure to bind to, specifically a hydrophobic pocket that is composed of α -helices on one side and β -sheets on the other, likely to bind to the relatively smooth α -face of the sterol and the methyl groups on the β -face, respectively (Bukiya and Dopico 2017). The sequences modeled from the ingroup (high confidence + low confidence clades) show an expected binding orientation and location for both cholesterol and coprostanone - where the catalytic triad binds to the sterol tail and the Rossmann folds hold the sterol body (Bukiya and Dopico 2017). Generally, the binding score for cholesterol goes up (i.e., binding becomes less efficient) as one moves from the high confidence to the low confidence clades, while outgroup models either can sometimes bind well but in the wrong location. Based on these model results, the low confidence clade is not likely to synthesize coprostanol, leaving only the vertebrate-specific high confidence clade containing likely IsmA proteins.



Figure 1.3

C27 5 β /5 α ratios from the literature. The coprostane:cholestane (three left panels) and coprostanol:cholesterol (right panel) ratios for different sources, with a dashed line at the 1:1 ratio.

In addition to studying the *ismA* gene, we analyzed C27 5 β /5 α ratios in the literature to get a better understanding of what is seen in the Ediacaran fossils. The results are summarized in **Figure 3**. All dickinsoniomorph fossils (*Andiva* n = 3, *Dickinsonia* n = 8, and *Yorgia* n = 2) have enriched levels (i.e., above a 1:1 ratio) compared to the other Ediacaran fossils (n = 2) and strata (n = 11). *Dickinsonia*, the most enriched and most sampled fossil, has ratio levels similar to immature bitumen, waste-polluted marine waters, modern vertebrate feces, and some microbial mats. Six ANOVA tests were conducted on the ratios (**see Table 3**), comparing: (1) *Dickinsonia* to *Andiva* and *Yorgia*, (2) dickinsoniomorphs to *Kimberella* and *Calyptrina*, (3) dickinsoniomorphs versus the strata, (4) *Kimberella* and *Calyptrina* to the strata, (5) Ediacaran strata to published immature bitumen data, and (6) Ediacaran strata to all bitumen data (including the immature data points from test #5). *Dickinsonia* is significantly different from the other dickinsoniomorphs, and dickinsoniomorphs are significantly different from the other Ediacaran fossils and strata. The difference between the non-dickinsoniomorph fossils and the surrounding strata were statistically insignificant. It is worth noting the single data points from

Kimberella and *Calyptrina* were combined, and this low sample size may impact the validity of Tests 2 and 4. Tests 5 and 6 showed that Ediacaran strata are statistically different from all bitumen (n = 72) but not immature bitumen. Turning to the sterol results, modern animal feces (n = 473) featured the most variability, ranging from 0 to 46. Modern marine environmental samples (total n = 233, unpolluted n = 119, polluted n = 114) span a wide range, with the polluted samples having a similar range to the modern animal feces. A C27 5 β /5 α ratio of 0.2 is considered the benchmark for fecal pollution in water, and anything above 2 is raw sewage effluent (Mudge and Ball 1964). Modern microbial mat (n = 20) ratios range from 0 to 4, with the highest values typically coming from hypersaline locations but did not keep any obvious trends with depth. In summary, amongst the Ediacaran fossils, dickinsoniomorphs show a significantly elevated level of coprostane compared to the rest of the fossil beds, with *Dickinsonia* having a significantly higher level of coprostane than any other fossil.

Test No.	Comparison	F-value	p-value
1	<i>Andiva/Yorgia</i> vs <i>Dickinsonia</i>	54.78	<2e-16
2	dickinsoniomorph vs non-dickinsoniomorph	39.52	1.32e-13
3	dickinsoniomorph vs strata	41.86	3.48e-14
4	non-dickinsoniomorph fossils vs strata	0.12	0.887
5	Ediacaran strata vs immature bitumen	0.636	0.531
6	Ediacaran strata vs all bitumen	34.9	2.04e-12

Table 1.3

ANOVA results comparing C 27 5 β /5 α ratio data published in Bobrovskiy et al. (2018a) and Bobrovskiy et al. (2022).

1.5 Discussion

In this study, we found no evidence for coprostanol biosynthesis in modern invertebrate guts. There are several possible interpretations of these results. Firstly, it is possible that some invertebrate microbiomes contain the IsmA enzyme, but they were missed in our analysis. Unlike genome sequencing, there is no *a priori* way to know when sufficient sequencing has been done to capture all genes in a microbial metagenome. We failed to identify IsmA proteins in several samples where it was

almost certainly present (e.g., several great ape microbiomes, including a human); so it is possible that the same is true in one or more invertebrates. It is also possible that invertebrates could use a different enzyme to catalyze the production of coprostanol, although we found no evidence for an undescribed invertebrate-specific SDR in our analysis. Still, without additional data, the most parsimonious interpretation is that modern invertebrates lack the necessary enzyme to generate coprostanol, and that this was also the case for prehistoric species. Our work supports the hypothesis of Bobrovskiy et al. (2018a); Bobrovskiy et al. (2022) that coprostanol biosynthesis was not occurring in the guts of these Ediacaran taxa.

In addition to the genetic evidence presented here, data from invertebrate anatomy and metabolisms suggests they are unlikely to produce coprostanol. Sterol auxotrophs, such as cnidarians, flatworms, and insects, are unable to biosynthesize sterols and are entirely dependent on dietary intake for use in membranes and modification into hormones (Goad 1981; Kanazawa 2001; Zhang et al. 2019; Haas et al. 2023). Presumably, auxotrophs would not support microbial symbionts that compete with the host for a necessary substrate (Robbins et al. 1961; Jing and Behmer 2020). Coprostanol has also been hypothesized to act as an estrogenic hormone in some bivalves, which may be another reason their gut microbiomes aren't promoting *ismA*-bearing bacteria (Gagné et al. 2001). Perhaps the largest reason that invertebrates may not be able to produce coprostanol via *ismA* is that, to our knowledge, such bacteria are strictly anaerobic. When vertebrates produce coprostanol, it is in large fermenting organs that are anoxic; this is in contrast to most invertebrates, which have digestive systems either open to the environment or have fluctuating external oxygen diffusion into the gut (Plante and Jumars 1992; Zimmer and Brune 2005; Gross et al. 2008; Steinmetz 2019). Another potential hindrance is the presence of a chitin-lined gut in some organisms, such as arthropods and tunicates, which, compared to mucin-lined guts, offer a poor surface for biofilm building that is crucial for Clostridiales; additionally, the periodic shedding of gut lining in arthropods may hinder a sustained biofilm (Nakashima 2018; Engel and Moran 2013). It is beyond the scope of this paper to consider how the above arguments speak to the 5 β -phytosterols reported in *Kimberella* and *Calyptolina* by Bobrovskiy et al. (2022); we simply note that the digestive production of 5 β -phytosterols is understudied in vertebrate guts and virtually

unknown in invertebrates (Eneroth et al. 1965; Blanco-Morales et al. 2019). In summary, invertebrate anatomy and physiology further suggest that the production of coprostanol in Ediacaran fossil taxa microbiomes is unlikely.

Before considering additional interpretations for the coprostanol biomarker, we want to address whether this signature is the result of contamination, as multiple colleagues and reviewers of this paper raised this possibility. Most of these concerns can be grouped into two major arguments: (1) that 5β steranes are thermodynamically unstable and should not exist in such abundance in Ediacaran rocks, and (2) that the rock samples were contaminated from either oil migration or modern vertebrate feces. Starting with issue (1): the logic is that β -position sterane stereoisomers are less thermodynamically stable than α position isomers and should reconfigure with sufficient diagenetic alteration (van Graas 1982; Peakman et al. 1992; Summons and Erwin 2018). However, the literature review we conducted shows a C27 $5\beta/5\alpha$ range of 0.223–2.270 for immature bitumen, which is consistent with the data reported in Bobrovskiy et al. (2018a); Bobrovskiy et al. (2022) and meets expectations that enriched proportions of the 5β stereoisomer can persist through geologic time if there are limited diagenetic forces. Recent studies detailing biomarker data from similar-aged Baltica and Siberian locations have all found the rocks to be immature, with mutually consistent and expected ranges for their C29 $5\beta/5\alpha$ ratios all <0.5 (Goryl et al. 2018; Pehr et al. 2018; Melnik et al. 2024). The $T_s/T_s + T_m$ maturity assessments from Bobrovskiy et al. (2018a); Bobrovskiy et al. (2022) range from 0.03 to 0.1 and places their Ediacaran samples well below the early oil window (which starts at ~ 0.5); therefore, any higher sterane $5\beta/5\alpha$ ratios could be interpreted as primary signals. A related concern is how greatly the Lyamtsa section strata vary in $5\beta/5\alpha$ values, ranging from 0.61 to 5.49 for C27 steranes and 0.21–3.7 in C29 steranes, with averages of 1.99 ± 1.65 and 0.98 ± 1.01 , respectively. Still, given that the samples are isomature by all other metrics and that the C29 isomers are in near equal proportions, we interpret the more disparate C27 $5\beta/5\alpha$ values to be of biological origin. Additionally, our ANOVA test does not suggest the difference between C27 $5\beta/5\alpha$ ratios in published immature bitumen and the Bobrovskiy et al. (2018a) strata samples is significant. All of this suggests that the environmental $5\beta/5\alpha$ values reported in Bobrovskiy et al. (2018a) and Bobrovskiy et al. (2022), while unusual, are not outside the

range of expected values. Regarding concern (2), we note that contamination with other petroleum sources should have widened the standard deviations for the maturity proxies that were calculated. Similarly, the hypothesis that the samples were contaminated by more recent vertebrate waste (such as livestock) should also elevate the relative proportions of C29 steranes, which is notably missing from *Dickinsonia* (Leeming et al. 1996). We think these are reasonable counters to the concerns raised by our peers, and no one has provided us a mechanism that would result in the specific statistical enrichment of C27 5 β steranes in *Dickinsonia* fossils. We therefore proceed with the hypothesis that the biomarkers reported in Bobrovskiy et al. (2018a); Bobrovskiy et al. (2018b); and Bobrovskiy et al. (2022) and thus require a biological explanation.

Despite our contention that the sterane signal reported by Bobrovskiy et al. (2018a); Bobrovskiy et al. (2018b); Bobrovskiy et al. (2022) is genuine, we admit that there are unusual aspects of the data that require further inquiry. For example, it is unclear to us why the environmental C27 5 β /5 α sterane signal reported in these papers hasn't been recovered in any other Proterozoic rocks with similar mat dominated ecosystems. Additionally, the C27 5 β /5 α ratio from *Beltanelliformis* and other benthic algae from Lyamtsa appears to be unusually high (up to ~2.0) (Bobrovskiy et al. 2018b), although, unlike *Dickinsonia*, C27 steranes are a minor portion of the *Beltanelliformis* sterane. It is possible that *Beltanelliformis* and the other macroalgae, like their modern counterparts, provided nutrient hotspots where *Clostridia* and microbial eukaryotes colonized, creating a potential source for coprostanol generation (Xing et al. 2011; Morrison et al. 2017; Margareta et al. 2020; Mills et al. 2025; Rehman et al. 2025) although coprostanol enrichment has not been observed in modern experiments (Bobrovskiy et al. 2024). The absence of some important data in these papers - including absolute sterane abundance, total organic carbon (TOC) and standard Rock-Eval data - makes it difficult to answer these questions or fully reject the possibility of contamination. Ratios like 5 β /5 α are common practice in geochemistry, but are typically accompanied by the absolute abundances so researchers can use them in other applications such as normalizing the data (e.g., 5 β /(5 α + 5 β)), which is more robust to noise and less subject to biases if one of the isomers is near zero. Background subtraction is another standard method, as seen in Bobrovskiy et al. (2018a); Bobrovskiy et al. (2018b); Bobrovskiy et al. (2022), but this data

also has limited utility. A potential risk of background subtraction comes from understanding the biology of the system: if *Dickinsonia* was consuming C29 sterols in the mat and integrating them into its tissue, that entire dietary signal may have been lost after subtraction and cannot be quantified to test. We recommend that future studies on these localities provide absolute sterane isomer abundance data alongside ratios and/or background subtractions, as this will allow for further hypothesis testing.

Assuming the coprostanol signature does not represent contamination or microbial activity from the gut, it seems most likely that the compound comes from the environmental mats. Bacteria in sediments have been known to convert sterols to stanols in terrestrial, freshwater, and marine locations, with some locations producing primarily coprostanol while others reduce all C27-29 5 β /5 α sterols in equal proportions (~ 0.64) (Mermoud et al. 1984; Peakman et al. 1992; Chen et al. 2024). Modern microbial mats produce detectable levels of coprostanol, and 5 β stanols generally, corresponding to layers with reducing conditions and methanogen bacteria (Boudou et al. 1986; Grimalt et al. 1992; Rontani and Volkman 2005; Allen 2006). The Kiritimati Island hypersaline mat analysis by Shen (2019) notes a rapid and direct conversion of cholesterol to coprostanol starting within a few centimeters of the surface, as cholesterol from surface and planktonic animals gets metabolized and reworked down into the mat. Other locations have described the transition from sterols to stanols within mats, but few report coprostanol, instead finding compounds like dinostanol and sitostanol (Bühning et al. 2009; Scherf and Rullkötter 2009; Słowakiewicz et al. 2016; Lee et al. 2021). Coprostane (sometimes with trace coprostanol) was found in a recently inactive hydrothermal vent's sediment; it is thought the sterols were microbially reduced from sinking detritus and vent animals, then abiotically matured with hydrothermal pyrolysis entirely through diagenesis (Brault et al. 1989). This East Pacific Rise vent location was noted to be petroliferous, so conceivably the coprostane could be a petroleum contaminant, but given the presence of both biologically immature and geologically mature isomers of a diversity of lipids, it is more likely to have been an *in situ* process. Since microbial mats found in freshwater, nearshore, and deep water can produce coprostanol, they represent a significant potential source in the biomarker record. Clearly, it is possible for coprostanol, and perhaps even coprostane, to accumulate in modern microbial mats, and presumably this was also true in the Ediacaran.

