SEASONAL DYNAMICS OF DECOMPOSITION ECOLOGY

By

Anthony Grigsby

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ABSTRACT

All organic life, by virtue of having ever been alive, is fated to end. Life and death represent opposing binary states: an organism is either alive and its biological processes functioning, or dead, with those processes halting entirely. The connotation of decomposition often betrays its nature as a complex, multivariate process that is mediated by far more than just the carcass enabling it. Among the decomposers responsible for nutrient cycling in carrion systems, bacteria and blow flies (Diptera: Calliphoridae) stand out as the most effectual. The objective of this study was to examine how the microbiota of carcasses, blow fly larvae, and larval masses developed as decomposition advanced. To account for abiotic factors that suspected to play a role in moderating these ecosystems, we evaluated the role seasonal weather dynamics play in influencing microbiome assemblage. We predicted that postmortem microbiome composition, diversity, and succession would be drastically dissimilar between seasons by seasonal weather conditions. It was hypothesized that colonization of carcasses by blow flies would significantly impact carcass bacterial community composition. Parallel predictions were made that season would, similarly, influence larval and lar microbiome assemblage. Ultimately, we found both season and larval presence jointly influenced postmortem microbiome succession, and that postmortem microbiome diversity was significantly greater in the summer than the fall or spring. Bacterial community composition differed significantly by season regardless of pairwise comparison. Blow fly colonization significantly influenced bacterial community composition in facilitating the introduction and subsequent dominance of insect-associated bacterial taxa like *Ignatzschineria*. These decomposition studies demonstrated the interconnectedness of life encompassed by the necrobiome and, in doing so, strengthened pretense to better evaluate interkingdom interactions observed in decomposition settings.

This thesis is dedicated to my grandparents†.
I'm glad you could see me start my journey.

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I'd be remiss if I managed to understate the role my colleagues played in helping me navigate this degree. Kelly Waters and Bethany Mikles are indispensable members of the Benbow Lab, and any success I might find in the coming future is in no small part a product of their support. I'd also like to thank Dr. M. Eric Benbow for providing me with the opportunity to pursue this degree. Thanks to his continued support and advice, I can confidently enter the final degree of my academic career with clear research goals in mind and experience to back them. A big thank you to Dr. Jennifer Pechal as well for accompanying Dr. Benbow at our numerous virtual interviews and confirming my suspicion that this lab had great minds behind it.

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LIST OF ABBREVIATIONS

ADD – Accumulated Degree Days

ADH – Accumulated Degree Hours

PMI – Postmortem Interval(s)

ANOVA – Analysis of Variance

PERMANOVA – Permutational Multivariate Analysis of Variance

ANCOVA – Analysis of Covariance

ANCOM-BC – Analysis of Compositions of Microbiomes with Bias Correction

PCoA – Principal Coordinates Analysis

LFC – Log Fold Change

MSU – Michigan State University

HTRC – Horticulture Teaching and Research Center

HORT – Horticulture Station

KW – Kruskal-Wallis

PCR – Polymerase Chain Reaction

CHAPTER 1: LITERATURE REVIEW

Introduction to Decomposition

In this review, I will outline the underlying concepts and associated knowledge gaps that motivate this thesis project's objectives. The projects comprising this thesis were designed to focus on carrion decomposition, and communicating its intricacy is critical to understanding the importance of our work. Janaway et al. (2019) is an incredibly instructive chapter explaining the expected trajectory and presentation of decomposition as a continuous, putrefactive process. In first framing our historical interest in decomposition as a microbially mediated process, it does well to illustrate why our projects focus on the role of microbes in decomposition environments. Janaway et al. (2019) also highlights the broad use of the term "taphonomy" in modern forensics as the exploration of decomposition and the extent to which human bodies experience it. An important distinction must be drawn between the two categories of death we consider in our explorations of it: "somatic" death and "cellular" death. Where somatic death is contingent on the individual experiencing it being irrevocably rendered unconscious and thus non-functional despite exhibiting metabolic activity, cellular death is instead defined by the cessation of all biological processes (Armstrong & Fernando, 2013; Wyllie, 2987). Such a distinction is important in the context of human health, as only cellular death is immediately succeeded by the onset of decay. When cellular death is experienced by the human body, a series of generalizations can be made about the overlapping changes it experiences because of its now static circulatory system (Tsokos, 2005). Livor mortis, otherwise known as postmortem hypostasis, is characterized by the discoloration of areas in which blood finds itself pooling naturally (Poinkes, 2013). In many cases these are the lowest parts of the body, given that the only force experienced by stagnant blood is ultimately gravity. A distinct blue-purple color

indicates this blood's depletion of oxygen, and signs of livor mortis become evident just 1-2 hours after cellular death. Indicators are maximally expressed just 6 hours after death and become irreversible after 12 hours. 2-4 hours after death, while the body is experiencing livor mortis, rigor mortis typically commences (Krompecher, 2015). This rigor mortis, in concerning the muscles of the body, is a considerably more dynamic process than livor mortis. The breakdown of adenosine triphosphate (ATP) and subsequent accumulation of lactic acid in muscle tissues drives their gradual transition from flaccid, to rigid, to relaxed once more. The muscle's initial flaccidity is attributed to persisting cellular life; and as this cellular life dwindles, muscles becoming increasingly unable to contract. The ability of muscles to contract will never be recoverable thereafter. In temperate conditions, researchers find that signs of rigor mortis are most pronounced at 12 hours, decline towards 24 hours, and ease into total limpness at 36 hours after death. The third of the phenomena experienced by cadavers is algor mortis, which represents a direct consequence of body heat escaping with no anticipated steady state. Most of the putrefactive action we associate with decomposition is encompassed by algor mortis, wherein the conditions it creates are most promotive of soft tissue liquification (Hayman & Oxenham, 2016).

Microbial Decomposition

Another key part of decomposition is autolysis. While our studies focused primarily on the contributions that organisms independent of them make, autolysis is a process driven specifically by the body's own endemic enzymes (Gibbons & Reed, 1930). Here we must make another important distinction: autolysis refers specifically to the body's self-induced cellular digestion; but putrefaction refers to what destruction can be attributed to bacterial action. Most anaerobic bacterial activity is relegated to the human gastrointestinal tract, meaning much of

what we see promoting putrefaction internally is native to the human body (Kennedy, 1992). Microbial activity aids in keeping this environment anoxic, which generally lends itself to anaerobic bacteria and not obligate aerobes (Rose & Hockett, 1971). In every instance of human decomposition, autolysis and putrefaction are the only driving forces we can expect to observe regardless of the context, and our studies emphasize the role of seasonal weather conditions as extrinsic, variable factors in these processes. Existing assertions that temperature heavily modulates the rate of enzymatic activity and overall microbial fitness is what motivated our heightened awareness of season as an oft underrepresented variable in datasets (Peterson et al., 2007). Where literature recognizes the role of temperature and humidity in cadaveric decomposition, we seek to elucidate the overarching impact of season as a concept wherein distinctions between spring, summer, fall, and winter are as much a matter of climatological conditions as they are a matter of temporality. While climates and thus seasons vary widely by geography, there is still value in characterizing the role they play in carrion ecology.

Classifying Decomposition & Related Issues

Janaway et al. (2019) do their best to categorize qualities researchers tend to attribute to a specific series of decomposition "stages;" but, carrion decomposition is a continuous process with no definite stages (Finley et al., 2014). As such, efforts to stratify this process are inherently limited by its exceptional co-morbidity among livor mortis, rigor mortis, and algor mortis. If these phenomena drive decomposition, and we know them to overlap and intersect almost pathologically, we cannot expect to create a reliably applicable criterion for decomposition staging. Despite this, many researchers can still demonstrate consistencies between many of the non-standardized taphonomic criteria put forth by experts throughout the history of forensic science (Anderson, 2015; Javan et al., 2018). Carcass decomposition begins with a "Fresh" stage

Fresh carcasses usually express the changes outlined by livor mortis, particularly as it regards discoloration. Adult fly activity is typical of this stage, and their ability to detect human remains is aided, in part, by the discharge of bodily fluids promoted by early putrefactive action (Goff, 2009). Around 24 hours after death, the body may transition from the fresh stage to the "Bloat/Bloated" stage. Larval blow fly activity can be seen in most cases; but the bloat stage is more defined by the accumulation of gas byproducts within the intestines than anything else (Goff, 2009). At this time (from around a day to a week after death) it is common to see instances of hair loss, skin slippage, and further discoloration, among other things. The bloat stage is unique in that, in most cases, the liquid discharge observed can be evacuated from the body with more intensity, or even imbued with a "frothy" quality because of mounting gas pressure. Bloat is followed by "Decay/Active" stages, which emphasizes the rupture of body cavities and escape of previously accumulated gas (Goff, 2009). Insect activity here is somewhat variable, but usually trends towards diminished. Skin breaks down and other tissues decay to expose the skeleton of the carcass. In this active stage alone, there are numerous factors that dictate its ultimate presentation, which complicates the existing ambiguity even further. After up to a month of decay, human remains will eventually find themselves in the "Dry" stage, which is essentially just whatever skeletonized remains are intact.

Decomposition is a complex, fundamentally multivariate biological process that borders on anecdotal in some cases (Cockle & Bell, 2015). Any number of seemingly minute disparities in victim health, clothing, or location can affect how decomposition progresses in ways that cannot possibly be predicted reliably. Discerning common, broad trends can lend themselves to the framework necessary to unravel the many factors at play, but we feel that it is and will remain unlikely that we can ever treat instances of death or decomposition as perfectly

analogous. Our attempts to mitigate this required the use of many experimental replicates; and even rigor of that level should not give researcher's the confidence to assert any discoveries absolutely.

The Necrobiome

As forensic science advances and technologies promise to strengthen our understanding of even its most fundamental concepts, Benbow et al. (2019) communicate the sheer breadth of carrion as transient ecological networks. In proposing this ecosystem, the "necrobiome," Benbow et al. (2019) do well to contradict the instinctive oversimplification of death that many scientists and non-scientists seem to default to despite its volatility. In cultivating such a rich and diverse network of decomposers, among other things, carrion presents us with something of an ecological problem. By reframing decomposition as a subject that lends itself to basic or non-applied science, the "necrobiome" framework additionally promises to strengthen forensic science at the most basic levels (Tomberlin et al., 2010; Tomberlin et al., 2011). Researchers posit that decomposition settings, as ephemeral ecosystems with sprawling networks of organisms at the micro- and macroscopic levels, inherently invite multidisciplinary research that characterizes how these networks overlap, interact, compete, etc. (Barton et al., 2012; Swan et al., 2021). As such, Benbow et al. (2019) broadly characterizes the necrobiome's biotic structure (and what ecological functions emerge because of this biotic structure) in carrion and plant matter systems. To this end, they define the necrobiome as "the community of organisms associated with necromass decomposition [including] their interactions with the necromass, with each other, and with their surrounding habitat and ecosystem" (Benbow et al., 2016; Tomberlin et al. 2011). The ultimate focus of our studies concerns specific parts of this necrobiome: the "necrobiome structure," which encompasses the internal, external, and saprophage communities,

and “necrobiome interactions and functions” (Benbow et al., 2019). This extends how microbes interact, navigate, are introduced, and disperse. Structurally speaking, it is best to characterize the necrobiome as the constitutive reflection of its endonecrotic (internal) and epinecrotic (external) microbial communities & scavenger communties directly affected by the process of decomposition (Pascual et al., 2017). It is the combination of these endonecrotic and epinecrotic microbial communities that comprises the overarching “microbiome” of the decomposing matter. In keeping with the ecological importance of the necrobiome, one must recognize that the postmortem microbiomes these communities represent can be comprised of any number of bacteria with any number of relationships with their host or even other microbes. Our initial emphasis on putrefaction and how it is driven chiefly by bacteria is important for the fact that how these bacterial communities change in structure during postmortem microbiome succession directly impacts the functions performed by that microbiome.

Invertebrate Decomposers

As time went on and research developed, forensic science came to recognize the voracity of many invertebrate decomposers as prolific colonizers of carrion in spite of the environmental or contextual circumstances that would ordinarily constrain their access to it (Ikeda et al., 2008). The presence or absence of invertebrates in the necrobiome can drastically influence the rate at which decomposition occurs and how the hypothetical “stages” of it present (Dadour & Harvey, 2008). The numerous invertebrate decomposers present us with yet another level of ecological complexity, as many instances of resource-portioning, niche overlap, and competition are observable between two or many species (Benbow et al., 2019). When you consider how organisms on completely different trophic levels could possibly interact with or influence each other, the true challenge of characterizing the necrobiome in earnest becomes much more

evident. Our studies were motivated the increasingly obvious knowledge gaps that have emerged as more questions are broached regarding the necrobiome; and to this end, we focus primarily on the bacteria comprising the postmortem microbiome and how blow flies (Diptera: Calliphoridae) interact with or impact them. Importantly, though, the necrobiome and the theories that it is built on have many important implications for not just decomposition overall, but fields that consider the process in applications they develop. Forensics was a primary focus in the development of the studies and its associated hypotheses.

Calliphoridae: Blow Flies

As explained by VanLaerhoven (2008), blow flies have long since seen utility in the field of forensic entomology as insects with exceptionally predictable growth rates and life histories. As such, the use of degree day or degree hour models built on blow fly developmental data and used to retroactively estimate postmortem intervals (PMI) rank among their most common use cases. In a comparison of three different variable combinations in the calculation of a PMI, it was found that they did not differ significantly in terms of efficacy (VanLaerhoven, 2008). While this method of PMI determination is contingent on the availability of multiple data types, it does tread water as a feasible means of approximating PMI with a moderate margin of error. Arias-Robledo et al. (2018) emphasize that blow fly activity on carrion is not a simplistic manner, keeping with the complexity outlined in the previously noted necrobiome framework. The blow fly's relationship with its environment and how those environments change as time goes on and seasons transition are important aspects of their life history (Ngoen et al., 2011). For instance, *Lucilia* species and *Calliphora* species have evolved such that they are represented in higher abundance during cooler months (Arias-Robledo et al., 2018). *Lucilia* species, additionally, appear to differentially motivated by humidity and light intensity when it comes to

oviposition and colonization (Amendt & Reckel, 2008). This serves as an excellent example of how species of Calliphoridae can differ behaviorally, despite the generalizations we tend to make regarding their life histories and development in decomposition settings.

One important concern in the colonization of carrion by blow flies lies in how they aggregate during these colonization events, where larvae can be seen forming larval masses. Rivers et al. (2011) keenly observe that this approach has advantages and disadvantages that directly influence how they experience the surrounding ecosystem and how that ecosystem is affected by them. While larval masses can improve extra-oral digestion of carrion tissue and produce heat that sustains or even promotes growth, they also make it difficult to discern the susceptibility of maggots to heat stress and—should this be a risk—whether premature dispersal of larval masses or resultant death can inadvertently change the trajectory of decomposition (Rivers et al., 2011). So, even when isolated, we can observe knowledge gaps in how these larval masses influence the microbial communities of the carcass they form on or in.

Forensic Microbiology

We should specify further that the subdiscipline our studies most directly address is that of "forensic microbiology," or alternatively, "microbial forensics." Spagnolo et al. (2019) outline the uses and general history of forensic microbiology, and immediately point out the relevance of the human microbiome to its emergence. Many popular forensic matters are ostensibly human ones. This indelible connection between the two made the Human Microbiome Project (HMP) an especial turning point in forensic science, as implications of the human microbiome as an indicator of health or even identity ushered in a still growing demand for practical microbiological applications in criminal/death investigation (Díez López et al., 2022). Given the recency of the HMP, it is not difficult to see why so little is known about the postmortem

microbiome and its changes despite the growing body of research contributing to its characterization. Weinstein (2003) is an important landmark in the overall characterization of the postmortem microbiome as something that can present with specific features or community structures somewhat consistently. While our study is more concerned with how the abundances of these bacterial taxa change as decomposition progresses and larval blow flies colonize carrion, it is still important to note the species or genera identified by foundational descriptive research that inspires our own. Pechal et al. (2013; 2018) provides us with a better understanding of what bacterial taxa comprise these postmortem microbiomes. These studies are two of many that corroborate the gradual rise in Firmicutes abundance as decomposition progresses while also noting the decline (and persistence) of bacteria in the phylum Proteobacteria. Many of the dominant families observed included *Moraxellaceae, Enterobacteriaceae,* and *Arococcaceae*. Papers like these ultimately serve as valuable points of reference for our own study results, as intersections or conflicts in observations can reveal crucial considerations to make with data collection or interpretation in future work. Spagnolo et al. (2019) make an excellent observation when they recognize that—while many studies have examined changes in bacterial communities of the skin or abdominal cavity exist to potentially extrapolate PMI—few studies have examined changes in bacterial communities of human organs specifically. This is a particularly interesting trend to note, as the absence of results means we cannot assume the irrelevance of these sampling sites as far as PMI determination attempts go. It proves to be an example of one technical knowledge gap in forensic microbiology that has emerged as an issue of convenience, despite the promise it could hold with the context of how intestinally focused putrefaction is. Fluids or tissues from these intestines are arguably the least environmentally influenced pieces of hypothetical evidence (Javan et al., 2016). This note serves mostly to demonstrate how far we

must go in rounding out the body of research that currently represents forensic microbiology as a discipline. Reviews of PMI determination efforts indicate several important methodological trends, regardless of efficacy. For instance, taxonomic resolution is not necessarily all that we should consider in the identification of PMI predictors of the postmortem microbiome. Indeed, lending more consideration to the functional expectations of bacteria at certain decomposition stages, or even making basic associations between the life history traits of bacteria seen driving decomposition and those observed in evidence, can all serve as valuable indicators of PMI. While there are consistencies between these communities across carrion models and sometimes causes of death, they are too beholden to the inherent stochasticity of decomposition to be meaningful at certain taxonomic levels. Oliveira & Amorim (2018) help identify even more uses of microbiological methods or concepts in ways that are less focused on human death. We feel it is important recognize the applications forensic microbiology has had in biocrime, bioterrorism, and geolocation (among others) to clarify that forensic microbiology is far from isolated to matters of PMI, cause of death (COD), or death in general.

Microbiome Analysis Considerations

Recognizing the difficulties of studies that seek to characterize microbiomes longitudinally is important in clarifying areas of research we feel need more support. Kodikara et al. (2022) has proven extremely helpful in outlining considerations for microbiome data analysis that should inform our approaches to characterizing the postmortem microbiome over time. One key feature of longitudinal microbiome data is its natural compositionality. Microbiome data being compositional is a direct result of it representing relative abundances rather than absolute abundances. Since longitudinal measures of the microbiome are ultimately reflective of individual, temporally dissonant samples with incongruous sequencing read counts, we must

recognize that the composite longitudinal profile of any given individual will always be uneven when compared to another's (Gloor et al., 2017). As such, it's important that measures we use in, say, assessing differential abundance of bacteria between two groups are considerate of the compositional data. Methods like Bayesian semi-parametric generalized linear modeling use counts and account for issues of sparsity, compositionality, covariation, and more—but while this method may have favorable qualities, it does not necessarily make it the most applicable one, nor the most accessible (Ren et al., 2022). This does not necessarily address the concerns of longitudinal data either, as datasets have long been plagued by issues of intra-subject correlation. Consequently, of the methods identified by the review that serve to mitigate or account for these issues, all may have subtler biases or characteristics of their own that make them inflexible and, as such, inappropriate for use in certain contexts. We are in an extremely early stage of microbiome data analysis, and many of the research that falls under this umbrella does not adhere to any specific standard of analysis for any statistical test or metric use. It is important to us that we make this lack of standardization clear to those using our findings to inform their own; and we suggest that studies in this field sacrifice methodological brevity for intricate explanations and justifications of the tests they use to produce their results.

Insect-microbe Interactions

Tomberlin et al. (2017) focuses primarily on blow flies of forensic relevance, and in doing so notes one of the single most important instances of bacterial interactions with blow flies as generators of volatiles that ultimately attract certain Calliphorid species to carrion. With the advent of microbiome research and in lieu of this increasingly important observation, further assessments of the role bacteria play in the life histories of blow flies were conducted. Of these, what stands out the most to us is the hypothesized impact of larval masses on carrion microbiota

as they increase the basicity of their immediate environment (Pinilla et al., 2013; Filippis et al., 2024; Thomas et al., 1999). In raising pH and potentially cultivating an environment unfit for the survival of select bacteria, they may be selecting for beneficial bacteria in the process, or in some way promoting the success of species like *Proteus mirabilis (*Tomberlin et al. 2017). *P. mirabilis* has long stood out as a species with unique blow fly associations and distinct antimicrobial properties, suggesting that it may have a role in sustaining blow fly life (Ma et al., 2012). For as many microbial interactions have been identified or researched throughout history, what is notably lacking among these investigations of blow fly-bacterial interactions are explicit studies of postmortem microbiome-specific influences on the blow fly microbiome and vice versa. Weatherbee et al. (2017) is one of few studies that address many of our own studies objectives with preliminary findings of their own. To better our understanding of how blow flies and bacteria interact as representatives from different kingdoms with overlapping functional niches, Weatherbee et al. (2017) used swine carcasses and repeated sampling of carcass and larval bacterial communities to illuminate how they might be interacting significantly. Their results indicated that there may very well be a significant interaction effect between the environment, postmortem microbiome, and blow fly larvae. Furthermore, compositional changes in larval mass composition as time goes on and the microbiome of the carcass itself changes indicated that there may be a presently unclear effect of carcass bacterial community succession on larval mass blow fly species succession. While few relationships or interactions were able to be identified in earnest, the speculation behind trends we observe in data like those collected by Weatherbee et al. (2017) are precisely how researchers downstream can conceptualize projects that rise to test those hypotheses.

CHAPTER 2: SEASONAL DYNAMICS OF POSTMORTEM MICROBIOMES

Abstract

The process of vertebrate decomposition is mediated by complex organismal networks whose scale and influence varies greatly by environment and resource. Members of the postmortem microbiome are vulnerable to environmental variation; but little is known how weather patterns contribute to changes in carrion microbial communities. The objective of this study was to characterize differences in carrion microbiota community succession over the course of seasonal decomposition studies. We hypothesized that postmortem microbial community succession would vary with season, in association with the dominate weather conditions (e.g., temperature and humidity). We anticipated that the colonization by blow flies would significantly impact the bacterial succession of the carcasses. In the spring, summer, and fall, six stillborn swine carcasses were arranged along a woodland tree line and allowed to decompose until their remains skeletonized and larval blow fly activity ceased. Carcass microbial communities were collected twice a day every day until studies concluded. Results indicated that bacterial diversity consistently increased between each successive stage of decomposition. Summer bacterial diversity was significantly higher than fall and spring. The community composition of postmortem microbiomes differed significantly by season. Interestingly, the insect-associated bacteria *Ignatzschineria* was in high abundance among all carcass bacterial communities once larval activity was notable. Investigating decomposition stage as a function of accumulated degree hours (ADH) and as a function of bacterial community composition showed that season was important in the modeling ADH and season. Data indicates that season and insect cocolonization are important in the analysis of postmortem microbiomes, especially when extrapolating information about the decedent and their circumstances, from microbial data.

Introduction

Decomposition is as ubiquitous as it is inevitable for living creatures of all shapes and sizes. The process of decomposition succeeds death but contradicts its nature by releasing the valuable resources our bodies contain back into the immediate environment. It is important to contextualize decomposition as multifaceted rather than an uncomplicated one; and nowhere is its complexity more apparent than in the ephemeral community it can sustain.

Decomposition ecology is unique in that many of its contributors are uniquely suited to compete in that environment and nowhere else (Fenoglio et al., 2014). This collection of decomposers and consumers, alongside the carcass itself, constitute what is referred to as the "necrobiome" (Benbow, 2016; Barton et al., 2019). The transient nature of decomposing remains is largely attributable to many members of the necrobiome—chiefly bacteria that mediate the breakdown of the organic materials and blow flies of the family Calliphoridae (Benbow et al., 2013; Hyde et al., 2014; Janaway et al., 2009). Beyond the ecological considerations we lend to carrion, the difficulty in studying decomposition stems from its multivariate nature and consequent complexity (Barton et al., 2012; Benbow 2016). This is especially prevalent in the field of forensics, where death investigation is constantly being improved upon. Researchers argue that forensic science should be held to a higher, more rigorous standard (Solomon & Hackett, 1996; Tomberlin et al. 2011); and the growing popularity of forensic microbiology promises to encourage that (Spagnolo et al., 2019). The applicability of a new science is a common pretense for forensic research, and the promise of the human microbiome as an indicator of health suggested to many that we can similarly derive determinations posthumously (Kaszubinski et al., 2019; Knight et al., 2017; Pechal et al., 2013). The collection of bacteria, fungi, and archaea that typically comprise the human microbiome are subjected to volatile

growth conditions following the death of their host organism, and this host death is precisely why microbiota come to constitute a "postmortem microbiome" instead (Pechal et al. 2018). Studies have demonstrated that trends in microbial community structure and succession follow predictable trajectories once the postmortem microbiome has been established (DeBruyn & Hauther, 2017; Dong et al., 2019; Javan et al., 2016; Liu et al., 2021). Additionally, the uniqueness of human microbiomes suggests that we can use their specificity to aid in determining the perpetrator of a crime (Oliveira & Amorim, 2018). Sequencing technologies that allow us to characterize portions or the entirety of a microbiome have also seen rapid improvements in terms of efficacy and accessibility (Wang, 2023). Many claims have been made as forensic microbiology and the body of research supporting it grows, but its suitability for practice is far from clear (Tozzo et al., 2022). Data trends seem to indicate the usefulness of the postmortem microbiome in postmortem interval (PMI) or cause of death (COD) determination. Despite this, the field is still well in its relative infancy. We believe that the postmortem microbiome may be useful in corroborating other forms of evidence (e.g., taphonomy, insect evidence) during an investigation. The use of forensic microbiology as a means of validation could prove pertinent to the creation or validation of taphonomic criteria, a matter for which there is no singular standardized measure (Pittner et al., 2020). If we demonstrate that microbial profiles can reliably coincide with the decomposition stages, it may further strengthen our understanding of postmortem microbiome succession. **Figure 1** outlines a hypothetical flowchart of common evidence types and how they can be processed with forensic microbiology in mind. Combining taphonomic assessments, postmortem microbiome sampling, and insect sampling when appropriate can provide multiple PMI determinations. Postmortem microbiome sampling in combination with the soil microbial profiles can even provide evidence of carcass

translocation. If the PMI determinations corroborate each other, we can be more confident that our inferences are correct. If one or two PMI conflict with others, then perhaps that inconsistency could elucidate an important element of the crime that warrants further consideration.

