THE EFFECT OF NEST ARCHITECTURE ON NEST MICROCLIMATE AND MICROBIOME ASSEMBLY IN TROPICAL BIRDS

by

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DISSERTATION ABSTRACT

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Animals interact with complex microbial communities (i.e., their microbiomes) throughout their lifetimes. Microbiomes can have important effects on their host's fitness and survival, but it is unclear how newborn hosts are initially colonized. This dissertation focuses on initial microbiome assembly and consists of three main parts: i) the description of a new perspective on the study of microbiome assembly (the *nidobiome* approach), which considers nests (and especially nest architecture) to be central to initial microbiome assembly; ii) a comparative study of the ability of tropical bird nests of various architectures to regulate temperature and humidity under natural environmental conditions, and iii) a study of nest architecture as a driver of initial microbiome assembly in the chicks of several species of tropical birds.

In the first part of this dissertation, I propose an integrative framework to study initial microbiome assembly, considering parents, nest and nestlings as an interacting unit: the nidobiome. In the nidobiome, nests have a central role at funneling parental inputs, by direct transmission and by indirect environmental modification. I propose the nidobiome framework as a way to better understand initial microbiome assembly.

In the second part of this dissertation, I provide evidence of important differences between the microclimate of temperate and tropical nests. Tropical nests do not appear to be insulative in nature, relying instead on evaporative cooling for avoiding the maximum environmental temperatures. To my knowledge, this is the first time that a nest has been suggested to utilize evaporative cooling to regulate internal conditions. This observation suggests a novel way for nests to alter their resident microbiome and alter initial microbiome assembly in tropical birds.

In the third part of my dissertation, I report that nest architecture affects the microbiome of the nest walls and the gut microbiome of nestlings from a number of different tropical bird species. To my knowledge, this is the first evidence that microbiome composition can respond to nest architecture. Overall, my results show that differences in nest architecture can impact both the abiotic conditions and the microbiome inside the nest.

This dissertation includes both previously published/unpublished and co-authored material.

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To you who never left me alone even when the dark was upon me
To my Hñähñu ancestors and all those native scientists
that carefully watch and learn

"Ra ngugagätu̱, xi ra tsani ha ra tsant'i;
xä nthoki ga ndinthe ha ga zanthe 'ne ga xi'yo"
Hñähñu (otomi language)

"El nido de la chuparrosa es muy pequeño y redondo;
está hecho de alga verde limón, de alga verde como cabello y de lana"

"A hummingbird nest is very small and round;
it is made of lime-green algae, hair-like green algae and wool"

Hñähñu dictionary – Indigenous vocabulary,
2010 Instituto Lingüístico de Verano, A.C.

TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

Table Page

CHAPTER I

INTRODUCTION

The unknown process of microbiome assembly in newborn animals.

Animals have profound interactions with the microbial world [1]. They host entire communities of microbes on their bodies (i.e., their microbiomes), with whom they interact in varying degrees of intimacy depending on the microbial species and the host's organ or tissue in question. There is growing evidence of the effect of microbes on the host's body, which include microbial aid in food digestion and fermentation, proper development of the adaptive immune system, protection against microbial pathogens, the ability to process and degrade environmental toxins, and even complex and long term effects such as neural development and social interactions [2]. Despite the evident importance of microbiomes in their host's fitness and development, the initial microbial colonization of a host remains obscure.

From a microbial perspective, initial microbiome assembly will depend on dispersal and the ability to find a suitable host to colonize, and from a host's perspective, it becomes a matter of microbial exposure. Environmental exposure has been an important factor shaping the microbiome of newborn hosts, making the initial environment where a host is born or hatches a pivotal factor in microbiome assembly. In this context, nests take a central place in early microbiome assembly, as they represent the first environment that a host will face after birth or hatching. In this dissertation I focus on the role of nests as microbiome modifiers during microbiome assembly in newborn animals.

The effect of nesting strategies on microbiome assembly

Nest construction is a widespread behavior in the animal kingdom, with nests traditionally considered as delimited structures that are built by one or both

parents and provide a different microenvironment in relation to the exterior. However, nests present a wide diversity of forms and designs. In this dissertation, I consider a nest continuum spanning from mere site selection to lay eggs or give birth to the complex and long-lasting nests of social insects. Each of these nests represent different degrees of environmental modification, influencing local factors such as exposure to wind and solar radiation, oxygen levels in aquatic nests, and thermal fluctuations.

The nest environment has been shown to be an important modifier for microbial communities. Parents can reduce microbial loads by using specific nest materials or body secretions with antimicrobial properties, and display incubating behaviors that reduce microbial loads inside the nest. Parents can also directly inoculate specific microbes into their nests, that will later colonize their eggs and newborns. This constant interaction between parental inputs and nest properties lead to the microbial colonization of newborns, setting the unique environment provided by nests under evolutionary pressure from a microbiome perspective. Therefore, parents, nest and offspring represent an ecological and evolutionary unit that strongly influences initial microbiome assembly, with fitness consequences for newborn animals and overall breeding success. I call this unit the "nidobiome" and invite other scientists to consider microbiome assembly under this integrative conceptual approach. The second chapter of this dissertation consists on a published perspective article coauthored with Dr. Brendan J.M. Bohannan in which I discuss in detail the importance of the nidobiome approach for understanding microbiome assembly in animals and how important it is to include a microbiome perspective along with other traditional factors (i.e., predation, competition for nesting sites) in order to understand nesting success and nest evolution.

Bird nests and microbiome assembly: a tropical perspective

Birds are an ideal model to study initial microbiome assembly given the presence of external embryonic development, nest construction, and parental care, allowing for a detailed exploration of the role of each component of the nidobiome

unit in microbiome assembly. Nest construction in birds is varied, spanning from shallow ground depressions such as the nests of plovers, to complex nests weaved with twigs and grasses such as the nests of orioles. In the third chapter of this dissertation I focus on the role of nests in modifying the nesting microenvironment, while in the fourth chapter I explored the interaction between nest and nestlings during microbiome assembly. Chapter III and IV consist of unpublished material that will be published in collaboration with Dr. Brendan J.M. Bohannan.

Another advantage of birds as a model to study microbiome assembly is the extensive body of literature regarding bird reproduction and nesting behaviors. However, despite the high attention that bird reproduction has had in the past, most detailed descriptions of bird breeding biology, and most particularly the studies of nesting conditions, have focused on temperate species. Such bias in research has led to assumptions and extrapolations of breeding biology to tropical species, often assuming that such biology is analogous to that of temperate birds. Although some of these patterns are undoubtedly shared by birds in general, there are important particularities that arise in tropical ecosystems. For example, temperate regions can offer a challenging environment for egg incubation, given that environmental temperatures can reach freezing conditions at night, risking egg viability and implying important metabolic costs of incubation. Such thermal variation is very rarely present in tropical ecosystems, and most tropical birds do not face the same temperate challenges for incubation and nest construction in relation to environmental temperatures. In the third chapter of this dissertation I explore important differences between the microclimates provided by tropical nests and temperate nests.

CHAPTER II
THE NIDOBIOME: A FRAMEWORK FOR UNDERSTANDING MICROBIOME ASSEMBLY IN NEONATES

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Highlights

An animal's associated microbes (its "microbiome") impact its fitness, representing a significant ecological and evolutionary factor. Animals begin life without a developed microbiome, making the first encounter with environmental microbes particularly important.

Nests have been increasingly recognized as important drivers of microbiome assembly in neonates.

The nidobiome concept integrates parents, nest and neonates to better understand initial microbiome assembly. This concept identifies the roles of parents, nest and neonates as microbiome modifiers, emphasizing their interactions and highlighting gaps in our knowledge of microbiome assembly.

It also recognizes the particular developmental stages during which microbial interactions can be especially important. Identification of such stages allows for comparison of microbiome assembly across animal species.

The need for a new conceptual approach to understanding microbiome effects on neonate fitness

The importance of microbial associations to animals' lives is widely recognized [1]. Microbes allow hosts to access nutrients [3], help develop functional organs and systems [4], process toxins [5], and resist pathogens [6], among many other fitness benefits [7]. Although microbes may colonize the reproductive tract of parents and can interact with both gametes since before fertilization [8,9], most healthy newborns begin with scarce microbial associations that rapidly increase in abundance and complexity after birth [10,11]. This entire collection of microbial inhabitants (i.e. the **microbiome**, see Glossary) goes through an assembly process with critical ecological and evolutionary implications [7,12–14].

Our understanding of **microbiome assembly** in **neonates** has greatly improved with the development of next-generation sequencing technologies [15–18], allowing in some cases for the identification of specific microbial sources during initial microbiome assembly (such as parents [19] or the rearing environment [20]). However, multiple individual sources interact during microbial colonization [16,21,22] and their contributions can vary through time [16]. Therefore, the explicit consideration of multiple factors and the dynamic nature of microbial colonization can broaden the perception of the process and better guide experimental designs. The **nidobiome** concept ("nee-doe-biome", nido=**nest**) integrates the combined effects of parents, nest and **nestlings** on initial microbiome assembly, their interactions over time, and the inherent feedbacks between hosts, their microbiomes and the surrounding environment [23].

As we detail below, the nidobiome concept extends beyond a nest's individual microbiome, using a multiscale approach that builds on other conceptual frameworks (including the holobiont concept [24] and the idea of microbiomes-as-metacommunities [23]). It brings together three key elements involved in the neonates' microbial colonization: 1) the nest as the **built environment** intermediate between the neonate and its primary microbial sources (parents and the environment), 2) the parents as both nest architects and microbial sources, and 3) the neonate, the new host environment to be colonized (Box 1). The nidobiome

framework not only considers the elements involved in initial microbiome assembly but also their interactive contribution at different stages of nestling development, as well as providing a framework for biologically meaningful comparisons across animal species, from model organisms to wild species.

The nidobiome concept hypothesizes that the quantitative contributions of host genetic variation, environmental variation (including nest construction) and variation in plastic host responses (e.g. learned nesting behavior) interact with each other to determine the neonate's microbiome and its fitness consequences. In this paper we focus on the contributions of environment and nesting behavior to microbiome assembly, leaving host genetics for a future discussion. The nidobiome framework addresses early microbial associations given their disproportionate effect on host fitness compared with associations at older ages [16]. Our framework can offer a template to study microbiome dynamics at older ages, and can be personalized to specific microbiomes (e.g. oral, skin, gut) when needed. Most studies of microbiome variation can explain only a small fraction of the variance in microbiome composition across individuals [e.g. 13,18,19]; the integrative perspective of the nidobiome framework could improve this, as well as lead to the development of new analytical approaches that take into account multiple simultaneous drivers of microbiome variation.

We propose the nidobiome concept fully recognizing that new concepts and their associated vocabulary risk hindering the development of a new field by losing connection with previous research. However, the potential benefits of the nidobiome framework outweigh these risks, because it provides a flexible developmental timeline based on previous knowledge about nesting ecology and microbiome assembly, a framework that can lead to a better understanding of the complex interactions that drive microbiome assembly. Although we focus primarily on birds as a model (given the extensive knowledge of bird breeding biology), our framework also applies to other taxa, since nest construction is a widespread behavior spanning aquatic and terrestrial animals [26,27].

The *nidobiome* concept: an integrated approach to understanding microbiome assembly

Nests are central to microbial colonization of neonates

Parents select, modify, and construct particular environments (i.e. nests) where their offspring will be born or hatched [28]. Nests represent the immediate built environment faced by newborns before being exposed to external conditions. Animal clades show different degrees of nest construction (Box 1), with complex nesting behaviors including both invertebrate (e.g. social insects) and vertebrate species (e.g. fish, crocodilians, rodents). At first glance, a nest consists of a defined structure for birth, or egg incubation and hatching, usually hosting at least part of the offspring's initial development [29]. However, nests are highly diverse structures that range widely in form, function, and parental involvement [26,30]**.** There is evidence that initial microbial colonization in animals can be driven by dispersal from the immediate environment [31–33], enhancing the role of nests as potentially important sources. Nests likely regulate microbial exposure by two mechanisms: acting as a microbial source via their constitutive materials [32], and acting as a microbial filter such that only some microbes can successfully establish within a particular nest microenvironment [21,34–36]. Therefore, nest features likely shape the first interactions between neonates and microorganisms [25,32,33].

Nests display a gradient of microbial filtering

Nests present a gradient of environmental modification, depending on parental involvement and structural complexity. On one extreme are species that provide extensive structural modification (i.e. nest construction) and care (e.g. woodpeckers, squirrels). At the other extreme are those species whose nests only consist on selecting a site to lay eggs or give birth, without subsequent structural modification or attendance (e.g. cane toads – *Rhinella marina*, catsharks; in Box 2 we discuss how

the nidobiome framework could apply to species lacking a nest). Following the nidobiome framework, we predict that neonatal microbial assembly on species without nest construction and little to no parental care, will resemble local environmental fluctuations in microbial pools. In contrast, we predict a differential effect on the order and timing of arrival, abundance, and diversity of early colonizing microbes with higher degrees of nest construction and parental involvement.