Although coprostanol is produced endogenously in microbial mats, we do not think that the elevated coprostanol signature found in dickinsoniomorphs is coming from microbial decay of the carcasses. The high levels of C27 5 β /5 α observed in *Dickinsonia* (**Figure 3**) are within the range of terrestrial carcasses, but *Dickinsonia* is very different from the well-studied vertebrate cadavers that would provide a basis for the “Animal Decomposition Index” (Evershed 1990; Gülacara et al. 1990; Evershed and Connolly 1994; von der Lühne et al. 2013). Intact carcasses are typically decomposed by their gut communities from the inside out, until they rupture and can be colonized by the larger environment (Briggs and Kear 1993; Briggs 2003; Hilal et al. 2021; Mason et al. 2023). Some mummies and peat bog cadavers, for example, have C27 5 β /5 α ratios that are comparable with *Dickinsonia*, demonstrating that you can have significant decomposition while preserving the external morphology of the specimen (Evershed 1990; Gülacara et al. 1990; Evershed and Connolly 1994). However, in these cases the source of coprostanol-producing bacteria is internal, while in *Dickinsonia* the coprostanol-producing organisms are coming from the external environment. At advanced stages of decay, when coprostanol is being widely produced in vertebrate carcasses, relatively little is preserved in the way of internal organs, but on a per-tissue basis there can be high levels of C27 5 β /5 α (Gülacara et al. 1990; von der Lühne et al. 2013; Ueland et al. 2021). Collagen-rich tissues (such as muscle) can be resistant to liquefaction during decay and contain high levels of coprostanol, while tissues that are highly labile generally have low to absent levels of coprostanol if they're preserved (Gülacara et al. 1990; Evershed and Connolly 1994; Nielsen et al. 2021). Such extensive decay appears to contrast with *Dickinsonia* being a member of the exceptionally preserved Ediacaran biota, where fine detail of soft-bodied macroorganisms and microbial filaments are recovered (Callow and Brasier 2009; Gehling and Droser 2009). It is possible that the organisms were cast rapidly after burial and prior to significant decay (Tarhan et al. 2016; Slagter et al. 2022, 2024) though this fails to explain the different C27 5 β /5 α ratios seen between different Ediacaran taxa as seen in **Figure 3**. There is an additional hypothesis, which suggests *Dickinsonia* fossils are the remains of an organic (non-mineralized) skeleton (Bobrovskiy et al. 2019); this would imply significant decay of the rest of the organism and is consistent with the “Animal Decomposition Index” hypothesis. However this interpretation appears inconsistent with

deformations of some *Dickinsonia* fossils, which indicate a thick and flexible epidermis, as well as putative trace fossils left by the organism which sometimes demonstrate the same morphology as the body fossils (Evans et al. 2019a; Evans et al. 2019b; Droser et al. 2020). Marine invertebrate carcasses that lack hard organic skeletons typically become “fluid-filled cuticle sacs,” where all tissue except the epidermis becomes liquified by gut bacteria and any disruption will result in a rupture and rapid disaggregation of the body (Briggs and Kear 1993). In addition, the broader rheology-based model used in Bobrovskiy et al. (2019) does not explain some Ediacaran fossil deposits with non-heterolithic strata, or why we fail to see Ediacara-style preservation of soft tissues throughout the Phanerozoic (Droser et al. 2019, 2020; Slagter et al. 2022). It is also unclear why other Ediacaran species analyzed in these biomarker studies (*Kimberella*, *Yorgia*, and *Andiva*) would lack a similar decay signal. The lack of elevated 5 β C27 stanols in *Kimberella* and *Calyptrina* could be a result of the low abundance of C27 steranes in these fossils, though a lack of information on the absolute abundance of C27 steranes makes it difficult to assess this hypothesis. It is also possible that the epidermises of *Kimberella* and *Calyptrina* were more resistant to bacterial infiltration compared to *Dickinsonia*, protecting them from decay. But this does not explain the difference between *Dickinsonia* and the other dickinsoniomorphs, particularly the specimen of *Andiva* presented in Bobrovskiy et al. (2018a), which has a low 5 β C27 signal despite being as large as the largest *Dickinsonia* specimens (Bobrovskiy et al. 2022). The relatively low level of 5 β C27 in *Andiva* is inconsistent with the general relationship between size and 5 β C27 signal in *Dickinsonia*, which is thought to represent the increased amount of biomass available for decay. As a final point, we note that biomarker studies on other fossils - including some vertebrates which presumably had *ismA*-bearing symbionts - have not recovered a coprostanol decay signal (Melendez et al. 2013; Thiel et al. 2014; O'Reilly et al. 2017; Plet et al. 2017; Brosnan et al. 2024). In summary, a decay-based explanation for the elevated 5 β C27 signal seen in *Dickinsonia* appears to conflict with the exceptional preservation of these fossils, and is inconsistent with what is seen in other fossils.

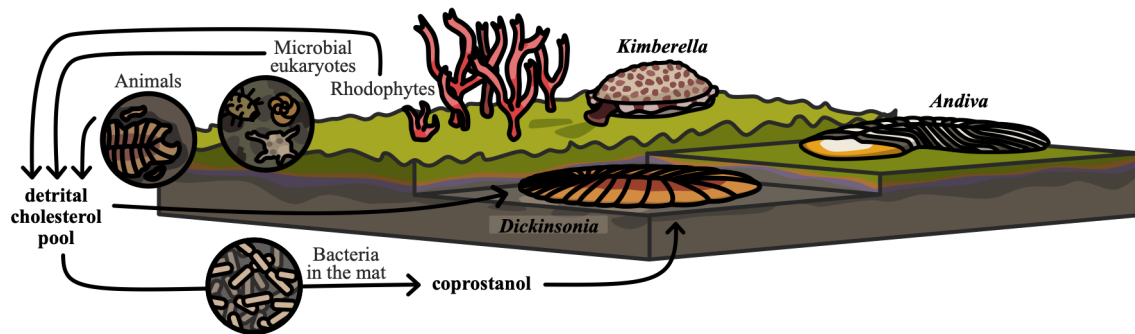


Figure 1.4

Our hypothesis for sterol cycling in the Ediacaran. Detrital cholesterol accumulates in the mat from diverse sources (including rhodophytes, microbial eukaryotes, and animals) and gets reduced by environmental bacteria to form coprostanol. Animals feeding on the microbial mats uptake differing amounts of coprostanol depending on the depth they feed to, with *Kimberella* only scratching the surface, while *Andiva* grazes lower, and *Dickinsonia* is the lowest.

In contrast to the prevailing interpretation, we hypothesize that the coprostanol biomarker is actually a unique dietary signal from dickinsoniomorphs feeding on the microbial mats (**Figure 4**). In this scenario, cholesterol built up in the microbial mat from the remains of C27-rich eukaryotes (e.g., animals, rhodophytes, and other microbial eukaryotes) and was converted into coprostanol by bacteria in the anoxic sublayer. The increase in cholesterol-rich eukaryote biomass during the Ediacaran likely represented a newly widespread metabolite for bacteria, which facilitated its decomposition until the Cambrian Substrate Revolution, when animals took a larger role as decomposers and disrupted mat ecosystems (Seilacher 1999; Brocks et al. 2017; Mills et al. 2025; Brunoir et al. 2023; Mitchell and Pates 2025). Dickinsoniomorphs ate these mats, preferentially absorbing the C27 sterols that animal tissues require. Although the microbial mat was dominated by C29 sterols, modern invertebrates can demonstrate differential absorption of sterols based on the mechanism they use to lyse the cells they eat; ctenophores, for example, often ingest algal cells but lack digestive fluids with a low enough pH to rupture the algal cell walls, preventing them from consuming the phytosterols within (Lobel 1981; Bumann and Puls 1997). Assuming *Dickinsonia* digested the mat with a ventral sole (Sperling and Vinther 2010), it could have had a similar filter for C27-rich material. Like living invertebrates,

dickinsoniomorphs would have utilized cholesterol in their cell membranes and may have even produced 5 α stanols during metabolism (Ballantine et al. 1976; Ballantine et al. 1977; Zhu et al. 1994; Kanazawa 2001; Blumenberg et al. 2002) leaving the biologically inert 5 β isomer (coprostanol) to accumulate. Coprostanol has been observed bioaccumulating in wastewater-exposed mussels and fish, meaning this is biologically plausible in dickinsoniomorphs (Gagné et al. 2001; Speranza et al. 2020). This bioaccumulation could also help explain the observed increase in C27 5 β /5 α ratio trending with *Dickinsonia* fossil width (Bobrovskiy et al. 2018a), as these molecules become more concentrated with time. In our scenario, the lower levels of coprostanol found in *Andiva* and *Yorgia* come from differences in feeding ecology. Trace fossil evidence suggests *Dickinsonia* leaves deeper impressions in the microbial mat than *Andiva*, which may suggest it fed more in the anoxic layer where coprostanol biosynthesis was occurring (Evans et al. 2019a). This interpretation could also apply to *Kimberella*; the lack of coprostane in this fossil could suggest surface-level feeding in the aerobic level of microbial mats. This hypothesis could be tested by studying the depth of putative *Kimberella* feeding traces (i.e., *Kimberichnus*, Gehling et al. 2014), although taphonomy and/or healing of the microbial mat after feeding might make this test untenable. If we are right, then the coprostane signal does not provide insight into how these ancient animals died, but instead provides insight into how they lived.

1.6 References

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Chapter 2: Coprostanol as a Biomarker of Gut Fermentation: A Reappraisal for Ecological, Paleontological, and Archeological Research

2.1 Abstract

Coprostanol is an important biomarker utilized in many fields, including archaeology, paleontology, conservation biology, ecotoxicology, and forensic sciences. Depending on the context, it is treated as a biomarker for humans, mammals, or general ‘higher-vertebrates’ and utilized in proxies ranging from distinguishing mammalian vs avian feces to reconstructing trophic levels. Despite the utility of the compound, a holistic review of the differentiating factors and biodiversity of coprostanol biosynthesis has not been done yet. In this study, we compiled 53 datasets (totaling 145 species) to gain a fuller accounting of its biological sources. The combined analysis of the vertebrate fecal data shows a clear pattern that prolific coprostanol production is linked to gut fermentation. By including multiple commonly reported sterols and their derivatives, specific gut fermentation strategies and taxonomic groups can be elucidated using principal components analysis. Lastly, several commonly used fecal proxy equations were evaluated for their accuracy using this large dataset, with some performing well while others did very poorly. The work presented here affirms that, while some previously published work may need to be reevaluated, fecal sterol data represents a powerful tool for both modern and paleontological studies.

2.2 Background

Coprostanol (or 5β -cholestan- 3β -ol) is the metabolic end-product of cholesterol (or cholest- 5 -en- 3β -ol) metabolism by bacteria in the guts of vertebrates and other anoxic environments (Subbiah et al. 1972; Hano et al. 2021; Kemp et al. 2022; Mulligan and Gold 2025). There are two established mechanisms for this conversion, one directly metabolizing cholesterol into coprostanol and one that is indirect, producing intermediate compounds first (Kenny et al. 2020; Netter et al. 2025). The cholesterol can either come from the animal host’s diet or its own tissues, and the conversion to coprostanol leaves it as a biologically inert compound that gets passed out in the animal's waste (Seidel et al. 1986; Juste

and Gérard 2021). The direct conversion has been identified in several bacterial groups (including Bacillota, Acidobacteria, and Cyanobacteria), involving a 3β -OH-D⁵⁻⁶-cholesterol-5 β -reductase (5 β ChR) enzyme (Netter et al. 2025). The 5 β ChR enzyme has been isolated in anaerobic fermenting microbiomes, including as commensals in healthy human gut microbiomes and from fermented Himalayan yak milk (Netter et al. 2025). The indirect conversion pathway uses enzymes called intestinal sterol metabolism A and B (ismA and ismB, respectively) to reduce cholesterol and is so far restricted to Clostridiales, which are a very important group of anaerobic obligate fermentors found in 98% of vertebrate gut microbiomes (Youngblut et al. 2019; Kenny et al. 2020; Mulligan and Gold 2025; Tinoco et al. 2025). Sterol metabolism can be generalized across all sterols (see **Figure 1A and 1B**), where the intact Δ^5 sterols (e.g. cholesterol and sitosterol) can be ultimately reduced to either 5 α stanols (e.g. where the hydrogen at carbon number 5 goes into the plane like cholestanol and sitostanol) or 5 β stanols (e.g. coprostanol and coprostanol, where the carbon 5 hydrogen goes out of the plane), with potential intermediate steps depending on the enzymes involved (such as 3-keto stanones/stenones). These stanols can also be further altered by bacteria into stereoisomers where the alcohol group at carbon 3 changes from going out of the plane (i.e. 3 β) to going into the plane (i.e. 3 α) and takes on the prefix epi-, such as epicoprostanol. Other microbial cholesterol metabolic end-products include sterol-sulfates, hydroxysterols, and sterolglucosides (Strott and Higashi 2003; Le et al. 2022; Li et al. 2024; Netter et al. 2025). Given the right conditions, these sterol compounds can all be preserved in the rock record as geochemical biomarkers after losing their functional groups, for example cholesterol is preserved as cholestane and coprostanol fossilizes to coprostane (Gill et al. 2009; Gill and Bull 2012; Zocatelli et al. 2017; Umamaheswaran et al. 2019; Summons et al. 2022; Tripp et al. 2022). The analysis of these sterol stereoisomers can be complicated by the fact that they often coelute in GC-MS spectra, requiring further steps to differentiate then quantify them (Adachi et al. 1997, Brosnan et al. 2025). There are two broad groups of sterols that can be generalized by which organisms commonly produce them: zoosterols are produced mainly by animals (e.g. cholesterol and its derivatives) and phytosterols that are produced by plants and algae (e.g. stigmasterol and sitosterol, see **Figure 1C**) - this provides the basis for distinguishing host diet in animal waste.

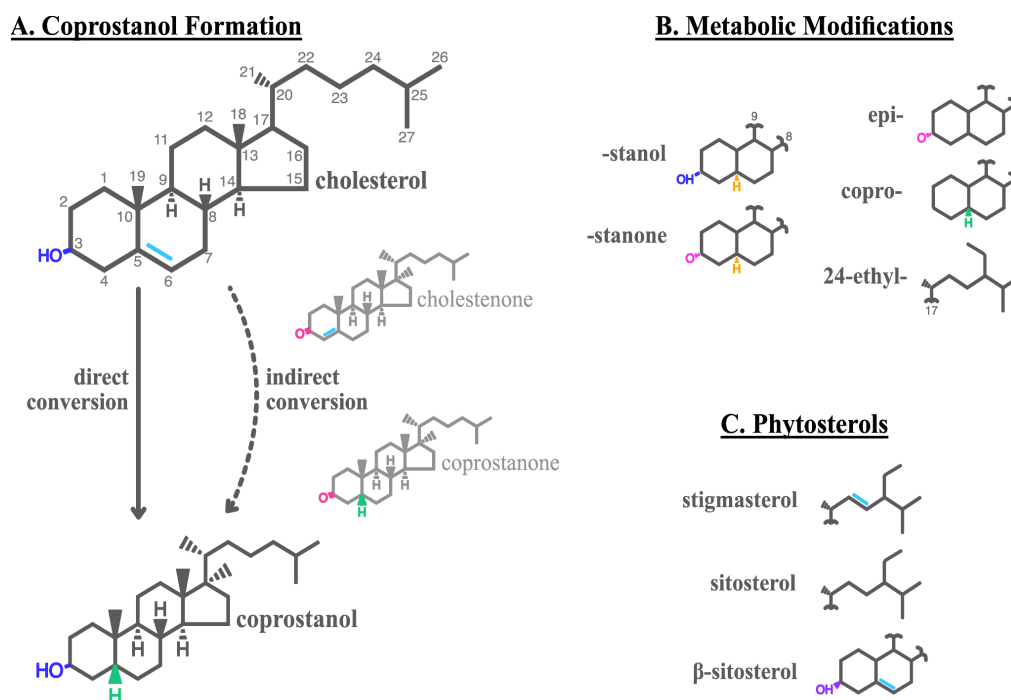


Figure 2.1

Chemical structures of sterols mentioned in this paper. **A.** Coprostanol production, starting with a cholesterol molecule with carbon numbers labelled and noting the direct versus indirect metabolic paths. **B.** Metabolic modifications of sterols - these are additive, for example 24-ethylepicoprostanol has all of the associated changes (i.e. an alcohol group at C3 in the α position, the H at C5 is in the β position, and an ethyl group at C24). **C.** Differences in phytosterols compared to cholesterol.