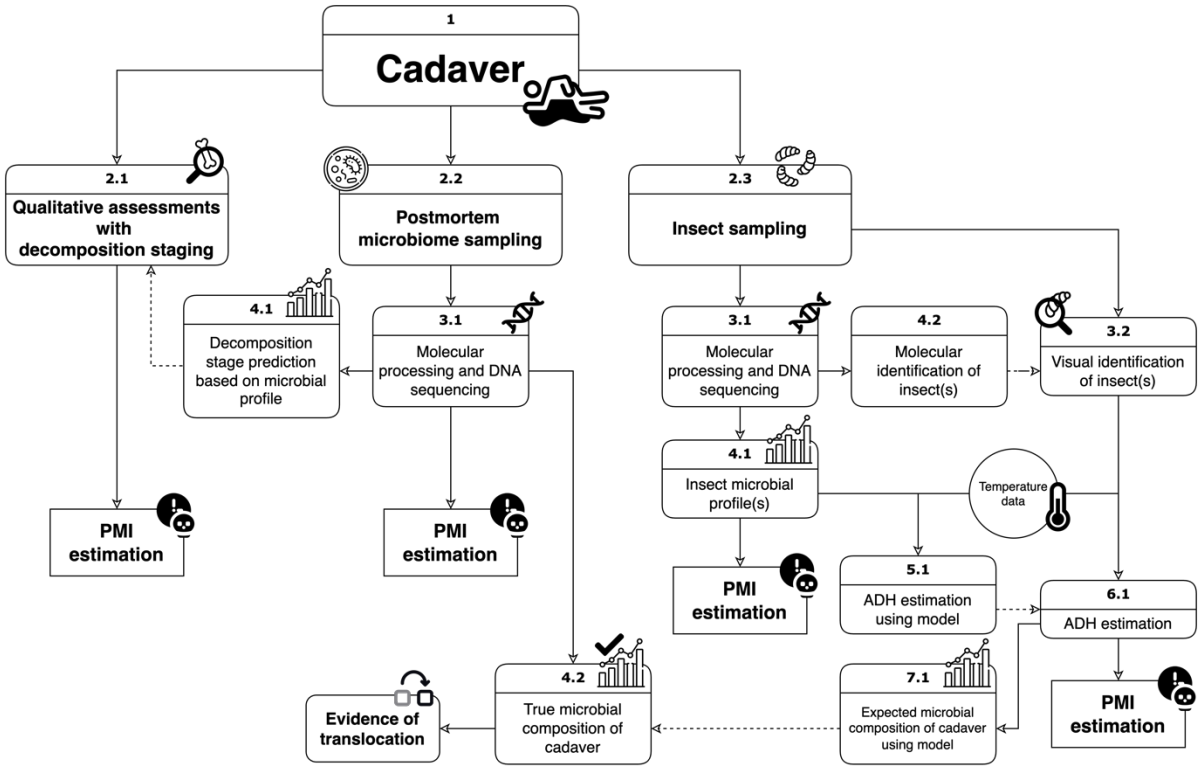

Figure 1. Hypothetical evidence processing pipeline that incorporates microbial data as complementary validation for traditional forensic techniques. This concept takes advantage of evidence type flexibility in the determination of multiple postmortem intervals (PMI) when contextualized further by microbial profiles. Dashed lines represent additional applications of inferences to other evidence processing pipelines.

Preliminary findings reported in recent research highlight the capabilities of longitudinal decomposition data in the creation of models that can predict measures like PMI (Metcalf et al., 2013), the reliability and flexibility of these models stands to make marked improvements as research continues. Predictive modeling has seen great improvement with the advent of machine learning (Li et al., 2023), and understanding what the algorithms responsible for generating these models are sensitive to may prove crucial in evaluating the usefulness of the postmortem

microbiome in death investigation. As such, especial attention should be given to the underlying forces that drive decomposition. Weather conditions are among the most crucial contributors to the process of carrion decomposition, as temperature and humidity have been broadly implicated in modulating the rate at which it occurs (Englmeier et al., 2023). Despite this, little research exists on the role seasonal weather variables play in postmortem microbiome assembly. Few datasets succeed in capturing the full range of seasons representatively, if at all.

To address some of these research needs, this study aimed to thoroughly characterize bacterial communities of carcasses throughout the course of decomposition with repeated measures. In addition to performing decomposition field studies during different seasons, we sought to sample the microbiota of larval masses as blow fly larvae colonized the carcass and consumed it. Focusing on these two aspects of our study allowed us to better understand both the impact that seasonal dynamics play in the succession and structure of postmortem microbiomes and the microbial exchange with blow flies.

We hypothesized that season would have a significant effect on microbial communities over the course of decomposition, reflecting their incongruous abiotic conditions (e.g., temperature, humidity) and their influence the biological processes of bacteria and insects. We predicted that the weather conditions typical of fall and spring would limit the growth of taxa intolerant of lower temperatures and moisture. If it were the case that seasonal weather variables during cooler months only lent themselves to bacterial species suited for them, then we also assumed that the overall diversity of these postmortem microbiomes would be significantly lower than those of the summer.

Materials and Methods

Decomposition Studies

Decomposition studies were performed in the summer (2022), fall (2022), and spring of 2023 (**Table 1**). Start dates were selected based on historical weather data sourced from the Michigan State University Horticulture (MSUHort) and MSU Hancock Turfgrass Research Center (MSU HTRC) weather stations to represent the average conditions associated with each season. Both weather stations were approximately the same distance from our study site (2-3 miles). Sampling occurred twice a day: once in the morning (AM), and once in the afternoon (PM). A winter study was not performed due to the presumed difficulties that snow cover and access would impose on a consistent sampling schedule.

Table 1. A summary of decomposition study periods. Dates reflect the days the stretch of time during each study that samples were collected twice daily. Temperatures and humidity readings were averaged for each study.

Study	**Sampling period (YYYY-MM-DD)**	**Average temperature (°C)**	**Average humidity (RH%)**
Summer 2022	2022-08-05 to 2022-08-11	22.3 ± .19	76.6 ± .78
Fall 2022	2022-10-14 to 2022-11-06	8.27 ± .12	76.3 ± .40
Spring 2023	2023-05-15 to 2023-05-26	16.7 ± .18	69.6 ± .67

Stillborn American Yorkshire swine (*Sus domestica*) carcasses were selected as our carrion model and purchased from the MSU Swine Teaching & Research Center. Domestic swine have a long history of use in forensic decomposition studies as analogues to human cadavers (Connor & Hansen, 2017). Using smaller carcasses also promotes the emergence of smaller larval masses whose accumulations are measurably distinct from others (Leblanc & Strongman, 2002) and offers better replication (n = 6 for each season) than adults **(Table S1)**. Carcasses were bagged in groups of 2-3 and stored at -20°C until the beginning of their respective study, when they were thawed at room temperature (20-22°C) 24 hours before placement at the study site. The carcasses were sexed and weighed using a mechanical scale, and no measures were taken to alter or otherwise eliminate the existing microbiota of the carcasses.

Decomposition studies were performed on the grounds of the Box Woodland in East Lansing, MI (42° 41' 23.0712" N, 84° 29' 29.5476" W) with permission from the MSU Campus Natural Areas Committee. Each trial was performed in a different location with the Box Woodland split into three 60m sub-sections of tree line to accommodate all three studies (**Figure 2**). Carcasses (n=6) were arranged at least 10 m apart along a transect parallel to the woodland tree line. Carcasses were placed with their heads facing west and maintained this position throughout the duration of the decomposition studies. Anti-scavenger cages were $1m^3$ and placed over each carcass to prevent vertebrate scavenger activity. Cages were secured using 3 bricks (~8kg) and were a successful prevention measure.

Figure 2. Field site and placement of stillborn pig carcasses used in seasonal decomposition studies. Each sub-section spanned 60m and accommodated n = 6 stillborn pig carcasses, each 10m apart from each other. All sub-sections faced away from main or access roads and did not interrupt ongoing agricultural work.

Abiotic Data Sampling

Hourly weather data were collected using HOBO data loggers (Onset Computer

Corporation, Bourne, MA) in the summer and Kestrel DROP D2 wireless data loggers (Kestrel Instruments, Boothwyn, PA) in the fall and spring seasons. Data loggers were affixed to the anti-scavenger cages immediately following carcass placement. The HOBO data loggers only recorded temperature (°C), while the Kestrel DROP D2 measured both temperature (°C) and relative humidity (%). Weather station data from MSUHort and MSU HTRC were also collected for downstream analysis of historical weather trends and data representativeness.

Microbiota Sampling

Epinecrotic (carcass surface) microbial communities were collected using sterile, DNA-free cotton-tipped swabs (Puritan Medical products, Guilford, ME). A transect of the carcass abdominal skin was sampled with repeated strokes while rotating the swab. The oral cavity and rectum surfaces were sampled the same way, but with insertions rather than strokes (**Figure 3**). Aseptic field technique was employed to reduce contamination; and swab tips were stored in 1.5mL microcentrifuge tubes with 200uL of room temperature RNAlater. RNAlater was selected as a reagent that stabilizes and protects cellular RNA/DNA until further processing (Gorokhova, 2005). Sampling of larval mass microbiota accompanied observations of substantial and accessible aggregations (>50 larvae). The sampling protocol for larval masses differed only in that repeated insertions (n = 3) prioritized unsampled areas of the mass. If more than one distinct larval mass was present, they were sampled independently. Once sample collection concluded for any given sampling event, tubes containing the samples were stored in laboratory freezers at -20°C.

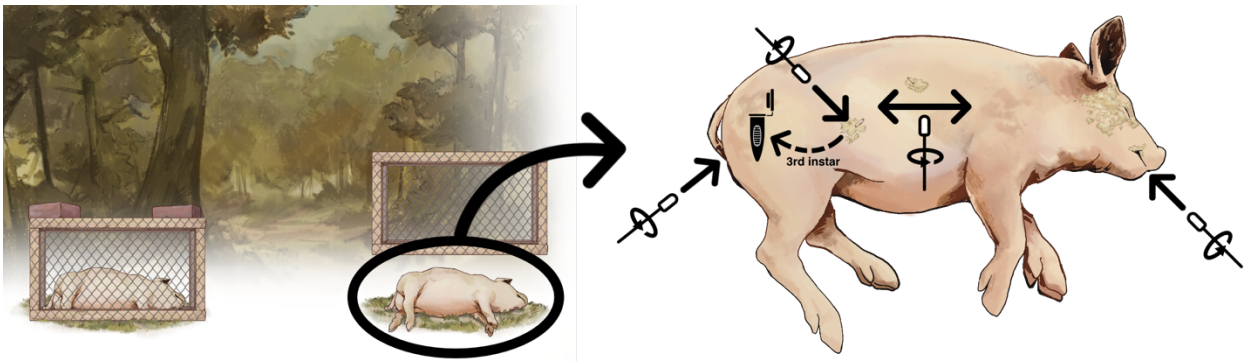

Figure 3. Scavenger cage configuration and sampling procedure, visualized. Body site sampling of the skin, mouth and rectum took place twice a day. Larval mass microbiota sampling was conducted when possible; and larvae were collected (n=10-30) provided they were confirmed to be third (3rd) instar based on the number of spiracles observed from individual to individual.

Insect Sampling

When distinct larval masses were present, a sterile, DNA-free tongue depressor was used to collect 10-30 large larvae for preservation in 50mL Falcon® Conical Centrifuge Tubes (Corning, Corning, NY) with 25mL of RNAlater. Larvae were visually inspected to family (Calliphoridae) and aged based on spiracle count (n = 3 spiracle slits for 3rd instar) after being collected. Only 3rd instar larvae were processed for analysis.

Taphonomic Assessments

Taphonomic assessments of the carcasses were performed using criteria outlined in Payne (1965) to approximate the following decomposition stages: Fresh, Bloat, Active, Advanced, and Dry. Photos of each carcass were taken throughout the study, once per sampling event (**Figure 4**). The Payne (1965) decomposition criteria were suited to our carrion model and experimental objectives in that they provided subjective, but commonly used, stages of a continuous process based on major biological activity (e.g., blow fly colonization, putrefaction).

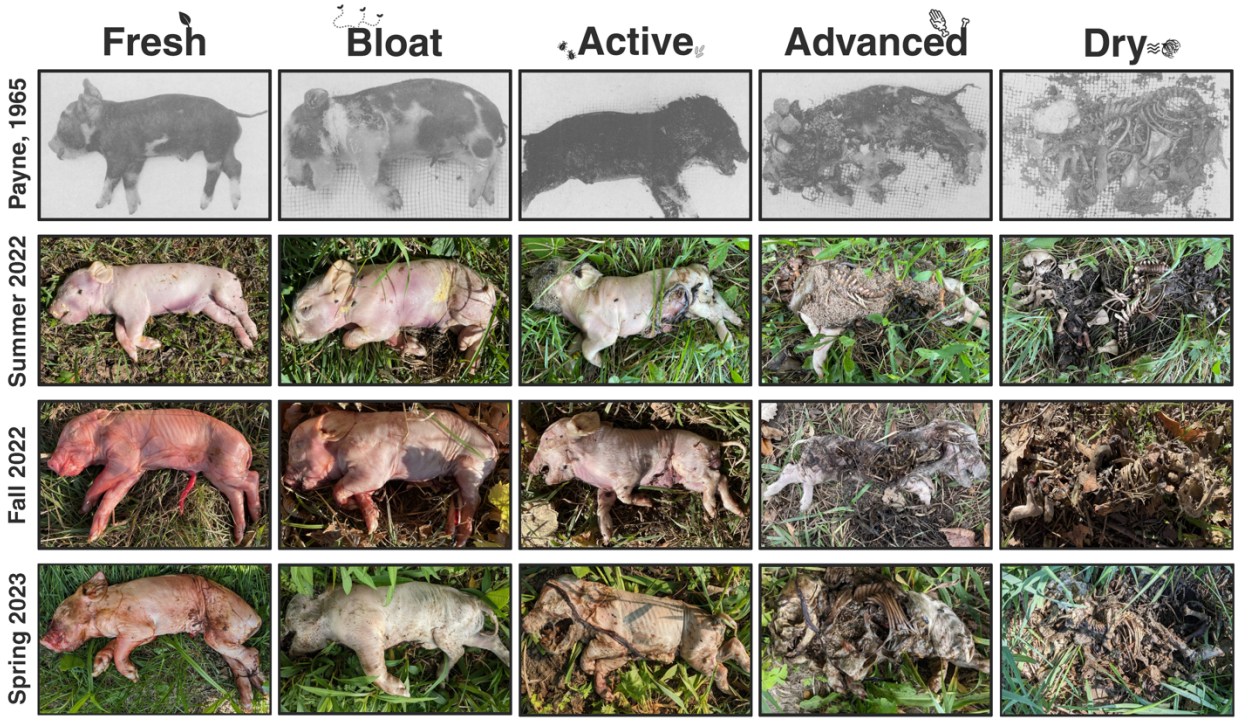

Figure 4. Comparative matrix of decomposition stages between studies. Includes visual representations of each decomposition stage (Fresh, Bloat, Active, Advanced, Dry) outlined by Payne (1965). The first row corresponds with example images from Payne (1965), subsequent rows correspond with taphonomic assessments made during each seasonal decomposition study.

DNA Extraction and Amplicon Sequencing

The prohibitively large number of samples collected across the fall and spring trials necessitated limited swab processing, so samples taken during evenly numbered sampling events were prioritized **(Table S6)**. Care was taken to ensure that the range of samples used encompassed all stages of decomposition. Isolation of genomic DNA was performed using the DNeasy® Blood & Tissue Kit (QIAGEN N.V., Hilden, Germany). The kit instructions were modified to include 15uL lysozyme (15mg/mL) prior to incubation, encouraging cell lysis (Salton, 1957). The cotton tips of the swabs were either removed from the stick entirely using sterile scalpel blades and forceps or beaten using 1.4mm ceramic beads (VWR International,

Radnor, PA) and a FastPrep-96™ high-throughput bead beating grinder and lysis system (MP Biomedicals, Santa ana, CA) prior to the 90-minute lysis step.

Individual blow fly larvae pooled during sampling events were introduced to new (separate) 1.5mL microcentrifuge tubes and homogenized using 1.4mm ceramic beads & the FastPrep-96™. Sampling events that 3rd instar larvae were present for were represented by 10 individuals, sampled randomly and such that all carcasses from which larvae were sampled are represented by 1-3 larvae (depending on the number of carcasses sampled). DNA extraction was performed identically to the protocol used for swab processing.

Eluted DNA was quantified using the Qubit™ dsDNA Quantification Assay Kit and a Qubit 2.0 fluorometer (Thermofisher Scientific, Waltham, MA). PCR amplification of the V4 region of the 16S rRNA gene using 515F/806R primers (5'-GTGCCAGCMGCCGCGG-3', 5'-TACNVGGGTATCTAATCC-3') was followed by gel electrophoresis. Samples containing viable DNA were submitted to the Michigan State University Genomics Core facility (East Lansing, MI) for Illumina MiSeq amplicon sequencing. Sequencing data was demultiplexed by the MSU Genomics Core before being made available for analysis.

Sequencing Data Processing

The bioinformatic software QIIME2 (v2023.7) was used to filter raw 16s amplicon sequencing data and generate diagnostic data important for quality and quantity control **(Table S4)**. Primer sequences were selectively trimmed from the sequencing data before amplicon error correction and ASV assembly was mediated by DADA2. Taxonomy was determined by a naïve Bayes classifier using the SILVA rRNA database (v138-99). Multiple sequence alignment (MSA) was made possible by MAFFT (v7), a tool native to the QIIME2 ecosystem. A final filtering step was performed to eliminate singletons and reads matched with mitochondrial or

chloroplast DNA. Additionally, rarefaction eliminated samples with sequencing read counts <150bp to better standardize the data and mitigate the risks resulting from their limited taxonomic resolution/profile. Samples above the 150bp threshold were significantly higher than 150bp and grew exponentially with each successive sample. >200bp samples were retained despite their size because they demonstrated greater degrees of homogeneity as far as their bacterial community compositions were concerned. The artifacts (outputs) corresponding with taxonomy, rooted phylogenetic trees, and filtered feature tables were imported into RStudio (v2023.06.0+421, RStudio Inc., Boston, MA) with the 'phyloseq' package for analysis.

Statistical and Bioinformatic Analysis

Statistical analyses were informed by recent standards for microbiome analysis (Kodikara et al., 2022; Silverman et al., 2018) that are non-parametric and accommodate compositional data.

To test for significant overall differences in biodiversity (alpha diversity) among factors (sample type, season, decomposition stage.) we used the Kruskal-Wallis rank-sum test (KW). Pairwise testing of alpha diversity metrics (observed species, species evenness, Shannon diversity, and Faith's phylogenetic diversity) was performed using a pairwise Wilcoxon rank-sum test and enabled direct comparisons of specific samples/groups. Analysis of covariance (ANCOVA) was used to test main and interaction effects of categorical variables combined with a third, continuous variable to track alpha diversity changes over time. Only Shannon diversity was prioritized in final analyses as a diversity metric that accounts for both species richness and relative abundance. Temperature data was tested using KW and post-hoc analysis was performed with Dunn's test.

Differences in beta diversity were tested among seasonal weather variables, sample types,

and season itself using the Adonis R package. Adonis is an alternative to permutational multivariate analysis of variance (PERMANOVA), a non-parametric alternative to multivariate ANOVA tests (MANOVA) that allows for the use of numeric and categorical variables in testing rather than just categorical variables. Principal coordinate analysis (PCoA) of UniFrac distance matrices were used to visualize community dissimilarity as a metric designed to compare microbial communities among different habitats and/or over time. It measures the phylogenetic distance of bacterial taxa within the communities and uses relative scoring to evaluate dissimilarity between samples. Weighted UniFrac (as opposed to unweighted UniFrac) accounts for the relative abundance of the taxa within our samples as well as the absence/presence of those taxa, much like Shannon diversity.

Analysis of compositions of microbiomes with bias correction (ANCOM-BC) is a method that estimates absolute abundances of bacterial taxa based on relative abundance data. It then uses these estimates to test for differential abundances of microbial taxa across different samples, groups, or variables. The bias correction accounts for issues that arise in non-normal data (like compositional microbiome data) and underlying issues of hypothesis testing.

Random forest predictive models (both regressors and classifiers) were developed using the 'randomForest' R package and default parameters **(Table S10).** Random forest is well suited to classification tasks like these as an algorithm that generates multiple decision trees from subsets of our data to create models whose accuracy and goodness-of-fit (GoF) can outperform those built on a single decision tree. This approach is especially appropriate for microbial data as a non-parametric modeling approach for complex, multivariate data. These models were designed to predict accumulated degree hours (ADH), season, and decomposition stage using the taxonomic feature and abundance data of samples contained within "phyloseq" objects, which

enable the import and analysis of phylogenetic sequencing data.

Accumulated degree hours (ADH) were calculated with HTRC data using the formula $ADH(h) = \sum_{i=1}^{h} \max(T_i - LDT, 0)$, where h is hours, Ti is the temperature (°C) for the *i*th hour, and LDT represents the lowest possible development threshold for which larval growth can be expected (Higley & Haskell, 2000). ADH is a measure of development that estimates the amount of accumulated heat necessary for larvae of a fly species to reach a particular developmental stage. It has also and has also been applied to postmortem microbiomes (Pechal et al 2018). The calculation assumes a linear relationship between developmental rate and temperature. Negative temperature values were entered as zero when necessary to prevent them from subtracting from the cumulative ADH. With ADH representing the interaction of time (hours) and thermal exposure, it was used in seasonal comparisons of decomposition to mitigate the visual and statistical challenges of our incongruous study timelines.

Results

Seasonal Weather, ADH, and Taphonomy

The duration of each decomposition study varied by season (**Figure 5**). From placement to skeletonization, the summer study took 7 days, the fall study 24 days and the spring study took 12 days.

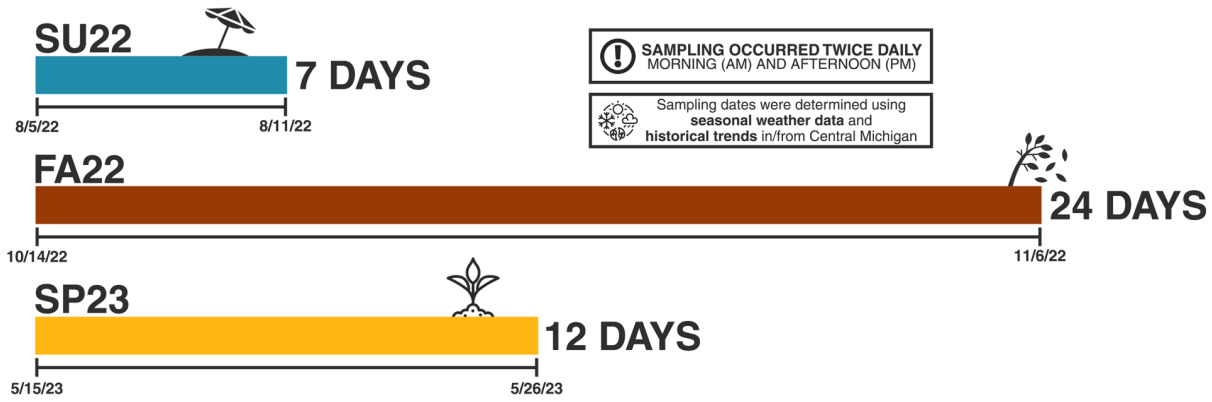

Figure 5. A timeline of seasonal decomposition studies. Bar length corresponds with the total number of days spanned by each study.

Due to instrument malfunctions, temperature and humidity data prior to the AM sampling event of May 21st were not recorded, resulting in more than half the spring 2023 decomposition study being unrepresented. To account for this loss, an ANOVA compared averaged hourly data logger readings from all seasons to data recorded hourly by the HTRC and MSU Horticulture weather stations. No significant differences were detected between any datasets (nor between the individual data logger readings themselves) **(Figure S1)**. Additional attempts to model the missing weather data from the spring study were made using HTRC and MSU Horticulture data successfully. Ultimately, weather station data offered more consistent and realistic snapshots of seasonal weather data; and HTRC Station readings were used for downstream analysis preferentially over data logger readings. ADH calculations showed that summer had the highest ADH of 1948. Fall reached 1558 ADH, and spring reached 1185 ADH.

Weather spanning the duration of each study was compared to three years of prior HTRC weather data. Summer and fall studies, having taken place in 2022, were compared to data from 2021, 2020, and 2019. The spring study took place in 2023 and was compared to weather data from 2022, 2021, and 2020. Hourly temperature and relative humidity were compared between these historical data for all seasons **(Figure 6)**.

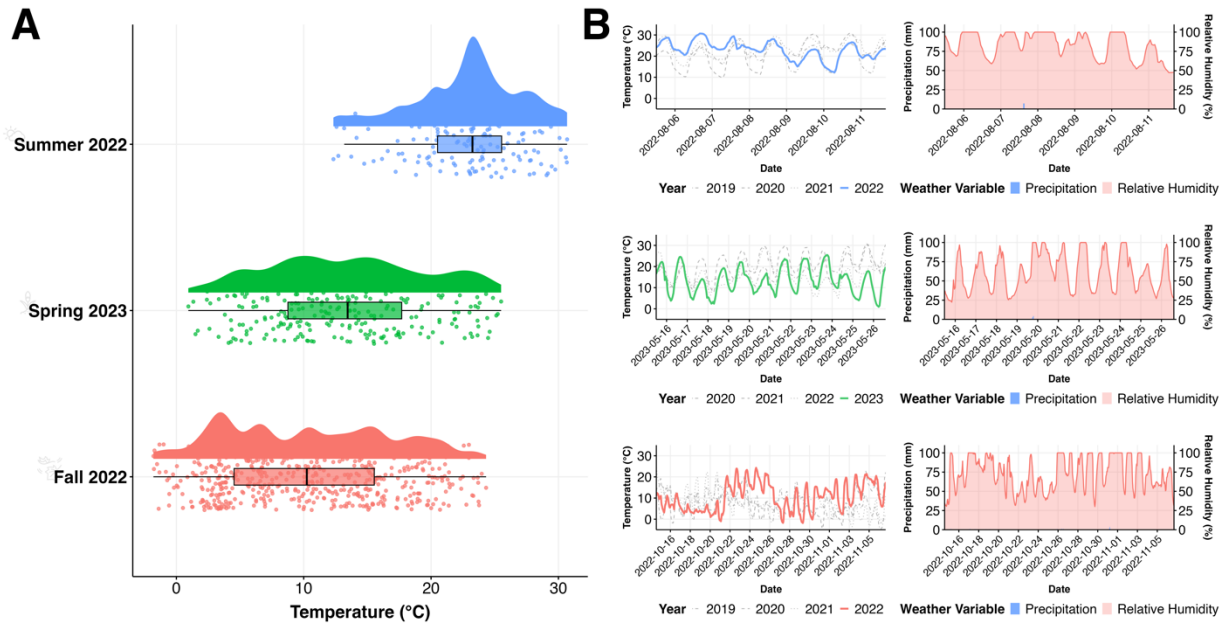

Figure 6. Decomposition study weather data. A) Rainfall plot of hourly temperature data between summer, spring, and fall. B) Plots of temperature over date between for the study year and the three years preceding it & relative humidity (RH%) and precipitation levels over the course of the study.

Temperature (adj. $p = 0.46$) and relative humidity (RH%) (adj. $p = 0.13$) did not differ significantly between summer 2022 and summer 2021. In the fall, however, both temperature (adj. $p \leq 0.001$) and RH% (adj. $p \leq 0.001$) were significantly different between the study year (2022) and 2021. Spring too was significantly different between 2023 and 2022 in terms of both temperature (adj. $p \leq 0.001$) and RH% (adj. $p \leq 0.001$) **(Table S7, Table S8, Table S9).** Cooler months (fall and spring) temperatures and humidity that are often highly variable (e.g., low temperatures, sporadic RH%, precipitation events) were significantly different between successive years i. Summer, however only differed significantly by temperature (adj. $p = 0.0018$) and humidity (adj. $p = 0.0029$) between 2020 and 2021. Otherwise, periods of weather within the span of the summer 2022 study remained relatively consistent.

Comparing just the weather data from the three decomposition studies in the summer, fall, and spring reinforced how dissimilar their conditions were **(Table 2)**. All differed

significantly from each other in both hourly temperature (adj. $p \leq 0.001$, all pairwise comparisons) and RH% (adj. $p \leq 0.001$, all pairwise comparisons) & represented extremely different climatological snapshots of seasonal weather. Lower test statistics between the fall and spring temperatures (-5.4) indicate that the differences between these two seasons was less that what was observed between summer and either month.

Table 2. Post-hoc analysis (Dunn's test) of Kruskal-Wallis rank sum tests comparing decomposition study (summer, fall, spring) temperature and relative humidity data.