The nest environment affects microbes

Nest architecture and nest materials act together to create particular conditions inside the nest that impact nestling development and survival [29,37,38]. Evidence from birds, frogs, and mammals show that parents alter their nests according to environmental pressures, for example modifying their nest's architecture to buffer the nest's microclimate against external temperatures [30,37–39]. However, the total impact of the nest environment on the nest's microbiome remains unclear. Nest architecture can modify microbial exposure in birds, as eggs placed in cavity nests showed lower bacterial loads compared to eggs on cup nests [40]. Structural features, such as the use of specific aromatic plants or feathers as nest materials, also decreased bacterial loads in avian nests [32,41,42], an important trait as lower bacterial loads are correlated with higher hatching success in birds [35]. In addition to altering the nest's architecture, parents can actively decrease the nest's microbial loads with behavioral responses such as incubation [43,44], nest sanitation [45], and by covering eggs with antimicrobial coatings as seen in birds, frogs, fish, and carrion beetles [26,33,46–48].

The combined effects of nest structure and parental inputs create a selective environment that contributes to the nest's microbiome, and eventually to the microbial colonization of nestlings. For example, in a cross fostering experiment with European cavity nesting birds the nestling's gut microbiome resembled the microbiome of the nest where they were reared instead of that of its own species

[25,49]. This is surprising given that animals usually host species-specific microbiomes [49–51]. Similarly, cuckoo nestlings (*Clamator glandarius*) parasitizing nests of other birds had gut microbiomes intermediate in composition between the parasitized species and the adult cuckoos [22]. We hypothesize that nesting strategies that lead to advantageous microbiomes would be favored by natural selection. The nidobiome framework provides an evolutionary perspective integrating the role of microbes as a factor influencing nest diversification and the evolution of mechanisms influencing initial microbiome assembly (Box 3).

The nest as an extended microbial cloud from parents to offspring

Nests have been considered extended phenotypes, where the fitness of the architect is assessed through multiple nest traits, especially during sexual selection in birds and fishes [26,52]. The nidobiome concept expands on this idea, proposing nests as an extension of the parent's "microbial cloud", essentially a microbiome-based extended phenotype. Parents are known to successfully inoculate their own microbes into their nests [33,53], making their nest a possible vector for microbial inheritance. Necrophagous beetles (*Nicrophorus vespilloides*) and Hoopoes (*Upupa epops* – a cavity nesting bird) constitute two examples of direct parental inoculation of the nest's microbiome. Adult necrophagous beetles spread oral and anal secretions into the carcasses where their larvae develop, successfully transmitting specific microbes to their young [33]. In a similar way, the nest walls and the eggshell of Hoopoes are spread with the mother's uropygial gland secretions [48], promoting a specific microbial community only present on incubating females and nestlings [54]. Microbiome similarity between mothers and their nests has been described in other systems, but the mechanisms underlying these patterns have not been described [19,53]. In species with parental incubation, parents will remain inside their nest during most of the day for a couple of weeks, likely transferring their associated microbes into the nest materials and ultimately to their offspring, making the nest an indirect mechanism of microbial inheritance.

The nidobiome concept also considers direct transmission as an important driver of microbiome assembly. Animal parents can share microbes with their offspring before birth, during egg formation or pregnancy [8–10,55]. Post-birth direct mechanisms of microbial inheritance include direct contact [12], mammalian milk [56], avian crop milk [57], and mouth-to-mouth feeding, as in avian regurgitation [58]. Such early colonizing microbes could have advantages inside the controlled nest environment, as the nest can be buffered against microbial dispersal from the exterior and decrease the probability of invasion from microbes outside the parental inocula. We predict that natural selection will favor nesting environments that increase the contribution of parental microbiomes to neonates, relative to the contribution of microbes from the surrounding environment.

The nidobiome concept is related conceptually to other perspectives regarding host-microbiome interactions, such as the Holobiont concept and the perspective of Hosts-as-ecosystems [59]. For example, within the Holobiont concept nests can be seen as an extension of the parental microbiome, representing a microbial extended phenotype. In the Hosts-as-ecosystems perspective, the nest would represent an environmental reservoir of microbes ready to colonize the neonate. Each of these approaches can generate a particular set of predictions within their view, but the pluralistic approach employed by the nidobiome framework combines the advantages of previous host-microbiome perspectives [59].

Nestlings as new environments to colonize

The simplified microbiomes of neonates [8,10,60,61] rapidly increase in complexity and abundance after birth or hatching [11,16,25,56,60]. Early microbial colonizers can elicit physiological and anatomical responses in the newborn host, shaping its microbiome and influencing its future fitness [13,56]. Studies in model organisms such as mice (*Mus musculus*), zebrafish (*Danio rerio*), and bobtail squid (*Euprymna scolopes*) have shown that neonates do not develop properly under axenic conditions, even when exposed to microbes later in their lives [1,2]. This highlights

the importance of microbial exposure during specific host developmental windows, and the time sensitive interactions between the newborn and its microbial sources.

The *nidobiome* across time: using key developmental events to understand microbial assembly and compare diverse life histories

Merging multiple microbial sources in a time-sensitive context presents a great challenge to understanding microbiome development in neonates. Considering environmental exposure and parental transmission independently simplifies experimental design but introduces conceptual limitations, failing to account for the combined effects of the nest, parents and the environment on initial microbial colonization. An alternative way to simplify experimental design is to partition microbiome assembly into particular stages aligned with the nesting process. Then at each stage, one can explicitly examine the microbial interactions between nest, parents and neonates.

Microbiome assembly is a dynamic process

Microbial exposure during early juvenile development presents a constant influx of microbial taxa [25,60]. In humans, developmental changes such as shifts in diet from initial breast feeding to solid food, and the onset of puberty, have been linked to changes in microbiome composition [14,16,62]. Similarly, shifts in gut microbiome composition of young zebrafish correlate with developmental changes such as mouth opening, the onset of adaptive immunity, and the onset of sexual maturity [61]. These dynamic interactions highlight the importance of host development and the identification of factors influencing microbiome assembly, at the time of sampling.

Microbial exchange between the nidobiome elements vary across the nesting season

The interactions between the nidobiome elements vary in length and degrees of intimacy across the high diversity of nesting strategies in animals. Parents of some species only indirectly interact with their offspring by selecting a site at which to lay their eggs or give birth (e.g. some snake species) while parents from other species will stay in contact with their offspring long after birth (e.g. macaws). In species with parental incubation, the immediate time after hatching represents the highest level of superficial microbial exchange across every element of the nidobiome, as the newborn will be in direct contact with the brooding parent and the nest materials [63]. With nestling development and the onset of thermoregulation, the presence of the parent as an incubator will decrease [63]. This will reduce the length of direct contact between parent-nest and parent-nestling, likely decreasing microbial exchange via direct contact between them. Regardless, parents will remain as microbiome modifiers by determining the nestlings' diet [25,56] and by cleaning the nest [64]. At this later stage, direct contact will mostly occur between nestlings and the nest until nest departure. In species without parental care, other parental traits (i.e. egg coatings) can still influence the microbial associations of neonates [33,46] but their direct effect will be restricted to a particular nesting stage.

Comparing microbiome assembly using key developmental events

Instead of assuming a standardized life history model as a baseline, we propose the use of key events with important implications for microbiome assembly and offspring development as landmarks to compare microbiome assembly across the high diversity of animal species. We provide a timeline template to compare microbiome assembly at similar developmental stages across animal species, regardless of potential differences in life history traits such as developmental rates, nest architecture, and parental involvement (Box 4). This flexible timeline can be expanded to include new developmental events in a chronological way or delete them if not experienced by the species of interest. Our timeline aims to integrate information from highly studied species (e.g. model organisms) to guide hypothesis

testing in unexplored systems (e.g. wild species), while accounting for differences in life histories.

Integrating the environmental context in microbiome assembly

When comparing initial microbiome assembly across different life histories, the environmental context in which they occur should be taken into account [18]. For example, the gut microbiomes of Mesquite lizards (*Sceloporus grammicus*) varies with altitude [65] and the gut microbiomes of howler monkeys (*Alouatta pigra, A. palliata*) and moose (*Alces alces*) varies across seasons [66,67]. Such environmentally-driven changes in microbial composition can have functional consequences that could potentially effect fitness; for example, the summer gut microbiome of moose can be inadequate for digesting their winter diet [67]. Since the microbiomes of adult animals can act as a microbial source for their offspring, such seasonal variation could have implications for neonatal microbiome assembly.

Animals generally have a defined breeding season, usually peaking within the period of highest resource availability, and triggered by environmental cues such as photoperiod, temperature, and plant phenology, as seen in birds [63]. Given that our general understanding of microbial responses to seasonal environmental changes remains incomplete, we encourage environmental characterizations at each stage of the nidobiome timeline to integrate environmental characteristics into microbial colonization. Reporting environmental conditions when sampling host-associated microbes can facilitate the comparison of patterns of microbiome assembly between seasons within the same location or across sites.

Concluding remarks

The goal of the nidobiome concept is to provide a flexible yet detailed framework for the study of initial microbiome assembly. The clear differentiation of elements, processes and key developmental events should make the nidobiome approach a

useful tool for hypothesis testing and experimental design. At the same time, it provides a flexible framework for successful comparisons between different clades from the wide diversity of life histories, allowing the integration of model systems and wild organisms. Framing the microbial colonization process through universal developmental stages can allow for predictions even in complex scenarios, such as under field conditions. The integrative nature of the nidobiome framework has the potential to improve our understanding of initial microbiome assembly and disentangle the mechanisms involved in microbial inheritance, inspiring future research (see Outstanding Questions).

Outstanding Questions

How prevalent are effects of the nesting environment on the neonate's microbiome? In what ways is microbiome assembly affected by the nesting environment?

How do effects of the nesting environment on microbiome assembly affect the neonate's survival and fitness?

Could selection for microbiomes that increase neonate fitness drive nest diversification? How strong is such selection relative to other drivers of nest diversification, such as predation or environmental buffering?

Do different host lineages rely to different degrees on nests to shape microbiome assembly in their neonates? Are the specific mechanisms used to modify the neonate's microbiome via nests phylogenetically conserved?

Does the nest environment select for particular microbial functions instead of particular microbial taxa?

Does the nidobiome have lasting effects on neonates (even after they have left the nest)?

Can we predict patterns of neonatal microbial colonization by measuring specific nest characteristics? If so, should we expect higher levels of microbial inheritance between parents and offspring in complex or highly maintained nests?

Are parents capable of assessing microbial colonization of the nest (e.g. via chemical signals detected by olfaction or taste) and respond accordingly?

Does the nidobiome influence microbiome assembly differently at different body-sites? Are external microbiomes (skin, feathers) more susceptible than internal microbiomes (gut, lung)?

Does the nidobiome's influence on microbial colonization scale with the density of nests?

Box 1. The nidobiome elements

The nidobiome concept creates a framework integrating multiple elements influencing microbiome assembly in newborns. Nest and parents act as **microbiome donors** through direct contact, but also as **microbiome modifiers** by jointly creating an incubation chamber that establishes a selective environment where only certain microbes can survive. The nest environment is determined by its materials and architecture [32], which widely vary across animal species. However, nests share three common features: they involve some level of environmental modification, they provide an extra level of safety for the newborns, and they funnel parental care into a single location (Fig. I). These features are complemented with incubation, sanitation, egg coatings, and other parental inputs, impacting microbial establishment [33,43,46].

Nests presumably play a central role in microbiome assembly (Fig. I) since the interactions between newborns and parents occur primarily within the nest. These interactions vary throughout the nesting season and their impact on initial

microbiome assembly should likely vary as well (see Box 4). Parental behaviors such as site selection and nest construction, represent important sources of innovation, given the potential learning from one nesting attempt to the next and from neighboring individuals [52]. Parents also represent microbial vectors when feeding their offspring, as diet greatly influences microbiome composition [22,25,56,62].