Coprostanol can be used for chemotaxonomy - the technique of using taxonomic-specific biomarkers to identify the presence of different clades - by quantifying and comparing it to other sterols in a sample. The proportions of fecal sterols in the environment has large applications for tracing biological sources, ranging from waste water indicators (Grimalt et al. 1990; Bull et al. 2002; Tyagi et al. 2007; Larson et al. 2022), to conservation biology (Leeming et al. 1996; Harrault et al. 2019; Kemp et al. 2022; Wendt et al. 2024), and archaeology and forensics (von der L  he et al. 2018; Ueland et al. 2021; V  zquez et al. 2021; Gryczewska et al. 2025). Coprostanol data can be analyzed in many ways, such as the raw counts or percent of the total chromatogram, normalized to a different compound, or through a more complex ratio involving many compounds. Recent reviews of fecal proxy ratios can be found in V  zquez et al. 2021, Larson et al. 2022, and Gryczewska et al. 2025; relevant proxy examples

involving coprostanol are provided in **Table 1**. The first is a ratio between coprostanol and cholesterol, **Ratio 1**. This ratio has been used extensively in the field of marine sewage contamination with a benchmark at 2.0 establishing raw effluent, and is also reported to distinguish cattle/horses at ~1 and sheep between 1 to 3 (Mudge and Ball 1964; Leeming et al. 1996). **Ratio 2** divides coprostanol by epicoprostanol, and a value >1.5 has been established to show human-specific contamination (Fattore et al. 1996; Patton and Reeves 1999). Dividing cholestanol by the sum of cholestanol + coprostanol + epicoprostanol in **Ratio 3** has been used to identify avian fecal presence with values >0.5 (Devane et al. 2015). **Ratio 4**, which is coprostanol normalized to 24-ethylcoprostanol, has been hypothesized to show values <38 for herbivores, 38-73 for omnivores, and >73 for humans (Leeming et al. 1997). **Ratio 5** combines zoosterols and phytosterols, such coprostanol + epicoprostanol / 5 β -stigmastanol + 5 β -epistigmastanol, which can help to distinguish omnivores versus ruminants at ratios above or below 2 (Bull et al 1999). **Ratio 6** uses (coprostanol + epicoprostanol) / (24-ethylcoprostanol + 24-ethylepicoprostanol) to distinguish humans/pigs/carnivores as higher than 1 and herbivores as less than 1 (Gryczewska al. 2024). **Ratio 7** is stigmastanol divided by stigmasterol + β -sitosterol, and is proposed in this paper to distinguish ruminants at a value >1.5, based on a trend also observed by Kemp et al. 2022. **Ratio 8** is proposed in this study by normalizing coprostanol to cholesterol and hypothesized to distinguish autoenzymatic and fermentive animals generally at a value of 0.01.

Although these biomarker proxies have been frequently used, and some have been reviewed for their shortcomings (such as Larson et al. 2022 and Gryczewska al. 2024), a broad biological review of the differentiating processes has never been done nor an evaluation of these ratios across more than a relatively limited number of common species. Many of these ratios were developed using only a handful of species and shown to work at a local level - but this is rarely reverified before the proxy is used in a different location where there are vastly different organisms present (such as proxies in Leeming et al. 1996 that were developed using only 6 bird species and 9 mammals from Australia, and has now been used in 600+ studies worldwide).

Ratio	Proxy calculation	Proxy interpretation
Ratio 1	$\frac{\text{coprostanol}}{\text{cholesterol}}$	cow/horse ~1, sheep 1–3 (Leeming et al. 1996)
Ratio 2	$\frac{\text{coprostanol}}{\text{epicoprostanol}}$	human >1.5 (Fattore et al. 1996; Patton and Reeves 1999)
Ratio 3	$\frac{\text{cholestanol}}{\text{cholestanol} + \text{coprostanol} + \text{epicoprostanol}}$	Aves >0.5 (Devane et al. 2015)
Ratio 4	$\frac{\text{coprostanol}}{\text{coprostanol} + 24\text{-ethylcoprostanol}}$	herbivore <38, omnivore 38-73, human >73 (Leeming et al. 1997)
Ratio 5	$\frac{\text{coprostanol} + \text{epicoprostanol}}{5\beta\text{-stigmastanol} + 5\beta\text{-epistigmastanol}}$	omnivore >1, ruminant <1 (Bull et al 1999)
Ratio 6	$\frac{\text{coprostanol} + \text{epicoprostanol}}{24\text{-ethylcoprostanol} + 24\text{-ethylepicoprostanol}}$	>1 human/pig/carnivores, <1 herbivore (Gryczewska al. 2024)
Ratio 7	$\frac{\text{stigmastanol}}{\text{stigmasterol} + \beta\text{-sitosterol}}$	ruminant >1.5 (proposed in this study)
Ratio 8	$\frac{\text{coprostanol}}{\text{cholesterol} + \text{coprostanol}}$	autoenzymatic digesters <0.01, fermenters >0.01 (proposed in this study)

Table 2.1

Example sterol ratios involving for the identification of fecal presence and origins. It is important to note that these ratios are necessarily unitless and can be taken from any measurement, so long as they are internally consistent.

Since the production of coprostanol has been linked to major groups of fermenting gut symbionts, special interest in the coprostanol levels of fermenting species with diverse anatomies is warranted (Krause and Khafipour 2010). The presence of coprostanol in animal feces is dependent on the host's microbial community, digestive anatomy, and their diet. Animals can be divided into two groups based on their enzymatic strategy: autoenzymatic digesters, who can supply all of the necessary enzymes to digest their food, versus alloenzymatic digesters, who rely on a microbial community to

digest their food; the latter is especially common in fiber fermentation (Karasov 2013). Fiber is defined as any polymer carbohydrate that a host animal cannot digest, but their microbiome may be able to break it down into its constituent sugars and allow the host to regain those otherwise lost nutrients. Microbes that ferment fibers can biosynthesize short chain fatty acids (SCFAs), namely acetate, butyrate, and propionate, as metabolites by a variety of reaction pathways (Koh et al. 2016). There are a few general morphological strategies for species that are dependent on these fermentation products: having a warm body temperature, maintaining low energetic costs, and/or adapting for ways to recapture lost nutrients (like rumination or coprophagy, which will be discussed below). Fermentating microbial communities can vary quite a bit across host taxonomy and organs, but major symbiont consortia include: firmicutes (like *Clostridia* mentioned above), lactic acid (LAC) bacteria, and Ruminococcaceae (Oliphant and Allen-Vercoe 2019). Other common fermenting symbionts in these microbiomes can include: methanogenic bacteria, archaea, ciliates, and anaerobic fungi (Hackstein and van Alen 1996; Hume 1997, Gill et al. 2010; Krause and Khafipour 2010; Newbold et al. 2015; Meili et al. 2023). Animal digestive tracts can be divided into three sections: pregastric (foregut), gastric, and postgastric (hindgut). A major delimiting factor in nutrient recovery between foregut and hindgut fermentation is capturing material before or after it passes through the stomach, meaning foregut fermenters can specialize on breaking down relatively nutrient-poor material prior to passing it to the absorptive postgastric organs, while hindgut fermenting species recover proteins and carbohydrates in their stomach before the more refractory components of their diet enter their intestines (Janis 1976; Alexander 1993). Foregut fermentation organs involve a development of organs preceding the stomach, either as outpocketing from the esophagus or partitioning the stomach into multiple chambers. Hindgut fermentation can occur in both the small intestine (known from only 2 species: a turtle and emus, Bjomdal and Bolten 1990; Kim et al. 2023) and most commonly in the large intestine. Large intestines are further subdivided into two sections: the colon, which is the direct continuation of the intestines, and the cecum, which is a blind-end outpocketing from the intestines (Hume 1997). Fermentation can also occur across multiple organs, and can span across pre- and postgastric sites. Herbivory and fermentation can be seen in every class of vertebrates, who employ diverse strategies and microbial

communities to process their diets and will be reviewed briefly in the paragraphs below by taxonomic class (see **Table 2** for summary).

Fishes occupy many diverse niches, with varying consumption of plant and algal material. There are four digestive strategies for herbivory in fishes, as described by Horn (1989): 1. highly acidic chamber (e.g. some surgeonfish, rabbitfish, cichlids, and blennies), 2. gizzard-like stomach (e.g. mullet, some surgeonfish, and southern seacarp), 3. pharyngeal mill (e.g. parrotfish, cales, and halfbeaks), and 4. hindgut fermentation (e.g. sea chubs and the bonnethead shark). Several species in Kyphosidae, the family name for sea chubs, have been shown to ferment in their hindgut to process their algal diet using both microbiome and metabolomic evidence (Horn 1989; Fidopiastis et al. 2005; Clements et al. 2016). Bonnethead shark (*Sphyrna tiburo*) diets can constitute 40-60% seagrass, while the rest is made up of a more typical shark diet such as crabs and cephalopods (Bethea et al. 2007). Microbiome and SCFA analysis along their digestive tract suggests that the spiral and distal sections of the bonnethead shark's intestine may be comparable to a colon and feature limited fiber fermentation (Leigh et al. 2021). Some fish, such as herring cale (*Odax cyanomelas*) and sea carp (*Crinodus lophodon*), may also ferment as suggested by SCFA analysis (Horn 1989, Clements et al. 1994). Although only a few species of fish are able to ferment, they are an important bracket for vertebrate gut microbial ecology from an evolutionary perspective, and if they are found to produce coprostanol in any appreciable amount then input from fish may be confounding variables in marine archaeological analyses.

Amphibians all have carnivorous diets that do not rely on fermentation, with a few notable exceptions. The greater siren (*Siren lacertina*) has been shown to ferment plant and algal material in their colon that they ingest via suction feeding, which has been confirmed by direct observations of feeding and digestion of plant material (Pryor et al. 2006; Hill et al. 2015; Schwarz et al. 2021). While there are no published fecal sterol studies for salamanders, the metagenome from a Chinese giant salamander (*Andrias davidianus*) was found to contain the *ismA* enzyme suggesting they may be capable of reducing cholesterol to coprostanol (Mulligan and Gold 2025). American bullfrog (*Rana catesbeiana*) tadpoles have herbivorous diets and have been observed to ferment in well-developed

colons, which ultimately disappear during metamorphosis as they transition to their carnivorous adult diets (Bjorndal 1997; Pryor and Bjorndal 2005). While coprostanol is generally biologically inert, it can serve as a precursor for some toad bufadienolide poisons (Garraffo and Gros 1986). Coprostanol generation from select amphibians is an open question if it is in fact a fermentation product, and would mean bullfrogs and larger salamanders could impact environmental data; and the suggestion of functionally omnivorous diets in large salamanders would present interesting questions about their care and conservation.

Herbivory is uncommon in living reptiles and seen only in some lizards, all tortoises, and a few turtles. Testudine herbivores ferment in their proximal colon, which has been enlarged to such a degree they have been considered functionally a cecum (Guard 1980; Bjorndal 1997). A unique departure from this trend is the Florida red-bellied turtle, *Pseudemys nelsoni*, which is a rare example of small intestinal fermentation (Bjorndal and Bolten 1990). Of the lizards, herbivory is restricted to the families of Iguanidae (e.g. rhinoceros iguana and chuckwalla) and Agamidae (e.g. bearded dragon and *Uromastyx*), and additionally the prehensile-tailed skink (*Corucia zebrata*) from the family Scincidae. Most lizard fermenters process material in partitioned colons - the segmentation slows the passage of food and mucosal folds to increase surface area (Iverson 1980; Bjorndal 1997; Stevens and Hume 1998). Fermentation in these ectotherms requires access to warm temperatures and adaptations to make the process more efficient, like gastroliths and colons limiting outflow (Hong et al. 2015). Herbivorous reptiles may produce coprostanol, and if they do this could impact environmental analyses in the tropics and could also provide insight to evolutionary trends such as dinosaur digestion when combined with avian data.

Herbivorous birds may employ a wide range of fermentation strategies, while nectivores, frugivores, carnivores, etc. use autoenzymatic digestion for their easier to metabolize diets. Bird foregut fermentation can involve two areas of the stomach: the proventriculus (a glandular section after the esophagus) and the ventriculus (equivalent to mammalian stomachs) (King and McLelland 1984). The only confirmed species to ferment in their proventriculus is the hoatzin (*Opisthocomus hoazin*, Grajal

et al. 1989), although there is metagenomic data to support it in screamers (García-Amado et al. 2023), and there are speculations of two other species: the great blue turaco (*Corythaeola cristata*, Johnston 1999) and kākāpō (*Strigops habroptilus*, Vispo and Karasov 1997; Waite et al. 2012). The only species known to ferment in their ventriculus is the speckled mousebird (*Colius striatus*) - these birds have unique anatomical adaptations to opportunistically bask in the sun to heat their stomach and promote microbial fermentation (Downs et al. 2000). Anaerobic crop fermentation may be widespread among granivorous birds (such as turkeys, pigeons, finches, and some parrots) but at a lower efficiency than the specialized folivores mentioned (Pacheco et al. 2008). Bird hindgut fermenters can use several organs - most commonly their cecum (single or paired organs stemming from the end of the small intestine) but some use their large or small intestines. Birds have diverse cecal anatomy regardless if they use it as a primary site of fermentation, and can be divided into 5 groups following Clench and Mathias 1995: 1. absent (e.g. parrots, woodpeckers, and hummingbirds), 2. vestigial-type (e.g. pelicans and petrels), 3. glandular-type (e.g. owls and nightjars), 4. lymphoid-type (e.g. pelicans, flamingos, falcons, and most passerines), and 5. intestinal-type (e.g. some ratites, geese, ducks, and shorebirds). Species that ferment primarily in their cecum feature the intestinal-type morphology, where there is a greater volume to process materials in. Some species, such as ostriches and screamers, ferment in their well developed colons (Garrod 1876; Swart et al. 1993), while emus ferment in their cecum and jejunum (Kim et al. 2023). Screamers, ostriches, and rhea are noted for having sacculated ceca and screamers have a spiral twist at the end of it (Garrod 1876), and elegant crested tinamous (*Eudromia elegans*) have been noted to have uniquely ornamented ceca (Clench 1999). While coprostanol production in birds has been of explicit interest for a few studies (Telesford-Checkley 2014; Cheng et al. 2016; Chen et al. 2024), the research has generally focused on agriculturally important species. The potential impact of birds, especially nesting rookeries, to environmental data remains largely unknown. Further research into avian coprostanol production can open up new lines of research into conservation and paleoecology, as will be discussed below.