Comparison	Test Statistic	Adjusted p-value
Temperature (°C)		
Summer 2022 vs Fall 2022	-17.46777	≤0.001***
Summer 2022 vs Spring 2023	-11.82683	≤0.001***
Fall 2022 vs Spring 2023	-5.404205	≤0.001***
Relative Humidity (%)		
Summer 2022 vs Fall 2022	-4.873887	≤0.001***
Summer 2022 vs Spring 2023	-9.093190	≤0.001***
Fall 2022 vs Spring 2023	6.443789	≤0.001***

*$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***,* (ns) = *not significant*
p-values adjusted using the Bonferroni method, normality of data tested using Shapiro-Wilk

Figure 7 illustrates both the average duration of decomposition stages throughout each study and how long each individual carcass was classified as each stage relative to their cumulative ADH. A significant difference in the amount of time spent by spring carcasses in the "Fresh" stage is immediately apparent when compared directly to the averages of summer and fall. Summer carcasses also spent considerably more time in the "Dry" stage of decomposition than either fall or spring, but the average duration of each stage was not skewed towards it in the same fashion that spring averages are by "Fresh." Summer and fall have comparable average durations between similar ADH timepoints; but the only overlap their stage durations have with

spring were the “Active” and “Dry” timeframes.

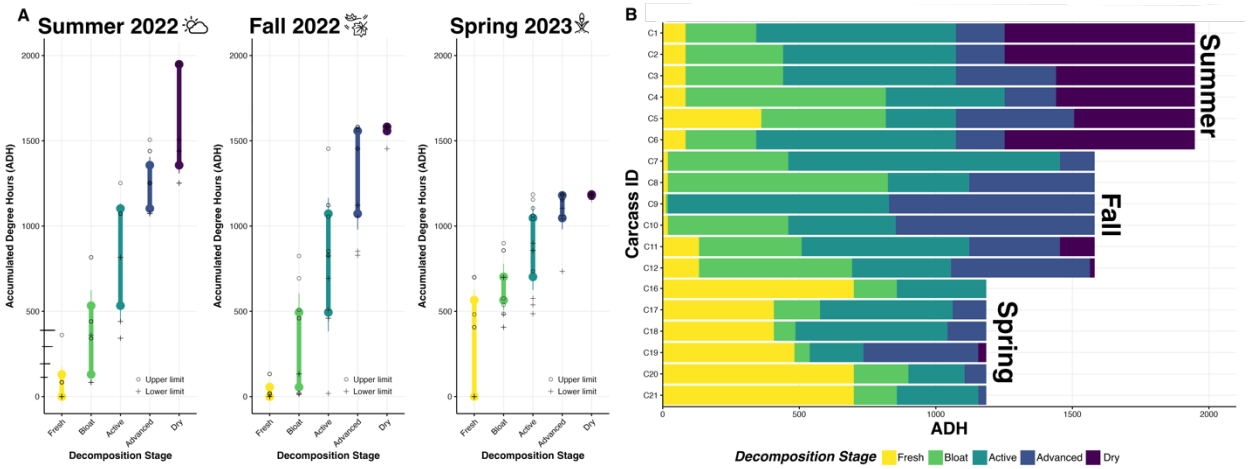

Figure 7. Differences in decomposition stage duration across accumulated degree hours (ADH). A) Average duration of each decomposition stage across all carcasses for each season. B) Intra-carcass variation of decomposition stage duration across all seasons.

Seasonal Microbiome Diversity

Shannon diversity of carcass postmortem microbiomes significantly increased with ADH over the course of decomposition (adj. $p \leq 0.001$ for each season) (**Figure 8, Figure S8**). The slope of this increase was greatest in the summer, while the slopes of fall and spring were comparable. The initial Shannon diversity typically started between 1.5-2 and ended at 2.5-3 for all seasons. ANCOVA testing revealed that the rate at which alpha diversity increased in the summer was significantly ($p = 0.045$) higher than it did in the fall. While the summer diversity growth rates were higher than the spring, it was not to a significant extent ($p = 0.062$). The slope for spring did not differ significantly ($p = 0.707$) from the slope for fall. Evidently greater diversity in the summer than in other studies. In any case, both ADH and Season have a significant relationship with alpha diversity.

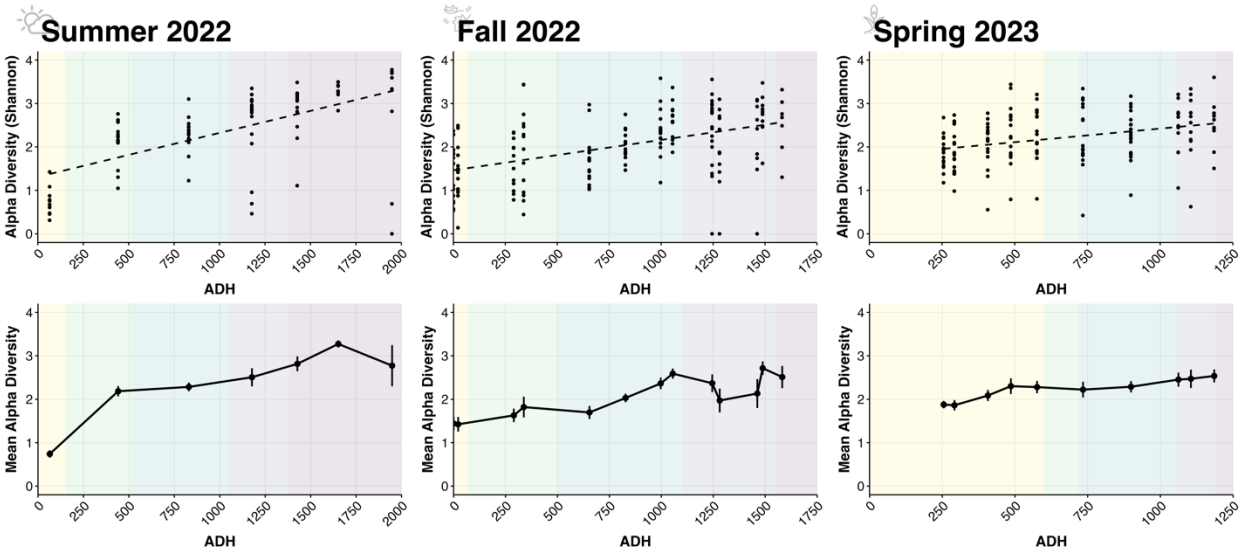

Figure 8. Linear regressions of the relationship between alpha diversity (Shannon) and ADH. The top row shows alpha diversity for all carcasses, while the bottom row reflects the mean alpha diversity of all carcasses. The gradient of colors overlaying the plot grid represent approximate decomposition stage associated with ADH range ("Fresh" → "Bloat" → "Active" → "Advanced" → "Dry").

Comparisons of postmortem microbiomes **(Figure S3, Table S3)**, larval microbiomes, and larval mas microbiomes by season revealed several trends **(Figure 9)**. Summer postmortem microbiomes were significantly more diverse than the fall microbiomes (adj. $p = 0.0054$) and the spring microbiomes (adj. $p = 0.033$). Fall and spring microbiota, however, did not differ so significantly in terms of alpha diversity (adj. $p = 0.051$). Larvae alpha diversity differed significantly by season, with fall larvae being significantly more diverse than both summer (adj. $p \leq 0.001$) and spring (adj. $p \leq 0.001$) larvae. Spring larvae were more diverse than summer larvae as well (adj. $p = 0.002$). Larvae sampled during the summer study were ultimately the least diverse by a large margin, despite it having the most diverse carcass postmortem microbiomes. Conversely, the fall study had the least postmortem microbiome diversity and the greatest larval diversity. Comparisons of larval mass alpha diversity trend similarly, with fall and spring larval masses being more diverse on average than those in the summer. Spring larval masses ranked as the most diverse, significantly moreso than the summer larval masses (adj. $p <$

0.001). Consolidating all sample types and comparing their overall diversity across each study shows no significant differences in alpha diversity.

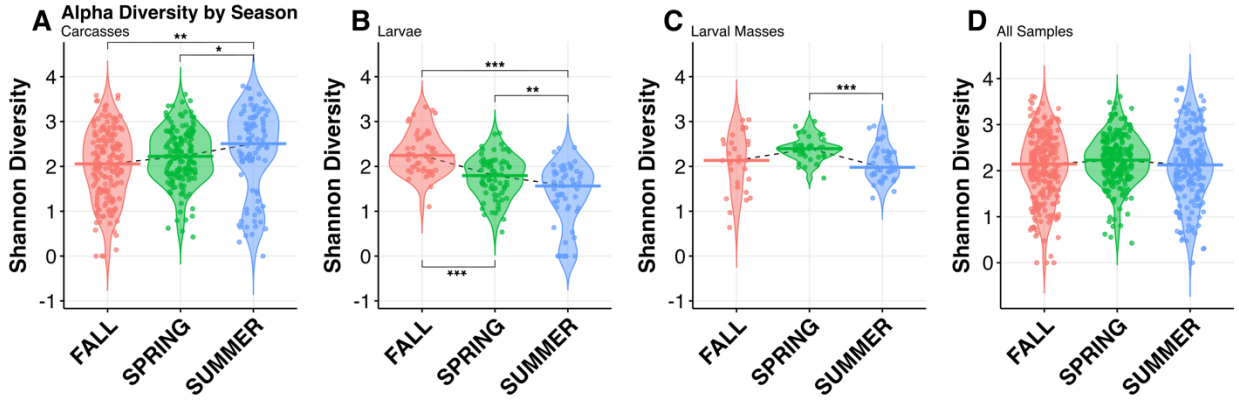

Figure 9. **Comparisons of season Shannon diversity faceted by sample type.** A) Violin plots of carcass postmortem microbiome diversity grouped by season. B) Violin plots of larval microbiome diversity grouped by season. C) Violin plots of larval mass microbiome diversity grouped by season. D) Violin plots of all sample types grouped by season. Colored horizontal lines denote median Shannon diversity. Dotted lines between these medians serves as a visual guide for positive or negative trends between seasons. Brackets with tails indicate pairwise Wilcoxon rank-sum tests of significance. Significance thresholds are denoted by * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

In a combined seasonal dataset, the diversity of carcass postmortem microbiomes (adj. p = 0.002) and larval mass microbiomes (adj. p = 0.002) were significantly higher than those of the larvae **(Figure 10)**. Where carcass diversity and larval mass diversity do not differ significantly from each other in the combined seasonal dataset, sample type diversities all vary significantly in the summer. In fact, summer carcass microbiomes were significantly greater than that of larvae (adj. $p \leq 0.001$) and larval masses (adj. $p \leq 0.001$). Larval masses were still more diverse than larvae, however (adj. p = 0.0035). No significant differences existed between sample types in the fall; but larvae were the most diverse. Spring trends matched those of the combined dataset, with carcasses (adj. $p \leq 0.001$) and larval masses (adj. $p \leq 0.001$) both more diverse than larvae. Interestingly, larval masses were more diverse than carcasses, but not significantly so.

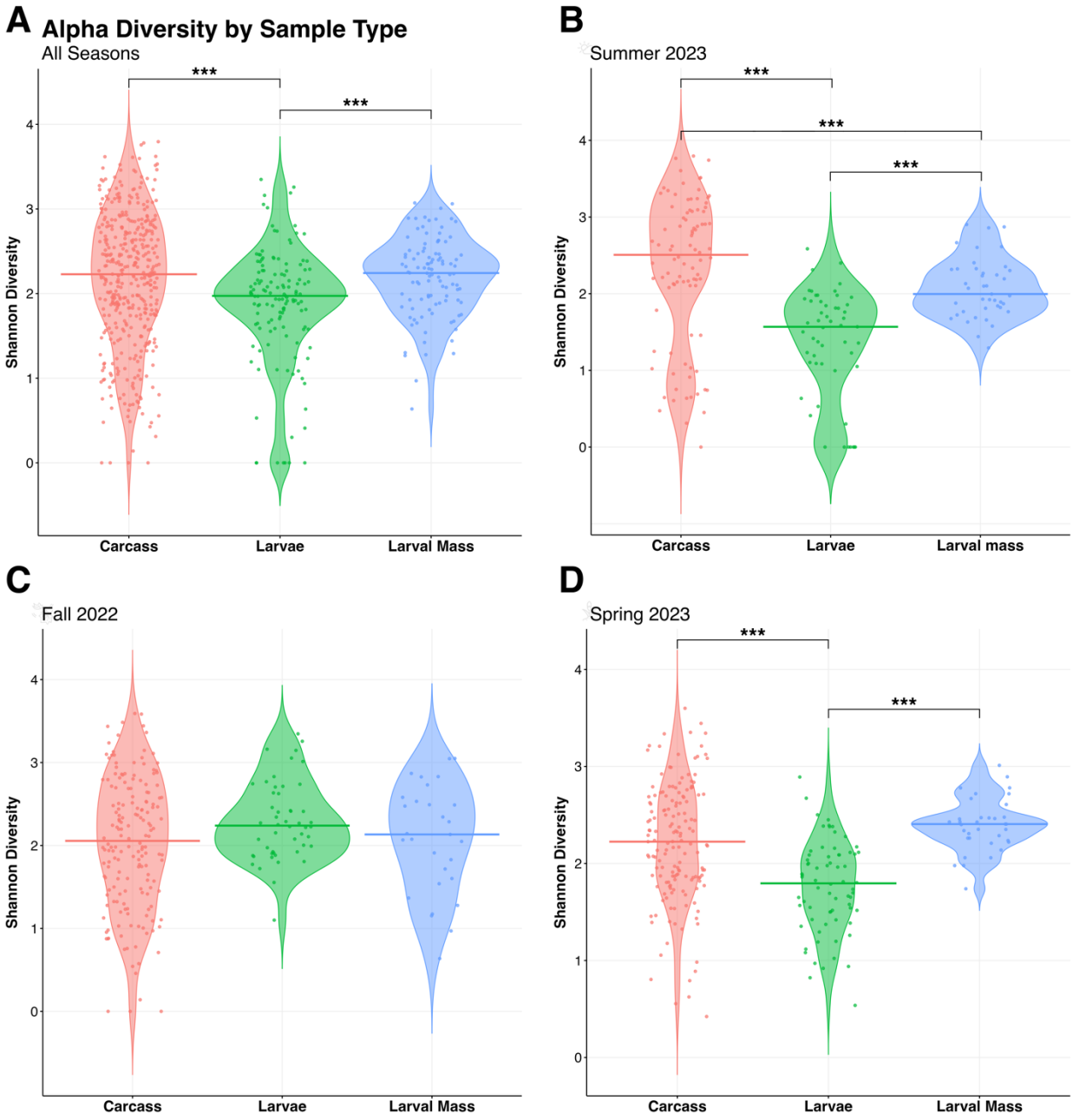

Figure 10. **Comparisons of sample type Shannon diversity faceted by season.** A) Violin plots of sample type microbiome diversity in a combined dataset of all seasons. B) Violin plots of sample type microbiome diversity in summer 2022. C) Violin plots of sample type microbiome diversity in fall 2022. D) Violin plots of sample type microbiome diversity in spring 2023. Colored horizontal lines denote median Shannon diversity. Brackets with tails indicate pairwise Wilcoxon rank-sum tests of significance. Significance thresholds are denoted by * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Seasonal Microbiome Community Composition

Bacterial community composition differed significantly overall in the summer among body sites ($p \leq 0.001$), but pairwise comparisons revealed that this was largely driven by differences between the carcasses and larvae (adj. $p \leq 0.003$) as well between the carcasses and larval masses (adj. $p = 0.039$). Summer larvae and larval mass communities were not significantly dissimilar from each other.

Fall sample type communities were significantly dissimilar to the same extent between each pairwise comparison (adj. $p \leq 0.003$). Spring carcass communities did not differ significantly from those of the larval masses (adj. $p = 0.390$), but differences between those sample types and the larvae were significant (adj. $p \leq 0.003$). Interestingly, when all seasons are combined into a singular dataset, larval mass communities do not differ significantly from carcass communities to an even greater extent than what was observed in the summer (adj. $p = 0.702$). Larvae communities differ from carcasses (adj. $p \leq 0.003$).

Each axis of a PCoA is a principal coordinate that represents a direction of maximum variance in our seasonal sample community data; and using them, inferences can be made about the percentage of variation each one explains (**Figure 11)**. The proportion of variation that either axis can account for is additive, such that the summer PCoA axes (PC1 + PC2) can explain a total of 94.8% of variation within the data. The incredible explanatory power of the PC1 axis indicates that it captures major differences in community composition, meaning that it may be possible to implicate a specific abiotic or biotic carriable in driving bacterial community dissimilarity between summer samples. Fall shares similarly high PC1 percentage of variation explained at 74.9%, meaning a dominant trend may be similarly driving sample dissimilarity during this season as well. Spring varies considerably from summer and fall, as the PC1 of its PCoA can only account for 51.5% of the variation while PC2 accounts for 23.5%, which is

significantly more than the PC2 axis of summer and fall PCoA were able to. Spring appears to be more complex in that its sample dissimilarity is driven by more than one influential factor rather than one dominant one. The PCoA corresponding with the combined seasonal dataset has the lowest explanatory power, with PC1 accounting for just 38% of variation and PC2 accounting for 22.4%. In considering the whole of our seasonal data, it appears that there are more factors at play driving these differences than in any of the individual seasonal datasets. Its complexity is most suggestive of the fact that there is no single dominant trend driving the significant dissimilarity we can observe between samples.

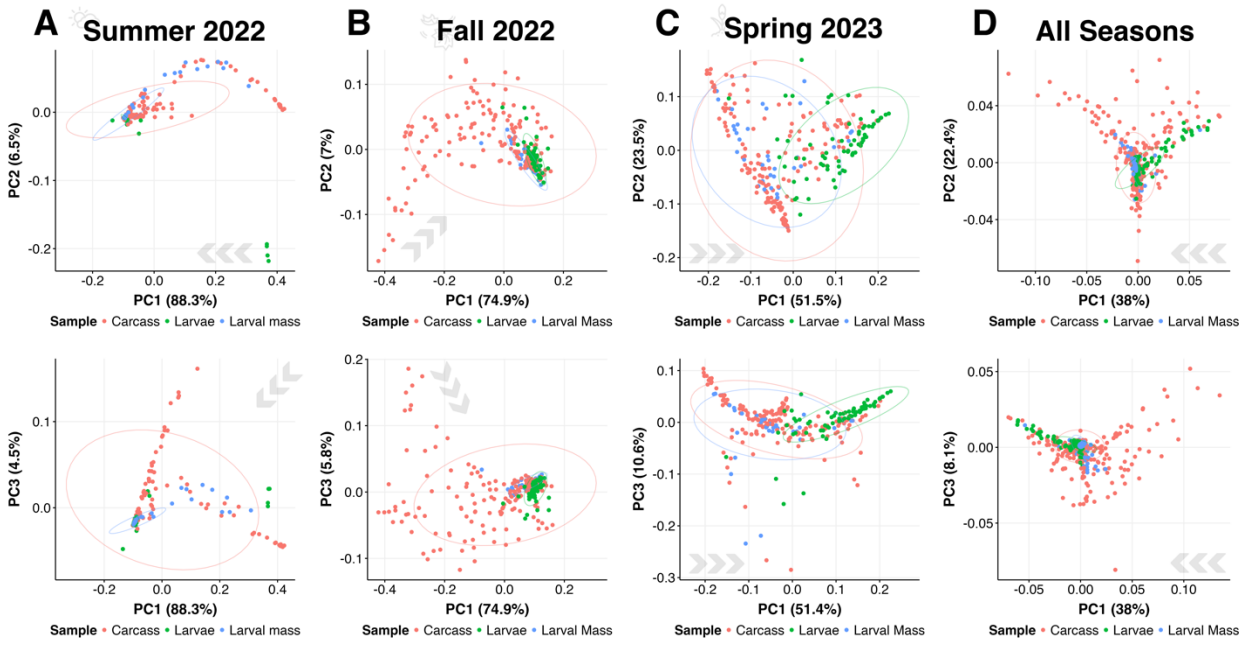

Figure 11. PCoA of seasonal weighted UniFrac scores grouped by sample type. A) Summer 2022 PCoA, first of axes PC1 and PC2 and then by axes PC1 and PC3. B) Fall 2022 PCoA, first of axes PC1 and PC2 and then by axes PC1 and PC3. C) Spring 2023 PCoA, first of axes PC1 and PC2 and then by axes PC1 and PC3. D) PCoA representing all seasons combined, first of axes PC1 and PC2 and then by axes PC1 and PC3. The percentage of each axis confers with the proportion of variation it can account for. Ellipses indicate the 95% CI of a specific sample type. Arrows indicate the approximate directional trend of samples as ADH increases.

Specifically comparing sample community dissimilarity of postmortem microbiomes

revealed that carcass microbiomes appeared to differ significantly by season (adj. $p \leq 0.003$). PC1 of the PCoA (weighted UniFrac) accounts for 82.4% of variation in our data, meaning that it's likely season itself is the primary driver facilitating the observed sample dissimilarity **(Figure 12)**. A density plot visualizing where sample types are most abundant relative to their UniFrac score shows that there is a lot of overlap between community composition despite that. Fall has the most overlap with summer and spring, but significant portions of both densities fail to overlap with the fall density itself.

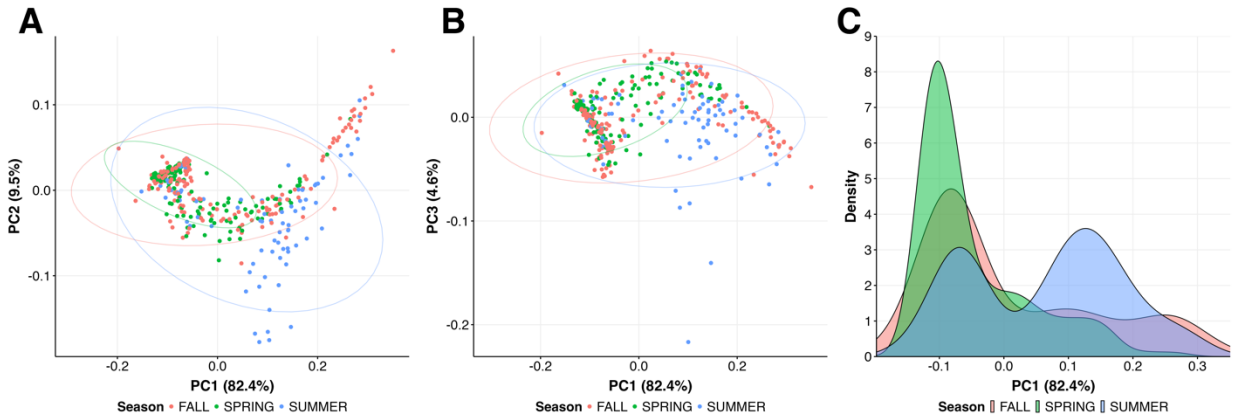

Figure 12. PCoA of carcass postmortem microbiomes among all seasonal studies. A) PCoA of PC1 and PC2. B) PCoA of PC1 and PC3. C) Density plot of UniFrac sample scores grouped by season.

There was also a significant effect of season on larval microbiomes (adj. $p \leq 0.003$). Interestingly, the density plot indicates overlap of fall and spring communities, despite this dissimilarity. Summer is extremely different from either season by any measure. PC1 explains significantly less of the variation than the PCoA of postmortem microbiomes was able to at just 51.3%. PC2 itself accounts for 25.4% of the variation **(Figure 13)**.

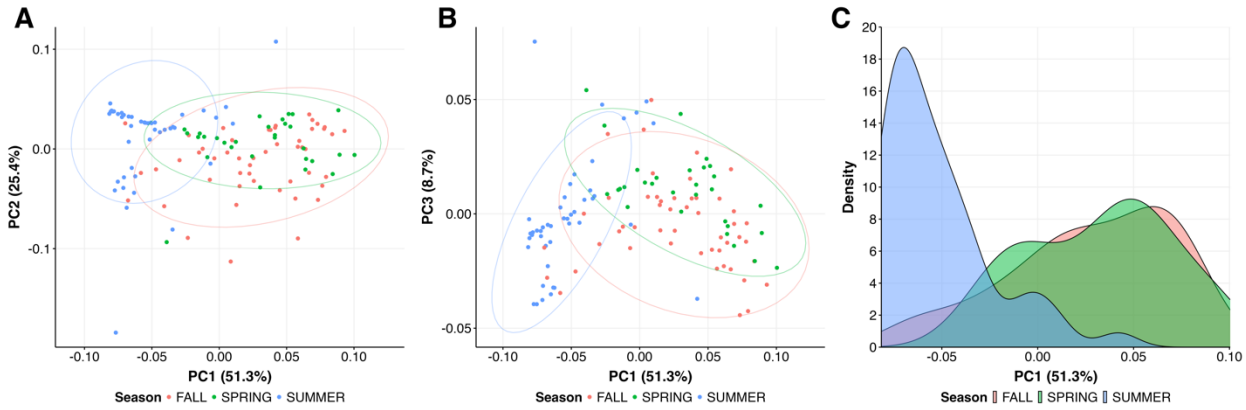

Figure 13. PCoA of larvae microbiomes among all seasonal studies. A) PCoA of PC1 and PC2. B) PCoA of PC1 and PC3. C) Density plot of UniFrac sample scores grouped by season.

Larval mass microbiomes were significantly different among seasons (adj. $p \leq 0.003$). Fall larval mass communities overlapped significantly more with summer larval mass communities than spring larval mass communities. PC1 explains 73.1% of the variation we can observe in our larval mass microbiome data, meaning that there is more than likely one significant factor in particular driving much of the dissimilarity we see between samples **(Figure 14)**.

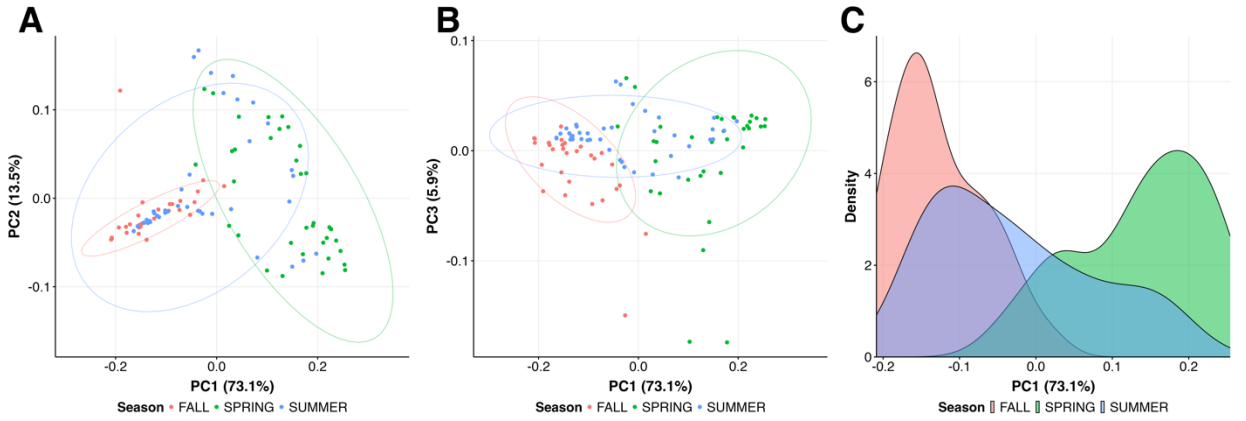

Figure 14. PCoA of larval mass microbiomes among all seasonal studies. A) PCoA of PC1 and PC2. B) PCoA of PC1 and PC3. C) Density plot of UniFrac sample scores grouped by season.

Diversity and Community Composition by Decomposition Stage

Overall alpha diversity increased over the course of decomposition in each season (**Figure 15**). Additionally, the median alpha diversity for each decomposition stage was consistent among all seasons. Despite this, there were significant differences in alpha diversity between the "Fresh" and "Bloat" stages as well as "Bloat" and "Active" stages for summer and fall **(Figure S2, Table S2)**. In the spring, median alpha diversity increased over decomposition, but did not differ significantly by stage. In all cases, median diversity increases between each stage of decomposition: most significantly between "Fresh," "Bloat," "Active," and "Advanced;" but substantially less so between "Advanced" and "Dry."