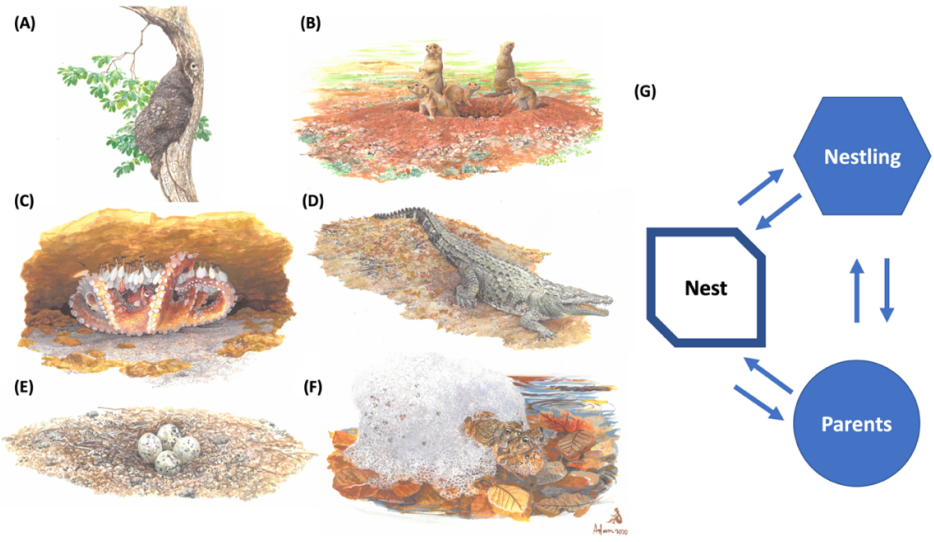

Figure I. Nest features. Environmental modification, protection, and directed parental care are clearly evident on nests of certain species, as in A) the controlled climates of termite nests [68,69], B) the underground nests of prairie dogs (*Cynomys sp*), or the parental protection of C) octopi eggs [70,71] and D) the leaf litter nests of crocodilians [72]. However, even non-apparent environmental modifications impact the conditions around eggs and neonates, such as E) the small ground depressions of snowy plover (*Charadrius nivosus*) nests that reduce the egg's temperature [73], and F) the mix of proteins providing insect and antimicrobial protection to eggs and tadpoles in the untended foam nests of Túngara frogs [*Engystomops pustulosus,* 22,35]. (G) Parents, nest, and nestlings interact as a unit during microbiome assembly.

Newborns represent the new host environment to be colonized and the final indicator of nesting success. **Nestlings** constantly act as microbial recipients and

microbiome modifiers, given their physiological and behavioral responses to microbial colonization. Their individual growth rates set specific time windows for certain developmental processes, some of which are highly influenced by microbes and cannot be fulfilled once the developmental window is closed [2].

Box 2. The nidobiome framework at the boundaries of nest construction

Nests are central within the nidobiome framework, providing a physical environment that develops its own microbiome while mediating microbial transfer between parents and their offspring. However, some species lack physical nests and give birth to offspring that must be immediately self-sufficient (e.g. spiny lizards – *Sceloporus sp.*). In the absence of a physical nest, parent-offspring interactions will be directly surrounded by the broader environment, increasing the chance that random processes will influence microbiome assembly. Even without a physical nest, parental care can lead to additional mechanisms impacting initial microbiome assembly.

For example, gregarious animals (e.g. mammalian herds, schooling fishes) present particular opportunities for microbial transmission between adults and early juveniles, as rates of social interactions can impact microbiome composition [75] potentially decreasing the effect of vertical transmission and increasing horizontal transmission from conspecifics. In cases where the parent's body functions as a "nest" (e.g. marsupial pouches, anurans carrying their tadpoles on their backs or mouth-brooding fish), we expect microbial transfer to mostly depend on direct contact between parent-offspring with minimal microbial input from the birth site. Lastly, providing no physical nest or parental care restricts the chances of microbial inheritance to pre-birth mechanisms and to any colonization through the birth canal. In all of these cases, the absence of nests precludes the direct application of the nidobiome framework. However, this framework remains useful as a guideline to account for multiple sources during microbiome assembly and highlight the importance of developmental stages during this process.

On the other extreme, some animals construct nests where multiple subsequent generations are born. Multigenerational nests can be occupied only during the breeding season (e.g. macaw nests) or all year round (e.g. prairie dog colonies, termite nests). We predict constant occupation to increase the host-associated microbes inside the nest, including pathogens. However, hosts can regulate microbial growth inside their nests in different ways, including the inoculation of bacteria with antifungal secretions of beewolf (*Philanthus triangulum*) nests [76] or restricting microbe-rich items to a section of the nest (latrines of prairie dog colonies). We predict multigenerational nests to enhance direct microbial inheritance and display constant mechanisms of microbial control, due to pathogenic risks. For multigenerational nests, the nidobiome framework could be modified to study microbiome dynamics later in host development (e.g. adulthood, senescence), considering host-host interactions inside the nest and changes in the nest microbiome itself.

Box 3. Evolutionary implications of the nidobiome

Microbial associations can alter host fitness [7,13,35,74], potentially making initial microbiome assembly subject to considerable evolutionary pressure. The nidobiome provides an integrative approach to identify adaptive innovations shaping initial microbiome assembly. We explore below the implications of the nidobiome, focusing on two areas of evolutionary thought: niche construction theory and life history evolution.

Niche Construction Theory

Niche construction theory states that organisms can alter their evolutionary trajectories by modifying their environments, creating an "ecological inheritance" [77]. In the nidobiome framework, the nest represents a constructed environment that hosts only a portion of the available environmental microbes, promoting specific microbial associations and modifying microbial inheritance. As the ability of

the nest to create its own environment increases, a distinctive form of ecological inheritance should increase as well. Therefore, we predict that complex nesting strategies will lead to more stable microbial transmission rates from parents to offspring, increasing fitness gains on neonatal development and survival. Also, the nidobiome framework incorporates non-genetic evolutionary novelties with fitness implications, such as changes in microbiome assembly due to behavioral learning from one nesting attempt to the next.

Life history evolution

Given that microbiomes can alter their host's fitness [7,13,35,74], we consider that microbial exposure should be included among other evolutionary factors shaping life histories, such as predation, competition, and environmental selection. There is already evidence for a diverse set of life history traits that influence microbial exposure of eggs and neonates, including incubation behavior, antimicrobial coatings, and nest sanitation [45,46,78]. The resultant microbiomes can impact early life stages by providing microbial protection [33], higher growth rates [60], and complex advantageous phenotypes, such as enhanced social behaviors [2]. The nidobiome provides a framework within which to identify life history traits that may be under natural selection during reproduction, due to their impact on initial microbiome assembly. We hypothesize that the advantageous aspects of such traits at least partially led to the diversification of nesting strategies.

Box 4. Using nesting stages to compare microbiome assembly

Comparing microbiome assembly across species using time units (e.g. days after birth) can be confusing, given differences in developmental rates. We provide four basic stages of nesting biology as a more informative alternative. Given that host development and microbial colonization interact [13,61], we provide examples of developmental events with important implications for microbiome assembly that can be adapted to any study system.

1. Nest site selection to nest construction. Microbes must be able to disperse into the nest to colonize nestlings. Nest location and nest architecture determine the nest's microenvironment by modifying environmental variables such as solar radiation, humidity levels, and wind exposure in terrestrial nests, including water flow and oxygen levels in aquatic nests, as seen in crocodiles, birds, frogs and fish [26,30,38,79,80]. Variations in nest architecture and nest materials can directly impact the nest's microbiome [32,40]. Nest reuse must be considered as well, as old nests may contain microbes from previous nesting attempts [78].

2. Nest completion to egg laying. The particular environment inside the nest gets established with nest completion and the onset of parental incubation, if present [29]. Most microbial colonization of the egg surface occurs after egg laying [19,32,48,81], and microbial communities are shaped by the nest environment [32,43], parental behaviors [43,44]**,** and the egg's antimicrobial defenses [78]. Each of these factors present variations across species. For example, parental care can be provided throughout development, as in male fish constantly attending their eggs with antimicrobial secretions [82], or just early after egg laying, as in adult Túngara frogs allocating antimicrobial peptides into their untended nests [46].

3. Hatching-birth and development within the nest. Birth increases the rate of interactions between the neonate and environmental microbes, even if such encounters do not develop into long term associations [83]. The neonate's microbiome responds to important systemic changes, such as the onset of the adaptive immune system [64], and dietary shifts [16]. Given their overall impact on the neonate's physiology, we suggest the onset of thermoregulation, and hair and feather emergence as potentially important events for microbiome assembly. We expect eye and mouth opening to be especially important for aquatic taxa, as they likely encounter a higher proportion of active microbes than terrestrial taxa.

4. Fledging. After nest departure, nestlings will be exposed to a broader diversity of microbes outside the nest. The fledging's microbiome will be shaped by its individual behavioral and physiological responses, and parental care.

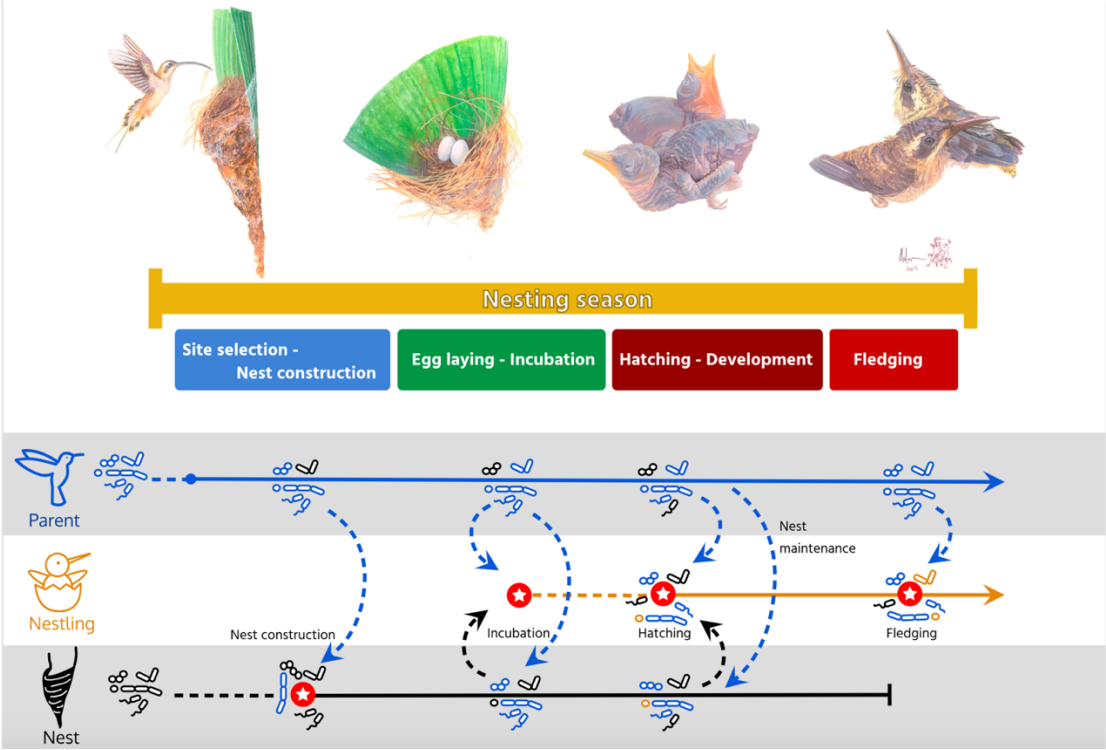

Figure I. General nidobiome timeline. Important events of nesting biology for microbiome assembly. Arrows represent the direction of expected microbial transmission between elements, leading to mixed microbiomes from several microbial sources. Color of microbes represent their source. Parental and nestling's microbiomes will continue to change after nest departure, but nests may not persist for the next reproductive event. These events represent a baseline to be adapted to other systems, where the nidobiome elements (parents, nest, offspring) involved at each stage may vary.

Glossary

Built environment: a modified environment that creates particular conditions that differ from the non-modified environment. In nests, examples of these modifications can be physical, with walls changing thermal and hydric conditions inside the built environment, or chemical, when providing secretions that prevent microbial growth.

Microbiome assembly: the process of dispersal and establishment of microbes associated with a host. Nesting strategies can determine environmental exposure and local microbial availability.

Microbiome donor: anything that acts as a source of microbes to the neonate. This includes the nest and the parents, as their associated microbes can be transferred to the neonate.

Microbiome modifier: an element that shapes the microbial community to which the neonate will be exposed. Both parents and nest create a particular microenvironment where only a fraction of the external microbes are present, successfully modifying the microbial pool available to colonize the neonate.

Microbiome: the community of microbes associated with a host, including microbes inhabiting its internal organs and its external surface. Although a majority of the animal microbiome literature is focused on the gut microbiome, the nidobiome framework applies to multiple host microbiomes (e.g. skin and feather microbiomes).

Nest: the concept of "nest" can refer to a variety of animal constructed structures. We consider a "nest" as the particular microenvironment selected to lay eggs or give birth. In principle, a nest represents microenvironmental conditions that differ from the broader environment, even when such differences might be subtle or cryptic. Nests exist on a continuum of parental involvement, environmental modification, and temporal permanence, spanning from simple ephemeral structures such as shallow ground depressions to the complex nests of social insects that last for multiple generations.

Nestling: the neonate during its stay inside the nest. Most neonates remain in their nests for some time after birth or hatching (minutes to months). This period can facilitate microbial transmission from the modified nest environment to the neonate.

Neonate: the recently hatched or newborn animal, hosting few to no microbes (later called nestling). Neonates go from the essentially sterile conditions of the egg or womb, to a much richer microbial environment on the exterior. Neonates deploy responses that lead to either prevent or establish microbial associations that can impact its future development.