Fiber fermentation site/strategy	Example taxa	References
Autoenzymatic digestion	Most fishes/amphibians/reptiles, many birds, Carnivora, bats, cetaceans	
Foregut fermentation		
Proventriculus	Hoatzin, great blue turaco?, kākāpō?	Grajal et al. 1989; Johnston 1999; Vispo and Karasov 1997; Waite et al. 2012
Heterothermic ventriculus	Mousebirds	Downs et al. 2000
Monogastric stomach	Kangaroos, sloths	Hume 1989; Vendl et al. 2016
Neutral pH	Echidnas	Perry et al. 2022; Shaw 2023
Compartmentalized stomach		
Pseudoruminant (tripartite)	Camelids, hippos	Al-Jassim and Hogan 2012; Langer 1976
Ruminant (quadripartite)	Most artiodactyls	Reddy and Hyder 2023
Fore- and Hindgut fermentation		
Gastric-midgut-cecal	Hyraxes	Krause and Khafipour 2010
Pregastric-cecal	Voles, hamsters	Hörnigke and Björnhag 1980
Proventriculus and colon	Screamers	García-Amado et al. 2023
Pseudoruminant and cecal	Colobinae monkeys	Matsuda and Clauss 2022
Hindgut fermentation		
Cecum	Koalas, brushtailed possum, pigs, capybara, most primates	Hörnigke and Björnhag 1980
Cecum plus caecotrophy	Rabbits, pika	Hörnigke and Björnhag 1980
Cecum plus coprophagy	Most rodents, ringtailed possum	Hörnigke and Björnhag 1980
Intestinal-type cecum	Some ratites, Galloanserae	Clench 1999
Ornamented cecum	Elegant crested tinamou	Clench 1999
Cecum and colon	Elephants	Van Hoven et al. 1981; Clemens and Maloiy 1982
Cecum and hindgut	Kyphosids	Fidopiastis et al. 2005; Horn 1989
Colon	Some toad tadpoles, ostriches, Perissodactyla	Swart et al. 1993; Pryor and Björndal 2005
Colon plus coprophagy	Gorillas	Graczyk and Cranfield 2003
Partitioned colon	Most Iguanidae, Agamidae, Scincidae, Laurenti's whiptail?	Dearing 1993; Björndal 1997
Proximal colon (called a "functional" cecum in Guard 1980)	Tortoises, some turtles	Guard 1980; Björndal 1997
Facultative colon	Sirens, Cryptobranchidae?	Pryor et al. 2006; Schwarz et al. 2021; Hill et al. 2015; Mulligan and Gold 2025
Jejunum and cecum	Emus	Kim et al. 2023
Posterior intestine	Coles?, butterflyfish? sea carp?	Clements et al. 1994
Small intestines	Florida red-bellied turtle	Björndal and Bolten 1990
Spiral and distal intestine	Bonnethead shark	Leigh et al. 2021

Table 2.2

Summary of different fermentation strategies and organs in vertebrate clades. Species that have been speculated to ferment, but not confirmed, are listed with question marks.

Fermentation in mammals is much more common and diverse than in the other vertebrate classes, although there are many clades that are autoenzymatic (such as Carnivora and Chiroptera). Foregut fermentation can be subdivided further based on the anatomy and number of chambers in the

stomach. Most artiodactyls (e.g. deer, bovids, antelopes, giraffes, etc.) are ruminants, who use a compartmentalized four chambered stomach (i.e. quadripartite stomachs) to hold and selectively regurgitate digested material to further mechanically and chemically process it (Reddy and Hyder 2023). Ruminants can be further subdivided by their digestive physiology and diet preferences following Hofman 1989: concentrate feeders specialize in digesting the soluble contents of plant cells (e.g. giraffes and mule deer), intermediate feeders have a mixed diet that changes with the landscape but otherwise avoid fiber-dense materials (e.g. Thomson's gazelle and goats), and grazing/roughage feeders (e.g. cattle and sheep) that specialize in digesting structural carbohydrates like cellulose. The prevalence of archaea in foregut fermenting microbiomes has led to the establishment of archaeol (a membrane lipid found only in archaea) as a biomarker for foregut fermenting mammals (Gill et al. 2010). Pseudoruminant fermentation (e.g. hippos, camelids, Colobinae monkeys) have tripartite (i.e. two blindsacs on the stomach) or quadripartite stomachs to stage the digestion of food but do not regurgitate food like ruminants, with the exception of the proboscis monkey (*Nasalis larvatus*) which does regurgitate and remasticate (Langer 1976; Al-Jassim and Hogan 2012; Matsuda and Clauss 2022). Monogastric stomach fermentation is done in a single chamber (e.g. echidnas, kangaroos, sloths, Hume 1989; Vendl et al. 2016), and echidnas do this with a neutral pH (Perry et al. 2022; Shaw 2023). Cetaceans have replaced their ancestral foregut capacity to ferment fiber for fermenting chitin, and will be classified in this paper as autoenzymatic digesters (Olsen and Mathiesen 1996; Sanders et al. 2015). Mammalian hindgut fermentation occurs in the cecum (e.g. koalas, brushtailed possum, pigs, capybara, and most primates) or colon (e.g. gorillas and *Perrisodactyla*), with some interesting behavioral variations. To recover lost nutrients, some species re-consume their feces (analogous to rumination but postgastric) - if the material passes through the full digestive system it's called coprophagy (e.g. ringtailed possums, most rodents, and gorillas), but if the material is specifically coming from the cecum and timed for reingestion it's called caecotrophy (e.g. rabbits and pika) (Graczyk and Cranfield 2003). Some species can have more complicated fermentation systems, such as cecal-colonic (e.g. elephants, Van Hoven et al. 1981), pregastric-cecal (e.g. voles and hamsters, Hörnicke and Björnhag 1980) and gastric-midgut-cecal (e.g. hyraxes, Krause and Khafipour 2010). Mammals make up the majority of wildlife biomass and represent significant contributors of modified sterols to the environment -

understanding the diversity and capacities of different mammals should be a prerequisite before evaluating environment fecal ratios.

Our goal is to improve the understanding of which groups of vertebrates actually have enriched proportions of coprostanol in their stool, and test if there is a connection between coprostanol production and gut fermentation type that can help inform the overall utility of fecal biomarker proxies.

2.3 Methods

All code and files are available on GitHub:

https://github.com/DavidGoldLab/2026_Coprostanol_Taxonomy

The first step was to collect published animal fecal sterol information. This search prioritized papers that reported both cholesterol and coprostanol, but all quantified sterol data from these papers was included in the database. Since the field primarily uses unitless ratios, all published data was accepted regardless of the measurement type, including dry mass, normalized mass, or a percentage of the chromatogram spectra. All raw data, including metadata such as taxonomy and sterol measurement units, is provided in **Supplemental Table 1**.

Statistical analysis of the sterol data in R was done with several approaches. An ANOVA test (R Studio v2024.4.2.764, R Core Team 2021) was run comparing the ratio of *coprostanol* / *coprostanol* + *cholesterol* against the effects of: simplified diet (carnivore, omnivore, herbivore, omnivorous primate, herbivorous primate), simple digestion strategy (autoenzymatic digestion, foregut fermentation, hindgut fermentation), detailed digestion strategy (see Table 2), and taxonomy (clade, class, order, family, species). All variables from the ANOVA were significant, meaning the next step was a *post hoc* analysis - a Tukey's test was done on the detailed digestion strategies and binned the into 5 mixed types (A-E), although primates were split into primate vs non-primate, pseudoruminants were separated into the two families (Camelidae and Hippopotamidae), and monogastric fermentation was omitted since there is only one sample. Plots (ggplot2 v3.5.1, Wickham 2016) were made to

visualize the ratio of *coprostanol* / *coprostanol* + *cholesterol*, in addition to the ratios discussed above in Table 1. Two PCA analyses were done: the first with the 5 most commonly reported zoosterols (cholesterol, coprostanol, epicoprostanol, cholestanol, and coprostanone), and a second with the addition of 3 common phytosterols (adding stigmasterol, stigmasterol, and β -sitosterol to the previous list), recovering 60 and 30 species for analysis, respectively. The PCA plots that were grouped by digestive system had cecal fermenters separated by class (e.g. Aves and Mammalia), and the ones grouped by taxonomic order broke up Artiodactyls and Primates into their respective sub-clades.

The primary use of sterol data is in proxy ratios - these were evaluated for their accuracy on the expanded dataset collected here. Each individual ratio test was limited to a restricted subset that had all of the required compounds. The ratios (see Table 1) were evaluated in R for their misclassification rate, precision, and accuracy. True positive (TP) and true negative (TN) values were counted where those expectations were met, while false positive (FP) and false negative (FN) samples are those where the data and categories were incorrectly matched. The misclassification rate was calculated with $(FP + FN) / total\ samples$. Accuracy is calculated by $(TP + TN) / total\ samples$. Lastly, precision is determined by $TP / (TP + FP)$.

2.4 Results

The *coprostanol* / *coprostanol* + *cholesterol* values varied considerably by taxonomy, diet, and digestive system, with the digestive system being strongly predictive (see **Figure 2**). When the data is separated by diet, carnivores overwhelmingly cluster at a ratio of zero, primate herbivores averaged 0.78 ± 0.17 , and non-primate omnivores, non-primate herbivores, and primate omnivores range from 0-0.97. When the data is binned by simple digestive strategies, autoenzymatic digesters averaged 0.04 ± 0.15 , foregut fermenters were 0.51 ± 0.15 , and hindgut fermenters were 0.58 ± 0.28 . Detailed digestive strategies show a lot of variability. The types of foregut fermentation strategies had the following ratios: ruminants averaged, the single monogastric fermenter (i.e. *Macropus* spp.) was 0.51, and the two pseudoruminants have a split distribution, where hippos cluster at 0.13 ± 0.01 and camelids at 0.66 ± 0.11 . Hindgut fermentation strategies have the following results: cecal-colonic fermenters (i.e.

Elephantidae) averaged 0.44 ± 0.14 , cecum fermenters were 0.5 ± 0.32 , and colon fermenters averaged 0.7 ± 0.12 . Pregastric/cecal fermenters (i.e. hamsters) averaged 0.6 ± 0.2 . Microbiomes, and therefore metabolites, can vary across individuals - intraspecific variation in sterol data is an important consideration with the results presented here (Derrien et al. 2011). Many species vary only ± 0.1 for their *coprostanol* / *coprostanol* + *cholesterol* ratio, while some (namely humans, rats, and one species of bat) varied by ~ 0.75 - 0.9 (see **Supplementary Table 2**).

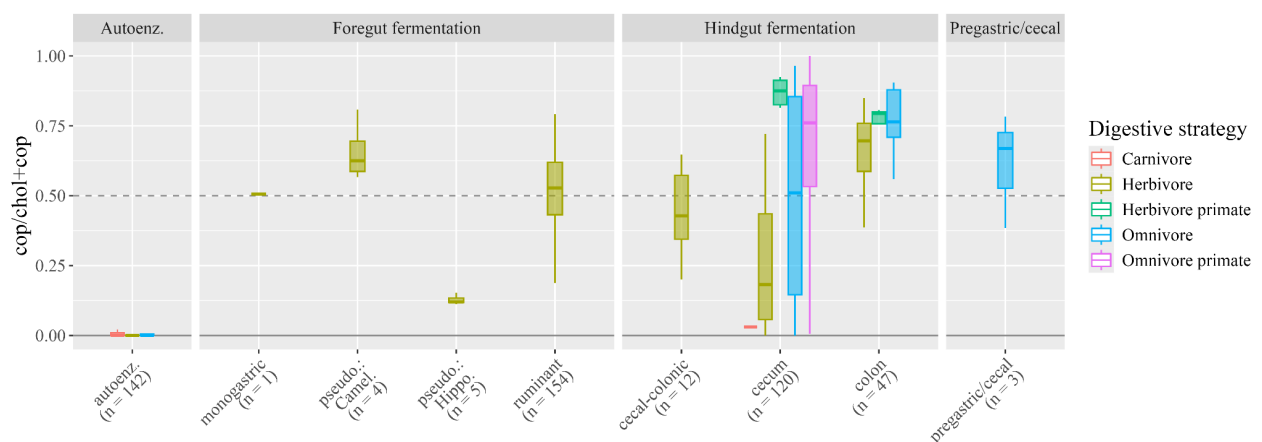


Figure 2.2

coprostanol / *coprostanol* + *cholesterol* ratio (abbreviated here as *cop/chol+cop*) in different digestive strategies. The dashed line represents a 1:1 ratio, and it is colored by the general diet. Primates are separated from animals due to their high coprostanol values, and pseudoruminants are split into the two families due to their markedly different ranges. Autoenzymatic digesters is abbreviated to ‘autoenz.’ and pseudoruminants are shortened to ‘pseudo.’ then followed by the abbreviated family on the x-axis.

Class	Clade	Order	no. families	no. species	no. samples	digestive strategy	avg cop/chol+cop
Actinopterygii	Actinopterygii	Characiformes	1	1	1	Autoenzymatic digestion	0.01
Actinopterygii	Actinopterygii	Cichliformes	1	1	1	Autoenzymatic digestion	0
Actinopterygii	Actinopterygii	Gadiformes	1	1	1	Autoenzymatic digestion	0
Actinopterygii	Actinopterygii	Salmoniformes	1	1	7	Autoenzymatic digestion	0
Actinopterygii	Actinopterygii	Spariformes	1	1	4	Autoenzymatic digestion	0
Reptilia	Crocodylia	Crocodylia	2	2	4	Autoenzymatic digestion	0.1 ± 0.187
Reptilia	Squamata	Squamata: Lacertidae	1	1	1	Autoenzymatic digestion	0
Reptilia	Squamata	Squamata: Trogonophidae	1	1	2	Autoenzymatic digestion	0
Aves	Paleognathes	Struthioniformes	1	1	2	Hindgut fermentation - colon	0.751 ± 0.059
Aves	Galloanserae	Anseriformes	1	8	13	Hindgut fermentation - cecum	0.05 ± 0.069
Aves	Galloanserae	Galliformes	1	5	8	Hindgut fermentation - cecum	0.223 ± 0.218
Aves	Neoaves	Accipitriformes	1	1	1	Autoenzymatic digestion	0
Aves	Neoaves	Cathartiformes	1	1	2	Autoenzymatic digestion	0.253 ± 0.004
Aves	Neoaves	Charadriiformes	1	3	4	Autoenzymatic digestion	0.005 ± 0.008
Aves	Neoaves	Columbiformes	1	1	2	Autoenzymatic digestion	0.002 ± 0.003
Aves	Neoaves	Falconiformes	1	1	1	Autoenzymatic digestion	0.003
Aves	Neoaves	Passeriformes	1	1	1	Autoenzymatic digestion	0.006
Aves	Neoaves	Pelecaniformes	2	2	12	Autoenzymatic digestion	0.019 ± 0.028
Aves	Neoaves	Procellariiformes	1	1	1	Autoenzymatic digestion	0
Aves	Neoaves	Psittaciformes	1	1	1	Autoenzymatic digestion	0.001
Aves	Neoaves	Sphenisciformes	1	2	2	Autoenzymatic digestion	0
Aves	Neoaves	Strigiformes	1	2	3	Autoenzymatic digestion	0.005 ± 0.004
Aves	Neoaves	Suliformes	1	1	1	Autoenzymatic digestion	0.002
Mammalia	Marsupials	Diprotodontia: Macropodidae	1	1	1	Foregut fermentation - monogastric	0.506
Mammalia	Marsupials	Diprotodontia: Phalangeridae	1	1	2	Hindgut fermentation - cecum	0.572 ± 0.105
Mammalia	Marsupials	Diprotodontia: Phascogasteridae	1	1	1	Hindgut fermentation - cecum	0.341
Mammalia	Marsupials	Diprotodontia: Pseudocheiridae	1	1	1	Hindgut fermentation - cecum	0.701
Mammalia	Afrotheria	Proboscidea	1	4	12	Hindgut fermentation - cecal-colonic	0.446 ± 0.143
Mammalia	Laurasiatheria	Artiodactyla: Camelidae	1	4	4	Foregut fermentation - pseudoruminant	0.657 ± 0.108
Mammalia	Laurasiatheria	Artiodactyla: Cetacea	1	4	4	Autoenzymatic digestion	0.02 ± 0.024
Mammalia	Laurasiatheria	Artiodactyla: Hippopotamidae	1	1	5	Foregut fermentation - pseudoruminant	0.128 ± 0.016
Mammalia	Laurasiatheria	Artiodactyla: Ruminants	4	19	154	Foregut fermentation - ruminant	0.515 ± 0.136
Mammalia	Laurasiatheria	Artiodactyla: Suidae	1	2	10	Hindgut fermentation - colon	0.763 ± 0.125
Mammalia	Laurasiatheria	Carnivora	6	23	44	Autoenzymatic digestion	0.077 ± 0.177
Mammalia	Laurasiatheria	Chiroptera	5	19	42	Autoenzymatic digestion	0.044 ± 0.195
Mammalia	Laurasiatheria	Perissodactyla	2	7	31	Hindgut fermentation - colon	0.668 ± 0.117
Mammalia	Euarchontoglires	Lagomorpha	2	3	13	Hindgut fermentation - cecum	0.324 ± 0.2
Mammalia	Euarchontoglires	Primates: Hominidae	1	2	36	Hindgut fermentation - cecum	0.71 ± 0.291
Mammalia	Euarchontoglires	Primates: non-Hominidae	4	9	34	variable	0.681 ± 0.198
Mammalia	Euarchontoglires	Rodentia	3	4	19	variable	0.699 ± 0.228

Table 2.3

Summary of sample diversity and average *coprostanol* / *coprostanol* + *cholesterol* per clade. Note that the ‘no. species’ column includes unknowns as a unique species (e.g. ‘unknown elephant’).