Trends in beta diversity (weighted UniFrac) somewhat mirror those of alpha diversity. Broadly speaking, sample community composition differed significantly across most decomposition stages. Across all seasons, "Fresh" and "Bloat" communities were significantly different from each other (adj. $p \leq 0.003$). Samples are similarly dissimilar between "Bloat" and "Active" stages in the summer (adj. $p \leq 0.003$) and the fall (adj. $p \leq 0.003$), but not the spring (adj. $p = 0.919$) **(Figure 15)**. It is noticeable that the most significant shifts in alpha diversity between stages corresponded with the two stages that demonstrate the most sample dissimilarity between each other. As was noted earlier, many spring postmortem microbiome samples are classified as being "Fresh," and few of them are classified as "Bloat." It's likely that spring "Bloat" and "Active" samples are less dissimilar because samples classified as "Bloat" were collected nearer to the "Active" stage transition. "Active" and "Advanced" samples did not differ significantly from each other in any season (adj. $p \geq 0.8$), nor did "Advanced" and "Dry" samples. (adj. $p \geq 0.8$).

Histograms that show the number of samples that fall within a specific range of PC1

weighted UniFrac scores (frequency) are useful for visualizing trends in distribution between decomposition stages. The further apart histogram bars conferring with a specific stage of decomposition are, the more dissimilar they are from the other stages plotted. In the cases of the summer, fall, and spring, the distribution of "Fresh" samples is very distinct from those of "Active," "Advanced," or "Dry." In most cases, "Fresh" samples are even distinct from "Bloat" samples. In the summer and fall, you can see that the distribution of "Bloat" samples practically closes the gap between "Fresh" samples and those belonging to later stages. Communities make their most dramatic transitions during "Bloat," it seems. "Active," "Advanced," and "Dry" samples all overlap so significantly that destringing them from each other visually is made somewhat difficult. Importantly, the PC1 axis of all PCoA, regardless of season, explains most of the variation observed in the postmortem microbiome data (>73%).

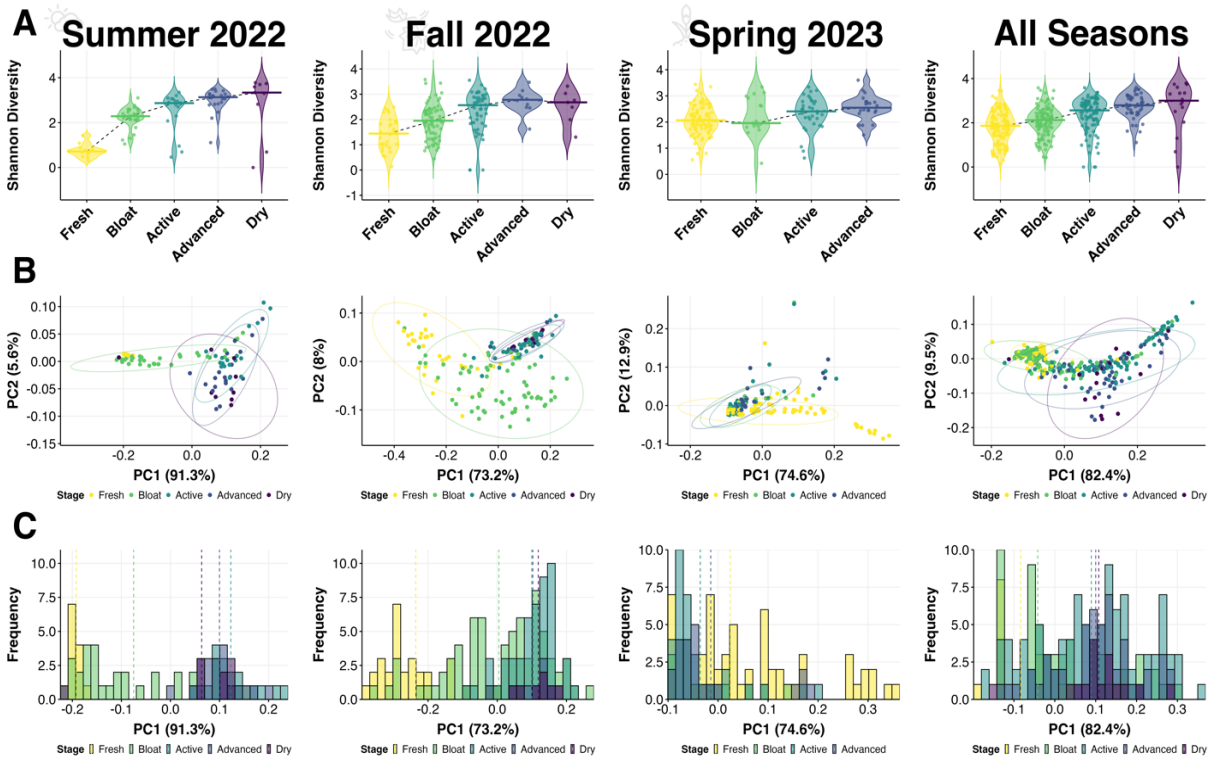

Figure 15. Postmortem microbiome diversity and community composition between decomposition stages. A) Violin plots of Shannon diversity by decomposition stage and faceted by season. B) PCoA of weighted UniFrac scores comparing sample community composition across stages of decomposition. C) Histograms showing the frequency of PC1 score distribution by decomposition stage.

Bacterial Community Structure and Succession

Analysis of compositions of microbiomes with bias correction (ANCOM-BC) testing can help identify bacterial taxa that potentially help drive some of the dissimilarity we observe between postmortem microbiome communities **(Figure S4, Figure S5)**. In comparing the postmortem microbiomes during decomposition, there were consistent trends for all seasons **(Figure 16)**. In the summer, fall, and spring, the genus *Ignatzschineria* ranked as the greatest increase in abundance. This means that, over the course of decomposition, the abundance of *Ignatzschineria* in the postmortem bacterial communities grew more than any other taxa comprising it. Similarly, the genus *Wohlfahrtiimonas*—a close relative of *Ignatzschineria*—also grew significantly in abundance as time went on. Conversely, the genera *Clostridium* and

Escherichia-Shigella (a combination of two closely related general) saw the greatest decline in abundance throughout decomposition.

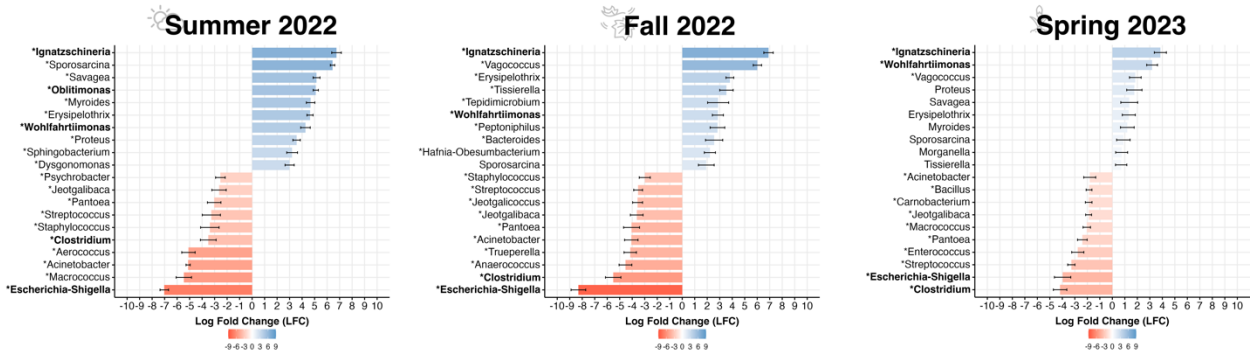

Figure 16. Bar plots of the 20 greatest ANCOM-BC log fold changes (LFC) in genera over the course of decomposition. Changes are relative to the "Fresh" stage of decomposition. Taxa whose abundances changed significantly (as indicated by $q < 0.05$ and $p < 0.05$) are preceded by an asterisk. Limited to postmortem microbiomes.

Among seasons there are many features that appear to drive the dissimilarity in bacterial community composition between the summer, fall, and spring (**Figure 17**). *Hafnia-Obesumbacterium* were significantly higher in abundance in the fall and spring than in the summer. *Lactococcus, Lactobacillus,* and *Leuconostoc* all also stand out as being significantly more abundant in the fall and spring than any of them are in the summer. Conversely, the abundance of *Wohlfahrtiimonas* and *Oblitimonas* in the summer far outweighed what was observed in the fall or spring. It seems that *Hafnia-Obesumbacterium*, among other taxa, may be preferential to cooler months with less extreme heat than what is seen in the summer. Conversely, *Wohlfahrtiimonas* and *Oblitimonas* being insect-associated may suggest that the summer, in being more promotive of insect activity than the fall or spring, may differentially assemble its bacterial community as a result. The drastic decline in log fold change magnitude in the comparison of fall and spring (compared to comparisons of either month with summer) is also notable, as it indicates that these differential abundances are less substantial, and that community structure may overlap more between the fall and spring.

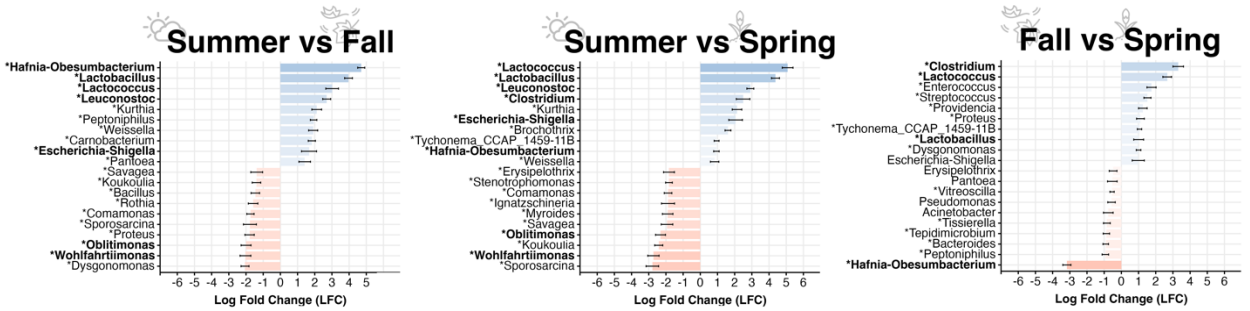

Figure 17. Bar plots of the 20 greatest ANCOM-BC log fold changes (LFC) in genera between seasons. Positive changes in LFC are relative to the first season in each title. Taxa whose abundances changed significantly (as indicated by $q < 0.05$ and $p < 0.05$) are preceded by an asterisk. Limited to postmortem microbiomes.

Evaluating longitudinal trends in phyla abundance over ADH helps to highlight more consistent trends in succession that manage to span multiple seasons. In all studies, Proteobacteria and Firmicutes reach an equilibrium after Proteobacteria declines in relative abundance while Proteobacteria increases, both ultimately balancing out around ~50% relative abundance in postmortem microbiomes **(Figure 18)**. The point at which equilibrium is reached varies between seasons, with summer reaching it around 500 ADH, fall around 1000 ADH, and spring around 1100 ADH. In the summer and fall Proteobacteria comprised nearly 100% of the observed phyla.

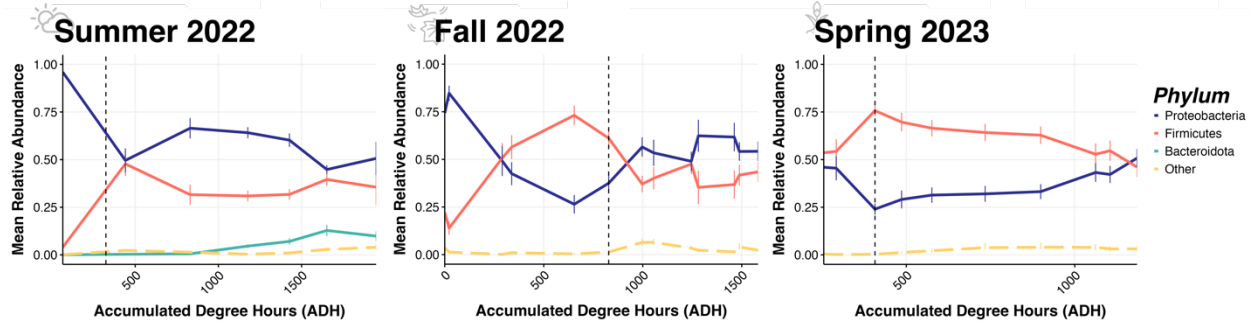

Figure 18. Mean relative abundance of phyla over ADH, by season. The dotted line represents the earliest observation of larval colonization on the carcasses.

The relative abundances of genera over time showed that *Acinetobacter* was the most prevalent genus at the beginning of decomposition **(Figure 19)**. In all cases, *Acinetobacter* sees its most drastic decline in relative abundance before 500 ADH, a period of decomposition that

coincides most closely with the transition of the “Bloat” stage to the “Active” stage. Firmicutes was largely represented by *Ignatzschineria*, whose relative abundance increased from 25% and 50% by the time larvae were observed to be colonizing the carcasses. Trends in both genera help explain trends at the phylum level, as the compensatory influx of *Ignatzschineria* making up for the rapid and massive decline of *Acinetobacter* abundance is what allows Proteobacteria to be represented in such high relative abundance. Interestingly, *Vagococcus* also appears to peak in relative abundance slightly before or after 500 ADH in all seasons. It and other genera like *Enterococcus* and *Lactococcus* all belong to the phylum Firmicutes. Proteobacteria were represented by fewer genera in higher abundance, while Firmicutes were represented by more genera in smaller abundances.

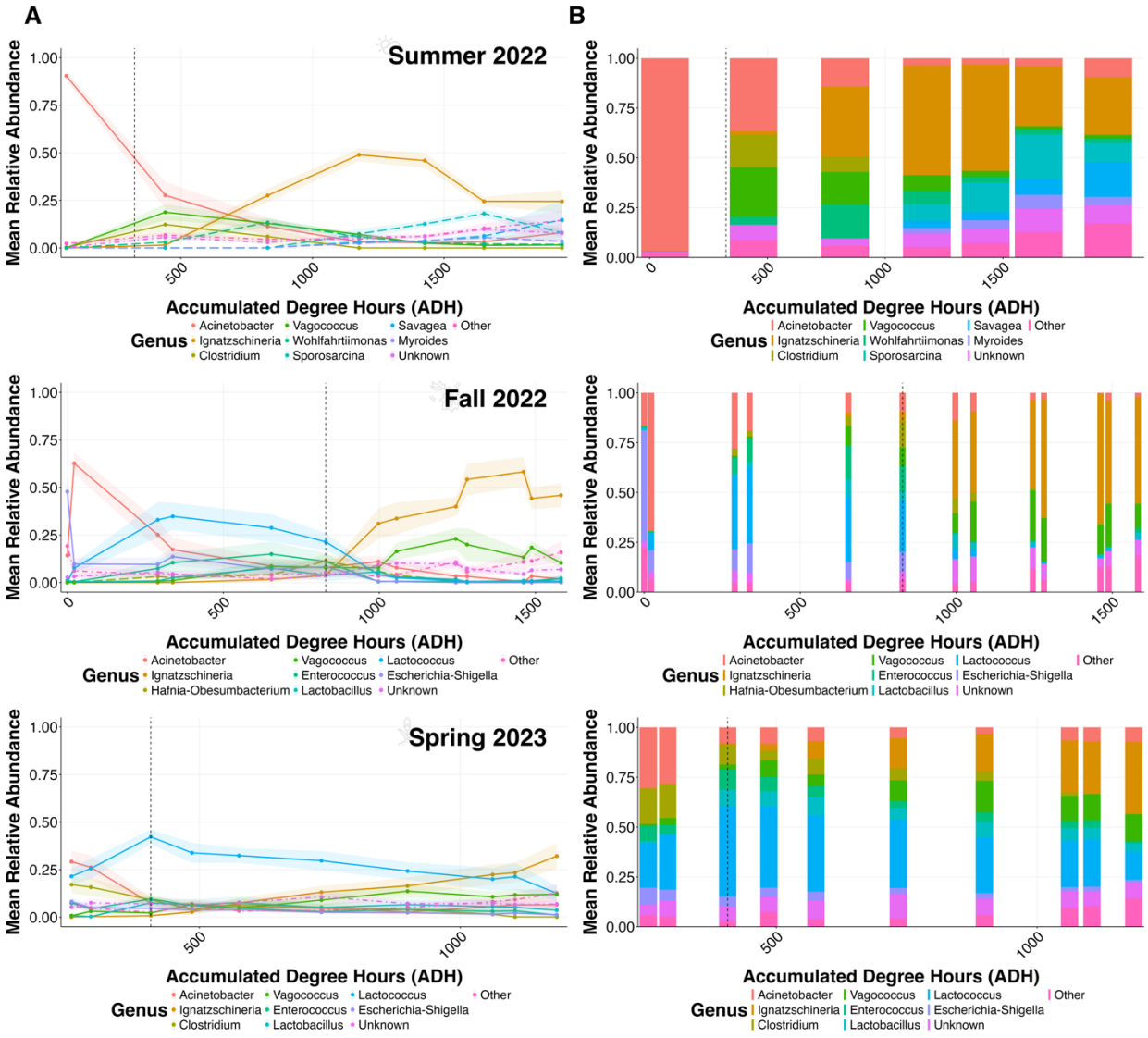

Figure 19. Mean relative abundance of genera over ADH, by season. A) Line plot of mean relative abundances over ADH. B) Bar plots of mean relative abundances grouped by ADH sampling point. The dotted line represents the earliest observation of larval colonization on the carcasses. Ribbons represent standard error.

Model Evaluation

Preliminary modeling in QIIME2 assessed each seasonal dataset independently to identify common or unique trends between microbial community changes during decomposition. Models had difficulty to discerning "Advanced" and "Dry" stages from the others regardless of season. Across all seasons, communities representing "Advanced" or "Dry" stages of

decomposition tended to represent a relatively low percentage of the overall samples that were able to be sequenced successfully. Learning classifier-derived models restricted to data collected during the same season had accuracies of 67.6% in the summer, 62.9% in the fall, and 65.6% in the spring (**Figure 20**). Heatmaps of the genera the supervised learning classifiers found most important for differentiating decomposition stages also demonstrated considerable variation between seasons, though all seasons had relatively higher abundances of *Ignatzschineria* at later stages of decomposition, and the absence of *Escherichia-Shigella* at earlier stages (**Figure 21, Figure S6, Figure S7**). Combined datasets that encompass all seasons represented in our decomposition studies yielded similar models to those built for each individual season, with an overall accuracy of just 61.2%.

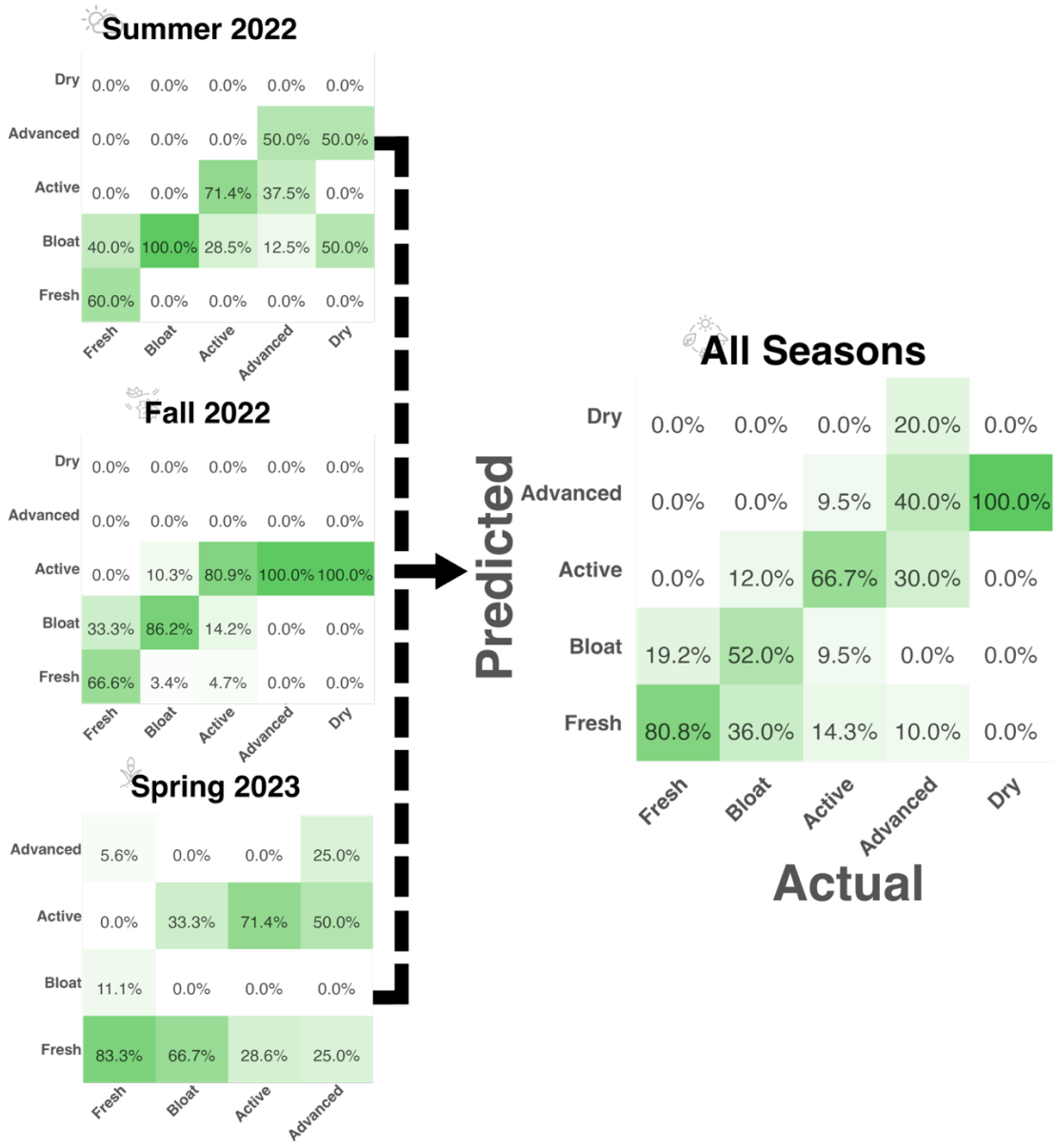

Figure 20. Supervised learning classifier confusion matrices for the prediction of decomposition stage based on postmortem microbiome sample profiles. Left-most matrices reflect the results of models built on individual seasonal datasets. The rightmost matrix reflects the results of a model built on a combined dataset comprised of all seasonal datasets. Percentages indicate what proportion of stage-specific samples were predicted to be by the model(s). Preliminary modeling attempt performed in QIIME2.

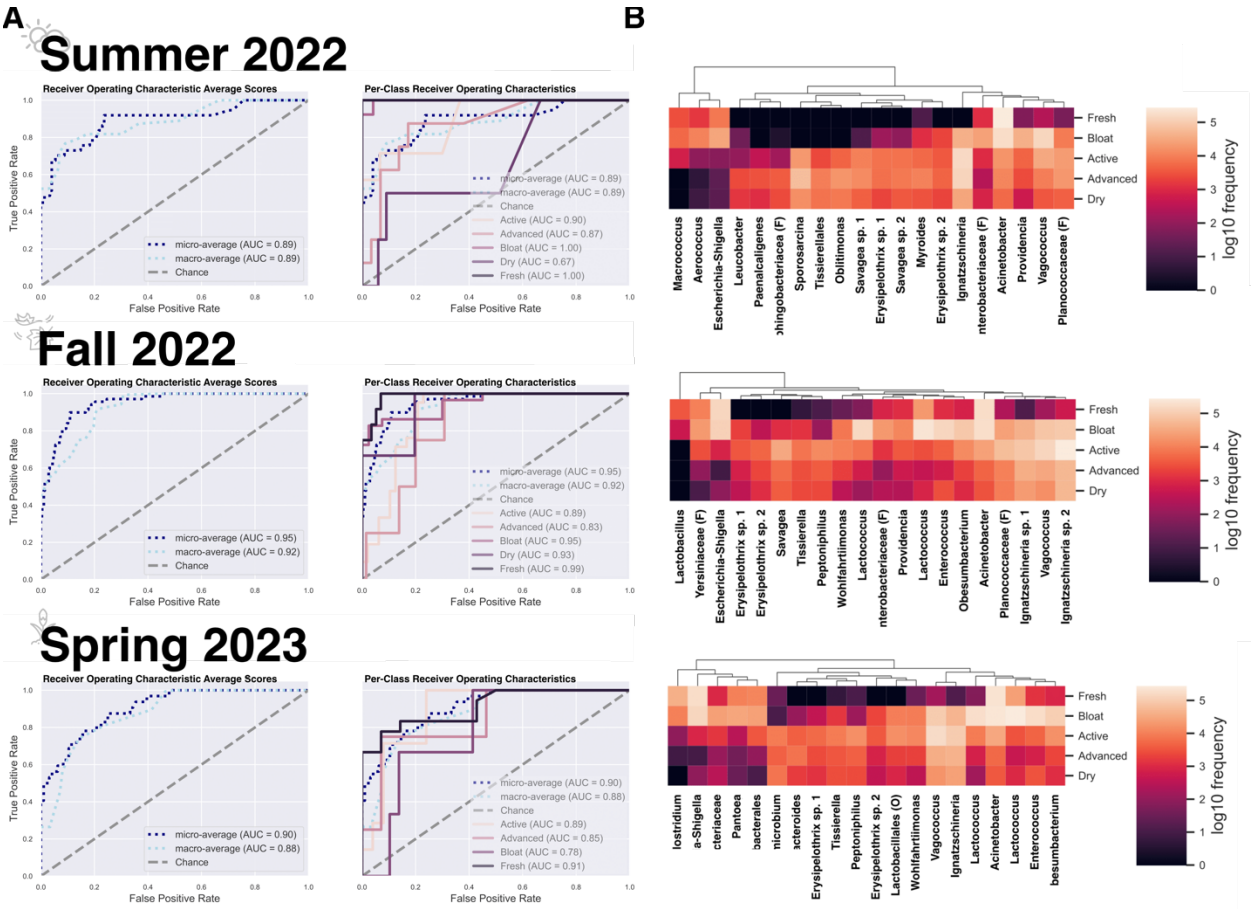

Figure 21. A) Area under the ROC Curve (AUC) plots and B) Genus-level taxaonomic abundance heatmaps for seasonal supervised learning classifiers. AUC plots measure model performance across all possible classification thresholds (decomposition stages). The dotted line denoting "Chance" refers to what the curve would look like if classification was randomly estimated. The closer the AUC curve is to a true positive rate of 1, the better the model's predictive power. The abundance heatmaps indicate predictor taxa that supervised learning classifiers associate with specific decomposition stages. Preliminary modeling attempt performed in QIIME2.

To improve future modeling attempts in R, analysis of variance using distance matrices (adonis) was used in combination with weighted UniFrac distance matrices of all microbiome samples to identify predictors that hugely impact community composition **(Table 3).** Testing highlighted the significance and impact of several important predictors, like season ($p \leq 0.001$), temperature ($p \leq 0.001$), humidity ($p \leq 0.001$), and sample type ($p \leq 0.001$). Interactions between season and sample type (Season:Sample type) were significant too ($p \leq 0.001$), as were

interaction effects between temperature and humidity (Temperature:Humidity) ($p \leq 0.001$). This suggests that the effect of season on microbial community compositions varies by sample type and vice versa. It also means that the effect of temperature on community composition varies humidity, and vice versa. Season was the most impactful of the significant predictors identified (F-value = 28.89), suggesting that it exerts the strongest influence over community variation as a concept encompassing several other variables, like seasonal weather conditions. Humidity (F-value = 20.22) alone is incredibly impactful as a variable that was shown to differ significantly by season, so it is possible that it represents the most impactful variable if season in general. Season was able to explain 7.1% of the variability observed among microbial communities, whereas humidity was only able to explain just 2.5%. The effect of sample type (SS) (F-value = 13.34, $R^2 = 0.03625$) was similarly significant, but this is to be expected. Confirmation that interaction effects between these predictors are themselves driving variability in our sample data is important to note as well.