Nidobiome (ˈni.ðo -ˈbaɪˌoʊm): the interconnected system shaping the microbial colonization of neonates. The nidobiome represents the ecological unit where parents, nest and offspring exchange microbes. Such microbial associations can affect the offspring's fitness and the overall breeding success of the adults. Similarly, the nidobiome functions as an evolutionary unit by maintaining and enhancing the interactions between members that lead to initial microbiomes with fitness advantages in breeding success over evolutionary time.

BRIDGE

The nidobiome framework was presented in detail in Chapter II, where I covered how parents, nest, and offspring interact during initial microbiome assembly. The ecological and evolutionary implications of the integrative framework of the nidobiome were also discussed, suggesting future avenues of research. In Chapter III, I explore the role of tropical nests in providing a nest-specific environment that differs from the external environment, one that could impact not only eggs and nestlings but also the nest microbiome. In Chapter III I provide the first detailed description of the microclimate provided by tropical bird nests and how microclimate patterns of tropical bird nests deviate from that of temperate nests.

CHAPTER III

TROPICAL NESTS CAN AVOID EXTREME HEAT BY EVAPORATIVE COOLING

This chapter involved the combined effort of multiple people. Sebastián Montejo, Rosi Ramos Pinto, Jesús Ramos Pinto and Oscar Díaz helped during field work. Dr. Brendan J.M. Bohannan reviewed the current manuscript and verified data analysis. I was the primary contributor, doing all data analysis and all the writing. This chapter will be published in the Journal Biotropica.

1. Introduction

Nest construction is a widespread strategy that modifies the immediate environment where eggs and hatchlings will develop (Mainwaring, Hartley, Lambrechts, & Deeming, 2014; Méndez-Narváez, Flechas, & Amézquita, 2015; Refsnider, 2016). Nests provide a protective environment that enhances breeding success by mechanisms such as decreasing predation rates and providing a controlled microclimate (Kesler & Haig, 2005; Purdue, 1976), which increases the chances of embryonic survival (Griffith, Mainwaring, Sorato, & Beckmann, 2016). Such controlled microclimate is a result of parental incubation and the structural properties of the nest architecture, such as thicker nest walls and insulating lining materials (Akresh, Ardia, & King, 2017; Mainwaring et al., 2014).

Nest architecture can provide an important buffer from external temperatures (Akresh et al., 2017), especially in environments where high daily thermal fluctuations and extreme temperatures can be detrimental for embryonic development and nestling survival (Michielsen et al., 2019; Wiebe, 2001). In general, thermal properties of bird nests have received ample attention in temperate environments but little is known for tropical species, especially for basket and cup nests. Given the important differences in tropical vs temperate climates (i.e., daily thermal fluctuation, mean, maximum and minimum temperatures), it is

unreasonable to expect tropical nests to mimic the microclimatic properties of their temperate counterparts as those properties might not be advantageous in a tropical climate.

Although temperature is one of the most frequent climatic factors to be addressed, humidity is also an important climatic factor influencing the nesting environment (Biddle et al., 2019). Eggs seem to be well equipped to deal with low humidity levels (Booth & Rahn, 1990), but high humidity levels may lead to heat loses or developmental problems that reduce hatching success (Biddle et al., 2019; Heenan, 2013). Taken together, the high temperatures and high humidity common in the tropics can represent an important challenge for the nest microclimate. In this study, I focused on the structural properties of tropical nests and how such structures impact temperature and humidity inside the nest. I considered a gradient of environmental exposure across nest types, with cup nests being highly exposed, basket nests experiencing intermediate exposure given the presence of walls and a roof made of sticks and twigs, and cavity nests as the more isolated nest type, having solid walls and roof. I hypothesized that a) nests modify their internal microclimates and buffer the external environmental conditions, b) basket nests provide a stronger microclimatic modification than cup nests, given their enclosed structure, and c) cup nests show higher climatic variation given their increased environmental exposure with respect to basket nests.

2. Methods

2.2 Study site

An intensive search for bird nests was performed in 2015 from April to August in Palenque National Park, Chiapas, the southernmost state of Mexico. The park encompasses ~700 ha of mature tropical rain forest on top of an ancient Mayan city, surrounded by a mosaic of pastureland and secondary forest (Patten, De Silva, Ibarra, & Smith-Patten, 2011). Annual mean temperature is 26ºC (range 22-29ºC),

with heavy rains occurring between May and December, decreasing in frequency and intensity from January to April, for an overall mean annual precipitation of 2,200 mm (Estrada et al., 2002). Palenque represents an example of the tropical evergreen rainforests that expand from Southern Mexico to South America.

2.3 Data collection and analysis

We sampled 28 nests, consisting of 16 cup nests, 9 basket nests, 2 cavity nests and 1 ground nest from a total of 16 species (Table 1). Given their low numbers, ground and cavity nests were not included in the analysis, but their climatic patterns are available for contrast. After nest abandonment or fledging, temperature and humidity (Relative Humidity %) inside the nest were automatically recorded every 5 min during three consecutive days using automatic sensors (Hygrochron iButtons DS1923). A second sensor was simultaneously placed in the vicinity to register the temperature and humidity outside the nest. Individual measurements were averaged into 1 hr periods, resulting in a time series of 72 consecutive measurements of temperature and humidity per nest. To compare the conditions between the inside vs the outside of the nest I focused on the following characteristics of the time series, following Rhodes et al. (Rhodes, O'Donnell, & Jamieson, 2009): daily mean values, daily extreme values (maximum/minimum), overall daily fluctuation (range: max - min), and the hourly rate of change, equivalent to an AR1 transformation (value of hr_2 – value of hr_1, etc.), which accounts for the temporal autocorrelation of the data. For a more comprehensive analysis, I also included other descriptive variables of the time series such as daily variability (variance), time of the day when extremes occurred, and length of time that extreme conditions persisted (accounting for the sensor's sensitivity: 0.5 ºC, 0.5º RH). To test if nests provided different microclimates than the external conditions, I considered basket and cup nests separately and performed a mixed model with each of the previous variables as response variables, including the nest ID, date, and day (day 1, 2, 3 of sampling, nested within nest ID) as random effects, and the origin of the data (Inside/Outside) as a fixed effect. To compare the mean

temperatures and the overall rate of change (AR1 transformation) I used the 72 consecutive measurements (24 data points per day) and included "hour" as a random effect to account for the paired nature of the internal and external measurements ("Hour" was nested within day, day nested within nest ID). If a variable showed differences between the inside vs the outside for both nest types, I tested if either nest type provided a stronger effect by first calculating the difference between the internal and external values (inside – outside) and then comparing these values between nest types (mixed model: differences as response variables, nest ID, date, and day nested within nest ID, as random effects, nest type as fixed effect). This procedure accounts for the paired nature of the inside/outside data and maintains the degree of microclimate modification provided by each nest (SAS, 2020). All statistical tests were performed in JMP (JMP Pro 14; SAS Institute, Cary, NC, USA 2020).

Given that I had simultaneous values of temperature and relative humidity, relative humidity was transformed to Vapor Pressure Deficit (VPD) following Paw U et al. (Paw U & Gao, 1988), as a better indicator of biological humidity (Anderson, 1936). Relative humidity misleads the interpretation of water vapor saturation in the air, as it does not take into account the effect of temperature on the air's capacity to hold water: hot air can present higher percentages of relative humidity without representing a higher availability of water vapor (Anderson, 1936). Using a value of negative pressure, Vapor Pressure Deficit explicitly states how much water can still be held by the surrounding air, and therefore, provides a value of how much water can be evaporated from a surface (e.g., the eggshell, the nest walls) into the air. In this case, an environmental value of zero equals to air completely saturated with water or the point where condensation starts.

3. Results

3.1 Temperature

Mean temperatures inside the nest did not differ from the external temperatures regardless of nest type (Table 2, Figure 1). However, nest temperatures had lower variances than the external temperatures, with no differences between basket and cup nests (Table 2). Although extreme temperatures (maximum/minimum) occurred at similar times of the day and were kept for a similar amount of time than the exterior, maximum temperatures were lower inside nests with no differences between basket and cup nests (Table 2). Minimum temperatures did not differ between the outside and basket nests, while cup nests presented warmer minimum temperatures than the outside (Table 1). Thermal fluctuations were measured by two variables: the daily range (maximum temperature - minimum temperature) and the hourly rate of change in temperature (AR1 transformation). Both showed that basket and cup nests had smaller daily fluctuations in temperature in relation to the exterior, with no differences between nest types (Table 2, Figure 2)

3.2 Humidity

Mean humidity levels inside the nest were higher than the exterior in both basket and cup nests, although neither nest type was different with respect to each other (Table 2, Figure 3). Humidity inside the nests showed lower variation compared to the exterior, with no difference between basket and cup nests (Table 1). At peak moisture, basket nests reached similar levels to the exterior, while cup nests remained drier than the exterior (Table 2). At their driest point during the day, both nest types remained with higher humidity levels than the exterior, without differing between each other (Table 2). Basket and cup nests reached their peak and lower moistures around the same time as the outside, with basket nests remaining at peak moisture for the same time compared to the outside while cup nests lasted 1 hr less at peak moisture in relation with their exterior (Table 2). Humidity fluctuations were measured by two variables: the daily range (maximum VPD - minimum VPD) and the hourly rate of change in VPD (AR1 transformation). Both showed that basket and cup nests had smaller daily fluctuations in humidity in relation to the exterior, with no differences between nest types (Table 2, Figure 4)

4. Discussion

To my knowledge, this is the first detailed description of the microclimate of tropical bird nests across natural environmental fluctuations. Tropical nests shared some similar patterns with temperate nests, such as a lower environmental variability compared to the exterior and smaller ranges between the maximum and minimum environmental conditions. In temperate nests, thermal stability has been linked to higher breeding success, improving embryonic development and hatching success (Heenan, 2013; Mainwaring et al., 2014). Although tropical ecosystems do not present the same levels of environmental fluctuation observed in temperate ecosystems, even changes of 1ºC can be deleterious for developing embryos (Ospina, Merrill, & Benson, 2018) and incubating adults (Uehling, Taff, Winkler, & Vitousek, 2020). My data suggest that microclimatic stability is important in the nest chamber, although the thermal tolerances of tropical eggs and nestlings remain to be determined.

Nests have been traditionally considered as insulating structures in temperate zones, where insulation against the external temperatures (Deeming & Mainwaring, 2015; Heenan, 2013; Wiebe, 2001) result in fitness gains via increased hatching success, proper nestling development and enhanced post-fledging survival (Michielsen et al., 2019). Most of these studies have tested the insulation properties of nests by following two main methodologies: using isolated temperature readings encompassing single time points of the daily fluctuations in the field (Austin, 1976; Tiainen, Hanski, & Mehtälä, 1983), or by exposing nests to specific temperatures in laboratory conditions and recording the rates of heat loss through time (Akresh et al., 2017; Botero-Delgadillo, Orellana, Serrano, Poblete, & Vásquez, 2017; Deeming & Mainwaring, 2015). I followed a different methodology by continuously recording the daily changes in temperature and humidity under field conditions, successfully registering the microclimate provided by the nest chamber under natural environmental fluctuations. This approach has been previously used to show that cavity nests maintain a more stable microenvironment than unused cavities or the

external environment (Kesler & Haig, 2005; Rhodes et al., 2009; Wiebe, 2001). My data from two cavity nests also present a similar pattern (Figure 1, Figure 3) which seems to be a general trend in temperate (Maziarz & Wesołowski, 2013; Wiebe, 2001) and tropical cavities (Dechmann, Kalko, & Kerth, 2004; Kesler & Haig, 2005). Aside from the present study, this approach has not been applied to other nest types such as cup or basket nests. Birds from temperate regions tend to build thicker and heavier nests when facing low environmental temperatures (Akresh et al., 2017; Deeming & Mainwaring, 2015; Heenan, 2013), which suggest that nests would be insulated against the minimum temperatures of the exterior. In contrast, I found that basket nests often reach similar minimum temperatures as the exterior, which could be explained by taking into account the humidity inside the nest chamber.

I was able to uncover evaporative cooling as a potential mechanism of nest microclimatic regulation by simultaneously measuring temperature and humidity. Both basket and cup nests experienced lower maximum temperatures than the exterior, but only cup nests remained warmer during the minimum environmental temperatures of the day. This excludes the possibility of thermal insulation by the nest walls as seen in temperate nests (Dickinson, Goodman, & Deeming, 2019; Wiebe, 2001; Windsor, Fegely, & Ardia, 2013), given that insulation should act in both directions, preventing reaching the maximum and minimum temperatures. Noticeably, warmer temperatures in my data almost exactly coincide with the drier periods of the day, when nests remained more humid than the exterior. Given this difference in water saturation between the inside vs the outside of the nest, nests were still capable of losing water and lowering their temperatures by water evaporation during the hotter hours of the day. On the other extreme, only cup nests were able to stay warmer during the colder periods of the day given that cup nests were less humid than the exterior at that time and likely experienced lower rates of thermal loss by water mediated conductivity. Basket nests remained as humid as the exterior, which potentially lead to thermal equilibrium between the inside and the outside of the nest. Although, evaporative cooling has been reported as a mechanism of thermoregulation in adult birds (O'Connor, Wolf, Brigham, & McKechnie, 2017;

Smith, O'Neill, Gerson, McKechnie, & Wolf, 2017) and as an incubation strategy (Austin, 1976; Walsberg & Voss-Roberts, 1983), I present the first direct evidence of a nest structure as a system capable of evaporative cooling to avoid reaching external maximum temperatures.