In addition to the digestive system, major taxonomic clades can be distinguished based on coprostanol ratio data. Broadly, clades that had ratios near zero were Actinopterygii, Reptilia, Neoaves, and Carnivora (see summary **Table 4** and **Supplemental Table 1**). Enriched (i.e. above a 1:1 ratio) clades include Struthioniformes, Rodentia, Perrissodactyla, Primates, and Artiodactyla (see **Figure 3**). Among the high carnivore outliers are the California condors (*Vultur gryphus*), an American alligator (*Alligator mississippiensis*), a Brown bear (*Ursus arctos*), two bats (*Saccopteryx bilineata*, *Pteronotus mesoamericanus*), and a California sea lion (*Zalophus californianus*). Humans (*Homo sapiens*, n=31) span from 0.006 to 0.977, while Neanderthal (*Homo neanderthalensis*, n=5) ratios range from 0.5-0.9. Artiodactyla consistently split into five groups (roughly by family and by digestive strategy) across all analyses, following low to high values: cetacea (autoenzymatic), hippos (pseudoruminant), camelids (pseudoruminant), ruminants, and suids (colon fermentation).

To evaluate the results, several statistical analyses were completed: an ANOVA and Tukey test to test the roles of metadata, and PCA plots to examine how the distribution of chemical compounds correlated to digestive strategy and taxonomy. All ANOVA variables (diet, digestion strategy, and taxonomy) were significant, with a majority of them (simple digestion, detailed digestion, diet, class, and clade) had p-value near or below $2e-16$, except for: 0.00107 for order, $1.15e-11$ for family, and $1.51e-05$ for the species level. A Tukey test was done comparing the ratios between the detailed digestion strategies, and grouped: A as colon fermenters and primate cecal fermenters, B as ruminants, E as autoenzymatic digesters, and the remaining digestive types were combinations of letters (see **Supplemental Figure 1**). Two PCA plots were done for the commonly reported zoosterols and a combined dataset with both zoosterols and phytosterols, showing clearly distinct clusters and explain a combined 94.55% and 91.04% of the data variance, respectively (see **Figures 4 and 5**). The zoosterol PCA pulled out to two large groups, one with overlapping autoenzymatic digesters, Galloanserae, and hippos, with the other group a gradient from hindgut fermenting mammals, to foregut fermenting mammals, to primates, and with ostriches in the middle of the series and humans at the end. The phytosterol PCA separated out ruminants as one group, elephants, hippos, and anseriformes as another, and all remaining taxa fall between these two.

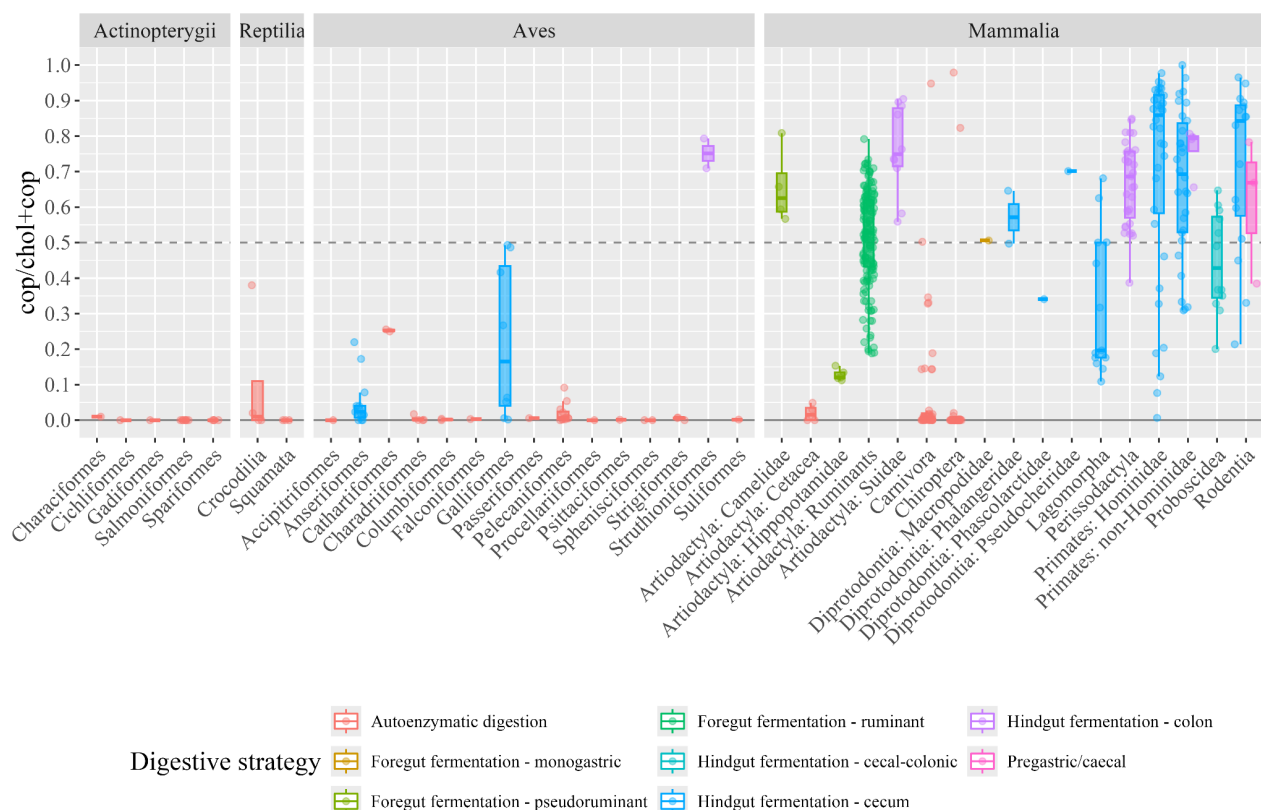


Figure 2.3

coprostanol / *coprostanol* + *cholesterol* ratio (abbreviated here as cop/chol+cop) grouped by taxonomic order, and at the family-level in some groups to show either notably different ranges or different digestive strategies. The dashed line represents a 1:1 ratio, and the figure is color coded by the digestive strategy. See Table 2.3 for more details.

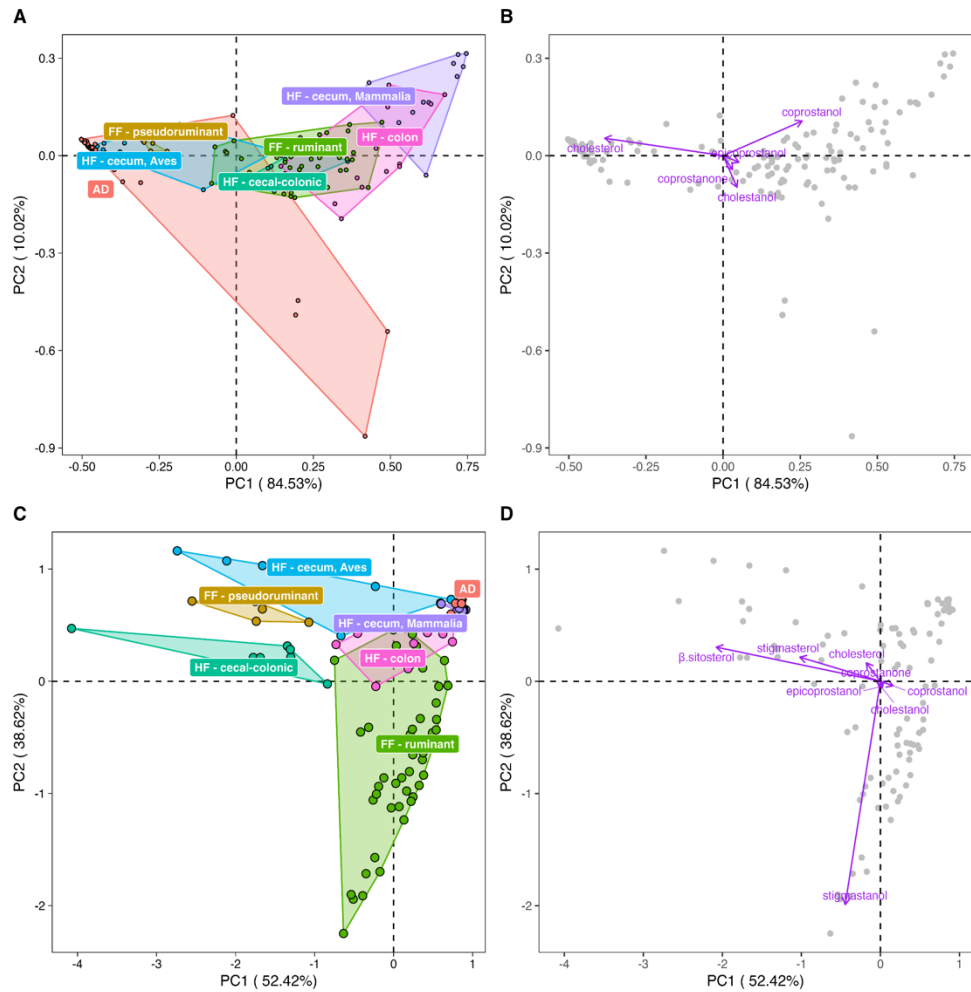


Figure 2.4

PCA plots of sterols normalized as a proportion of their summed values: **A** and **B** show zoosterols (cholesterol, coprostanol, epicoprostanol, cholestanol, and coprostanone). Panels **C** and **D** show the previous zoosterols in addition to common phytosterols (stigmaterol, stigmastanol, and β -sitosterol). Panels **A** and **C** show the data grouped by fermentation strategy, while panels **B** and **D** show the corresponding eigenvectors. The digestive strategies have the following abbreviations: AD = autoenzymatic digestion, FF = foregut fermentation, and HF = hindgut fermentation.

Each ratio proxy was evaluated for its misclassification rate, accuracy, and precision (see **Table 4**) - some performed well (such as Ratio 6), while others did poorly (such as Ratios 2 and 3). Ratio 1 should have identified sheep, cows, and horses (Leeming et al. 1996), but did not show any specific clustering of taxa along the specified levels, with Artiodactyla ranging from 0-9.45 and Perrissodactyla

ranging from 0.63-5.62. Ratio 2, which was suggested to separate out humans at above a ratio of >1.5 (Fattore et al. 1996; Patton and Reeves 1999), had an accuracy rate of 10%, owing to an average ratio value of 136.37 across all taxa (with only 9 of the 353 samples being human). The avian ratio, Ratio 3, did have many birds at an elevated level, but with a misclassification rate of 91% it failed to capture ostriches, many Galloanserae, and many Pelecaniformes - while incidentally retaining all of Crocodilia, Carnivora, Chiroptera, and many Artiodactyls, Perrisodactyls, and Lagomorphs. A separate ratio meant to identify avian feces from Cheng et al. 2016, $\text{cholesterol} / (\text{cholesterol} + \text{sitosterol})$, had a remarkably similar pattern of identifying birds, bats, and mammalian carnivores in addition to a lizard and several hominids. The Ratio 4, which should distinguish between herbivores, omnivores, and humans, had mixed results with an accuracy rate of 62% - humans indeed clustered tightly around 73, but the rest of the diets had wide values across the entire 0-100 range. Ratio 5, which divided ruminants < 1 > omnivores, left all animals below 1 except for a red grouse (*Lagopus lagopus scotica*, the only bird amenable for the analysis) and Neanderthals (the only amenable hominid) having values up to ~100 for a misclassification rate of 69%. The proxy for human/pig/carnivores versus herbivores, Ratio 6, had an accuracy rate of 92%. Ratios 7 and 8 are proposed in this study, and successfully identify ruminants and autoenzymatic digesting species with 92% and 93% accuracy, respectively. Interestingly, dividing Ratio7/Ratio8 can identify felids, canids, and hyenas very well, and Ratio8/Ratio7 isolates just non-human primates (although only vervet monkeys and olive baboons were included). It is worth noting that the ratio evaluations presented here are tested against a globally diverse dataset - each proxy may produce better results in more restricted contexts.

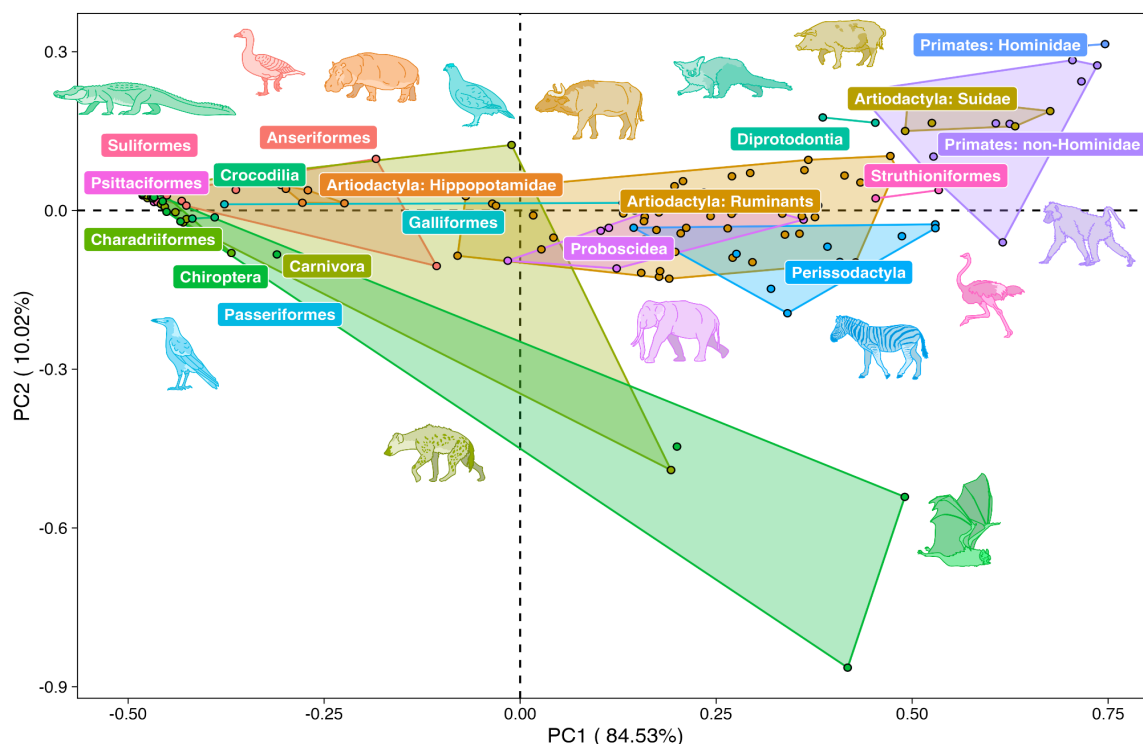


Figure 2.5

PCA plots of zoosterols normalized as a proportion of their summed values (this is the same data as Figure 4A and B), grouped and color-coded by taxonomic order.