Table 3. Analysis of variance using distance matrices (adonis) results for the Weighted UniFrac Distance Matrix of all sample types (carcass, larvae, larval masses) across all seasonal decomposition studies (summer 2022, fall 2022, spring 2023).

Predictor(s)	Sum of Squares (SS)	R^2	F-value	p-value
Main Effects				
Season	0.04607	0.07072	28.89	≤0.001***
Temperature	0.00490	0.00753	6.15	≤0.001***
Humidity	0.01612	0.02475	20.22	≤0.001***
Sample type	0.01708	0.02622	10.71	≤0.001***
Sample type (SS)	0.02127	0.03625	13.34	≤0.001***
Interaction Effects				
Season:Sample type	0.01982	0.03042	6.21	≤0.001***
Season: Sample type (SS)	0.01550	0.02379	4.86	≤0.001***
Temperature:Humidity	0.00519	0.00797	6.51	≤0.001***

*$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***,* (ns) = *not significant*
(SS) = *site specific carcass samples (skin, mouth, and rectum)*

With a better understanding of our predictor variables, additional modeling attempts were made using 'randomForest' **(Table S10).** The first of these new models was a Random Forest predictive classifier tasked with predicting ADH using phyloseq object feature data **(Figure 22)**. Models made using individual seasonal datasets as well as a model built using a combination of all seasonal data were relative performant **(Table 4)**. All models were able to explain >79% of the variance in the response variable. The summer model performed the poorest (RMSE = 245.73, $R^2 = 0.79$), the spring the second best (RMSE = 142.02, $R^2 = 0.84$), and the fall the best (RMSE = 130.58, $R^2 = 0.94$) when comparing just the individual seasonal models. The combined model outperformed both the summer and spring models (RMSE = 160.91, $R^2 = 0.91$). Adding "Season" as a predictor worsened the combined model performance (RMSE = 164.43, $R^2 = 0.90$), but introducing humidity (RMSE = 154.16, $R^2 = 0.92$) and temperature (RMSE = 158.67, $R^2 = 0.91$) independently improved it significantly. Accounting for all predictors (season, temperature, humidity) yielded the strongest combination model (RMSE = 148.38, $R^2 = 0.92$), but it was still outperformed by the fall model.

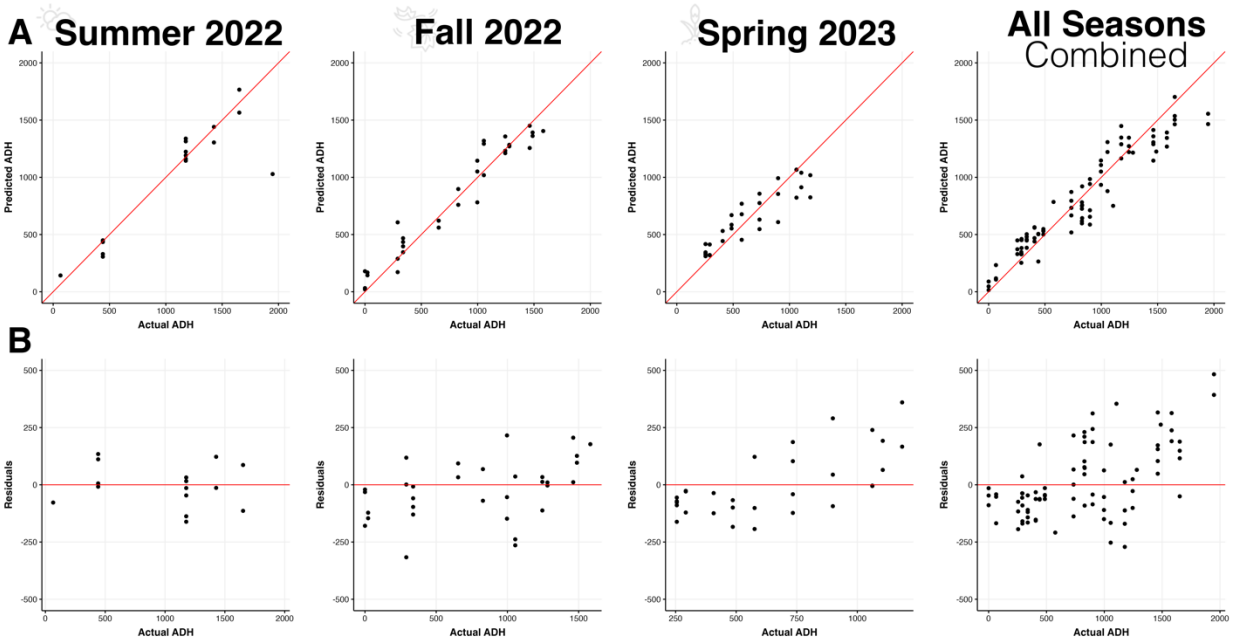

Figure 22. Supervised learning regressor model accuracy in the prediction of ADH from postmortem microbiome bacterial composition.

Table 4. Performance of Random Forest predictive regressors using postmortem microbiome data (carcass samples) to predict accumulated degree hours (ADH). Models were built using individual seasonal datasets or a combined dataset encompassing all seasonal data.

Model	Mean Squared Error (MSE)	Root Mean Squared Error (RMSE)	Mean Absolute Error (MAE)	R^2
Summer 2022	60380.81	245.73	125.05	0.79
Fall 2022	17050.77	130.58	101.22	0.94
Spring 2023	20171.04	142.02	115.79	0.82
Combined	25891.07	160.91	130.39	0.91
+ Season	27036.28	164.43	132.84	0.90
+ Temperature	25174.70	158.67	125.20	0.91
+ Humidity	23765.96	154.16	125.01	0.92
+ All variables	22017.50	148.38	116.46	0.92

The Random Forest models built to predict seasonality from postmortem bacterial communities was excellent was able to correctly classify ~96% of the communities correctly (OOB error = 4.41%) **(Figure 23)**. The fall and spring have relatively low error rates (1.45% and 2.34% respectively), but the classifier appears to have more difficulty predicting summer communities (13.51% error rate). Certain features specific to the summer may be more challenging for the classifier to capture or identify. The genera identified as being important in the classification of season included *Lactococcus,* and *Hafnia-Obesumbacterium* that include genera most differentially abundant between the cooler months (fall and spring) and summer. *Escherichia-Shigella* and *Acinetobacter* are prevalent in every season, but the rates at which they decline and grow in abundance—as well as the abundance they reach—aid in distinguishing the seasons from each other.

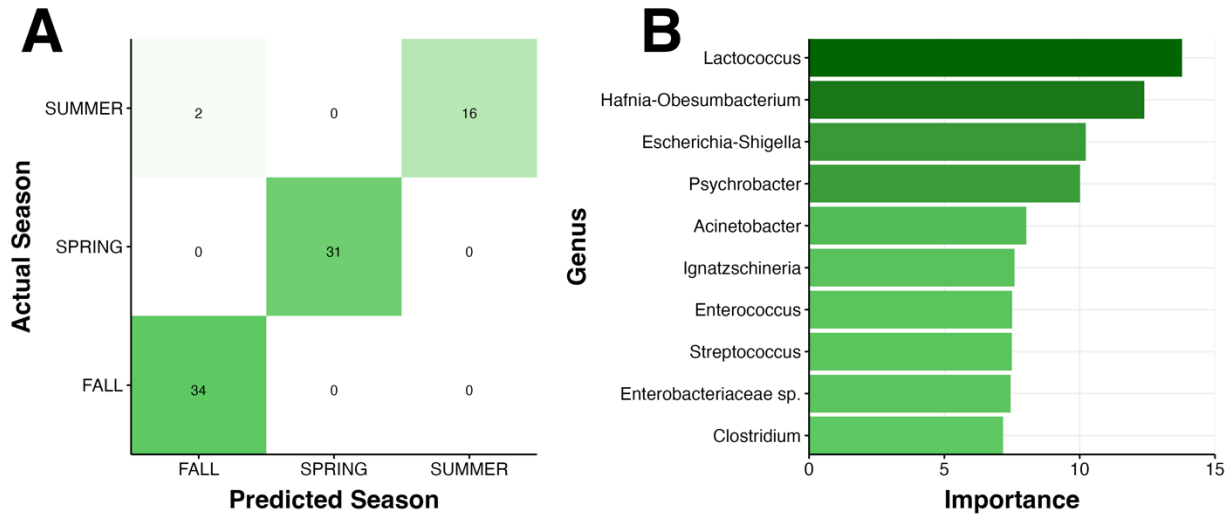

Figure 23. Supervised learning regressor model accuracy in the prediction of ADH from postmortem microbiome bacterial composition.

Discussion

The aims of this study were to better understand the potential utility of the postmortem microbiome in the forensic sciences. As such, we wanted to characterize how the postmortem microbiome interacts with other members of the necrobiome (Benbow et al 2019), with a focus on blow flies. To do this we characterized the postmortem bacterial communities by season to better understand how they change by decomposition stage and ADH. The results of this study demonstrate the importance of broadening our knowledge of decomposition and the role that abiotic and biotic variables play in the process.

The results supported our prediction that conditional growth restraints would impact the diversity and composition of carcass microbiomes. Summer carcass diversity significantly outgrew what was observed in fall and spring carcasses; but fall larvae microbiomes were significantly more diverse than larvae from the summer or spring despite this. Larval mass microbiomes were technically more diverse in the spring than any other season, but not as significantly as the other sample type disparities. This aside, alpha diversity steadily increased with each successive decomposition stage; but this trend was only significant between the "Fresh" and "Bloat" stages as well as the "Bloat" and "Active" stages. Importantly, none of these statistically significant trends in postmortem microbiome diversity growth were observable during the spring, despite its median increase in diversity over time. In any case, there are studies that have succeeded in demonstrating the tendency of the postmortem microbiome to increase in diversity over time in several different carrion models. Deel et al. (2021) highlight diversity growth in postmortem human rib microbial communities across both summer and spring, and Zhao et al. (2021) see a non-linear increase in diversity as far as postmortem oral microbiomes of mice are concerned. Burcham et al. (2024) highlighted differences between human cadavers in

three different environments, reporting that microbial community ecology was impacted by decomposition stage and geographical location. Studies like these better our understanding of the impact environment has on postmortem microbiome succession; and several corroborate that climactic variables could play a role in driving community composition. Given the heightened rate of change in alpha diversity observed during the summer, significantly warmer weather conditions may have played an important role in promoting bacterial community diversification. Bacteria benefit greatly from high temperatures and high relative humidity (Ratkowsky et al., 1982; Scott & Hwa, 2011; Zwietering et al., 1991); and conditions like those of the summer encourage microbial and insect activity more than those typical of spring or fall. The trajectory of seasonal weather variables like temperature and humidity, affects carcass necrobiome biodiversity, as fall temperatures typically decline with time as it gradually transitions into the inhospitable climate expected of winter (Benbow et al., 2013; Vrac et al., 2013). Spring, conversely, represents a month wherein conditions like temperature and humidity promise to improve with time as summer nears. It appears that seasonal influences on microbiome development extended to the sampling sites themselves, too. Carter et al. (2015) concludes that the effect of swine decomposition on soil microbiota differ between summer and fall and emphasize the importance of seasonality in future analyses.

Many decomposition stages varied considerably in terms of microbial community composition (beta diversity). Bacterial communities grow in dissimilarity more significantly towards the beginning of decomposition than towards the end; and transitions from “Fresh” to “Bloat” and “Bloat” to “Active” were characterized by significant levels of this dissimilarity. Extreme dissimilarity driven by these two stage transitions is consistent with results put forward by Parkinson et al. (2009), which found that community changes were most radical between the

earliest stages of human decomposition. Substantive bacterial community succession only appeared to extend to these two stage transitions, however. Season also impacted community composition much the same way it did alpha diversity. With the weighted UniFrac distance metric accounting for rare taxa and lending itself to questions of community structure, it gives us a better idea of how these seasonal bacterial communities manage to exhibit such significant dissimilarity from each other (Chen et al., 2012; Lozupone et al., 2011). Seasonal comparisons indicated that the spring samples are the least dissimilar from each other, while the summer samples are the most dissimilar. It is possible that the spring's resistance to many of the significant changes in diversity or community composition observed in the summer and fall are a consequence of seasonal weather conditions and prolonged decomposition timelines. The body of research implicating seasonality in alpha diversity growth over time is somewhat lacking, making comparisons difficult. Seasonal trends in temperature growth and consistency enabled carcasses in the spring to spend more time at temperatures above 20°C than those in the fall, and we suspect that spring's consistent temperature ranges lent themselves to the significantly shorter decomposition timeline it had in comparison to fall. We also suspect that lower spring temperatures modulated bacterial growth and succession less drastically than the intensely conditions of the summer or fall.

The bacterial genera *Ignatzschineria* and *Wohlfahrtiimonas* were present across all seasonal decomposition studies, with the LFC of *Ignatzschineria* being the highest positive shift in abundance between the start and end of decomposition. These stand out among the other bacterial genera as having distinct invertebrate associations, since both are closely related genera belonging to the same order, Lysobacterales, and class, *Gammaproteobacteria* (Gupta et al., 2011; Kumar et al., 2019; Snyder at al., 2020). Blow flies like *Lucilia sericata* and *Chrysomya*

megacephala have been linked to the introduction of *Wohlfahrtiimonas* to myiatic wounds (Barker et al., 2014; Heddema et al., 2016; Le Brun et al., 2015; Mejias et al., 2016). *I. larvae* as well as *I. indica* are species isolated from the gut contents of Sarcophagidae (flesh flies). It is unknown why these bacterial taxa have close associations with blow flies and flesh flies. Importantly, Deel et al. (2022) identifies flies as one of the human postmortem microbiome's microbial sources across studies performed in the winter, spring, and summer; but they were less able to implicate a seasonal effect in what extent these flies influence the postmortem microbiome. In keeping with our supposition that spring seasonal conditions mitigate drastic shifts in diversity and community composition, it displays similarly mild changes in LFC among all significant ANCOM-BC genera. Seasons also varied in terms of their bacterial abundances, which is reflected in its incongruous measures of alpha and beta diversity. However, a direct comparison of the fall and spring revealed exceptionally low levels of differential abundance—a stark contrast to comparisons of the summer to either season. Few of the genera identified by the test varied significantly, but of those that did, *Hafnia-Obesumbacterium* stood out as being in much higher abundance in the fall than spring. Conversely, *Clostridium* saw a much higher positive LFC in spring when compared to fall. Most species of these genera are frequently associated with mammalian guts, but *Clostridium* are unique in that they are gram-positive and obligate anaerobes (Stevens et al., 2015).

Visualizing changes in relative bacterial abundance as decomposition progressed revealed that fluctuations we observe in phyla composition are relatively well-conserved between each season. Postmortem microbial communities reach an equilibrium of mostly Proteobacteria and Firmicutes, which is consistent with literature characterizing key bacterial features of postmortem microbiota (Buresova et al., 2019; Metcalf et al., 2015; Pechal &

Benbow, 2016;). In the cases of the summer and fall, Proteobacteria began with high relative abundance before rapidly declining, while Firmicutes had low relative abundance that gradually increased to that of Proteobacteria. The genus *Acinetobacter* was the most abundant Proteobacteria at the start of decomposition. The presumed simplicity of the stillborn pig microbiota is the most likely explanation for this domination by *Acinetobacter* and several unrelated genera with extremely low relative abundances. While the circumstances of carcass stillbirth were not investigated, it is known that genera like *Acinetobacter* increase in intestinal and vaginal co-transmission during periods of heat stress, which could have contributed to the premature death of neonatal pigs (He et al., 2020). Research has even suggested that species like *Acinetobacter baumannii* can induce preterm delivery if the mother carrying them has an infection (Aivazova et al., 2009; Jung et al., 2021). Ultimately, we cannot discount the life histories of these stillborn swine carcasses nor their mothers in discerning what established the high *Acinetobacter* abundance in postmortem microbiome communities, especially considering how common it is as an environmental contaminant. Genera belonging to Firmicutes, like *Vagococcus* and *Lactococcus*, only rise to prominence after abundances of *Acinetobacter* begins to fall. Genera like *Wohlfahrtiimonas, Ignatzschineria, Acinetobacter,* and *Vagococcus* are all were reported by Burcham et al. (2024) using a microbial network that included bacterial abundance and functional profiles. The insect-associated genus *Ignatzschineria* replaces Acinetobacter as the only other relatively abundant genus of Proteobacteria. Where Proteobacteria are represented by a few genera with little diversity, Firmicutes are instead represented by a more diverse array of bacterial genera with low levels of relative abundance. This, in combination with other discoveries, is evidence that invertebrates play a significant role in shaping the microbial community of decomposing remains.

Attempts to use the seasonal carcass microbial data in mathematical modeling for predictive purposes had three objectives. The first aim was to evaluate the ability of machine learning-assisted models to properly identify a carcass' decomposition stage with microbial data and minimal modeling parametrization. The second objective concerned developing more specific Random Forest predictive models—a regressor and a classifier—to predict ADH from & the seasonality of sample types. The third objective was to validate the notion that the multivariate nature of decomposition necessitates the inclusion of factors like season in modeling. The initial QIIME2 Random Forest classifier struggled to identify later stages of decomposition, regardless of whether it was trained on independent seasonal data or a combined seasonal dataset. *Vagococcus* appeared to be a useful indicator in differentiating "Fresh" from "Bloat" despite the overlap of *Acinetobacter*. *Ignatzcshineria* proved particularly useful in separating the "Active" stage from either of its predecessors, meaning the insect activity itself is a crucial consideration in these modeling attempts. Later stages are where identifying predictive taxonomic features becomes more difficult, so the model instead opts to find genera whose absence is unique to stages like "Advanced" and "Dry". *Escherichia-Shigella* stands out consistently, as its decline in abundance is consistent between all three seasons. Performant Random Forest regressors were quick to indicate the importance of *Ignatzschineria*, *Wohlfahrtiimonas*, *Acinetobacter*, and *Escherichia-Shigella*. These regressor models were markedly more successful at predicting ADH than a classifier was at limiting postmortem microbial samples to just the generalized stages of decomposition. A seasonality classifier built on a combined seasonal dataset also vastly outperformed the decomposition stage classifier, reinforcing that taxon like *Lactococcus, Hafnia-Obesumbacterium,* and *Clostridium* are among the most differentially abundant and temporally important predictive genera. Interesting, genera

like *Lactococcus* and *Hafnia-Obesumbacterium* were noted to be significantly more abundant in the cooler months of fall and spring than the summer. However, even in comparisons of the fall to the spring, *Hafnia-Obesumbacterium* and *Clostridium* are significantly more abundant in the spring to a lesser extent. The model is implicitly sensitive to the inherent nuance of relative and approximated absolute abundances, which is promising for the future of predictive modeling applications. As for the regressor: a combined seasonal model outperformed the majority of models built on just a singular seasonal dataset; but introducing impactful predictors like season and humidity sharply improved its performance. In fact, including all three factors despite the potential of collinearity between season and the weather variables improved the performance of the model. We suspect that both humidity and season contribute unique types of information, being that humidity can be more specifically implicated in bacterial moisture content needs, while season technically encompasses temporal associations as well as weather variables that span much further than just temperature and humidity.

Evidence suggests that seasonal considerations are a must when considering postmortem microbiota assemblage and their use in modeling. Our seasonal decomposition studies all differ in such significant ways that there is little else to implicate than the seasonal conditions in most cases. Where season may account for an overwhelming majority of the variation observed between sample types in postmortem microbiomes and larval mass microbiomes, ordinations of larvae samples indicates that the factor or factors driving their dissimilarity may be significantly more complex—perhaps a matter of species, life stage, or any number of variables unaccounted for by these studies. Stage appears to play a similarly significant role in driving sample variation among samples in individual seasonal postmortem microbiome datasets. Explicit significance testing suggests that season as a predictor only captures about 7% of the variation observed,

which is considerable given the complexity of carrion ecology. Ultimately these variables reflect very few aspects of the overarching necrobiome's ecology; but even if only a small amount of complexity was captured, it can be argued that the role these specific factors play in the survival of bacteria drastically impacts their fitness in seasonally affected growth conditions. It is likely that bacteria suited to harsh conditions or cold temperatures would fare better than those ill-adapted to them; and life history traits may play an important role alongside seasonal weather constraints in differentiating the suite of dominant bacteria between sample types. Insect activity itself is modulated by seasons, too, meaning that only certain species of blow fly or certain numbers of insects will even be able to access and colonize a carcass depending on the time of year (Babcock et al., 2019; Campobasso et al., 2001; Brundage et al., 2011; Weidner, 2016). This, alongside the restrictions seasonal weather may impose on bacterial communities, is a highly suspected cause for the prolonged decomposition timelines we observed in fall and spring.

The implication of seasonal weather effects in the succession of the postmortem microbiome, we feel, will prove invaluable as more breakthroughs are made in the realm of forensic microbiology. Emphasizing the importance of approaching theories we intend devise applications for with their multivariate natures in mind will only serve to strengthen the science and improve the standards we hold it to. Seasons are just one of many contributing factors to the observations we make of decomposition throughout its process, and as such, we must account for the significant variability it is responsible for if we ever intend to create a tool or framework for forensic application. In performing these three decomposition studies, we have revealed that these effects may very well extend to factors beyond just the microorganisms driving decomposition, as the process is an effort orchestrated by a complex ecological system we do not yet entirely understand.

CHAPTER 3: SEASONAL DYNAMICS OF LARVAL MASS MICROBIOMES

Abstract

Calliphoridae (blow flies) larvae are principal decomposers in the complex ecological network of the "necrobiome." Their prevalence as voracious consumers of decomposing remains is well documented in literature; but little is understood about how the colonization of carcasses by blow flies is influenced by environmental weather variables. Additionally, little research exists that examines the microbial interactions of carrion and larvae themselves. The objective of this study was to better characterize the overall differences in carrion microbiota as decomposition progresses and, in doing so, clarify the potential dynamic between two of decomposition's most important contributors: blow flies and bacteria. We hypothesized that larval masses and larvae microbiome composition would vary significantly between seasons. We also suspected that larval microbiomes will gradually increase in complexity as bacteria residing on/within the carcass are consumed alongside its soft tissue. To address these hypotheses, decomposition studies were performed in the summer, fall, and spring using six stillborn pigs each as carrion models. Carcass microbial profiles were collected twice daily until skeletonization occurred and larval activity could not be observed. Larval masses and larvae were similarly sampled when available. Results indicated that overall larval mass bacterial community composition overlapped significantly with the microbiota of the larvae or the microbiota of carcasses differentially. Larval diversity was the lowest among all sample types in all seasons but the fall; and larvae microbiota experienced significant shifts in bacterial community composition when carcasses underwent similarly significant periods of succession. Most importantly, the data strongly suggested that the fly-associated genera *Ignatzschineria* and *Wohlfahrtiimonas* significantly moderated bacterial community succession on carrion after colonization by larvae was observed.

Introduction

Dipterans belonging to the family Calliphoridae, colloquially known as blow flies, have long been known to colonize carrion (Carter et al., 2006). Their proclivity for detecting and accessing vertebrate carrion has been of interest for forensic experts for centuries, with observations of blow fly behavior seeing written observation as far back as 1248 (Benecke, 2001). Few invertebrates can safely consume carrion as larval blow flies do, and occupying this unique ecological niche plays an important role in promoting vertebrate decomposition (Smith & Wall, 1997). In brief, blow fly larvae voraciously consume necrotized flesh and grow as quickly as possible before dispersing. The remainder of their lives are spent pupating in the nearest substrate as pupae before emerging as adults. The predictability of blow fly life histories allows scientists to model their growth based on temperature and humidity, and these data can often be useful in forensics (Stevens & Wall, 2001). Despite their relevance to death investigation and naturalistic decomposition, little is known about the ecology of these larval masses (Weatherbee et al., 2017). Recent research described the succession of blow fly larval microbiota along with the communities outside of the larval mass that represents decomposition fluids and larvae excretions; however, there are few studies that quantify the relationships of bacteria native to the carcass and those brought into the system by adult blow flies during colonization. Additionally, it is not yet known what role seasonal weather conditions may play in the emergence of these larval masses—or on their potential impact to postmortem microbiome succession. Understanding the nature of these larval masses is important for better informing the use of insects in forensic investigation. Emerging technologies and methods in the field of forensic microbiology, for instance, could provide a flexible new use for larvae collected from carcasses.

We hypothesized that blow fly larvae colonizing the stillborn carcasses used in our

experiments would significantly impact the bacterial succession of the postmortem microbiome. Blow fly larvae are known to secrete an enzymatic cocktail that aids in the digestion of dead tissue (Hobson, 1931), and the reported antimicrobial properties could prove impactful on the success of microbial communities within or around the body site these larval masses form (Hobson, 1931). Research has demonstrated the ability of these secretions to differentially kill bacteria or disrupt biofilms—primarily in the context of maggot debridement therapy—and we assumed that this may uniquely influence carcass microbiota (Harris et al., 2009). Additionally, multiple bacterial genera have been reported in scientific literature to be associated with blow flies (Tomberlin et al., 2017). Blow flies, like many insects, are known to harbor their own microbiomes; and if it is the case that members of their microbiota resist being broken down by their enzymatic secretions, then it may suggest that they are well-equipped to compete for resources or space in decomposition settings (Weatherbee et al., 2017). It is possible that some bacteria may have adapted over time in response to the blow fly life cycle, making them uniquely suited to compete in decomposition environments (Burcham et al., 2024). We similarly predicted that blow flies, in colonizing the carcass and consuming it, would introduce their own bacteria to the carcasses.

We also suspected this of the microbiomes of the larval masses. Additionally, the fluids of the larval masses (hereafter larval mass represents the external fluids of a mass generated by the larvae during feeding and growth on the carcass) and the microbiomes of the larvae would be similar in overall bacterial diversity and community composition. Importantly, we anticipated that neither larval microbiota nor larval mass microbiota would differ significantly between seasons. It was also assumed that significant, mutual exchanges in bacterial taxa could be made between larvae and carcasses but would vary by season. We suspected that inclement seasonal

weather conditions would lower the overall microbial diversity of larval masses and larvae, particularly when compared to those sampled during the summer. In keeping with the potential impact larval masses could have on their respective carcasses, our final hypothesis stated that the disruption of postmortem microbiome succession caused by blow fly colonization would be useful in the identification of colonized vs uncolonized remains by predictive models.

To test these hypotheses, we used stillborn pigs equipped with anti-scavenging cages. The microbiota of the carcasses and larval masses were sampled twice a day. Third instar larvae from these larval masses were also sampled when present. Sampling concluded when the remains had completely skeletonized, and larval activity was no longer observed. These studies were undertaken in the spring, summer, and fall; and each study used 6 stillborn pig carcasses for a total of 18. DNA extraction and 16S amplicon sequencing helped characterize the bacterial communities found on or in the carcasses, larval masses, and larvae throughout decomposition.

Materials and Methods

Decomposition Studies

Decomposition studies were performed in summer 2022, fall 2022, and spring 2023 within date ranges representative of different seasons in Central Michigan. Relevant weather data (temperature, humidity, precipitation) was sourced from a nearby weather station owned by Michigan State University (See Materials and Methods of CHAPTER 2: SEASONAL DYNAMICS OF POSTMORTEM MICROBIOMES).

Because of their small size, stillborn pig carcasses were chosen as accessible vertebrate carrion models that could be well replicated in different locations for three seasons. Decomposition studies were conducted in the Box Woodland, located in East Lansing, MI (42° 41' 23.0712" N, 84° 29' 29.5476" W). Three sides of this woodland were facing farmland with one side facing a dirt road, so the three seasonal decomposition studies were assigned one vacant (60m) length of tree line each. Carcasses maintained 10 meters from each other, and their anti-scavenging cages were secured with 4-5 bricks each.