These observations suggest that more studies are needed to explore the variety of ways that bird nests utilize to maintain distinct microclimates in relation to their exteriors. Although I found similar climatic patterns across nests from different species, the architectural details and mechanisms of climatic regelation of each of these nests are still unknown. Additional studies are especially critical in tropical ecosystems, which host the majority of bird species (Hawkins, Diniz-Filho, Jaramillo, & Soeller, 2007), and exhibit a broader array of nesting strategies than temperate ecosystems. This diversity could contain multiple unknown mechanisms for maintaining adequate brooding environments in the nesting chamber. It is also important to remember that incubation conditions are provided by the combined effect of parental inputs and the architectural properties of the nest (Healy, Morgan, & Bailey, 2015), and therefore their independent and combined effects should also be considered. By holistically approaching nesting biology we would not only better understand the role of nests as microclimatic modifiers, but also as antipredation strategies, (Beier & Tungbani, 2012; Mainwaring et al., 2014) and as important players during microbiome assembly of newborns (Campos-Cerda & Bohannan, 2020); see Chapter IV of this dissertation).

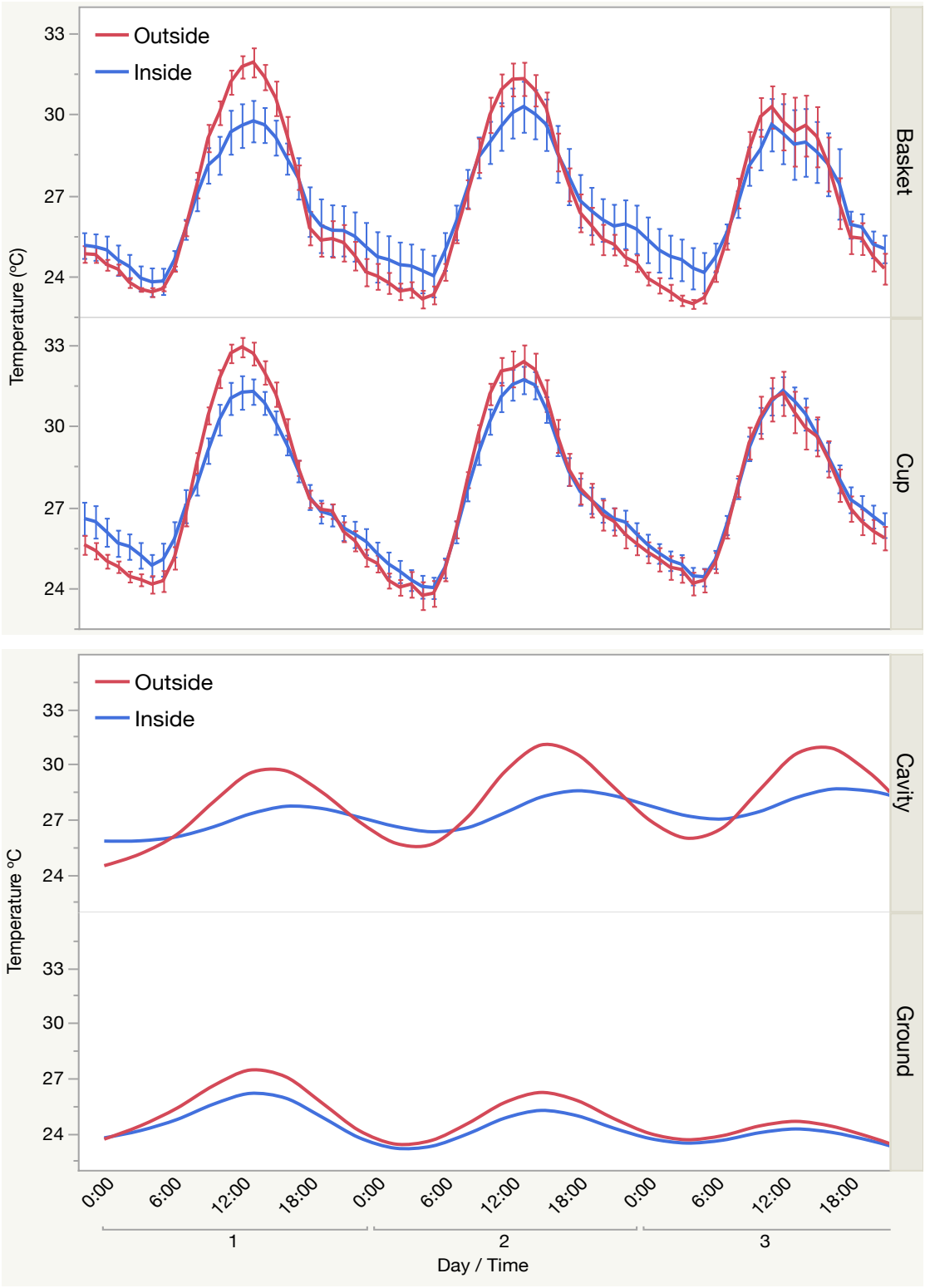

Figure 1. Daily nest temperatures by nest type. Each error bar is constructed using one standard error from the mean (Basket n = 9, Cup n = 16, Cavity n = 2, Ground n = 1).

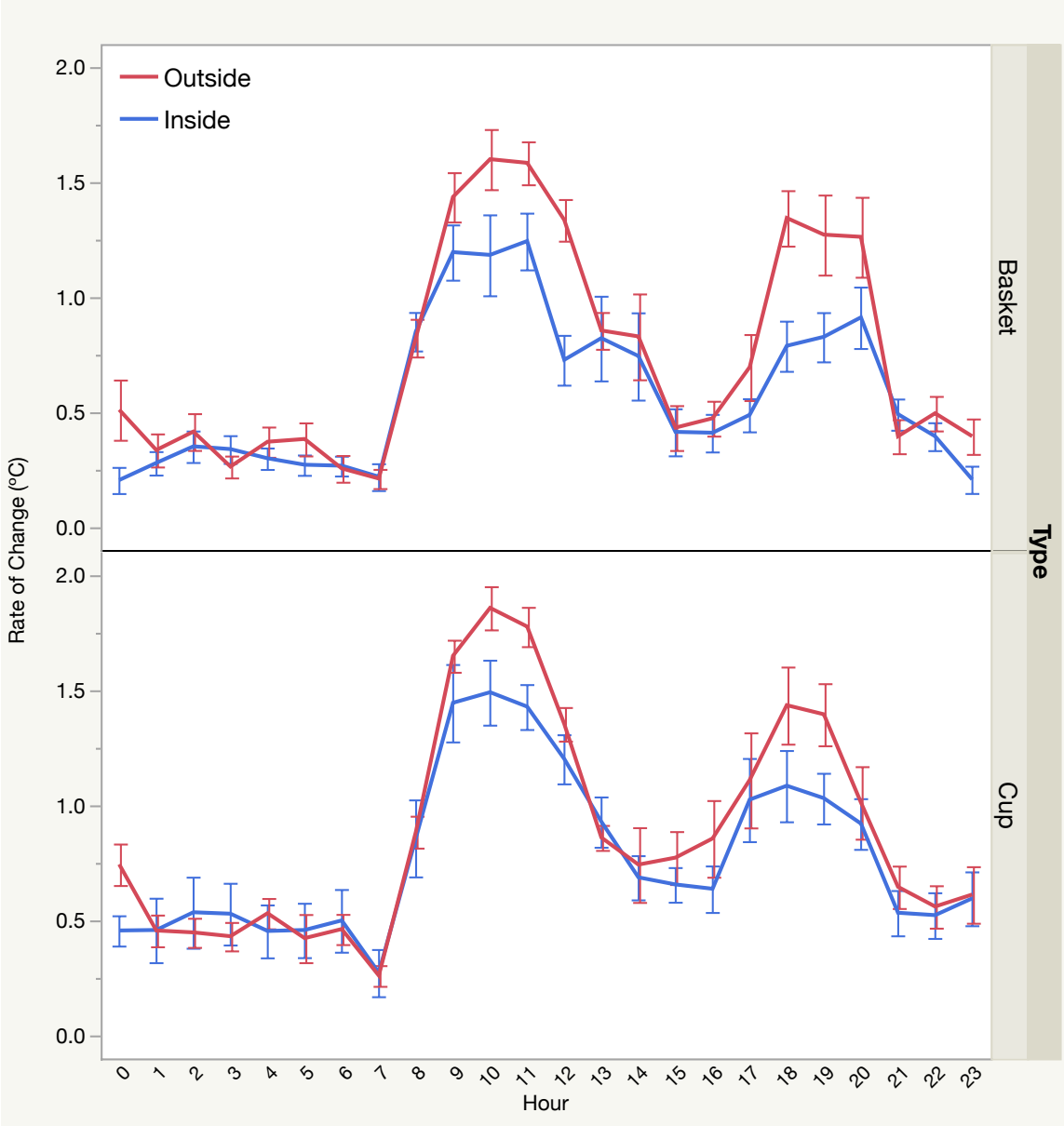

Figure 2. Temperature rate of change. Each error bar is constructed using one standard error from the mean.

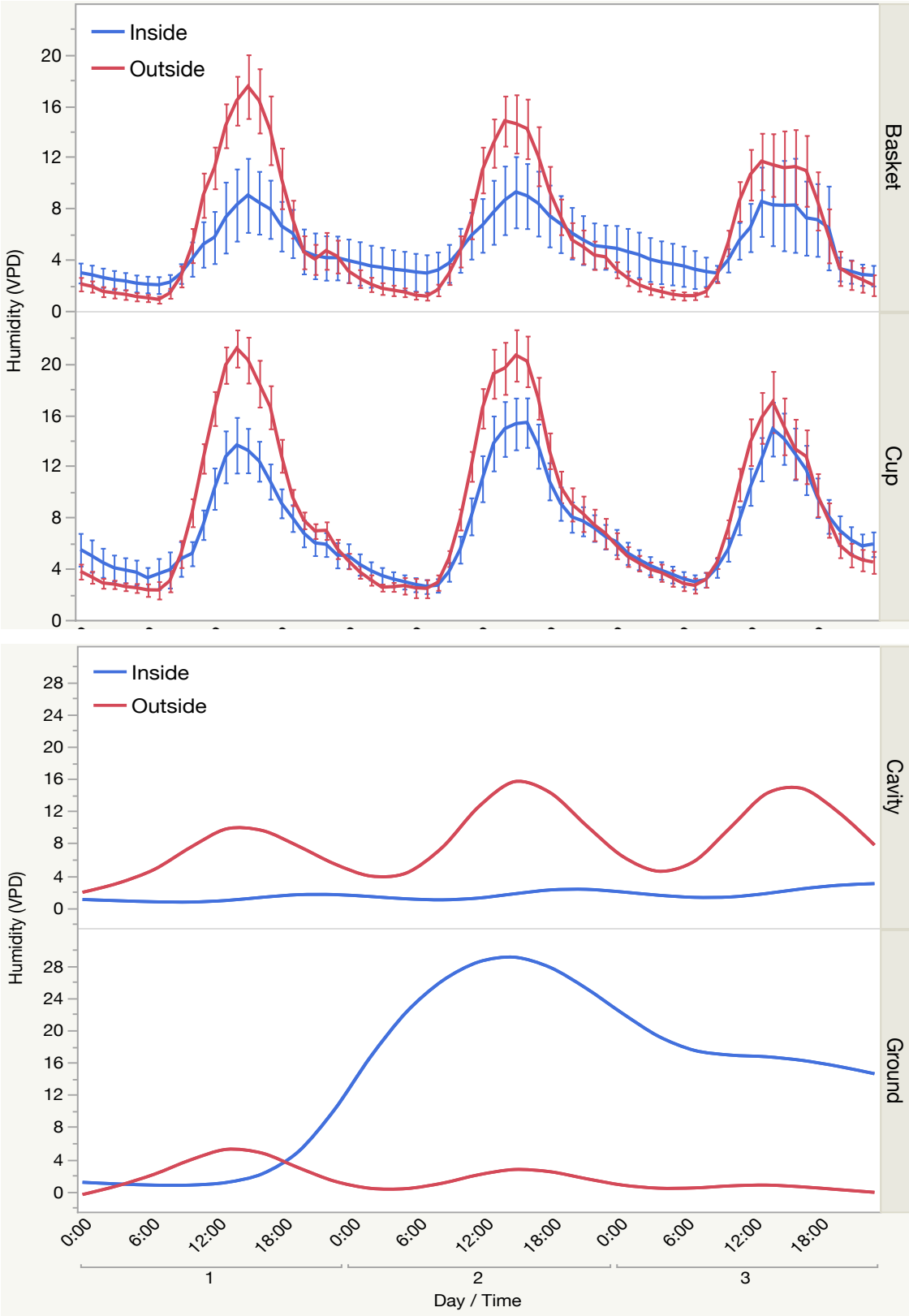

Figure 3. Daily nest humidity by nest type. Each error bar is constructed using one standard error from the mean (Basket n = 9, Cup n = 16, Cavity n = 2, Ground n = 1).