Ratio	n species	TP	TN	FP	FN	total	MisR	Acc	Prec
Ratio 1	127	7	313	99	34	453	0.29	0.71	0.07
Ratio 2	100	5	31	315	1	352	0.90	0.10	0.02
Ratio 3	100	26	7	316	3	352	0.91	0.09	0.08
Ratio 4	46	125	0	53	24	202	0.38	0.62	0.70
Ratio 5	18	11	0	17	8	36	0.69	0.31	0.39
Ratio 6	26	117	0	1	9	127	0.08	0.92	0.99
Ratio 7	39	101	92	3	14	210	0.08	0.92	0.97
Ratio 8	127	420	0	0	33	453	0.07	0.93	-

Table 2.4

Results of the ratio evaluations (see Table 1 above for the ratios themselves). Column ‘n species’ shows the total number of species amenable for each ratio’s analysis. TP = true positive, TN = true negative, FP = false positive, FN = false negative, total = total number of individual samples, MisR = misclassification rate, Acc = accuracy, and Prec = precision.

2.5 Discussion

Coprostanol production varies significantly between species - the host's diet and fermentation strategy play large roles in determining the levels of *coprostanol* / *coprostanol* + *cholesterol* in their stool. Trends by diet roughly followed: carnivores have low values, herbivores generally have higher values, and omnivores span the full range. Carnivores can produce low amounts of coprostanol but in a ratio it is completely overprinted by high amounts of dietary cholesterol - unlike many other lipid biomarkers, coprostanol can be assumed to have no trophic modifications beyond the initial reduction from sterol to stanol and changes in concentration. The minimal coprostanol values in autoenzymatic digesters (i.e. carnivores) are likely due to their short digestive retention times and overall fewer fermenting microbes. Higher *coprostanol* / *coprostanol* + *cholesterol* levels are found in gut fermenting species, although the separate types cannot be distinguished by this ratio alone. The addition of other sterols can be used to distinguish fermenters quite well, especially stigmastanol for ruminants vs β -sitosterol and stigmasterol for other herbivores, as exemplified in Ratio 7. The higher coprostanol content in fermenters is likely due to physicochemical differences in the organs, stable microbial communities (with high proportions of Clostridia), long retention times, and reduced competition with other bacteria focused more on carbohydrate and protein fermentation. Another potential cause of elevated coprostanol in fermenters is that the byproducts of fiber (propionate and other volatile fatty acids) can inhibit reductase enzymes and hepatic cholesterol synthesis genes, reducing overall cholesterol levels (Hara et al. 1999; Den Besten et al. 2013).

Many of the species had relatively predictable sterol compositions based on their diets or fermentation strategy, but there were several species that defied expectations. While some of the high-coprostanol carnivore outliers are certainly a direct signal from their diet (e.g. the condors and bear) or potentially their unique habits (such as the anomalously high bat ratio from *Saccopteryx bilineata*, whose males ferment material in their wing pouches, see Voigt and Von Helversen 1999; Voigt et al. 2005), but others are less easily explained, like a captive alligator that was fed a consistent diet of fish (Tint et al. 1981). The dung of a few autoenzymatic digesters (caspien seals, *Pusa caspica*, and three bat species: *Myotis keaysi*, *Pteronotus mesoamericanus* and *Saccopteryx bilineata*) had much lower

proportional cholesterol levels than other carnivores and higher proportions of epicoprostanol, cholestanol, and coprostanone (as seen in the PCA plot of **Figure 4A-B** and **Figure 5**), potentially suggesting microbiomes that are specialized in metabolizing cholesterol differently or ones with a slower production of coprostanol along the indirect metabolic route. Hippo (*Hippopotamus amphibius*) fecal sterols presented an unexpected distribution - both their *coprostanol* / *coprostanol* + *cholesterol* ratio and their zoosterol PCA results plotted them closer to carnivorous mammals and Anseriform birds than the rest of Artiodactyla, but their combined zoosterol and phytosterol PCA plot places them more expectedly closer to elephants. Hippo dung contains a much higher portion of cholesterol than any other herbivorous mammal (see **Supplemental Figure 2**) - this may be explained both by their understudied microbiomes (that are relatively dry and ciliate-diverse) which may produce different metabolites, and a recent reappraisal that they are not purely grazers but actually facultative omnivores that consume much more animal tissue than previously appreciated (Thurston et al. 1968; Dudley et al. 2016). Largely, birds and bats had similar sterol compositions (as noted in the results section with Ratio 3 and *cholesterol* / (*cholesterol* + *sitosterol*) from Cheng et al. 2016) - one possible explanation is that their microbiomes are incredibly similar due to their shared metabolic requirements from flight (Song et al. 2020).

The large, biodiverse dataset analyzed here prompts a reevaluation of the fecal sterol ratios and the overall state of coprostanol as a biomarker, in agreement with similar evaluative studies such as Larsen et al. 2022 and Gryczewska et al. 2025. Several papers have now-erroneously attributed the stand-alone presence of coprostanol to humans (such as Dagodzo et al. 2025), mammals (such as Leeming et al. 1996), or carnivores (such as Ardenghi et al. 2024; Linseele et al. 2013; and Umamaheswaran et al. 2019) - these claims need to be reinterpreted to more nuanced speculations. Most published fecal sterol ratio proxies were built on very specific datasets with low sample diversity, and cannot be immediately generalized across ecosystems or contexts. Many variables need to be understood before a conclusion can be drawn from environmental ratio data, such as the ecosystem's biodiversity, sediment deposition rates, time averaging, and seasonal effects (such as pulses of fecal input after snow thaw, as described in Wendt et al. 2024). These ratios can be applied to single-producer

dominated areas (e.g. wastewater) or simple ecosystems, such as human settlements in remote areas. Wastewater monitoring in locations with high volumes of fecal input is generally feasible, although there are two major caveats. First, high fecal input from local livestock (including rabbit and ostrich farming) may carry a very similar signature to humans, while other spatially dense species won't have an effect (such as bird/sea mammal rookeries and bat colonies). Secondly, human enterotypes may need to be taken into consideration to use sewage pollution ratios properly, since the proportion of Clostridia in human microbiomes is variable across different populations, and this dataset's wide ratio range (from 0.006 to 0.977) for *coprostanol* / *coprostanol* + *cholesterol* values strongly undermines the global utility of coprostanol-based sewage pollution proxies. Third, ratio proxies likely cannot be done on environmental samples from in biodiverse locations such as tropical regions, since there may be many producers. Finally, there are other sources of coprostanol that may complicate interpretations, such as microbial mats and carcasses (Boudou et al. 1986; Grimalt et al. 1992; Rontani and Volkman 2005; Allen 2006; von der L  he et al. 2018; Ueland et al. 2021; Dagodzo et al. 2025; Shen 2019; Mulligan and Gold 2025). Environmental applications of coprostanol, such as archaeological and pollution monitoring analyses, need nuanced interpretations and should incorporate as many other biomarkers as possible before determining the presence of humans or other animals (such as lithics, PAHs, aDNA, and *Sporormiella*) - anywhere with a diverse ecosystem means we need to be wary of additive effects and time averaging (especially for environmental processes converting coprostanol to epicoprostanol).

Despite these issues, the fecal sterol biomarkers still have many applications to paleobiology, evolutionary biology, and conservation biology. We have shown that fecal sterols can help disentangle large clades and fermentation strategies - while the proxies to identify more specific species sources remain dubious, prior characterization of the ecosystem and making assumptions of which species were present can help alleviate the course-resolution ambiguity of sterol signals. Fecal sterol data is still missing from species with unique or relatively unknown fermentation strategies, including: fermenting fishes (including the bonnethead shark, *Sphyrna tiburo*, and Kyphosids), Cryptobranchidae salamanders, any herbivorous reptiles, foregut fermenting birds (especially hoatzin, *Opisthocomus hoazin*), and various mammal clades (such as afrotherians, xenarthrans, colobid monkeys, and pandas).

The work presented here suggests future research questions, such as work on hippopotamus fermentation and feeding ecology, working towards dinosaur metabolisms and fermentation strategies, comparing captive versus wild animal microbiomes and metabolomes, and case studies comparing fossil and modern species. Further work on the sterol-stanol conversion enzymes (i.e. 5 β ChR and ismA/ismB) could allow 16S data to open up metagenomic research for more unknown bacterial and host species potentially producing coprostanol. This clearer picture of sterol fecal proxies can be used for conservation with modern chemotaxonomy monitoring programs as a more robust but less detailed alternative (or accompaniment) to methods like eDNA and camera traps. Working with vulnerable species may provide novel dietary information for reintroduction and captive breeding programs (including giant salamanders and bonnethead sharks) and rewilding work involving functional substitutes for missing keystone species (such as dodos, Temple 1977; marsupial megafauna, Dawson 2006; and ungulates, Smith et al. 2022). Sterol metabolomic research can be an important tool to help both our understanding of past life and also for the future of modern wildlife.

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Chapter 3: Monitoring Seagrass Meadow Health Using Coastal Bird Fecal Fatty Acid

Biomarkers

3.1 Abstract

Lipids have the potential to trace trophic interactions through an ecosystem, but their use has been largely limited to predator/prey-scale studies. Carnivore fecal lipids offer an unexplored way to strategically monitor the health and spatial distribution of ecosystems that are difficult to access. This hypothesis is explored here in seagrass meadows, which are a globally significant long-term carbon storage reservoir. This study tested the hypothesis that lipids can be used to passively monitor seagrass meadow health by tracing fatty acids transferred up the food chain and onto nearby beaches via coastal bird stool. By comparing the lipids produced by seagrasses and those made by diverse microbial eukaryotes, such as diatoms and dinoflagellates, our analysis could distinguish between healthy and collapsed meadows in a pilot dataset. A metabolite that is produced by the seagrass wasting disease pathogen was also recovered in our data, providing a potential diagnostic for seagrass health. The advantages to this non-invasive monitoring approach using lipids are that beaches are far more accessible than seagrass meadows, and birds spatially ‘sample’ a larger area than researchers could do efficiently. Although this pilot study was limited in samples and field sites, our results provide compelling reasons to pursue carnivorous fecal dietary lipids as a source of ecosystem data.

3.2 Background

Seagrass ecosystems are incredibly important on a global scale but are vulnerable to disturbance. Seagrass meadows provide hunting grounds for many animals including seals and birds, act as nursery habitats for several species of fish and invertebrates, stabilize coastal erosion, and play a significant role in mitigating climate change both from their efficient carbon sequestration and by buffering ocean acidification (Hyndes et al. 2018; Unsworth et al. 2019; Ricart et al. 2021). Despite the multitude of benefits these ecosystems provide, there are many threats to meadows globally (Unsworth et al. 2019; 2022). Like many ecosystems, seagrass meadows are facing a combination of risks, including increasing seawater temperatures, pollution and runoff, and wasting disease infections caused

by the pathogen *Labyrinthula*. Collectively these threats can result in the collapse of seagrass meadows, leaving either bare mudflats or algal/biofilm dominated communities in their wake (Unsworth et al. 2019; 2022). The loss of these important ecosystems leads to a loss of carbon storage and all of the other benefits they provide. There are many long-term seagrass observation stations across the globe, most of which fall under the SeagrassWatch and SeagrassNET organizations. Monitoring can include many variables, including area coverage, shoot density, visual ID of lesions from wasting disease, C/N/P elemental ratios in the shoots, and environmental information such as light, depth, and temperature (Unsworth et al. 2014). Some typical methods for meadow health and extent include intertidal walking and plant sampling, freediving, towed videos, sonar, and helicopter (Unsworth et al. 2014). While these techniques are the field standard for monitoring seagrass meadows, they can be timely, exhausting, and expensive to carry out for long term records, especially at short intervals. Tracking seagrass carbon sequestration is typically done with isotopes from the total carbon pool, but some of those individual molecules are very biologically specific and that information is often lost with isotopic analyses (Ward et al. 2021). Given the importance of seagrass meadows and the difficulty of reaching them, additional monitoring methods would be scientifically valuable.

A possible alternative approach to monitoring seagrass meadows involves tracing their lipids through the coastal ecosystem. Fatty acids (or FAs) are a common lipid class in cell membranes composed of hydrocarbon chains, usually 14 to 24 carbons long, with a terminal carboxyl headgroup. FA compound notation is structured using the following notation: the total number of carbons in the compound, the number of saturations, and the position of the first saturation starting from the carboxyl end. For example, 22:6n-3 is 22 carbons long with six double bonds beginning at the third carbon from the carboxyl end. FAs are used in cells for membrane stability and permeability, energy storage, cell signaling, and as the building blocks for more complex lipids (de Carvalho and Caramujo 2018). Lipid biomarkers have been used in many studies to track ecological interactions by taking advantage of tax-specific compounds that get incorporated into the tissues of consumers, thus bypassing the limitations of observation or gut contents (Jordan 2005; Nielsen et al. 2018; Galloway and Budge 2020; Jardine et al. 2020). FAs are particularly useful for distinguishing bacteria, phytoplankton, and algal prey (Jardine

et al. 2020, Kelly and Scheibling 2012). Since the FAs of autotrophs are chemically similar to the consumer's FAs they require relatively little (and usually predictable) modification before incorporation into cell membranes (de Carvalho and Caramujo 2018). While there is a wealth of information that could be learned from seagrass lipidomics, several key FAs might be particularly useful to monitor their health (Sbrizzi et al. 2024). Seagrasses can be characterized using 18:2n-6, 18:3n-3, and 28:0 FAs; while these are typical FAs in flowering plants, they can be useful when paired with additional lipid analyses that calculate terrestrial versus marine biomass (Dalsgaard et al. 2003; Alfaro et al. 2006; Colombo et al. 2017; Dahl et al. 2025). Long chain FAs (LCFAs), particularly those longer than 24 carbons, are also used to identify plant leaf waxes from flowering plants, including seagrasses (McClymont et al. 2023). In contrast, green algae such as sea lettuce (*Ulva* spp.), which often dominate an area after seagrass meadows collapse, can be identified by diverse n-6 polyunsaturated FAs (PUFAs) (Galloway et al. 2012). Many other clades found in coastal ecosystems, such as kelps and other brown algae, diatoms, and golden algae, also have distinct FAs and can be reliably identified using lipids (Galloway et al. 2012). One commonly used class of FAs are omega FAs, also called essential FAs, which cannot be synthesized by most animals but are critical for their musculature and nervous systems. The omega fatty acid docosahexaenoic acid, or DHA (22:6n-3) is highly associated with marine ecosystems but is not produced by macroalgae or seagrasses, instead being biosynthesized primarily by microbial eukaryotes such as diatoms, dinoflagellates, cryptomonads, and notably the eelgrass pathogen *Labyrinthula zosterae* (Kharlamenko et al. 2001; Dalsgaard et al. 2003; Veloza et al. 2006; Chu et al. 2008; Coelho et al. 2011; Galloway et al. 2012; Peltomaa et al. 2017; Yoshioka et al. 2019). DHA is highly retained trophically, with nearly a 1:1 transfer rate between prey and predators (Jardine et al. 2020). These major groups of autotrophs that form the foundation of marine ecosystems have distinct lipid profiles, making lipidomics a powerful tool to study offshore communities.