All seasonal studies used n = 6 carcasses, where carcasses were bagged in groups of 2-3 and stored at -20°C up to a week before each study. At 24 hours before the start of their respective decomposition studies, carcasses were set out at room temperature to thaw after being weighed and sexed.

Microbiota Sampling

Carcass postmortem microbiomes and larval mass microbiomes were sampled using sterile cotton swabs of skin, mouth, and rectal communities at each sampling event. To sample the skin microbial communities, a transect of the carcass' exposed abdomen was rubbed repeatedly while rotating the head of the swab. The mouth and rectal communities were sampled

by inserting the swab repeatedly and rotating it. Larval masses were sampled by inserting just one swab into three distinct areas of the mass and rotating it for 15 seconds in each spot. External larval masses were only sampled once aggregations were significant enough estimated to be >50 larvae; and if more than one larval mass was present during a sampling event, the masses would be sampled independently. Individual third instars were also sampled from the masses to investigate internal microbiomes during decomposition, and compared to the larval mass microbiomes Aseptic field technique was used every sampling event and swab tips were stored in 1.5mL microcentrifuge tubes containing 200uL of room temperature RNAlater. Larvae were stored in a 50mL Flacon tube with 25mL of RNAlater. All samples were then stored at -20°C following sampling. To assign stages of decomposition of the carcasses, taphonomic assessments followed Payne (1965) criteria.

DNA Extraction and Amplicon Sequencing

Isolation of genomic DNA contained in our samples was performed using the DNeasy ® Blood & Tissue Kit (QIAGEN N.V., Hilden, Germany). This was modified to include the introduction of 15uL lysozyme (15mg/mL) prior to incubation to encourage cell lysis. The cotton tips of the swabs were also either removed from the stick entirely using sterile scalpel blades and forceps or beaten using 1.4mm ceramic beads and a FastPrep-96™ high-throughput bead beating grinder and lysis system prior to the 90-minute lysis. Blow fly larvae pooled during sampling events were individually introduced to new 1.5mL microcentrifuge tubes and homogenized. Each sampling event was represented by n = 10 randomly selected, individually processed 3rd instar larvae when possible. DNA extraction was performed identically to the protocol used for swab processing.

Samples containing viable DNA were submitted to the Michigan State University

Genomics Core facility (East Lansing, MI) for Illumina MiSeq amplicon sequencing. Sequencing data was demultiplexed by the MSU Genomics Core before being made available for analysis.

Sequencing Data Processing

The bioinformatic software QIIME2 (v2023.7) was used to filter raw 16s amplicon sequencing data and generate diagnostic data about the quality and quantity of our reads. Primer sequences were trimmed from the sequencing data before amplicon error correction and ASV assembly with DADA2. Taxonomy was determined using a naïve Bayes classifier with the SILVA rRNA database (v138-99). A final filtering step was performed to eliminate singletons and reads conferring with mitochondrial or chloroplast DNA. Samples whose sequencing read counts were >150 were truncated from the data.

Statistical and Bioinformatic Analysis

To test differences in sample diversity (alpha diversity) among sample types, seasons, and different points in decomposition, we used Kruskal-Wallis tests with pairwise Wilcoxon ran-sum tests. Testing for significant dissimilarity in bacterial community composition (beta diversity) was done using pairwise adonis. ANCOM-BC estimated absolute abundances of bacterial taxa based on their relative abundances. A thorough description of statistical analyses and modeling approaches can be found in the “Materials and Methods” of CHAPTER 2: SEASONAL DYNAMICS OF POSTMORTEM MICROBIOMES.

Results

Environmental Weather Variables

Temperatures over time during carcass decomposition were plotted to better assess the thermal conditions for blow fly colonization and growth (**Figure 24**). Since larvae used in analysis were only identified to family (Calliphoridae), standards for optimal growth of most blow fly species were generalized to be between 20°C and 30°C. The range for neutral growth is between just 15°C and 20°C. The poorest possible conditions that can sustain blow fly development range from 5°C to 15°C (Salimi et al., 2018; Roe & Higley, 2015; Voss et al., 2014). Anything below the minimum of this threshold is considered unfit for blow fly survival.

We observed that the temperatures experienced by carcasses and larvae in the summer were almost entirely within the "optimal" range—so much so that larval mass(es) were observed as early as 24 hours into decomposition. Summer larval masses also dispersed as early as 50 hours, while sparser groupings of larvae persisted without meeting our requirements for what constitutes a larval mass.

Fall temperatures differed from summer in that most readings fell into the "poor" growth range. Larvae were observed internally in carcass mouths at around 300 hours, better protected against volatile weather. Importantly, this observation was made after a brief stretch of time wherein temperatures ranged from poor, to neutral, to optimal. The temperatures immediately following our initial observations, however, were far from suitable for the larvae.

Spring temperatures were marginally better over the course of a decomposition being nearly twice the length of summer but half the length of fall. Spring differs from fall in that its initial temperatures were not exclusively relegated to the poorest possible growth range. Instead, spring reached temperatures within the neutral and optimal growth ranges periodically before

dropping to temperatures in the poor growth range. Larval masses were not observed until after 100 hours into the spring study.

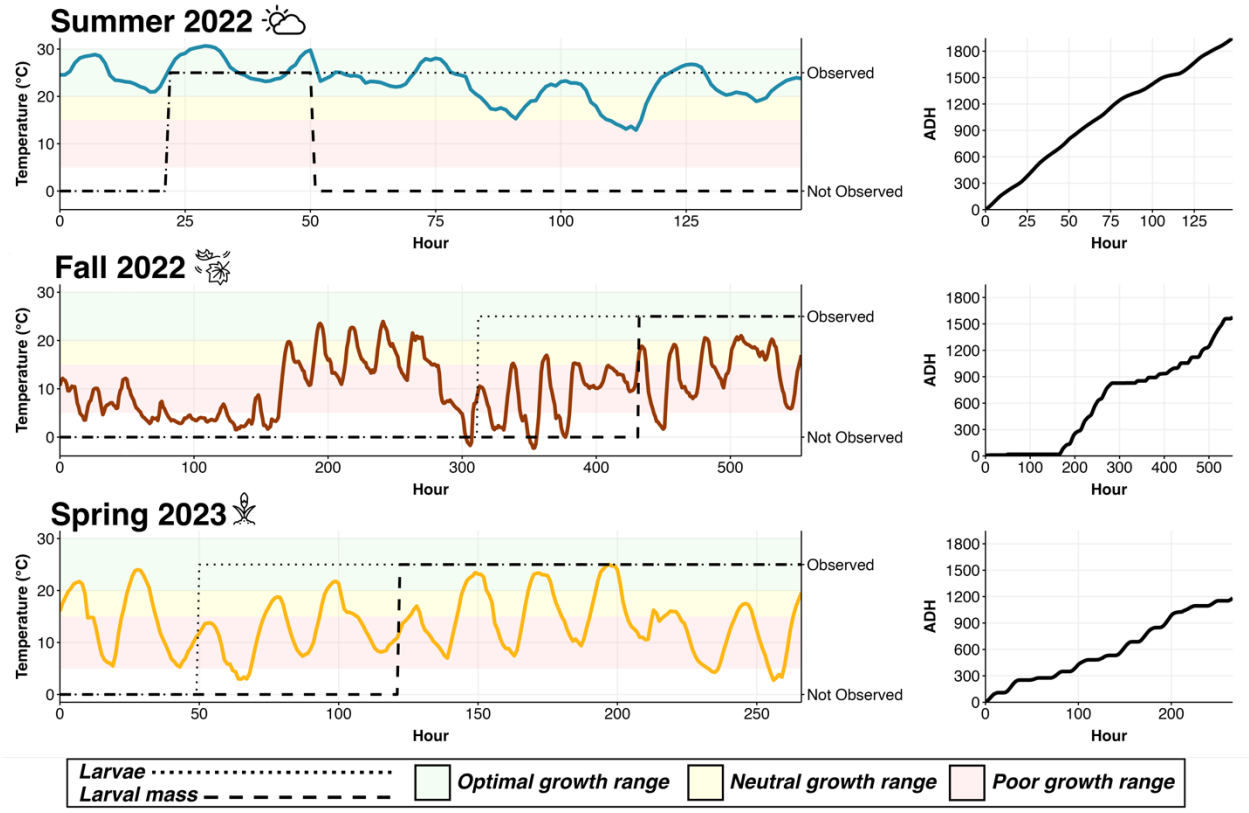

Figure 24. Seasonal temperature readings and accumulated degree hour (ADH) growth. The leftmost line plots represent hourly temperature readings over the course of seasonal decomposition studies. Dotted lines signify whether larvae or larval masses were observed and when. Calliphorid growth ranges are signified by the green (20°C - 30°C), yellow (15°C - 20°C), and red (5°C - 15°C) areas of the grid. Rightmost plots show ADH growth over the course of each seasonal decomposition study.

Microbiome Community Composition and Structure

Weighted UniFrac distance matrices were used to estimate sample type community composition dissimilarity. The extent of this overlap was then visualized using PCoA **(Figure 25)**. The distribution of larval mass scores is very uniquely overlapping carcass samples and larval mass samples almost equally. Larval samples themselves make significant departures from the carcass sample communities. The PC1 axis explained 34.1% of variation while the PC2 axis explains 24.7%, suggesting that there are many complex factors at play driving the dissimilarity of samples and sample types.

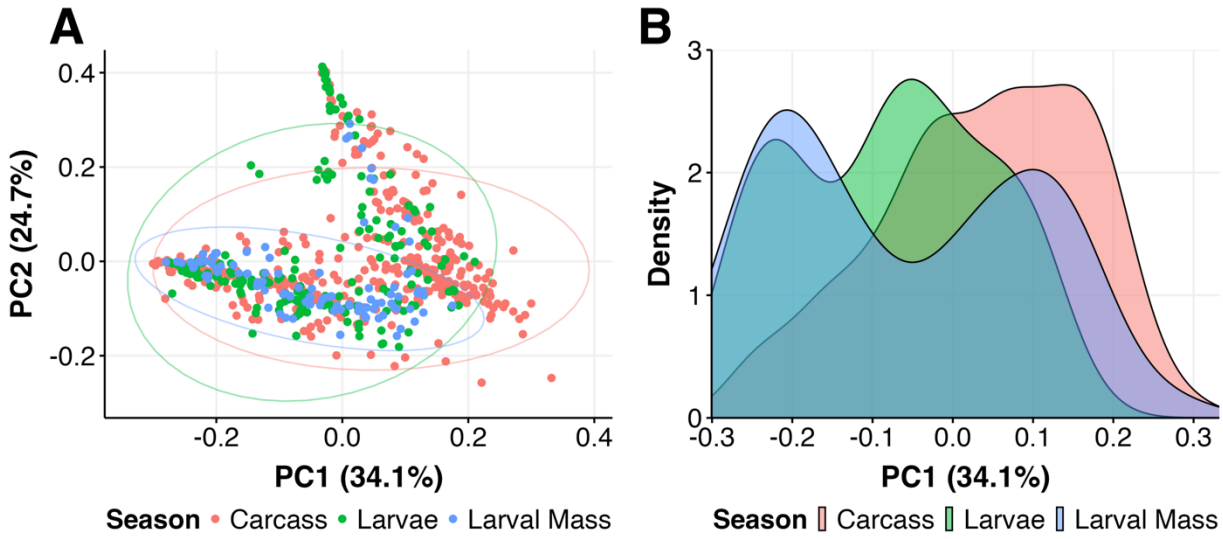

Figure 25. Principal coordinate analysis (PCoA) for weighted UniFrac matrices of larvae, larval masses, and carcass samples across all seasons. Ellipses indicate 95% CI by sample type.

Pairwise PCoA and significance testing accompanied this to evaluate how specific sample types compared in a combined seasonal dataset **(Figure 26, Figure S9, Table S11)**. Seasonal data of specific sample types were also compared to see which seasonal differences drive the most dissimilarity. Of the sample types compared, only larvae and larval mass sample communities exhibited non-significant levels of dissimilarity (adj. $p = 0.398$). Carcass and larval mass sample community dissimilarity was significant (adj. $p = 0.017$), but less so than the

dissimilarity of carcass and larvae sample communities (adj. p = 0.001).

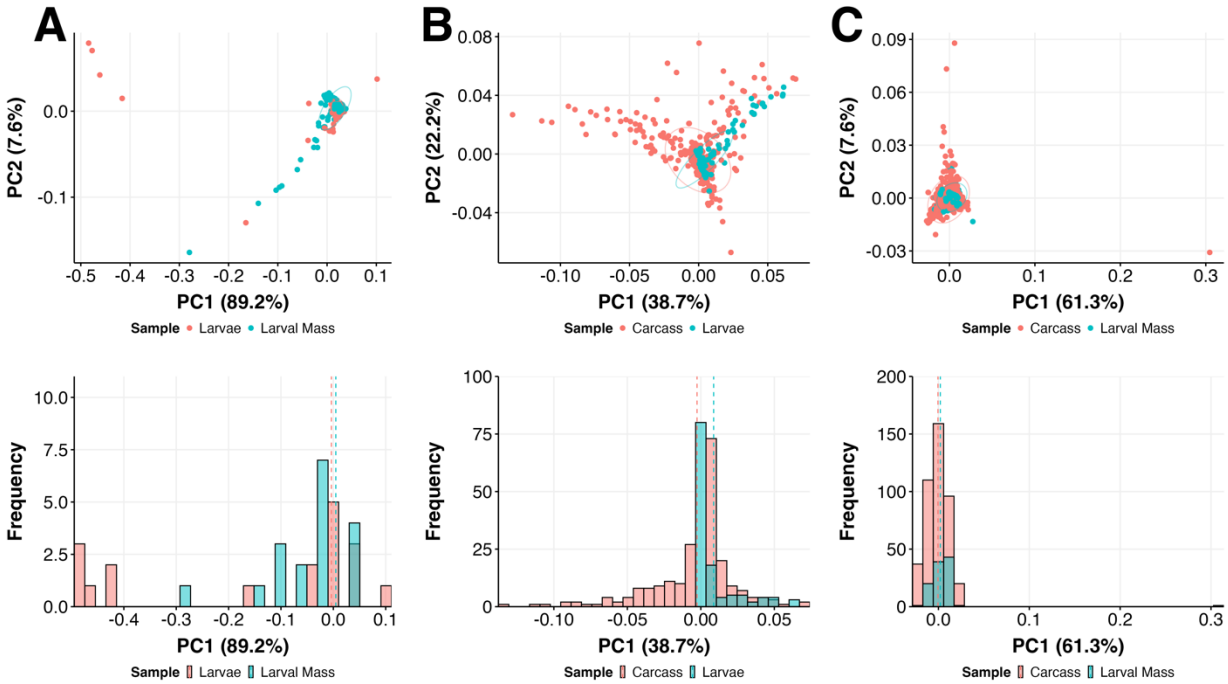

Figure 26. Principal coordinate analysis (PCoA) and weighted UniFrac score frequencies for pairwise sample type comparisons. Ellipses indicate 95% CI by sample type. Dotted lines indicate the mean score of a given sample type.

To see if substantial changes in the community compositions of larvae and larval masses could be visualized between timepoints, we examined the mean relative abundances of all larvae collected at a specific timepoint as well as the temperatures observed during and between those sampling events (**Figure 27, Figure S10**). The community overlap indicated by PCoA and significance testing is made much clearer here. Of note is that the mean relative abundance of *Ignatzschineria* among fall summer and larval mass samples was much greater than those of the spring, which may explain why spring larval masses appeared to be significantly more diverse than either of the other studies. Similarly, fall larvae compositions featured a greater mean relative abundance of *Vagococcus* than the larvae of the summer or spring, which may have driven its significantly higher larvae bacterial community diversity. In all cases, *Ignatzschineria*

either begins or becomes a dominant genus in larval mass samples; and is present in high abundance among larvae from the very start of their sampling in all seasons. Community dissimilarity seems to be largely driven by differences in *Ignatzschineria*, *Vagococcus*, and *Lactococcus* abundances, among others.

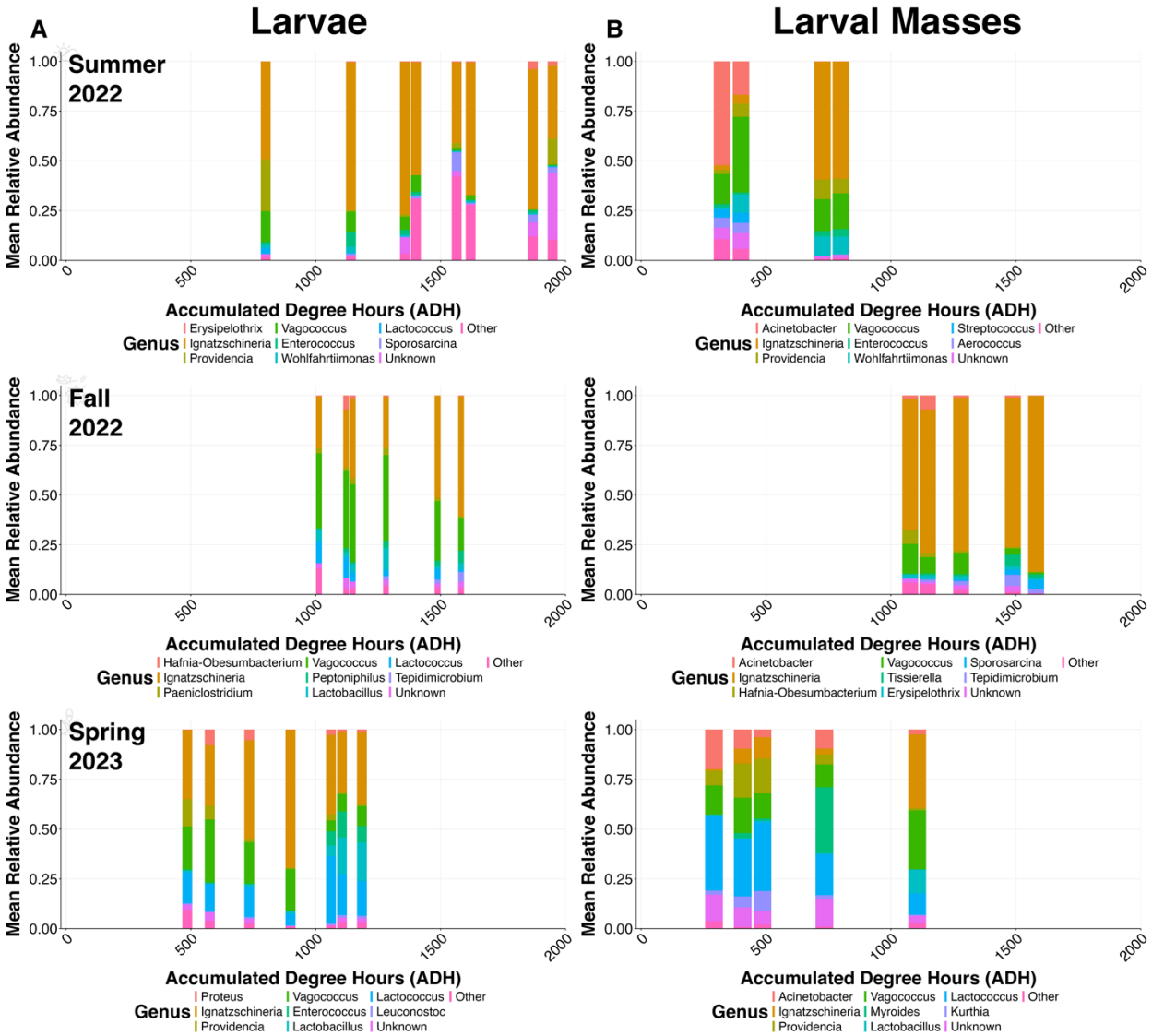

Figure 27. Mean relative abundance bar plots organized by ADH sampling point. A) Mean relative abundances of larvae bacterial communities. B) Mean relative abundances of larval mass bacterial communities.

Larval mass microbiota

Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) tested how the abundance of genera differed between seasonal groups of larvae **(Figure 28)** and larval masses **(Figure 29)**. Results indicated that abundances of *Lactobacillus*, *Lactococcus*, and *Leuconostoc* were particularly skewed towards the fall. *Wohlfahrtiimonas*, *Dysgonomonas*, and *Koukoulia* are in significantly higher abundance during the summer.

Like the fall, spring differs from the summer with high abundances of *Lactobacillus*, *Lactococcus*, and *Leuconostoc*. While both fall and spring share higher levels of *Hafnia-Obesumbacterium* and *Peptoniphilus*, they vary significantly in their abundance.

Wohlfahrtiimonas is noticeably greater in the summer when compared to the fall and spring. Fall and spring share many of the same genera disparities. Larvae from these two seasons were more similar than larvae collected in summer.

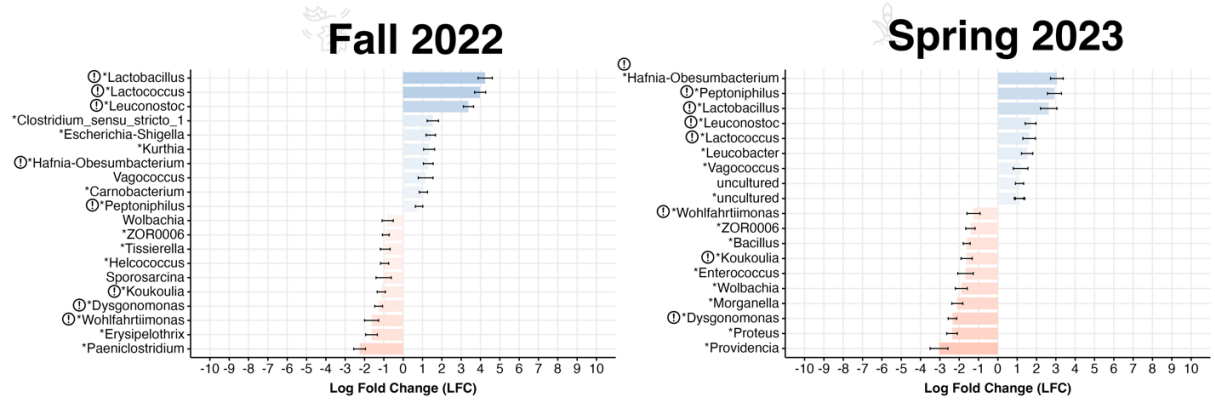

Figure 28. ANCOM-BC results for larvae grouped by season, with summer 2022 as the reference category. Genera experiencing significant log fold changes (LFC) in abundance are preceded by an asterisk. Genera experiencing shared trends between fall and spring, when compared to summer, are denoted by an explanation point symbol.

Interestingly, among larval masses, *Wohlfahrtiimonas* and *Lactobacillus* saw similarly significant, negative LFC in abundance in the fall and spring (with summer as a reference category). The overlap in these shared, significant taxa trends did not extend past these two genera, though. Like what was observed among spring larvae when compared to summer larvae,

Leuconostoc is similarly abundant in larval masses of spring, but not of fall. *Hafnia-Obesumbacterium*, despite its relatively low (positive) LFC in fall larvae, ranks as the highest (positive) LFC between summer and fall larval masses.

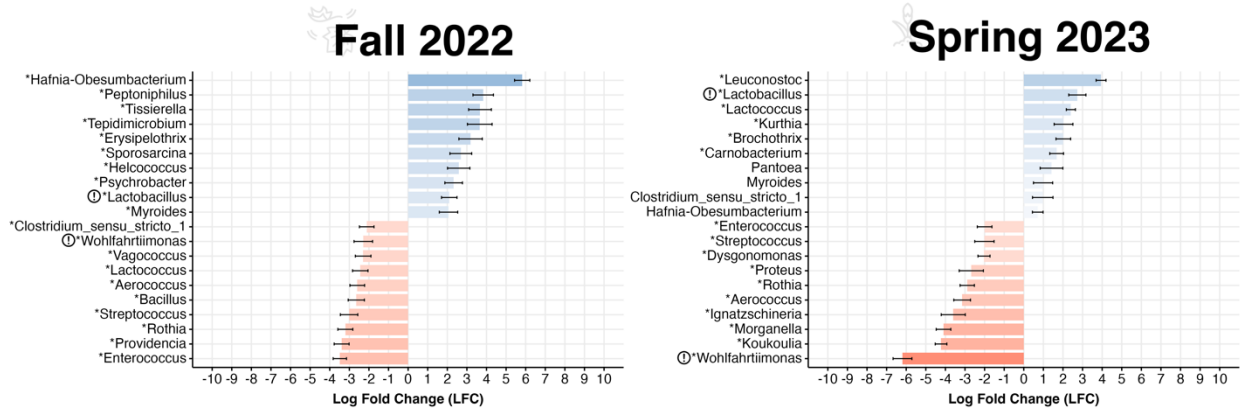

Figure 29. ANCOM-BC results for larval masses grouped by season, with summer 2022 as the reference category. Genera experiencing significant log fold changes (LFC) in abundance are preceded by an asterisk. Genera experiencing shared trends between fall and spring, when compared to summer, are denoted by an explanation point symbol.

Modeling

Random Forest models of microbial communities were used to predict carcass colonization by larval blow flies, with basic categorization tasks performed in QIIME2 for each season and for and all seasons combined (**Figure 30, Figure S11**). In the summer, where the process of decomposition took the shortest amount of time and larvae were seen colonizing the carcass and evacuating it earlier, models were able to discern whether carcasses were or were not colonized. The fall and spring, while mostly successful, both mistakenly identified 30-50% of carcasses as uncolonized when they were colonized. However, both performed better in predicting a colonized carcass. Combining all seasonal datasets and using it to construct a model it yielded better results than two of the three seasonal models, and ultimately proved more robust.

All models prioritized indicator taxa genera like *Ignatzschineria* and *Wohlfahrtiimonas* in associating sample compositions with fly activity. Similarly, it associated *Acinetobacter* with the

native postmortem microbiome, which corresponds with what has been observed in bacterial abundance data. Other genera also stand out as unique indicators, since their presence was often detected in carcasses of one status but not in the other. An example of this was *Trueperella*, which was seen in uncolonized carcasses but not in colonized carcasses. *Sporosarcina*, conversely, was only seen in colonized carcasses. Bacterial features like these played a significant role in the model's ability to correctly assess carcass states.