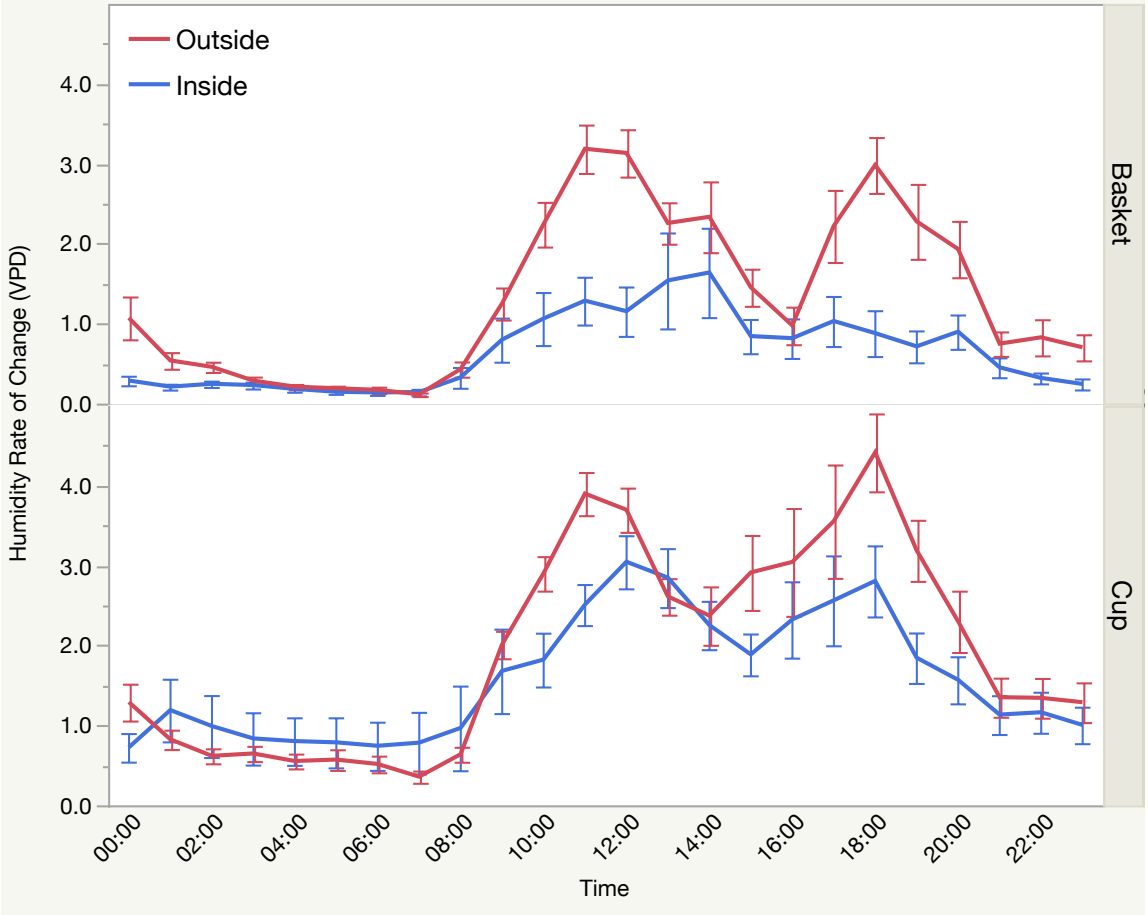

Figure 4. Humidity rate of change. Each error bar is constructed using one standard error from the mean.

Table 1. Bird taxonomy and nesting details of nests sampled (April – August 2015, Palenque, Chiapas, Mexico).

Order	Family	Species (nests)	Nests	Nest type
Apodiformes	Trochilidae	*Amazilia candida* *Campylopterus hemileucurus* *Phaethornis longirostris*	3 1 6	Cup Cup Cup
Caprimulgiformes	Caprimulgidae	*Nyctidromus albicollis*	1	Ground
Columbiformes	Columbidae	*Geotrygon montana*	2	Cup
Passeriformes	Furnariidae	*Lepidocolaptes souleyetii*	1	Cavity
	Pipridae	*Ceratopipra mentalis*	1	Cup
	Tyrannidae	*Tolmomyias sulphurescens* *Mionectes oleagineus*	1 3	Basket Basket
	Turdidae	*Turdus grayi*	2	Cup
	Troglodítidae	*Henicorhina leucosticta* *Thryothorus maculipectus*	1 1	Basket Basket
	Fringilidae	*Euphonia gouldi*	1	Basket
	Emberizidae	*Arremonops chloronotus*	2	Basket
	Formicariidae	*Formicarius analis*	1	Cavity
	Cardinalidae	*Habia fuscicauda*	1	Cup

Table 2. Mixed Model results on microclimatic conditions inside Basket (n=9) and Cup (n=16) nests compared to their immediate external environment. Palenque, Mexico summer 2015.

Variable	Nest Type	Inside[a]	Outside[a]	*df*	*F*	*P*
Mean	Basket	26.7 (2.0)	26.7 (1.4)	1, 609	0.525	0.469
Temperature	Cup	27.4 (1.6)	27.5 (1.2)	1, 1161	0.192	0.661
Thermal	Basket	5.5 (4.4)	8.9 (4.3)	1, 26	42.131	**<.0001**
variation[b]	Cup	6.5 (2.8)	10.4 (3.3)	1, 47	47.653	**<.0001**
	Basket vs Cup[c]			1,23.2	0.133	0.718
Max	Basket	30.3 (2.9)	31.5 (1.9)	1, 26	8.982	**0.006**
Temperature	Cup	31.8 (2.1)	32.9 (1.7)	1, 47	17.494	**0.0001**
	Basket vs Cup[c]			1, 22.76	0.022	0.884
Min	Basket	23.9 (1.7)	23.3 (1.2)	1, 26	3.267	0.082
Temperature	Cup[d]	24.2 (1.5)	23.4 (0.8)	-	z= 4.19	**<.0001**
Time of Max	Basket	13.6 (1.7)	13.7 (1.0)	1, 26	0.074	0.787
Temp	Cup	14.0 (1.3)	13.4 (2.7)	1, 47	2.384	0.129
Time of Min	Basket	5.6 (4.9)	6.6 (6.5)	1, 26	0.913	0.348
Temp	Cup	5.2 (4.0)	6.3 (5.3)	1, 47	1.891	0.176
Length of Max	Basket	3.26 (1.5)	2.8 (1.4)	1, 26	1.908	0.179
Temp	Cup	2.4 (1.3)	2.5 (1.2)	1, 47	0.975	0.328
Length of Min	Basket	4.3 (2.4)	4.0 (1.8)	1, 26	0.486	0.492
Temp	Cup	3.5 (1.9)	3.1 (2.3)	1, 47	0.986	0.326
Mean Humidity	Basket	5.1 (4.6)	6.2 (3.2)	1,609	21.390	**<.0001**
	Cup	7.1 (4.2)	8.4 (2.9)	1,1164	77.814	**<.0001**
	Basket vs Cup[c]			1,22.41	0.053	0.820
Humidity	Basket	14.6 (26.2)	31.8 (26.5)	1, 26	42.595	**<.0001**
variation[b]	Cup	22.9 (19.8)	49.7 (29.2)	1, 47	41.421	**<.0001**
	Basket vs Cup[c]			1,19.4	1.692	0.208
Min Humidity	Basket	10.2 (9.4)	16.2 (6.9)	1, 26	23.551	**<.0001**
	Cup	15.8 (8.0)	21.7 (6.7)	1, 47	30.673	**<.0001**
	Basket vs Cup[c]			1, 22.61	0.001	0.971
Max Humidity	Basket	2.3 (2.8)	1.3 (1.3)	1, 26	2.946	0.098
	Cup	2.4 (2.3)	1.5 (0.8)	1, 47	9.796	**0.003**
Time of Min	Basket	14.4 (4.8)	14.7 (1.6)	1, 26	0.101	0.752
Humidity	Cup	14.8 (2.8)	14.5 (2.9)	1, 47	0.271	0.605
Time of Max	Basket	7.9 (7.2)	6.7 (7.4)	1, 26	1.336	0.258
Humidity	Cup	6.9 (5.9)	7.8 (6.7)	1, 47	3.454	0.069
Length - Min	Basket	3:36 (6:00)	1:24 (0:30)	1, 26	3.771	0.063
Humidity	Cup	1:24 (0:48)	1:12 (0:30)	1, 47	1.500	0.227
Length - Max	Basket	5:54 (5:36)	5:42 (5:06)	1, 26	0.046	0.833
Humidity	Cup	4:12 (3:48)	3:12 (2:18)	1, 47	4.251	**0.045**
Temperature	Basket	6.4 (2.6)	8.3 (2.1)	1, 26	49.054	**<.0001**
Range	Cup	7.7 (1.6)	9.5 (1.5)	1, 47	38.827	**<.0001**
	Basket vs Cup[c]			1, 23	0.003	0.958
Humidity	Basket	7.9 (8.4)	14.9 (6.3)	1, 26	48.346	**<.0001**
Range	Cup	13.4 (6.8)	20.2 (6.3)	1, 47	43.122	**<.0001**
	Basket vs Cup[c]			1,22.7	0.012	0.913

Table 2. Continued

Variable	Nest Type	Inside[a]	Outside[a]	*df*	*F*	*P*
Temp Rate of Change	Basket	0.59 (0.61)	0.75 (0.69)	1,600	57.813	**<.0001**
	Cup	0.69 (0.57)	0.89 (0.88)	1,1139	90.556	**<.0001**
	Basket vs Cup[c]			1,23.4	0.304	0.587
Humidity Rate of Change	Basket	0.66 (1.37)	1.35 (1.62)	1,600	134.797	**<.0001**
	Cup	1.14 (1.39)	1.87 (2.29)	1,1144	154.755	**<.0001**
	Basket vs Cup[c]			1,23.4	0.096	0.760

[a] Overall Mean values (Standard Deviation)
[b] Variance
[c] Comparing the mean difference between the inside vs de outside across nest types
[d] Compared using a *z*-test to test if the difference between in/out was different from 0

BRIDGE

In Chapter III, I tested if the generalized assumption that nests provide an insulation chamber for egg incubation and nestling development also applies for tropical nests. I rejected insulation as an important mechanism regulating the microclimate of tropical nests and propose evaporative cooling instead. I provided evidence that tropical nests could avoid temperature extremes by losing water during peak environmental temperatures. This is the first time that the nest per se has been shown to utilize evaporative cooling as a mechanism for thermoregulation.

After confirming the efficacy of tropical bird nests at providing a different microclimate in relation to the exterior, I explored if tropical bird nests also had an effect on the microbial communities living on the nest and colonizing the gut of nestlings. Chapter IV presents the effect of nest architecture on the microbiome of the nest itself and the nestlings inhabiting it.

CHAPTER IV

NEST ARCHITECTURE AS A FACTOR INFLUENCING MICROBIOME ASSEMBLY IN TROPICAL BIRDS

This chapter received the contribution of multiple people. Sebastián Montejo, Rosi Ramos Pinto, Jesús Ramos Pinto and Oscar Díaz helped during sample collection and field work. Dr. Brendan J.M. Bohannan reviewed the current manuscript and verified data analysis. I was the primary contributor, performing all data analysis and writing. This chapter will be published in the Journal Frontiers in Microbiology with Dr. Brendan J.M. Bohannan as coauthor.

Introduction

Adult animals host entire microbial communities (their "microbiomes") which impact their fitness and survival in various ways (McFall-Ngai et al. 2013). There is growing evidence of the benefits granted by microbiomes, especially early in their host's life, aiding in nutrition (Treichel et al. 2019), immune defense (Fung, Olson, and Hsiao 2017), neural development (Phelps et al. 2017), and many other processes (Milani et al. 2017; Fraune and Bosch 2010). Variation in microbiome composition has been shown to be related to variation in these processes, but the drivers of microbiome variation remain unclear. Some of this variation may be due to differences in microbiome assembly early in life; most hosts acquire nearly all of their microbiome members after birth or hatching (Grond et al. 2017; Ferretti et al. 2018; Videvall et al. 2019) and early colonization can influence processes later in development (Phelps et al. 2017). However, the process of microbial colonization during early life and the factors shaping it remain largely unexplained.