It has long been recognized that animals take on a FA signature reflecting their diet, or their prey's diet, allowing researchers to estimate the trophic linkages using lipid tracers (Nichols et al. 1986; Alfaro et al. 2006; Budge et al. 2006; Coelho et al. 2011; Kelly and Scheibling 2012; Guerrero and Rogers 2020). The combined facts that seagrass meadows have distinct FA profiles and that FAs can

be transferred up the food web means that nearshore birds have the potential to retain FA biomarkers from these offshore ecosystems. In this system (illustrated in Figure 1) the lipids of seagrasses and other autotrophs get taken up by primary consumers (such as snails and amphipods) and move up the food chain into generalist predators. Each animal in this series will typically esterify their dietary lipids to remove the headgroup, then either incorporate them into their cell membranes or store them for energy in compounds like triacylglycerides (Giese 1966; Griminger 1986; Williams and Buck 2010). Once the marine animals are eaten by a bird, the prey's tissues and stomach contents are broken down and the FAs are released once again. While the majority of FAs are reabsorbed through the intestinal lining, carnivorous birds have short digestive tracts and relatively quick food retention times, meaning a portion of these diagnostic lipids are potentially passed out as waste and amenable for research (Williams and Buck 2010; Twining et al. 2021). FAs have been used extensively in birds, using tissues such as feathers, eggs, blood plasma, and adipose samples to reconstruct their diets (Williams and Buck 2010). Marine and coastal birds serve as important sentinel species, both their presence and health can be used as proxies for the larger ecosystem's condition (Boersma 2008; Grove et al. 2009; Wolf et al. 2010; Quinn et al. 2017; Mathot et al. 2019). One important use of birds is for ecotoxicology monitoring; as predators they biomagnify pollutants such as lead and microplastics that propagate up the food chain (Burger and Gochfeld 2004; Grove et al. 2009; Athira et al. 2024; Montgomery et al. 2026). Several recent studies have developed other novel techniques using data from wildlife to monitor ecosystems that are difficult to access by taking advantage of an animal's feeding habits and range coverage (Siegenthaler et al. 2019; Shao et al. 2021; Ji et al. 2022; Hartig et al. 2024; Beltran et al. 2025).

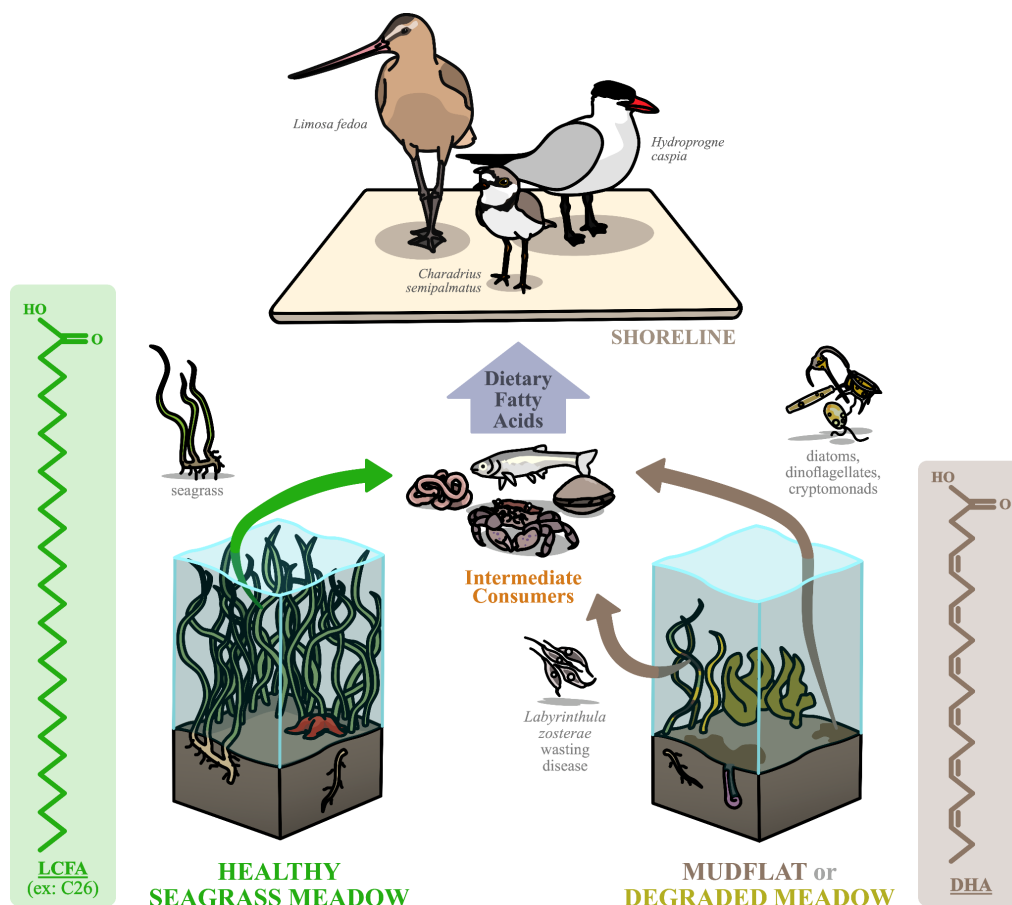


Figure 3.1

Transfer of fatty acids along trophic relationships proposed in this study, traveling from autotrophs, namely long-chain fatty acids (LCFAs) produced by seagrass shown in green and docosahexaenoic acid (DHA) rich microbial eukaryotes shown in brown, up through intermediate prey species and ultimately deposited onto the shoreline by birds.

This pilot study explored if coastal bird fecal lipids correlate to the local offshore ecosystems, a mudflat and a seagrass meadow, as confirmed through field observations. Our hypothesis was that FAs that were originally biosynthesized by seagrass and microbial eukaryotes can be traced up the marine food web and ultimately recovered in the bird feces on the beach. By leveraging shorebird ecology with lipid biomonitoring principles, it may be possible to employ wildlife to do a majority of the fieldwork for tracking seagrass meadow health without researchers ever leaving the shoreline.

3.3 Methods

The birds targeted in this project were carnivorous shorebirds in the order Charadriiformes (plovers and relatives), with additional samples from Anseriformes (ducks and their relatives) and Ardeidae (herons and relatives). Droppings from seagrass grazers and biofilm-specialists such as geese and small sandpipers were largely avoided since these birds prefer consuming autotrophs and therefore would have a biased signal against nonvegetated mudflats (Valentine and Heck 1999; Kuwae et al. 2012). In contrast, medium and large carnivorous shorebirds (e.g. scoters, avocet, plovers) consume a wide range of generalist invertebrates or fish that are present in all nearshore ecosystem types (Angarita-Báez and Carlos 2023; Correia et al. 2023).

Samples were collected from two field sites between May and July 2025. Millerton Point in Tomales Bay, California (approximately 38.314573, -123.056826) was chosen as a healthy seagrass site since wasting disease is uncommon until much later in the summer, based on many years of continuous monitoring by several groups at the UC Davis Bodega Marine Laboratory (BML). Gaffney Point in Bodega Harbor, California (approximately 38.105780, -122.849698) is a tidal mudflat with very small declining patches of seagrass that consistently have wasting disease lesions; for simplicity this will be referred to as the “mudflat” ecosystem. Samples were collected during the day under overcast to sunny conditions when the birds were most active. Sample collection was largely timed with mid- to low-level tides, when enough area was exposed for foraging but the tide was high enough to keep the birds close to the main shoreline. A rising tide will push the birds closer to the shore but comes with increased risk of losing samples to the ocean. Additional samples were collected without having observed the producing species but were ultimately omitted from most statistical analyses (i.e. PCA and ANOVA).

All field work was conducted with appropriate aseptic techniques and PPE out of caution for a regional outbreak of H5N1 Highly Pathogenic Avian Influenza (HPAI), of which many of this project’s target species are reservoir hosts (CDC, 2024). Birds were monitored along the shoreline, and fresh fecal samples were collected after observing the deposit, identifying the species, and monitoring for

visual symptoms of HPAI. Stools were sampled in a way that minimized urates and excluded any environmental sediment, even at the expense of stool material. When cecal droppings were found they were ignored since it would likely reflect the bird's long-term diet and not the last meal. Samples were collected with a sterile plastic scoop into pre-weighed and labeled borosilicate 20 mL vials containing ~10 mL of pre-aliquoted ethanol (EtOH). A square of furnace-baked aluminum foil was placed between the glass and vial cap. These vials were gently mixed to get the stool fully submerged before continuing with field work. Vial exteriors were thoroughly cleaned with Super Sani-Cloth® sanitizing wipes and allowed to air dry for at least 1 minute. All waste was double bagged, with the first bag being washed with sanitizing wipes and air dried before adding the second bag, which was also wiped down. All PPE (goggles, waders, and boots) were rinsed with fresh water both on location and at BML, then treated with wipes after each field day and allowed to air dry. A field control was collected by opening a vial in the intertidal zone of Gaffney Point, closing it, and storing/processing it with the other samples. Control samples of healthy seagrass (*Zostera marina*) and seagrass lesions infected with wasting disease (*Labyrinthula zosterae*) were collected from mesocosms grown at BML and stored in EtOH, courtesy of Serina Moheed.

Samples were stored at room temperature in a fume hood, then submitted to West Coast Metabolomics Center for extraction and analysis by ultra high performance liquid chromatography (UHP-LC) following the methods outlined in Matyash et al. (2008). The relative abundances of free FAs (FFAs) were normalized as a sum of the total FFAs to make the data directly comparable across samples. Analysis was limited to FFAs rather than intact lipids (i.e. those with a headgroup or part of larger storage molecules like triacylglycerides) for three reasons: 1) feces is chemically complex and fewer unique molecules meant a simpler analysis, 2) intact lipids typically represent living biomass, and we wished to exclude both the bird gut microbiome or any potential environmental contamination, and 3) dietary intact lipids may lose their biological specificity after being passed through multiple consumers, making the analysis of headgroup-specific affinities contentious. Principal components analysis (PCA) was performed on the full UHP-LC results (Supplementary Figure 1) as well as the

FFAs. Analysis of variance (ANOVA) tests using different lipid classes and proxies were completed to determine the significance of sample location.

3.4 Results

A total of 24 stool samples were collected: nine from Millerton Point and 15 from Gaffney Point. Samples were collected from seven species: great egret (*Ardea alba*, n=2), sandpiper (*Calidris* spp., n=1), semipalmated plover (*Charadrius semipalmatus*, n=2), Caspian tern (*Hydroprogne caspia*, n=4), marbled godwit (*Limosa fedoa*, n=4), Hudsonian whimbrel (*Numenius hudsonicus*, n=1), and a willet (*Tringa semipalmata*, n=1). It is worth noting that, unfortunately, there were no samples collected from the same species at both sites. Nine additional samples were opportunistically collected without observing the individual that produced them.

UHP-LC analysis returned 4,067 compounds of which 1,202 were characterized (Supplementary File 1) and 38 were FFAs (seen in Figure 2, with each as a proportion of the total FFAs; Supplementary File 2). Nomenclature for FFAs was standardized using both the Metabolomics Workbench (<https://workbench.sdsc.edu/databases/refmet>) and PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) databases. All code and data can be found at this GitHub repository: https://github.com/DavidGoldLab/2026_Seagrass_Monitoring/tree/main.

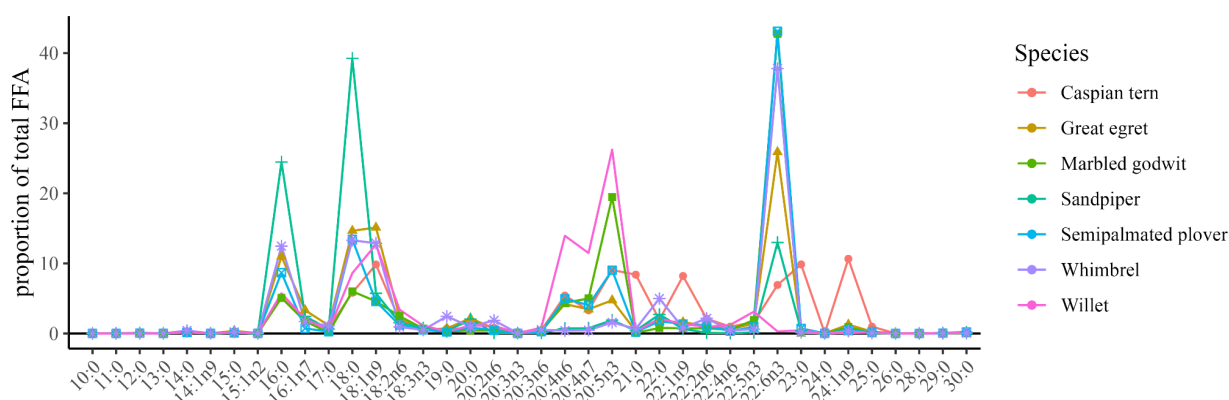


Figure 3.2

Line graph of average fecal free fatty acids (FFA) proportion for each bird species.

PCA results suggests that healthy and collapsed seagrass meadows can be distinguished by their FFA profiles (Figure 3). Principal component (PC) 1 and PC2 (Figure 3A-B), captured 55.6% of the variation and separated the two ecosystems, while PC2 and PC3 (Figure 3C-D), captured 34.65% of the variation and separated the bird species. PC2 differentiates the samples largely along axes of 16:0 + 18:0 and 21:0 + 23:0, while PC1 and PC3 were most strongly associated with 22:3n-6 and 20:5n-3, respectively. Following these results, barplots (Figure 4) were created showing the normalized ratio of DHA versus LCFAs (24:0 + 24:1n-9 + 25:0 + 26:0 + 28:0 + 29:0 + 30:0) for both fecal samples (including unknown species) and seagrass tissue controls from BML mesocosms. The healthy meadow's lowest values (Figure 4B) were associated with species observed feeding in the seagrass (i.e. the willet was foraging in an intertidal seagrass meadow and the terns were seen feeding for most of the day in a deep channel of subtidal grass) while higher values came from species feeding closer to shore in marginal nonvegetated mud areas (i.e. the plovers and sandpiper). The sandpiper values may also reflect the influence of biofilms in their diet, potentially explaining why their PCA values are so far removed from the other species (Kuwae et al. 2012; Schnurr et al. 2020).