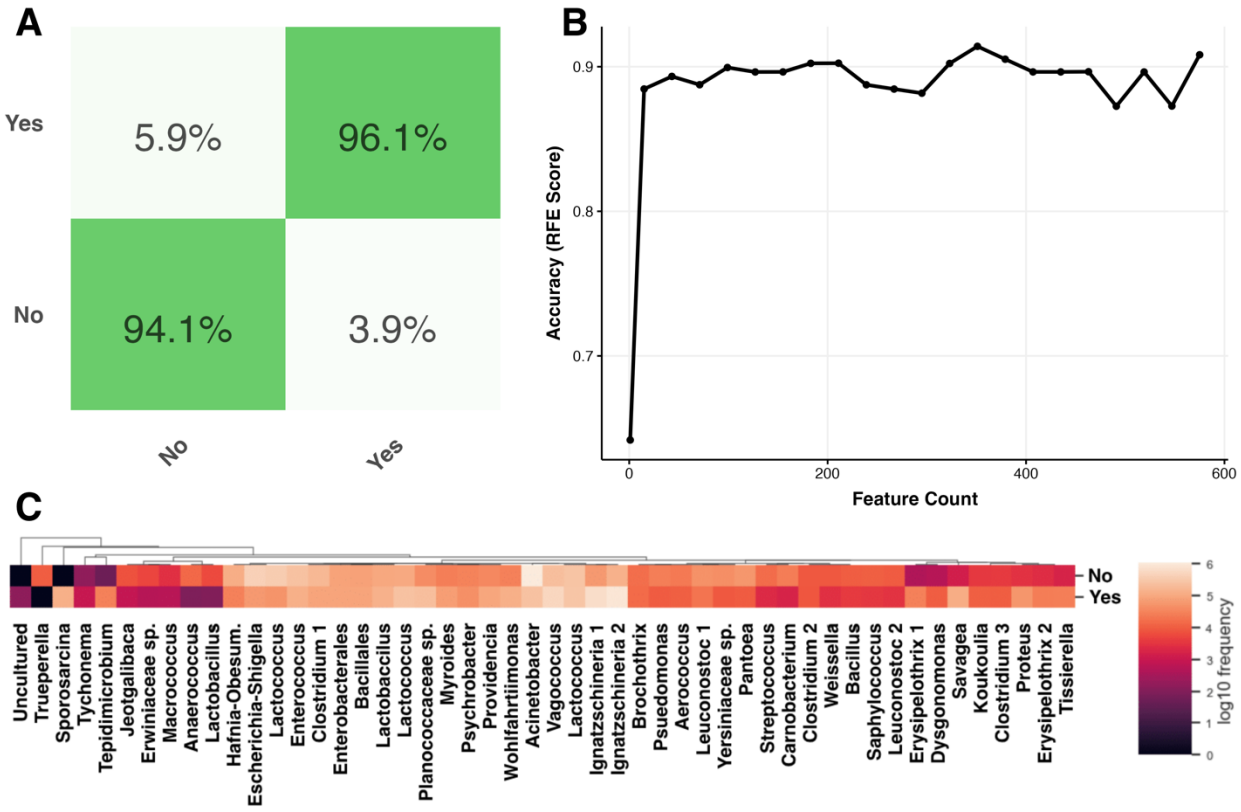

Figure 30. Random forest predictive classifier of colonized or non-colonized carcasses. A) Model accuracy for a consolidated seasonal dataset, represented as a confusion matrix, where x = actual and y = predicted. B) Model accuracy (recursive feature exclusion) as a function of sample feature count. C) Taxonomic heatmap indicating genera prioritized by RFE and used to indicate colonized and non-colonized samples.

Discussion

The objectives of this study were to communicate the underlying complexity that blow fly colonization ultimately brings to our understanding of the postmortem microbiome, bacterial succession within that microbiome, and how these communities change with insect colonizers. Researchers have long endeavored to thoroughly characterize decomposition as on the context of multiple carrion models (humans, deer, pigs, etc.) and associated invertebrate decomposers; but researchers have advocated for additional work into understanding the interactions of the complex "necrobiome" (Benbow et al., 2016). This, in large part, motivated our examination of blow fly larvae and bacteria, since both are recognized as chief decomposers during carrion decomposition. The also share many similarities as far as optimal living conditions are concerned. This venture serves as one of many recent attempts to unravel the fundamental mystery of the necrobiome's many overlapping macro- and micro-level communities and how their presence factor into each other's success and the trajectory of decomposition as a process.

Our principal hypothesis that larval colonization and mass formation would significantly influence postmortem microbiome succession proved extremely likely. Larvae and larval mass community compositions were not significantly dissimilar, despite carcasses being extremely dissimilar from both larvae and larval masses. Carcasses sample compositions were, however, more like those of larval masses than larvae. Blow fly microbiomes are known to be relatively simple, as is often the case with many insects (Junqueira et al., 2017; Mason, 2020) and well conserved. We have also established that the enzymatic secretions generated by blow fly larvae are beneficial to them in that their antimicrobial properties can kill harmful bacteria and differentially allow certain taxon into their microbiomes as they feed (Simmons, 1935). It is possible that the inherent limitations of the larval microbiome as well as the innate filtration of

microbes that occurs when feeding may limit the extent to which their microbiomes can be influenced by carcasses. Additionally, the slight community overlap larval masses have with postmortem microbiomes suggests that the overall matrix of the larval mass is dually influenced by the larvae comprising it and the carcass itself, but significantly more so by the larvae. The gooey film associated with larval masses is understood to be a homogenous combination of digestive enzymes, larval excretions, and organic matter contributed from the carcasses themselves (Rivers et al., 2011). This may account for the considerable community overlap larval mass microbiome samples share with larvae and carcasses. *Ignatzschineria* and *Wohlfahrtiimonas* have stood out as bacterial genera of interest due to their apparent relationship with blow fly larvae and presence in larval masses; and given what we know of larval life history, it's possible that these bacteria are introduced through the secretions or excrement of larvae and are uniquely suited to the volatile matrix. Random forest predictive classifiers were additionally able to evaluate whether a microbial sample presented to it was impacted by larval colonization; and by building these models on seasonal postmortem microbiome data, all managed to identify that *Ignatzschineria* was the single most important bacteria in distinguishing colonized carcasses from non-colonized carcasses. This further reaffirms out belief that the introduction of this bacteria by blow flies is significantly influencing the bacterial succession of postmortem microbiomes. Given that *Ignatzschineria* and *Wohlfahrtiimonas* were found to be among the most dominant bacterial genera in and on carcasses as decomposition progressed, and that it was found in high abundance within the larvae & larval masses themselves from the moment they were available for sampling, we can assume that their introduction of this bacteria is altering postmortem microbiome succession in some way. Burcham et al. (2024) used multi-omic approaches to microbiome characterization to develop a bacterial "decomposer" network

that, interestingly, includes both *Ignaztschineria* and *Wohlfahrtiimonas*. Functional analyses of the microbiota that were classified as "decomposers" implicated *Ignatzschineria* as a compatible cross-feeding partner for a species of *Oblitimonas*. They also echo our suggestion that insect-microbe interactions may be driving succession effects, since many lab-based decomposition studies lacked representation of either genus when invertebrate access was restricted. We cannot definitively say that the larval mass matrix is also modulating bacterial communities of the surrounding carcass, but we feel strongly that this may be the case.

Evaluating whether larvae bacterial communities differed by season seemed to indicate that the difference in compositions between summer and other seasons was substantive enough to be significant. The second evaluation testing the significance of larval microbiome disparities over time (ADH) yielded results that conflict with our initial prediction. One potential source of variation among larvae microbiomes could be the species succession of colonizing blow flies. Many studies have demonstrated the considerable temporal variation in Calliphoridae species abundances throughout different seasons (Benbow et al., 2013; Pechal et al., 2013), and it is possible that dynamics between different blow fly species could drive microbial community dissimilarity to different extends. While most larvae sampled did not differ significantly as time went on, significant differences did appear when carcass decomposition stages transitioned from "Bloat" to "Active" in both the summer and spring. The only season exempt from this was fall, whose larval bacterial communities were found overlap for the most part. Identifying potential causes is difficult, but we speculate the departure fall larval formations took from other seasons in terms of overall strategy (their preference for internal formation and growth) may have resulted in keeping larvae microbiota relatively consistent. The low temperatures observed in the fall promoted the emergence and survival of those larvae laid in spaces with some level of

protection from the elements. Consequently, larval masses were observed to form internally in all carcasses before external larval masses were ever detected. A study by Johnson et al. (2014) confirms that larval mass movement is not random, and further suggests that larvae may select for temperatures such that they will prefer the hottest part of larval masses. Data indicated that the overall diversity of mouth and rectal microbiomes was dwarfed by diversity observed on the skin, so perhaps the skin microbiota was so dynamic in its bacterial succession that it was able to dramatically alter the overall larval mass matrix (and external larval microbiome) as decomposition stages shifted from "Bloat" to "Active." We feel there is a strong possibility that larvae may not differ significantly by internal microbiome The likelihood of external microbiomes driving the slight disparity in bacterial community we observe across season and ADH is reasonable considering how significantly different the larval mass microbiomes were from each other. Studies of *Phormia regina* microbiomes have indicated significant differences between externally samples and internally samples microbiota, with the latter representing just a fraction of the diversity expressed by external communities (Deguenon et al., 2019). Also, while summer postmortem microbiome communities were significantly more diverse in the summer than other seasons, larval mass and larvae bacterial community alpha diversity remained strikingly consistent among all three **(Figure S8).** Comparisons revealed not only that larval masses were significantly more diverse than the larvae comprising them (except for fall), but also that the median differences of these sample types from a seasonal perspective remained relatively consistent.

The hypothetical utility of larvae outside of their current uses in forensic entomology has seen considerably speculation in recent literature (Sharma et al., 2015). Among these applications, the determination of a post-mortem interval (PMI) with larval development data

ranks among the most popular. This method stands out as being deceptively complex in the considerations it must make in the determination of a reliable PMI; and the inherent error that one can attribute to misrepresented or improperly measured variables required of the technique are only compounded by additional errors in evidence collection. An important means by which researchers believe this error can be mitigated is by providing accurate information outlining the PMI considerations that motivate entomological evidence collection, so advancements have ultimately emphasized identifying those analytical needs (Hall, 2021). As such, we have come to recognize the importance and interconnectedness of factors like insect succession in decomposition settings, how those insects develop, and how environmental conditions modulate both (Benbow et al., 2016).

In many ways our study was an extension of this principle insofar as it sought to characterize seasonal weather conditions reflecting their growth conditions and how they influence their life histories. What stands out about new and emerging techniques is how flexible they are, as well-designed evidence processing pipelines can do well to maximize the ways in which just a single piece of evidence can be used. With that in mind, we have conducted preliminary research to evaluate the importance of carrion in microbiome succession across blow fly development, and future analyses of these data may prove helpful in making a deliberation about how appropriate microbiome-centric approaches to age determination may be. We do feel, however, that using larvae or larval mass microbial profiles to predict decomposition stage is a relatively fruitless effort. Indeed, larval mass samples only spanned one or two stage transitions in every season, often providing us with binary predictions at best. Many larvae also did not exhibit any significant dissimilarity between successive, linear timepoints (ADH). This is a largely insubstantial observation, but it does beg the question of how significant and consistent

the rare taxa driving the few significant changes in larvae microbiota may be in nature. Our findings similarly fail to indicate that larval masses would be of particular use in predicting ADH for PMI determination. Despite this, our findings have enabled us to identify overlaps in larval, larval mass, and carcass bacterial communities throughout decomposition. Measurable trends in succession and indications that seasonal weather dynamics play a role in said succession are important considerations to make. Additionally, evidence that larvae significantly influence the microbial succession of their host carrion in a potentially reciprocal manner serves to illustrate why considerations like these are important in the evaluation of necrobiome-associated data.

This study is just one small step in better characterizing the necrobiome as an immense, complex web of life alongside the processes that sustain it. We are reaffirmed in our belief that elucidating significant interactions of organisms spanning various trophic levels or functional niches will help strengthen the proposed applications of postmortem microbial data. Despite the breadth of this study, there are still many questions whose answers remain unclear. In identifying the trends we have, we succeeded in bringing new concerns to light alongside the objective, measurable observations made in the process. We argue that studies such as these that emphasize longitudinal data collection across significantly different environments will provide us with a clearer understanding of what postmortem microbial data, among others, is communicating to us.

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APPENDIX A: RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species used in this research.

Voucher number: 2024-08

Author and Title of thesis:
Anthony Grigsby – Seasonal Dynamics of Carrion Decomposition Ecology

Museum where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Table 5. A list of individual voucher specimen as characterized by species, life stage, and preservation method.

Family	Genus-Species	Life Stage	Quantity	Preservation
Calliphoridae	*Phormia regina*	Adult	2	Pinned
Calliphoridae	*Lucilia ilustris*	Adult	2	Pinned
Calliphoridae	*Lucilia silvarum*	Adult	2	Pinned
Calliphoridae	*Calliphora vicina*	Adult	2	Pinned

APPENDIX B: SUPPLEMENTARY MATERIALS

Table S1. A summary of the identities, average weights, and sexes of stillborn carcasses used in the summer 2022, fall 2022, and spring 2023 decomposition studies.

Season	Carcass IDs	Average weight (kg)	Sex ratio (male:female)
Summer	C1-C6 (n = 6)	4.13	1:5
Fall	C7-C12 (n = 6)	2.92	4:2
Spring	C16-C21 (n = 6)	3	5:1
	C1-C21 (n = 18)	3.5	10:8

Table S2. A summary of significantly different decomposition stage transitions and the p-values of their respective alpha diversity metrics. Significance evaluated by pairwise Wilcoxon Rank-Sum tests. Significance thresholds denoted by * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Seasonal decomposition stage transition	Observed	Evenness	Shannon	FaithPD
Summer 2022; “Fresh” → “Bloat”	0.00053***	>0.001***	>0.001***	0.017*
Summer 2022; “Bloat” → “Active”	0.0022**	0.12	0.011*	0.00058***
Summer 2022; “Active” → “Advanced”	0.045*	0.47	0.025*	0.069
Fall 2022; “Fresh” → “Bloat”	0.62	>0.001***	0.0028**	0.0068**
Fall 2022; “Bloat” → “Active”	0.00093***	0.00076***	0.6605	0.00069***

Table S3. A summary of significantly different sample types and the p-values of their respective alpha diversity metrics. Significance thresholds denoted by * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Seasonal sample type comparison	Observed	Evenness	Shannon	FaithPD
Fall 2022; Skin vs Mouth	>0.001***	0.032*	0.00065***	>0.001***
Fall 2022; Skin vs Rectum	>0.001***	0.032*	0.00065***	>0.001***
Spring 2023; Skin vs Mouth	>0.001***	0.79	0.0015**	>0.001***
Spring 2023; Skin vs Rectum	>0.001***	0.74	>0.001***	>0.001***
Spring 2023; Mouth vs Rectum	>0.001***	0.74	>0.001***	>0.001***

Table S4. Distribution of biological sample types used in analysis across seasonal decomposition studies (summer 2022, fall 2022, spring 2023).

Sample Type	Summer 2022	Fall 2022	Spring 2023	Total
Carcass	94	189	159	**442**
Mouth	30	62	54	**146**
Skin	24	54	52	**130**
Rectum	40	73	53	**166**
Larvae	71	57	70	**198**
Larval mass	42	32	78	**152**
Total	**207**	**278**	**307**	**792**

Only the "Carcass" *total is considered in the calculation of the overall sample total.*

Table S5. A summary of significantly different seasons and the p-values of their respective alpha diversity metrics. Significance thresholds denoted by * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Season comparison	Shannon	FaithPD
Summer vs Fall	0.0054^{**}	0.0026^{**}
Summer vs Spring	0.033^{*}	0.00054^{***}

Table S6. The number and percentage of successfully sequenced samples classified by decomposition stage across all seasonal datasets.

Season	Fresh	Bloat	Active	Advanced	Dry
Summer 2022	12 (13%)	33 (35%)	18 (20%)	20 (22%)	9 (10%)
Fall 2022	31 (18%)	73 (42%)	51 (30%)	10 (6%)	7 (4%)
Spring 2023	87 (55%)	17 (11%)	33 (20%)	22 (14%)	0 (0%)

Table S7. Post-hoc analysis (Dunn's test) of Kruskal-Wallis rank sum tests comparing historical weather data (temperature and relative humidity) from 08-05 to 08-11 during summer 2019, 2020, 2021, and 2022.

Comparison	Test Statistic	Adjusted p-value
Temperature (°C)		
2019 vs 2020	-0.907138	1.00 (ns)
2019 vs 2021	-4.345112	≤0.001***
2019 vs 2022	-2.911824	0.011*
2020 vs 2021	-3.437974	0.0018**
2020 vs 2022	-2.004686	0.14 (ns)
2021 vs 2022	1.433288	0.46 (ns)
Relative Humidity (%)		
2019 vs 2020	-0.871118	1.00 (ns)
2019 vs 2021	-4.173899	≤0.001***
2019 vs 2022	-6.191031	≤0.001***
2020 vs 2021	-3.302780	0.0029**
2020 vs 2022	-5.319912	≤0.001***
2021 vs 2022	-2.017132	0.13 (ns)

*$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, (ns) = not significant*
p-values adjusted using the Bonferroni method, normality of data tested using Shapiro-Wilk

Table S8. Post-hoc analysis (Dunn's test) of Kruskal-Wallis rank sum tests comparing historical weather data (temperature and relative humidity) from 10-14 to 11-06 during fall 2019, 2020, 2021, and 2022.

Comparison	Test Statistic	Adjusted p-value
Temperature (°C)		
2019 vs 2020	-0.900968	1.00 (ns)
2019 vs 2021	-3.366803	0.0023**
2019 vs 2022	-8.381079	≤0.001***
2020 vs 2021	-2.465843	0.0410*
2020 vs 2022	-7.480119	≤0.001***
2021 vs 2022	-5.014275	≤0.001***
Relative Humidity (%)		
2019 vs 2020	4.849699	≤0.001***
2019 vs 2021	-5.233818	≤0.001***
2019 vs 2022	1.897688	0.1732 (ns)
2020 vs 2021	-10.08351	≤0.001***
2020 vs 2022	-2.952018	0.0095**
2021 vs 2022	7.131499	≤0.001***

*$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***,* (ns) = *not significant*
p-values adjusted using the Bonferroni method, normality of data tested using Shapiro-Wilk

Table S9. Post-hoc analysis (Dunn's test) of Kruskal-Wallis rank sum tests comparing historical weather data (temperature and relative humidity) from 05-15 to 05-26 during spring 2020, 2021, 2022, and 2023.

Comparison	Test Statistic	Adjusted p-value
Temperature (°C)		
2020 vs 2021	-5.237887	≤0.001***
2020 vs 2022	3.290787	0.003**
2020 vs 2023	7.244964	≤0.001***
2021 vs 2022	8.528674	≤0.001***
2021 vs 2023	12.48285	≤0.001***
2022 vs 2023	3.954177	≤0.001***
Relative Humidity (%)		
2020 vs 2021	8.151398	≤0.001***
2020 vs 2022	3.468432	0.0016**
2020 vs 2023	8.989569	≤0.001***
2021 vs 2022	-4.682966	≤0.001***
2021 vs 2023	0.838170	1.00 (ns)
2022 vs 2023	5.521136	≤0.001***

*$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***,* (ns) = *not significant*
P-values adjusted using the Bonferroni method, normality of data tested using Shapiro-Wilk

Table S10. Random Forest model overview of parameter settings.

	Value/Setting	
Parameter	Regression	Classification
Training set ratio	0.8	0.8
Decision tree quantity	500	500
Importance	TRUE	TRUE
No. variables tried per split	$\sqrt{p}$ where p = predictors	$\sqrt{p}$ where p = predictors
Node splitting criterion	Residual sum of squares	Gini index

Table S11. Analysis of variance using adonis, results for the Weighted UniFrac Distance Matrix of pairwise sample type (carcass, larvae, larval masses) and seasonal comparisons.

Comparison	Sum of Squares (SS)	R^2	F-value	Adj. p-value
Larvae vs Larval Masses	0.00554	0.00328	0.77	0.398 (ns)
Summer vs Fall	0.03254	0.01983	3.46	0.189 (ns)
Summer vs Spring	0.05030	0.03526	5.70	0.039*
Fall vs Spring	0.03230	0.11762	18.80	0.003**
Carcasses vs Larval Masses	0.00204	0.00828	4.38	0.017*
Summer vs Fall	0.00649	0.03591	12.37	0.003**
Summer vs Spring	0.01400	0.14294	54.04	0.003**
Fall vs Spring	0.01556	0.07700	32.71	0.003**
Carcasses vs Larvae	0.01781	0.02851	16.02	0.001**
Summer vs Fall	0.00939	0.03401	12.57	0.003**
Summer vs Spring	0.02908	0.07406	25.91	0.003**
Fall vs Spring	0.04107	0.07446	32.90	0.003**

*$p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***,* (ns) = *not significant*

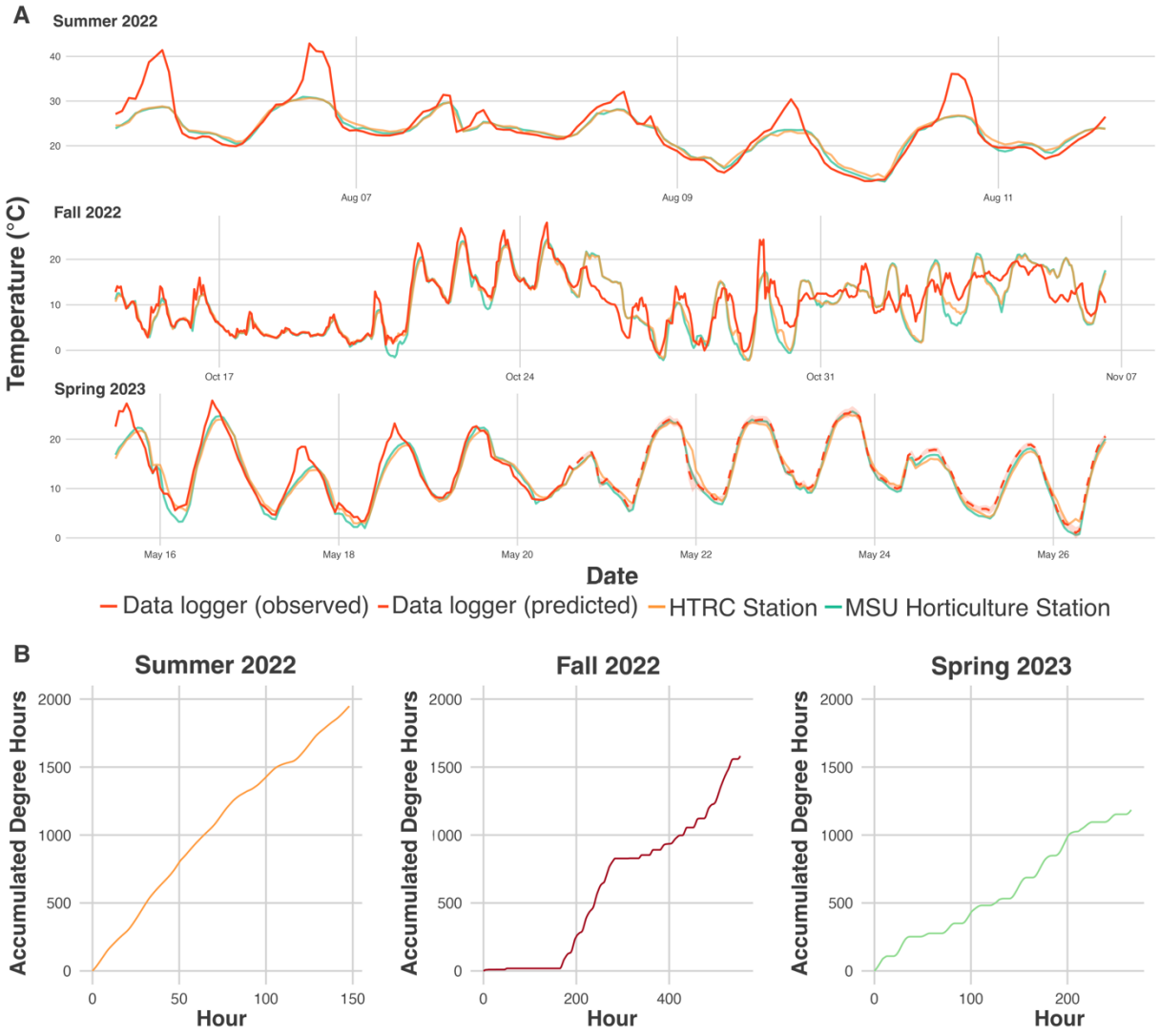

Figure S1. Environmental data and time over the course of seasonal decomposition studies in the order that they were conducted. A) A comparison of temperature data (recorded hourly) over time and between three different sources: Data loggers (including predicted data in Spring 2023), the HTRC weather station, and the MSUHORT weather station. B) Accumulated degree hours (ADH) over time in hours.

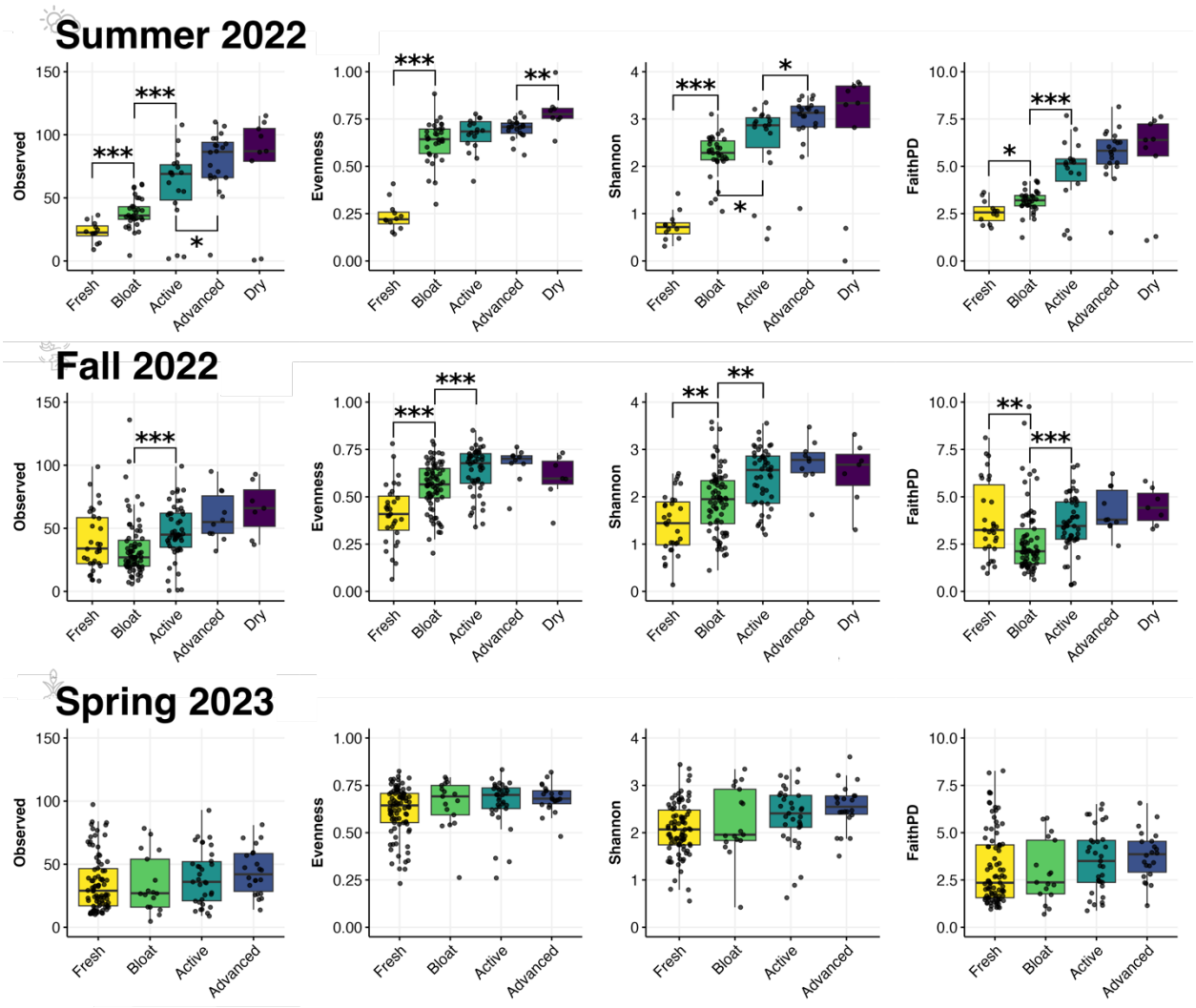

Figure S2. Alpha diversity metrics (observed richness, evenness, Shannon diversity, Faith's phylogenetic diversity) across decomposition stages. Pairwise significance was determined using Pairwise Wilcoxon Rank Sum Tests. Significance thresholds denoted by * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

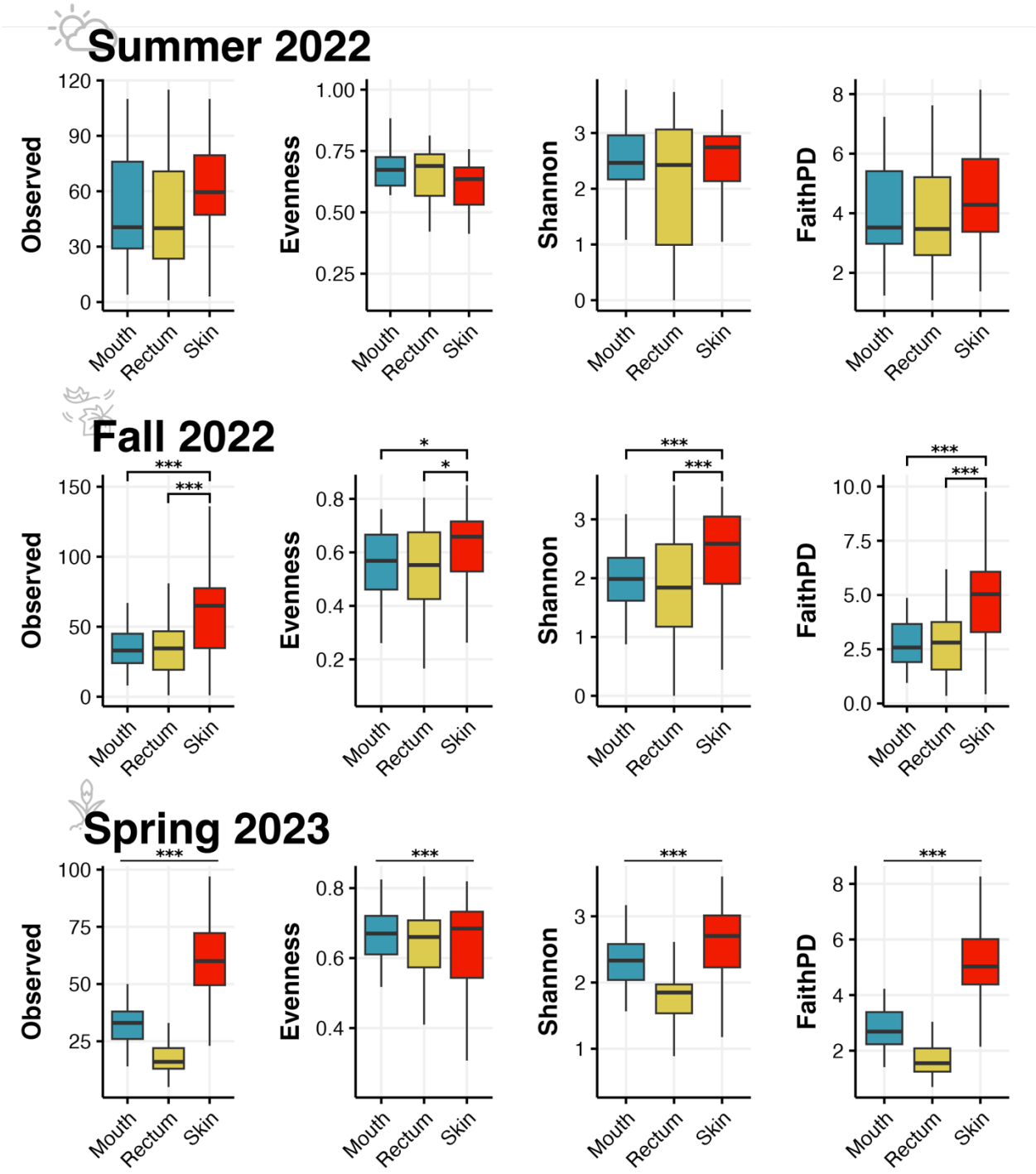

Figure S3. Alpha diversity metrics (observed richness, evenness, Shannon diversity, Faith's phylogenetic diversity) of biodiversity across different body sites. Pairwise significance was determined using Pairwise Wilcoxon Rank Sum Tests. Significance thresholds denoted by * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001. Brackets with tails refer to specific pairwise tests, brackets without tails encompass all pairwise testing.