As the immediate environment in which a newborn animal develops, nests have a key role in determining microbial exposure and subsequent colonization of eggs and neonates (Campos-Cerda and Bohannan 2020). Previous evidence has shown that

early exposure is an important factor in determining microbiome assembly (Burns et al. 2016; Ruiz-Castellano et al. 2016; Shukla et al. 2018). Considering that the nest, parents and nestlings form an ecological unit whose interactions drive the process of initial microbial colonization (i.e., the nidobiome), nest construction represents a mechanism of environmental modification that could regulate microbial exposure for early juveniles. Inside a nest, lower rates of exposure to environmental microbes would enhance mechanisms of direct or controlled microbial transmission from parents to offspring.

Evidence from studies of birds have shown that nests have the potential to be important drivers of microbial colonization of newborns. For example, studies have shown that eggs located in cavity nests have lower abundance of bacteria on their surfaces than eggs incubated in cup nests (Godard et al. 2007), that nest microbiomes are more similar to parental microbiomes than to adjacent environmental microbiomes (Goodenough et al. 2017), and nests can participate in the vertical transmission of microbes between mothers and nestlings (e.g., through inoculation of nest surfaces by parents; (Martín-Vivaldi et al. 2018)) . These effects on nestlings' microbiomes result from both parental inputs and the nest structure itself. Considering the nest on its own, we know that nest materials can shape its internal bacterial environment (Mennerat et al. 2009; Ruiz-Castellano et al. 2016) and that nests can host microbial communities that vary with the taxonomy of the nest-builder (e.g., by bird species) (Goodenough and Stallwood 2010). However, nests include a wide variety of structures with different characteristics that could affect a nest's microbiome and eventually influence a nestling's microbiome. The influence of nest architecture on microbiome diversity and composition of nests and nestlings is not known.

I propose that the extent of architectural modification within a nest determines the degree of microbial exposure of eggs and nestlings, by regulating external microbial inputs. In this study, I aimed to determine if a nest's architecture influences the microbial communities colonizing the nest walls and the guts of resident nestlings. I

considered a gradient of environmental exposure determined by nest architecture, with cup nests as the most exposed nest type given their lack of an upper cover, basket nests having intermediate levels of exposure given their semi-permeable walls and roof, and cavity nests being the least exposed nest type, having solid walls and roof. I hypothesized that 1) nest architecture will influence both the nest and the nestling's microbiome, and 2) this influence will lead to specific temporal changes in microbiome composition related to each nest type.

My study focused on tropical birds, for several reasons. Tropical regions host the majority of bird species (Hawkins et al. 2007)), which is also reflected in a high diversity of life history traits including breeding phenology and nesting behaviors (Stiles 1983). For example, it has been hypothesized that tropical cavity nesters breed during the dry season to avoid the risk of pathogens during the rainy season (Stiles 1983) and some tropical species remove their nestlings' feces from the nest (i.e., most passerines) while other do not (i.e., trogons, doves, hummingbirds, parrots). Also, the effect of tropical nests as environmental modifiers has received little attention, with the prevailing perception (almost entirely based on temperate nests) that nests are primarily insulating structures (Deeming and Mainwaring 2015). I have generated data that contradicts the assumption that tropical nests are insulating chambers (previous chapter of this dissertation), which supports the idea that a new perspective is needed when studying tropical birds. This chapter expands on my previous studies of tropical nests as environmental modifiers, extending this work to include the microbiome assembly of nestlings.

Materials and Methods

Study site.

An intensive search for bird nests was performed from April to August 2015 in Palenque National Park, Chiapas, the southernmost state of Mexico. The park encompasses ~700 ha of mature tropical rainforest on top of an ancient Mayan city,

surrounded by a mosaic of pastureland and secondary forest (Patten et al. 2011). The annual mean temperature is 26ºC (range 22-29ºC), with heavy rains occurring between May and December, decreasing in frequency and intensity from January to April, for an overall mean annual precipitation of 2,200 mm. At a continental scale, lowland tropical rainforests expand from South America to Southern Mexico, with Palenque representing an example of such ecosystems.

Microbial sampling

Every nest found was visited every 3 days until fledging or when its residents disappeared. We found 89 nests, most of which were predated at different stages of development. Nests were sampled only after hatching to lower the risk of nest abandonment. Sampling consisted of swabbing the nest's inner surface for 1 min using sterile flock swabs (Copan Minitip flocked swab - 23-600-950) moistened with a sterile solution of 0.1% tween20 + 0.15 M NaCl. Fresh fecal samples were collected from every chick present at the nest. When chicks did not defecate while being manipulated, a cloacal sample was taken by introducing the swab tip into the chick's cloacae for 1 min. Nitrile gloves were always used by the collector, and were regularly disinfected with 70% ethanol. After processing each nest, we exposed a moistened sterile swab to the air during one minute as a negative control. Samples were immediately kept on ice, until the end of the day, when Zymo RNA/DNA shield buffer (R1100) was added in a 1:2 sample-to-buffer ratio. Samples were kept frozen until shipped to the lab. Ice was added during shipping and total travel time was approximately 36 hr before being frozen again and kept at -20 ºC until DNA extraction.

DNA extraction and library preparation

DNA extraction was performed under a laminar flow hood using Zymo's Fecal/Soil DNA extraction kit (Zymo D6012). I performed DNA extraction on a sterile swab per every 50 samples as a negative control of my extraction procedure. Library

preparation included the amplification of the V4-V5 region of 16S rRNA gene (Primers 515F/806R) and the addition of Illumina adapters in a single PCR reaction. Illumina primers were provided by the University of Oregon Genomics Core. PCR was performed using NebNEXT Q5 mix with the following PCR conditions: 98º for 30s, with 26 cycles of 98º for 10s, 61º for 20s and 72º for 20s, with a final extension time at 72º for 2 min.

Sequence processing and (community) Microbiome analysis.

Sequence processing (i.e., sequence quality, removal of low quality reads and removal of artificial chimeras), phylogenetic distances across bacterial taxa and taxonomy assignment was performed following the QIIME2 pipeline using standard parameters. Three final elements were exported at the end of the QIIME2 pipeline: the final matrix of amplicon sequence variants (ASVs) by sample, the bacterial taxonomy table and the phylogenetic tree of the ASVs. The analysis of microbial communities was performed using the "Phyloseq" package (McMurdie and Holmes 2013) in R (R Foundation for Statistical Computing, Vienna, Austria, 2020). Samples with less than 100 reads were considered unreliable and were filtered out of the dataset. Bacterial richness was calculated at this point and exported to be analyzed using a Mixed Model approach via JMP Pro 14 (JMP Pro 14; SAS Institute, Cary, NC, USA 2020). To test for differences in bacterial richness of feces and nests across time between nest types I constructed a General Linear Mixed Model (GLMM) where sample type (feces/nest), nest type (cup, basket, cavity), visit number, and the (nest type * visit) interaction were entered as fixed effects, using the nest ID as a random variable to account for the repeated measures experimental design. A similar model was constructed to test if bird order had an effect on bacterial richness, replacing nest type with bird order as a fixed effect.

For microbial community analysis, samples with less than 100 reads were considered unreliable and were filtered out of the dataset. ASV abundances where transformed to relative abundances, filtering out ASVs that had a mean lower than

10^{-5} in the entire data set. This number was selected to remove ASVs with very low abundance, which are more likely to represent spurious taxa. In a similar sense, ASVs that only occurred in one sample where deleted. To test if fecal and nest samples contained different microbial communities I constructed a distance/dissimilarity matrix between samples based on Bray-Curtis distance and performed a PERMANOVA (via the Adonis function in vegan) with sample type as my predictor (fixed factor), running 999 permutations. To test if nest type or bird taxonomy had an effect on the temporal trajectories in community similarity between fecal or nest microbiomes, I calculated two separate dissimilarity matrices (one for fecal samples, one for nest samples) and performed the following PERMANOVA on each matrix:

adonis(dissimilarity_matrix ~ Predictor_variable*Visit_No + Nest_ID, strata = Nest_ID, data = metadata)

where I included nest type or bird taxonomy as my predictor variable, including its interaction with time (visit number) as fixed effects. Nest_ID was included as a variable to constrain permutations; this is recommended as a way to account for the repeated measures experimental design, and is considered as the equivalent of a "random effect" (J. Stephen Brewer, personal communication[1]).

Results

Sampled Nests

From the 89 nests that we initially found, we could only collect three consecutive sets of fecal and nest samples from 36 nests, given the high predation rates of my field site. Sampled nests included 21 bird species from 14 families (Table 1). From

[1] https://stat.ethz.ch/pipermail/r-sig-ecology/2013-February/003595.html

those, 21 were cup nests, 11 were basket nests and 5 were cavity nests. Number of nestlings per nest was variable (one to four), depending on the bird species.

Microbiome alpha diversity

I obtained an initial set of 7,568,989 raw reads, from which 5,725,024 reads remained after QIIME2 quality processing and were later grouped into 12,213 individual ASVs using the DADA2 pipeline. I removed samples with less than 100 reads as final quality control, ending with 145 samples out of 236 and a total of 2,952 individual ASVs. Actinobacteria (28.6%), Proteobacteria (23.7%), Firmicutes (19.1%), Cyanobacteria (2.4%), and Bacteroidetes (1.7%) were the five most abundant Phyla in fecal samples (Fig. 1), while Proteobacteria (23.2%), Actinobacteria (16.3%), Firmicutes (6.5%), Bacteroidetes (2.5%), and Chloroflexi (1.6%) where the five most abundant Phyla in nest samples (Fig. 1).

Species richness was higher in nest samples in relation to fecal samples ($F_{1,120.5} = 26.52$, $P < 0.0001$) with neither fecal nor nest samples showing any temporal trend ($F_{2,103.2} = 0.12$, $P = 0.89$) or a difference between nest types ($F_{2,116} = 1.07$, $P = 0.347$, Fig. 2) nor bird order ($F_{6,95.23} = 1.36$, $P = 0.240$, Fig. 3).

Microbiome beta diversity

Fecal samples had a distinct bacterial community composition relative to nest samples ($F_{1,143} = 13.2$, $R2 = 0.08$, $P < 0.001$, Fig. 4). Given this difference between fecal and nest microbiomes, each sample type was analyzed independently in order to reduce the noise in the ordination when performing subsequent PERMANOVA's. Fecal microbiomes of the same nest type were more similar to each other than to samples from other nest types ($F_{2,77} = 4.83$, $R2 = 0.10$, $P < 0.001$, Fig. 5A), and temporal trajectories in community similarity were also influenced by nest type ($F_{4,77} = 1.36$, $R2 = 0.06$, $P = 0.041$, Fig. 5A).

Fecal microbiomes from the same bird Order were more similar to each other than to fecal microbiomes of other bird Orders ($F_{3,74} = 8.19$, $R^2 = 0.22$, $P < 0.001$, Fig. 5B), and temporal trajectories in community composition were more similar between species sharing the same bird Order than to species from a different Order ($F_{6,74} = 1.57$, $R^2 = 0.08$, $P = 0.004$, Fig. 5B).

Microbiome composition of nest samples were more similar between the same nest type than across different nest types ($F_{2,50} = 2.79$, $R^2 = 0.09$, $P < 0.001$, Fig. 6A). In this case, similar nest types did not share similar temporal changes in community composition ($F_{4,50} = 0.90$, $R^2 = 0.06$, $P = 0.802$, Fig. 6A). Nest microbiomes were more similar to nests of the same bird Order than to nest microbiomes from a different order ($F_{3,47} = 2.38$, $R^2 = 0.12$, $P < 0.001$, Fig. 6B), but nests from the same bird Order did not share more similar trajectories in community composition through time in comparison with a different bird Order ($F_{6,47} = 0.75$, $R^2 = 0.07$, $P = 0.999$, Fig. 6B).

Discussion

To my knowledge, this is the first study of the potential influence of nest architecture on the microbiomes of nests and nestlings. I observed that nest architecture affected both the nest and the gut microbiome of nestlings. Previous studies of temperate bird species suggest that nest microbiomes could influence chick microbiome composition; for example, nest microbiomes and chick microbiomes have been reported to be more similar within a nest than across nests (Martín-Vivaldi et al. 2018; van Veelen, Falcão Salles, and Tieleman 2018; Teyssier et al. 2018). However, these studies did not sample across nests of different architectures, nor did they sample across time. My study builds on this previous work to document differences in nest architecture itself as a driver of microbial colonization of nestlings. My results support the view that nests provide a distinct microenvironment, not only in terms of temperature and humidity (Maziarz and

Wesołowski 2013; Biddle et al. 2019; Michielsen et al. 2019) but also in relation to the microbial colonization of neonates.