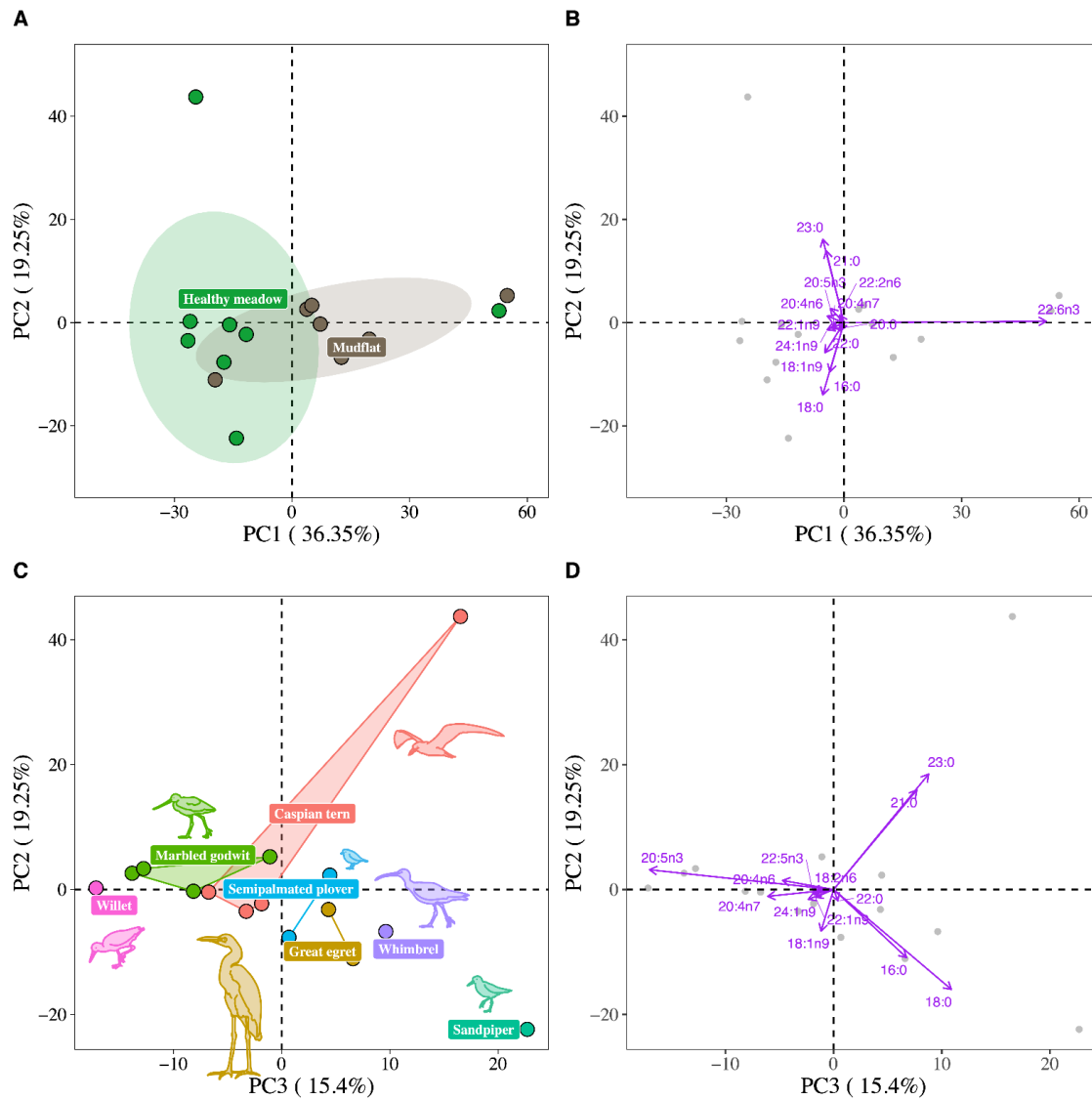


Figure 3.3

Free fatty acids (FFA) principal components analysis (PCA) results. Panel A) shows a Principal component (PC) 1 and PC2, with points colored by environment and ellipses drawn at a radius of 80%. Panel B) shows the eigenvectors for PC1 and PC2. Panel C) shows PC2 and PC3 with the points colored by bird species. Panel D) shows the eigenvectors for PC2 and PC3. For simplicity, only eigenvectors with $\cos^2 > 0.6$ are labelled in B) and D).

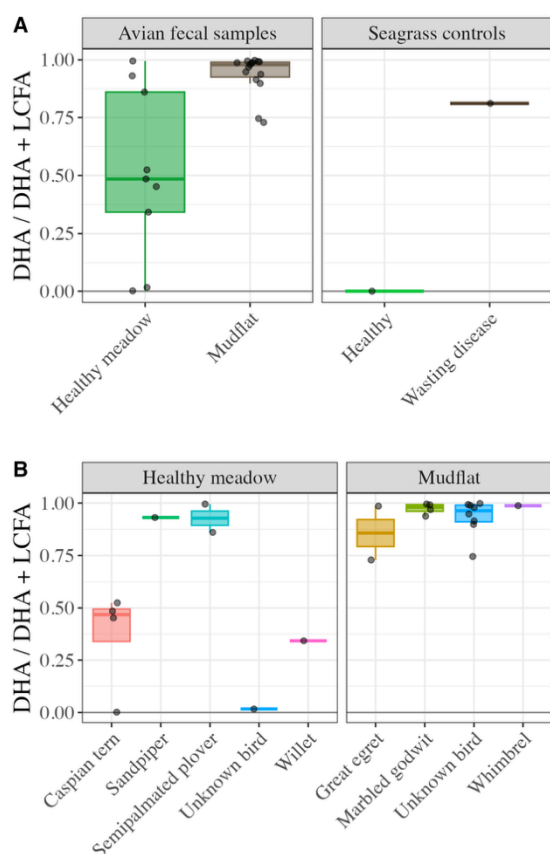


Figure 3.4

Barplots showing a ratio of docosahexaenoic acid (DHA) normalized to the total long-chain fatty acids (LCFAs ($24:0 + 24:1n-9 + 25:0 + 26:0 + 28:0 + 29:0 + 30:0$), including data from unknown species. Panel A) compares the two ecosystems and the two seagrass tissue controls. Panel B) shows the same information, isolating each bird species separated (including those that are unknown).

A total of eight ANOVA tests were performed between the two ecosystem types (see **Table 1**). Of these, only the $\text{DHA} / (\text{DHA} + \text{LCFA})$ differed significantly between ecosystems ($p = 0.0155$). The trends of each species were not tested with ANOVA since the sample sizes would violate the model assumptions. Animals are broadly efficient at metabolizing diverse FAs and at molecule-specific rates; it is therefore striking that we can correlate the fecal FA pattern to the ecosystem with such confidence.

Test	Mean Sq	F value	P value	Residuals Mean Sq
All FFAs	9.69E-28	1.404	0.257	6.90E-28
SFAs (0 saturations)	706.9	1.84	0.198	384.1
MUFAs (1 saturation)	204.2	1.006	0.334	203
PUFAs (>2 saturations)	1671	3.664	0.0779	456
LCFAs (>C24)	101.99	3.632	0.079	28.08
Seagrass biomarkers	0.222	0.063	0.805	3.499
DHA / (DHA + LCFA)	0.5058	7.747	0.0155*	0.0653
Terrestrial vs marine PUFAs	0.004783	0.969	0.343	0.004938

Table 3.1

Results of each ANOVA test. SFAs = saturated free fatty acids (FFAs), MUFAs = monounsaturated FFAs, PUFAs = polyunsaturated FFAs, LCFAs = long chain FFAs (24:0 + 24:1n-9 + 25:0 + 26:0 + 28:0 + 29:0 + 30:0). The FFAs for seagrass biomarkers were 18:2n-6 + 18:3n-3 + 28:0. A ratio of terrestrial (18:2n-6 + 18:3n-3) vs marine (20:4n-6 + 20:5n-3 + 22:6n-3) PUFAs was calculated following Colombo et al. (2017). An asterisk* denotes statistically significant P values below 0.05.

3.5 Discussion

While fecal samples from carnivores represent ecological black boxes with many unknown variables, the results of this pilot project suggest that they may be suitable for noninvasively monitoring ecosystems. Despite the limited dataset, fecal DHA normalized to LCFAs can distinguish between the healthy seagrass meadows and mudflats. It is possible that high LCFA values indicate terrestrial and freshwater input to the Millerton Point sample area, since it sits at the mouth of Lagunitas Creek. However, we can leverage the differential PUFA biosynthesis rates in terrestrial vs marine ecosystems to test for this bias (Colombo et al. 2017). The ratio of terrestrial/marine PUFAs, (18:2n-6 + 18:3n-3) / (20:4n-6 + 20:5n-3 + 22:6n-3), did not yield a significant ANOVA difference between either the ecosystems (Table 1). Likewise, high DHA values could be indicative of Gaffney Point being closer to the ocean than Millerton Point, but these concerns are again assuaged by the PUFA ANOVA test, which shows no discernible bias in ecosystem inputs. It remains unclear what the primary source of DHA was, since it is produced by diverse organisms via *de novo* synthesis and modification of other FAs (Kharlamenko et al. 2001; Dalsgaard et al. 2003; Veloza et al. 2006; Chu et al. 2008; Coelho et al. 2011;

Peltomaa et al. 2017; Yoshioka et al. 2019). Regardless of the specific source of DHA, the statistical significance holds, and it has been observed that healthy seagrass meadows can modulate their microbiomes to have much lower abundances of the microbial eukaryotes, especially diatoms and dinoflagellates (Jacobs-Palmer et al. 2020; Liu et al. 2022; Pan et al. 2022). The elevated presence of DHA in bird stool may suggest both the presence of the seagrass wasting disease and various microbial eukaryotes characteristic of a mudflat or collapsing meadow, and a low amount of DHA suggests a healthy seagrass meadow that is free of pathogens and/or healthy enough to remove diatoms and dinoflagellates from its microbiome.

The recovery of diverse, and presumably quickly metabolized, FFAs from bird stools lays the groundwork for broader research into avian biomonitoring techniques. Although it is a straightforward and common practice to identify host species from mammalian dung using DNA barcoding (such as 18S and COX1 genes), it is difficult to achieve the same results from bird fecal samples (Joo and Park 2012; Smith et al. 2023; Spence et al. 2025). The FFA approach here shows that lipid chemotaxonomy may offer a promising alternative for birds to traditional genomic barcoding methods when paired with fecal sample controls of known species. Moreover, FFAs from stool offer a snapshot of diet, which can be consumed and metabolized within ~10-60 minutes for small birds depending on the species (Kuwaie 2007). This represents a less invasive data source than typical FA samples taken from bird adipose tissue or blood plasma, as well as protocols that require euthanasia for samples of flight muscles, nervous system tissue, or stomach contents (Williams and Buck 2010). A larger implication of recovering essential FAs from bird stool is the previously unappreciated export of marine nutrients to terrestrial ecosystems via bird guano, in addition to classical elements such as nitrogen and phosphorus (Obrist et al. 2020; Appoo et al. 2024; Finne et al. 2024; Grant et al. 2024). Lastly, finding seagrass LCFAs in the food chain's top carnivores highlights the nutritional value of intact seagrass meadows, due to the seagrass's recalcitrant tissues it is likely that the LCFAs came from detritus rather than living biomass (Harrison 1989; Nichols et al. 1986; Kharlamenko et al. 2001).

While the results presented here are promising, there are many limitations to this study that need to be considered in future research. The field work was less efficient than traditional seagrass monitoring methods, especially with regard to the predictability of target bird species being present and the recovery of stool samples (~100 hours of field work for 24 samples). While the birds may have very local chemical signatures, their prey—especially larger fish eaten by the terns and egrets—represent much longer averages of the environment and have potential to travel long distances to the field site. This concern compounds with the unknown number of organisms that could be in the trophic chain. Despite being a keystone species, not many organisms directly consume live seagrass, which restricts the mobilization of seagrass FAs amenable for proxy work to mostly detritus. This may make our approach prone to false negative results in locations with limited appropriate detritivores (Harrison 1989; Nichols et al. 1986; Kharlamenko et al. 2001). An opposite way this could bias the results presented here is that the Tomales Bay seagrass prey community, specifically the Millerton Point area, is dominated by an invasive species of amphipod (*Ampithoe valida*) which directly consumes the seagrass blades and may be a driving force in the stronger seagrass signal than may be found elsewhere (Reynolds et al. 2012, Claire Murphy personal communication June 5th 2026). Estimating a bird's prey items from FAs is possible using Bayesian tools (such as the QFASA R package, Stock et al. 2018), which could help alleviate concerns about the ecosystem signal being biased by the species present. These advanced analyses need custom metabolic calibration coefficients and a strong local database - this is feasible to develop but would take extensive field work, prey taxa husbandry, and bird feeding trials to do properly (Iverson et al. 2004; Galloway and Budge 2020; Guerrero and Rogers 2020).

There are three main areas for future refinement for this monitoring technique: improved target species selection, larger datasets, and more cost-effective analyses. The efficiency of this new technique hinges largely on using species that can be predictably located, either by species (e.g. osprey nests and cormorant rookeries) or location (e.g. piers and buoys that birds tend to congregate around). Bird species that are too small or too terrestrial may not be as useful, as illustrated by the plover stool having a mudflat-type signal despite foraging in a seagrass-adjacent location. Picking better model species will require careful tradeoff consideration of their diet, foraging habits, and gut retention times. Validating

this work will require expanded datasets, both temporally (e.g. sampling throughout the disease progression cycle over a summer) or spatially (e.g. along a long shoreline that encompasses multiple ecosystem gradients). Pairing this approach with collaborators who can diagnose disease severity indices could generate a scale of wasting disease rather than mere presence/absence. The addition of isotope work would greatly improve our understanding of the ecological interactions, especially $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, which are used to estimate food chain length, and compound-specific isotope analysis on individual DHA molecules could disentangle their sources (Perkins et al. 2014; Dirir et al. 2025). While there is no equal substitute for mass spectrometry techniques for quantitative data, thin layer chromatography (TLC) or other gel electrophoresis techniques may offer a cheaper and easier qualitative method to identify target compounds now that their presence has been verified. The next steps for validating this lipid biomonitoring work will sample stools across the seagrass's growing season from several locations and targeting species that congregate at specific locations, such as cormorants and osprey.

The pilot study described here is the basis to developing a noninvasive tool that could be readily implemented into existing infrastructure (e.g. birding groups and established environmental study programs), which can both complement and augment current seagrass monitoring practices. Developing efficient tools for monitoring seagrass meadow health, and therefore its carbon sequestration capacity, is imperative for international climate change initiatives and meadow restoration efforts (Unsworth et al. 2022; Cooley et al. 2022; Briand et al. 2026). Ecosystem connectivity provides novel methods for monitoring keystone species beyond just seagrass - lipids proxies for autotrophs collected from carnivores allow researchers to pull on proverbial strings in a food web, affording us both a 'top-down' and 'bottom-up' views of the system as a whole when combined with traditional practices (Marneweck et al. 2022; Natsukawa and Sergio 2022; Briand et al. 2026). Many carbon dynamics are mediated by animals, especially carnivores like birds that can travel between different ecosystems; lipidomics is a powerful tool to efficiently reconstruct these endmember relationships (Schmitz et al. 2010; Schmitz et al. 2014; Buelow and Sheaves 2015). Once better validated, this new technique could be expanded to any inaccessible ecosystem with a keystone species that has unique lipids and a mobile animal that goes

in and back out to somewhere more accessible. For example, kelp forests are incredibly important and imperiled, but scuba-diving monitoring and restoration programs in California are often hindered by the white sharks that hunt there. Other potential ecosystems to monitor could include coral reefs, mangroves, and deep sea ecosystems. This animal-based lipid biomonitoring technique is a versatile and holistic approach to conservation biology: by tracing specific molecules up through ecosystems, an animal's natural behavior can bring samples directly to researchers.

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