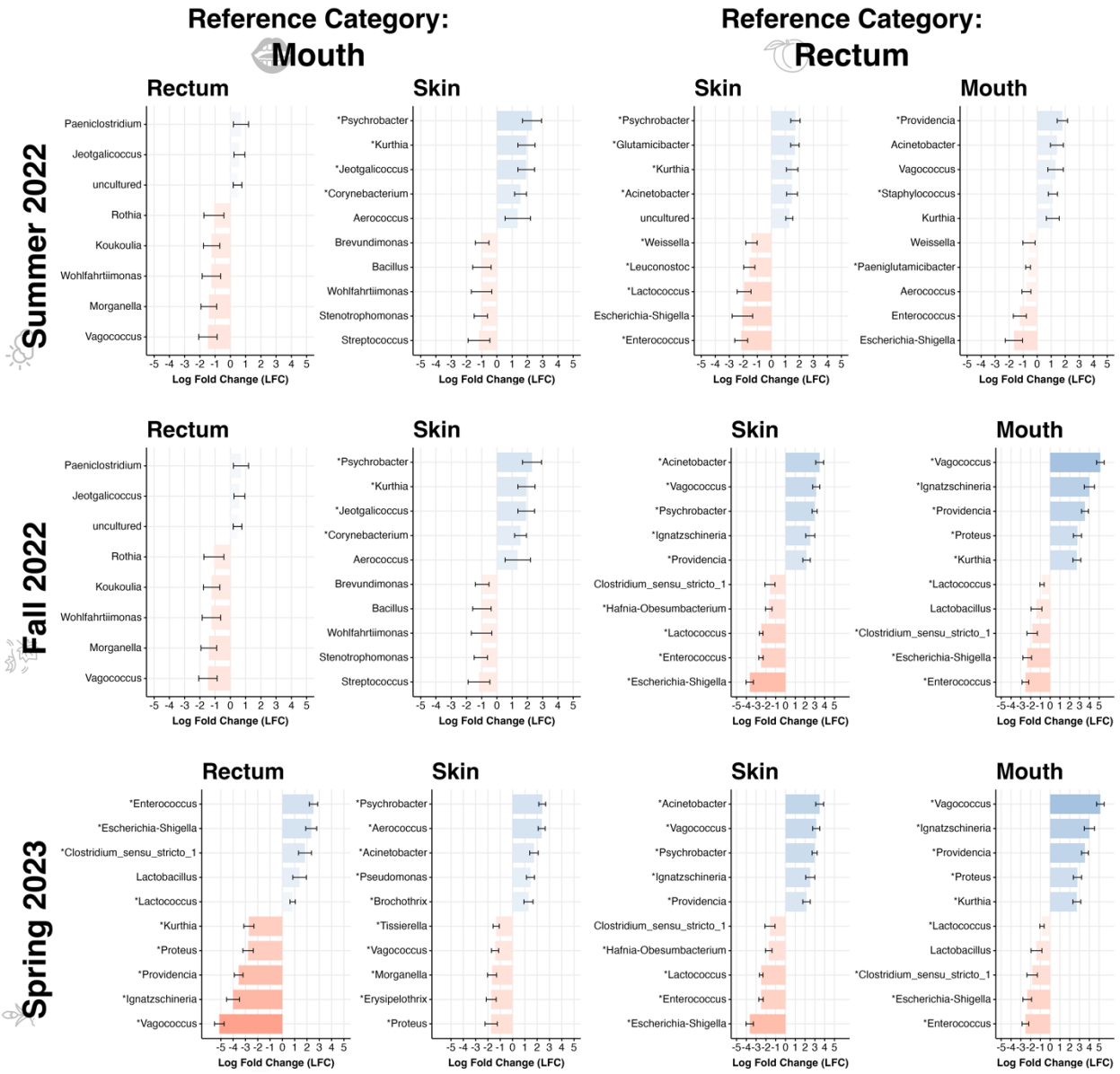

Figure S4. Bar plots of ANCOM-BC log fold changes (LFC), limited to the top five positively and negatively differentially abundant bacterial genera for each body site. Changes are relative to sample type indicated in the reference categories of mouth or rectum. Taxa whose abundances changed significantly (as indicated by $q < 0.05$ and $p < 0.05$) are preceded by an asterisk.

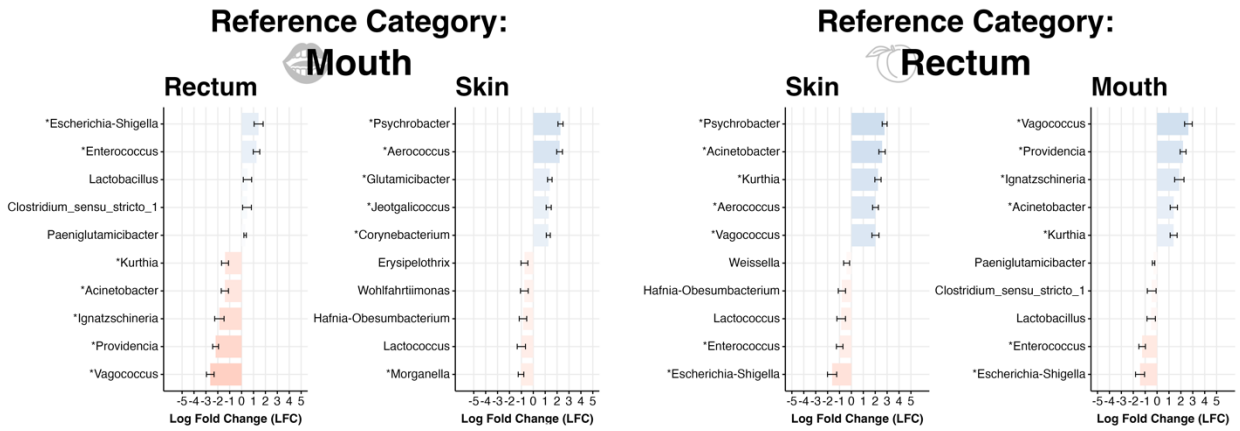

Figure S5. Bar plots of ANCOM-BC log fold changes (LFC), limited to the top 5 positively and top 5 negatively changing bacterial genera. All seasons are evaluated as a combined dataset. Changes are relative to sample type indicated in the reference category of mouth or rectum. Taxa whose abundances changed significantly (as indicated by $q < 0.05$ and $p < 0.05$) are preceded by an asterisk.

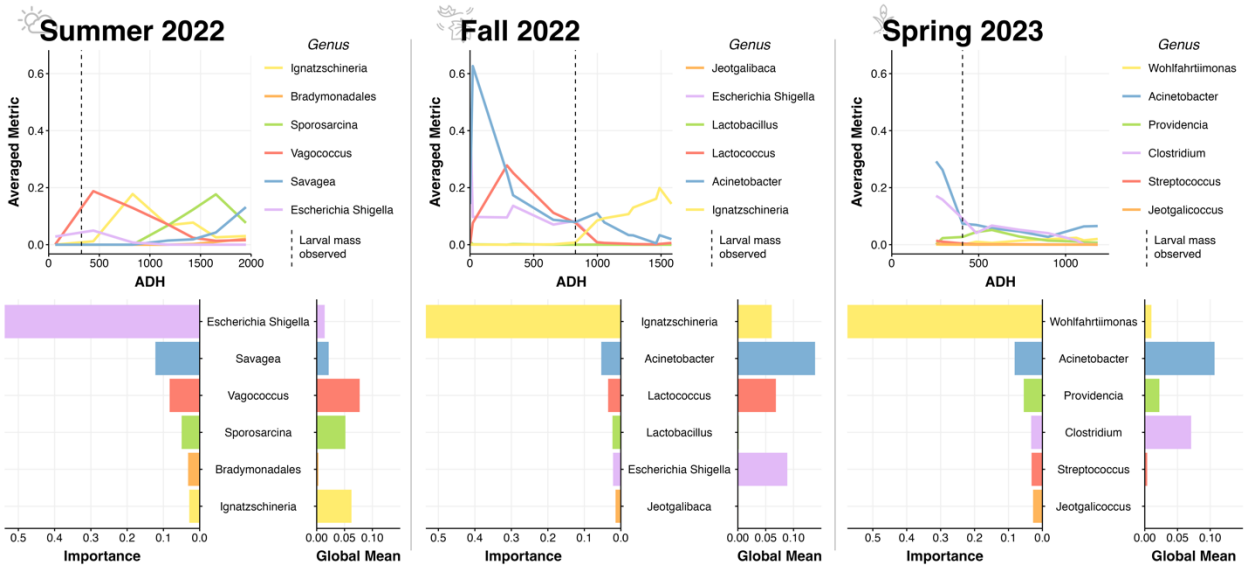

Figure S6. Feature volatility analysis of carcass microbiota among seasons Line plots represent the average prevalence of dominant genera among communities over time (ADH). Barplots represent the importance of those taxa in predicting ADH and their global mean among all samples. "Importance" quantifies the contributions made by each taxonomic feature to the model's overall predictive power.

Figure S7. Feature volatility analysis of body site microbiota. Line plots represent the average prevalence of dominant genera among samples over time (ADH) among different sample types. Bar plots represent the importance of those taxa in predicting ADH and their global mean of all body sites. Metrics used by the underlying supervised learning classifier are meant to emphasize relative importance.

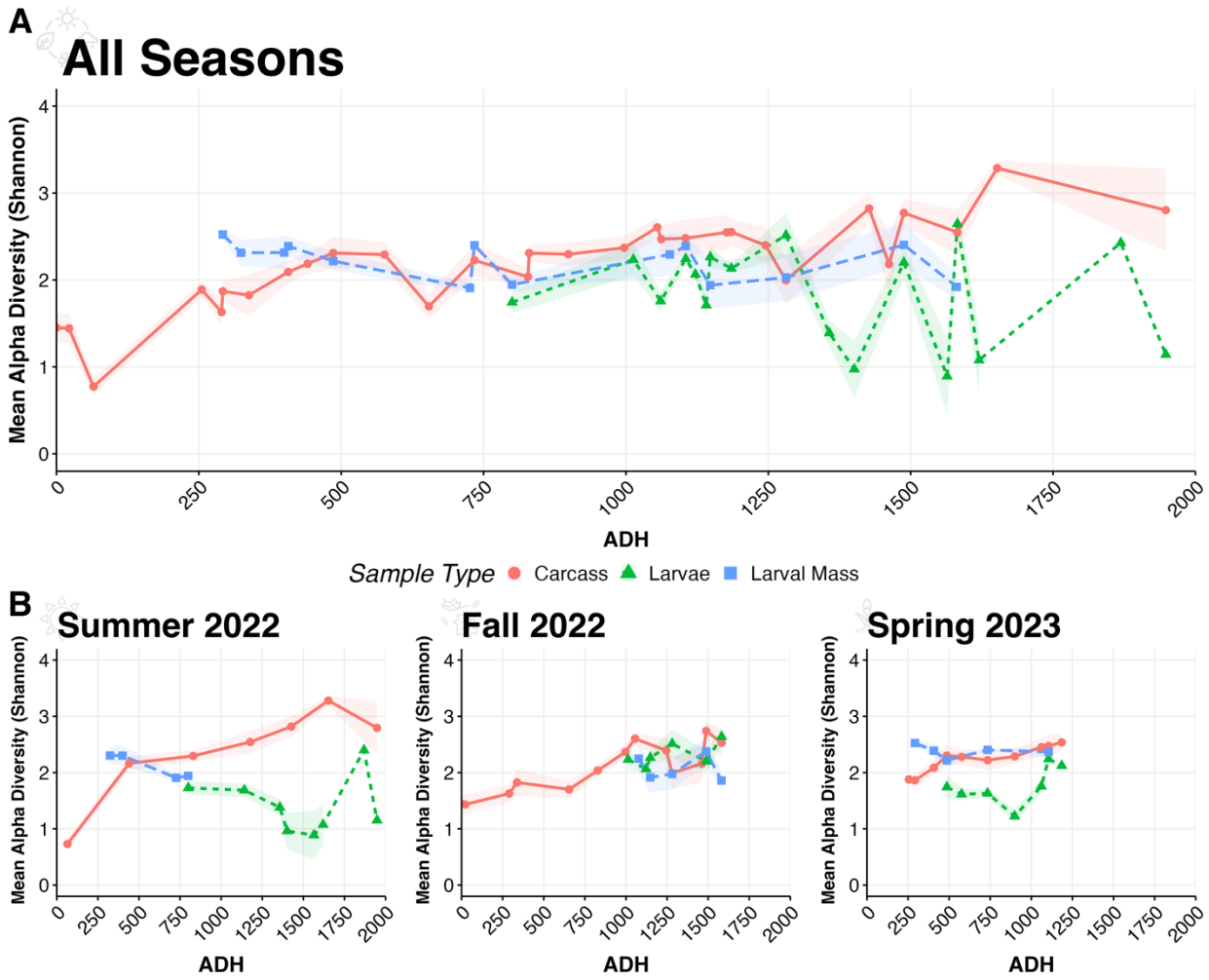

Figure S8. Line plots of mean alpha diversity (Shannon) over time (ADH). A) mean alpha diversity of samples across all seasons, B) mean alpha diversity of samples within independent seasonal datasets. Lines correspond with sample types. Ribbons represent standard error between averages samples.

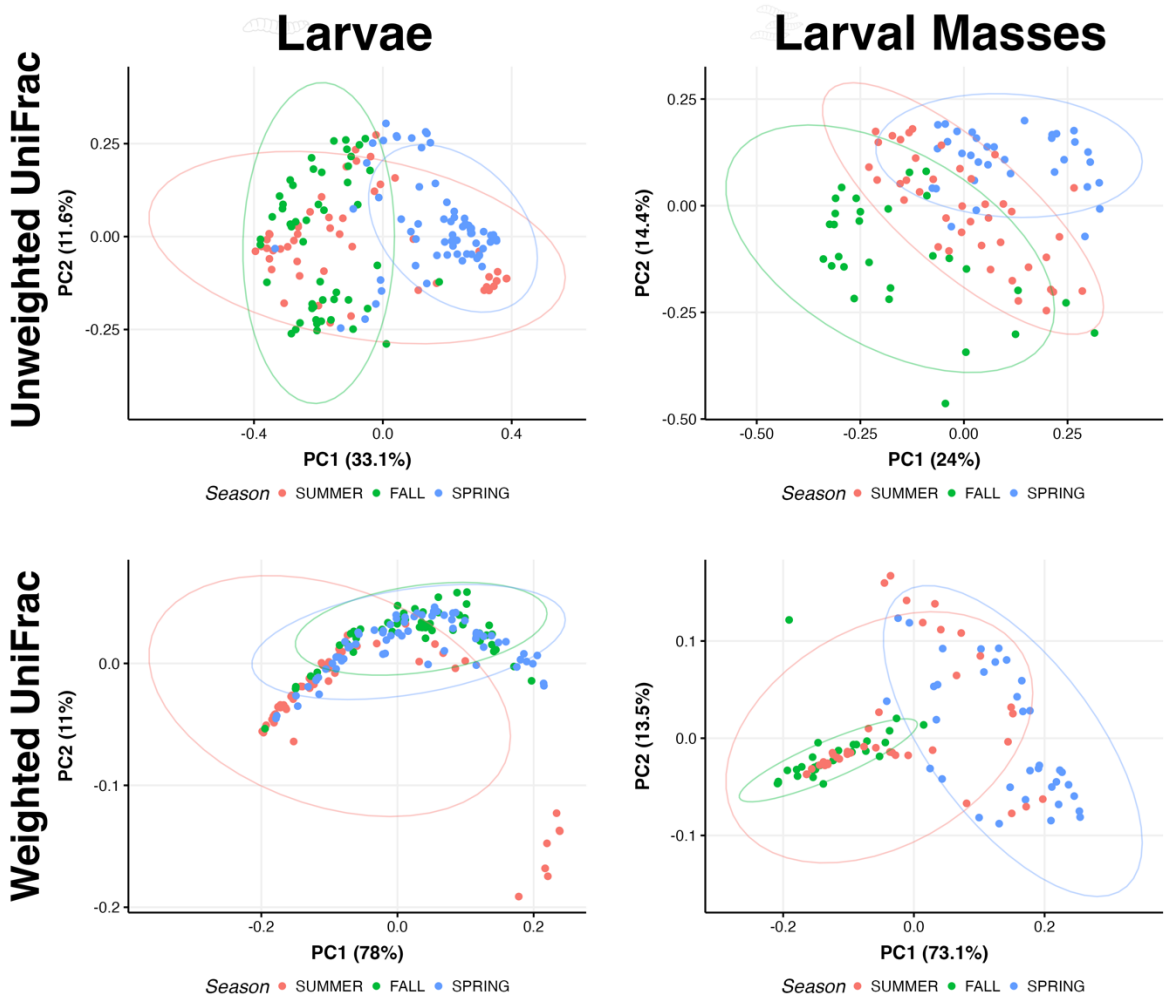

Figure S9. Principal Coordinate Analysis (PCoA) of weighted and unweighted UniFrac matrices for larvae and larval masses. The color of samples and ellipses correspond to the season they belong to. Ellipses indicate 95% standard error of seasons.

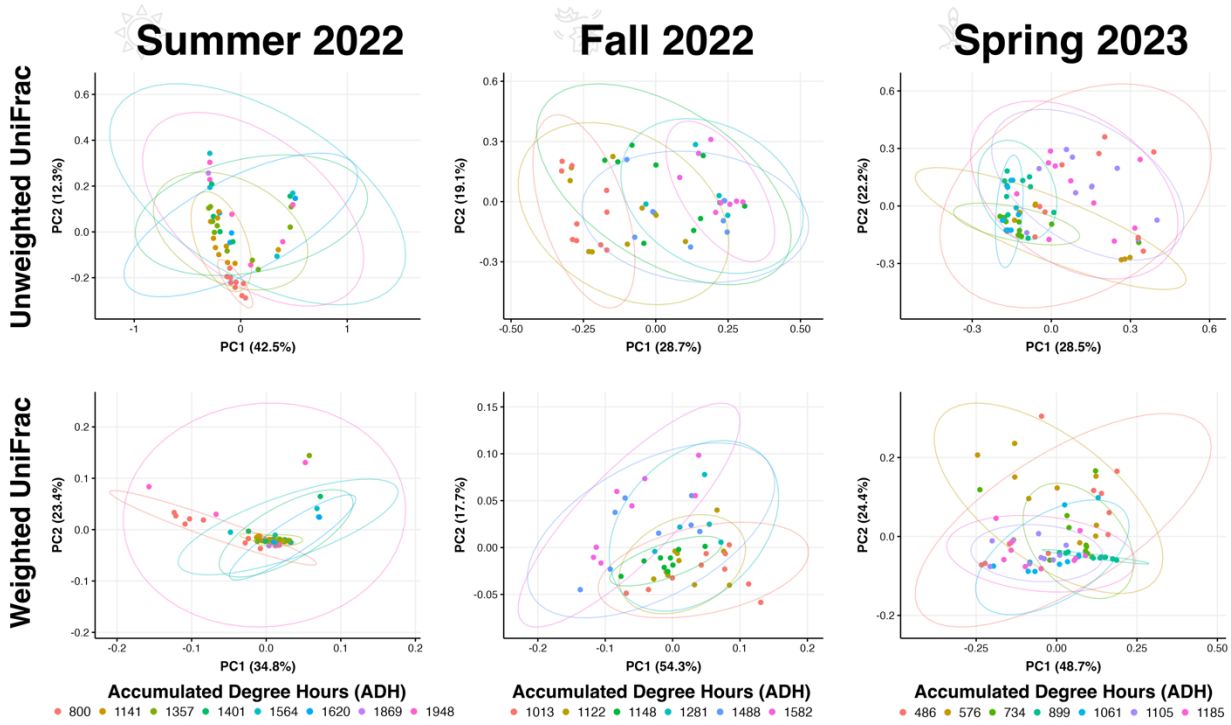

Figure S10. Principal Coordinate Analysis (PCoA) of larval microbiome samples (unweighted and weighted UniFrac) by season. Colors of samples and ellipses correspond with their respective accumulated degree hour (ADH) timepoints when collections occurred. Ellipses indicate 95% standard error of ADH timepoints.

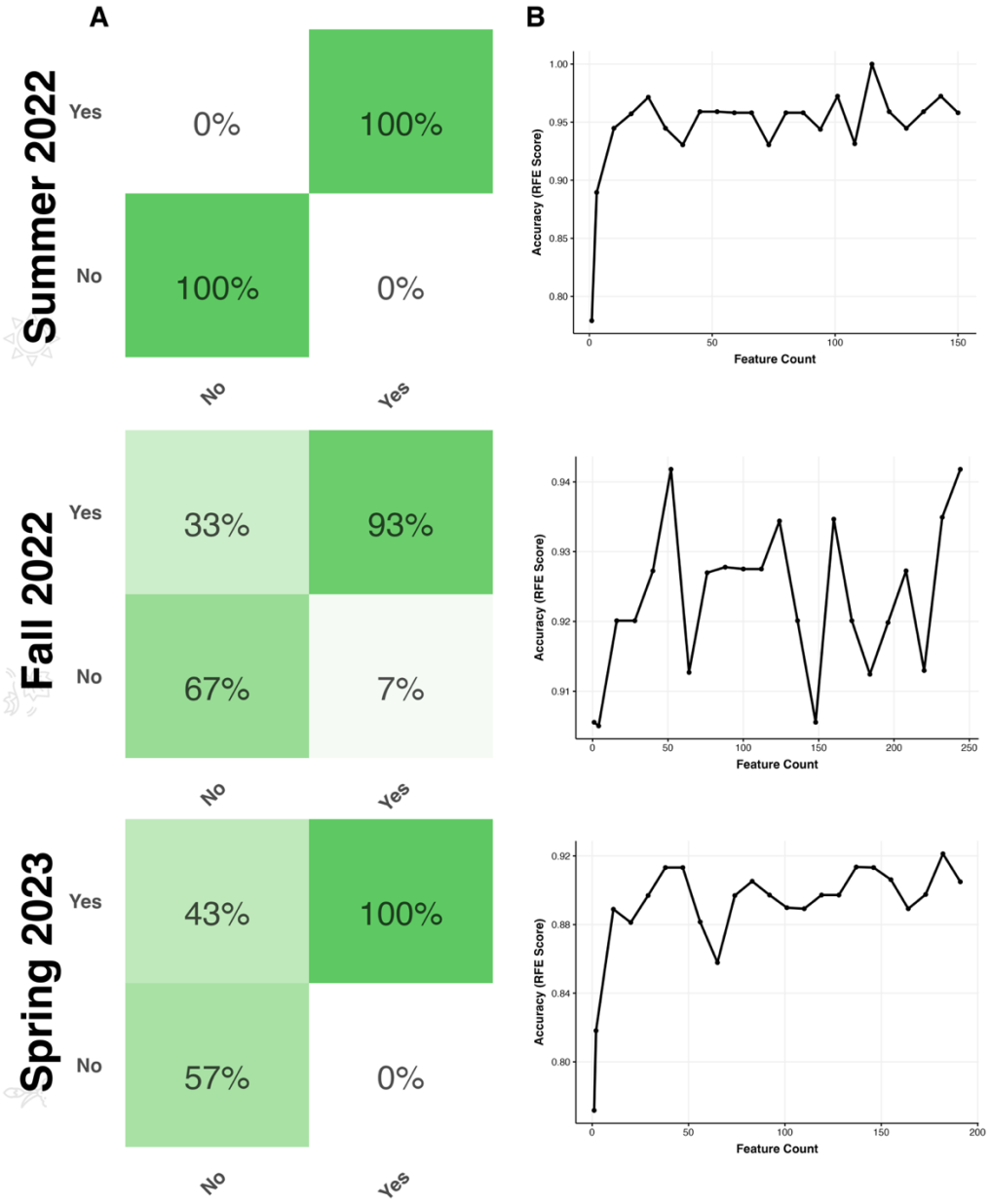

Figure S11. Supervised learning classifier accuracy as confusion matrices, by season. The leftmost column are the confusion matrices, where x = actual and y = predicted. The rightmost column expresses model accuracy as a function of sample feature count.