Nest microbiomes had higher bacterial richness than fecal microbiomes, overlapping their three most abundant Phyla (Proteobacteria: 23.2%, Actinobacteria: 16.3%, and Firmicutes: 6.5%) with the three dominant Phyla observed in gut microbiomes (Actinobacteria: 28.6%, Proteobacteria: 23.7%, and Firmicutes: 19.1%), just in different proportions. These Phyla have been previously reported in the few studies to date of the gut microbiomes of tropical birds (Hird et al. 2015; Godoy-Vitorino et al. 2008; San Juan et al. 2019) and are also common members of the gut microbiome of temperate birds (van Veelen, Falcao Salles, and Tieleman 2017; Grond et al. 2017; Ruiz-Rodríguez et al. 2018).

I did not detect an increase in gut bacterial richness through developmental time. This is in contrast to previous studies of temperate bird species that have reported a sustained increase in gut bacterial richness that can last up to three months after hatching (Videvall et al. 2019; Kers et al. 2018). We sampled across a time window of 9 days, which I expected to be long enough to detect increasing trends in bacterial richness during the nestlings' development if present. The fact that I did not find such a trend could respond to the different growth patterns of the various species that we sampled and the subsequent difficulty of comparing similar growth stages across species. My results could also represent a different pattern in microbiome colonization of tropical chicks, relative to temperate species.

I did not find evidence that nest type limits or increases bacterial richness, as expected from my exposure gradient by nest type (cup – basket – cavity nests). Previous findings using culture based methods and artificial nests reported lower colonization levels in cavity nests compared to cup nests (Godard et al. 2007), which led us to hypothesize that nest exposure would be reflected in an gradient of bacterial richness going from low exposure (cavity nests) to high exposure nests (cup nests). My results may be different because I used culture-independent

methods to determine bacterial richness, and the effect of rare and low abundance taxa might have been stronger than in previous studies. My results may also be different because I focused on occupied nests, which allow for interactions between chicks, parents and the nest environment. For example, there are likely differences in parental investment across nest types which could alter the impact of nest architecture on bacterial richness. Parental behavior strongly contributes to the nesting environment; for example, brooding behavior changes temperature and humidity and can reduce bacterial loads (Ruiz-Castellano et al. 2019). To fully disregard nest architecture as a potential modifier of bacterial richness I would need to account for parental care and brooding behavior as both can decrease bacterial abundances (Cook et al. 2005; Ibáñez-Álamo, Ruiz-Rodríguez, and Soler 2014). My observed similar levels of bacterial richness across nest types could be the result of differential investments in parental care, with exposed nests needing higher parental investment than closed nests.

I found that nest architecture affects the microbiome composition of the nest walls and the gut microbiome of tropical nestlings, even when bacterial richness was not influenced. Although studies of many animals (such as birds, frogs, and insects) have shown that the microbiomes of nests and their resident neonates can overlap in composition (Martínez-García et al. 2015; Warne, Kirschman, and Zeglin 2019; Shukla et al. 2018; Kaltenpoth et al. 2005), this is the first recorded case where differences in nest architecture are associated with differences in microbiome composition of the nest and nestlings. Gut microbiomes showed temporal differences in microbiome assembly related to nest architecture. This suggests that different nest types provide different conditions that shape the overall gut microbiome of nestlings, and that such influence is persistent over time. Broiler chickens and ostriches have shown a continuous and dynamic process of microbiome assembly from hatching to at least the first molt (Kers et al. 2018; Videvall et al. 2019; Grond et al. 2017; Teyssier et al. 2018). The influence of nest type on initial microbiome assembly was evident even though my data encompasses only a portion of the nestling period before fledging. In future work, a longer

timespan of study could provide more details on the relative impact of nest architecture at shaping the gut microbiome of nestlings, as this process has been shown to be non-linear in temperate birds (Teyssier et al. 2018). Interestingly, although each nest type had a different microbiome on its walls, nest architecture did not affect the temporal trajectories of these communities. This suggests that the effect of nest type on the microbial colonization of nestlings may interact with the chick's physiology and development, and is not solely driven by changes to the nest microbiome.

Bird phylogeny had a significant effect on the composition of the nest's and nestling's microbiome as well. Host's phylogeny commonly represents a strong factor shaping the host's microbiome, both in adults and newborns (Ruiz-Rodríguez et al. 2018; Knowles, Eccles, and Baltrūnaitė 2019). In closely related species, the nest's microbiome has been shown to override the effect of the host's phylogeny at shaping the gut microbiomes of foster chicks raised on the nest of their sister species instead of its own (Goodenough and Stallwood 2010). In that case, nestlings from the same nest shared similar microbiomes even when belonging to different species (Goodenough and Stallwood 2010). In contrast, species from different families maintained a different gut microbiome even when sharing the same nest, as seen in cuckoo chicks raised in magpie nests (Ruiz-Rodríguez et al. 2018). From the four bird orders that we were able to sample, only Passeriformes had species nesting in more than one nest type. Regardless of such limitation, I could still see how nest type was a factor influencing the nest and gut microbiomes of nestlings, and that the other orders clustered more closely to Passeriformes with the same nest type than to Passeriformes with other nest types. It would be worth pursuing a larger project to explore the interaction between host phylogeny and nest architecture, increasing sample sizes and species sampled. In addition, my work could be expanded in future studies by sampling parents, and explicitly testing how their inputs interact with the effect of the nest itself (Campos-Cerda and Bohannan 2020).

My results suggest that a new perspective may be necessary when studying the initial microbiome assembly in tropical bird species, given important differences between temperate and tropical nests (such as microclimatic properties; see previous chapter of this dissertation). Besides their unique effect on temperature and humidity, nests from tropical bird species could be interacting in unique ways with parental behavior. For example, predation rates of tropical nests are higher compared to temperate nests (Roper, Sullivan, and Ricklefs 2010) which would modify antipredation behaviors such as nest attendance and nest location (Martin, Scott, and Menge 2000), and in turn affect the rates of microbial exchange between parents and offspring. Future studies of microbiome assembly in tropical species should take into account the unique natural history of these species.

I have provided evidence of the effect of nest architecture on the microbiome of nest walls and importantly, the gut microbiome of chicks. My results support the assertion that microbiome assembly should be considered one of the intrinsic fitness benefits of nest construction in the animal kingdom (Campos-Cerda and Bohannan 2020). Our understanding of nest diversification and the evolution of nesting strategies would benefit from explicitly considering the fitness gains from microbial colonization through nest construction, which has the potential to be as important as factors that are commonly considered to drive nest diversification (such as predation and competition) (Beier and Tungbani 2012; Rhodes, O'donnell, and Jamieson 2009; Brightsmith 2005).

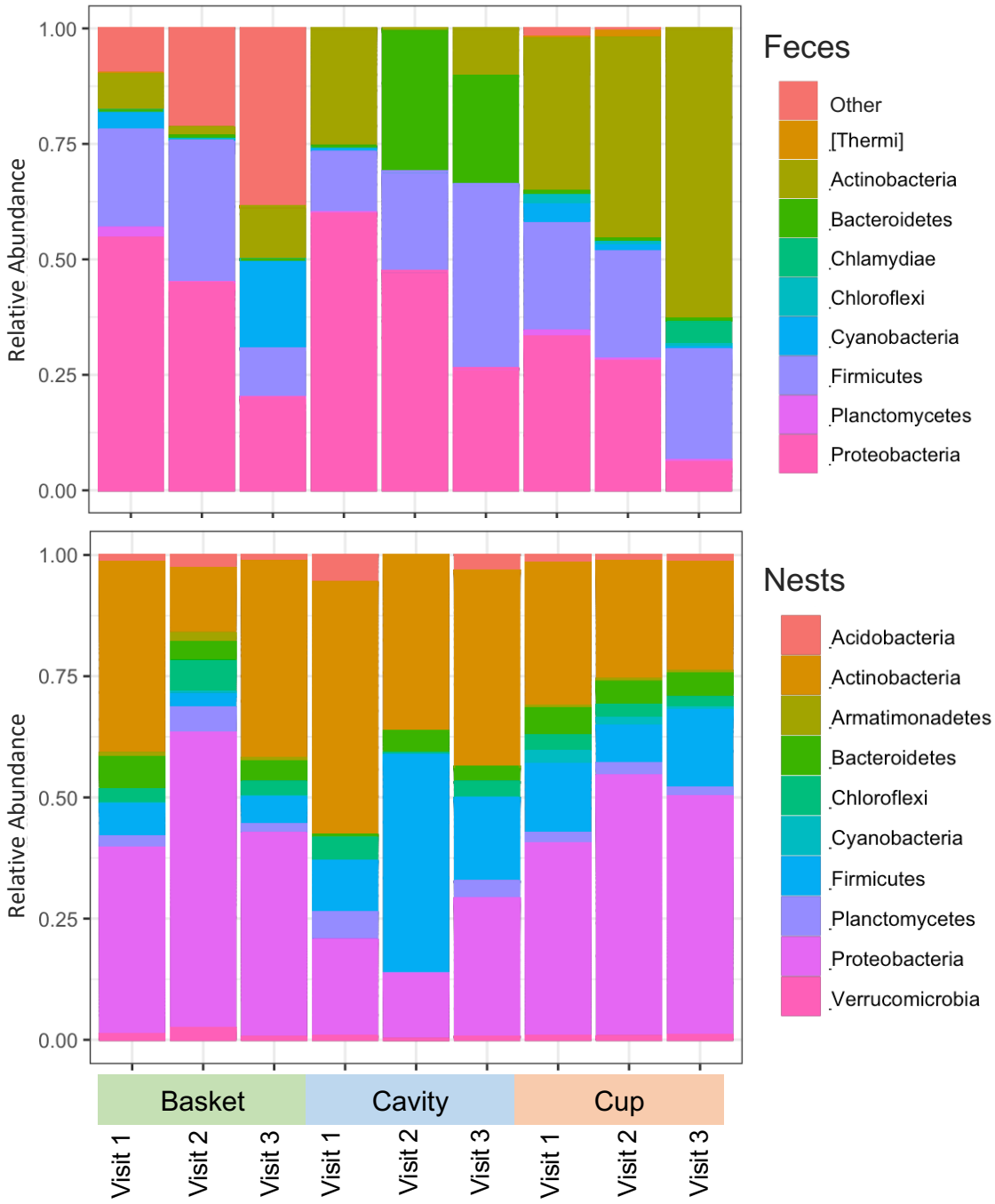

Figure 1. Taxonomic diversity of bacteria present in Fecal and nest samples of tropical birds (Palenque, Chiapas, Mexico 2015). Top 10 most abundant bacterial Phyla are shown, with all other Phylum aggregated as other.

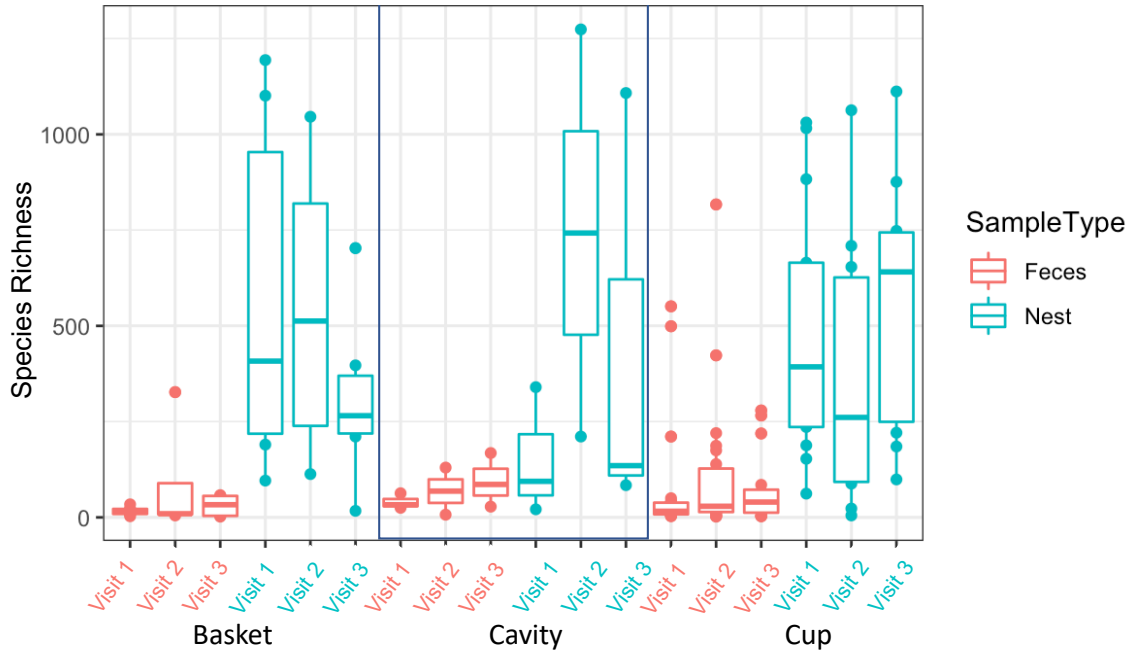

Figure 2. Bacterial richness of feces and nest samples from each nest type during the three visits. Nest samples showed higher bacterial richness than fecal samples.

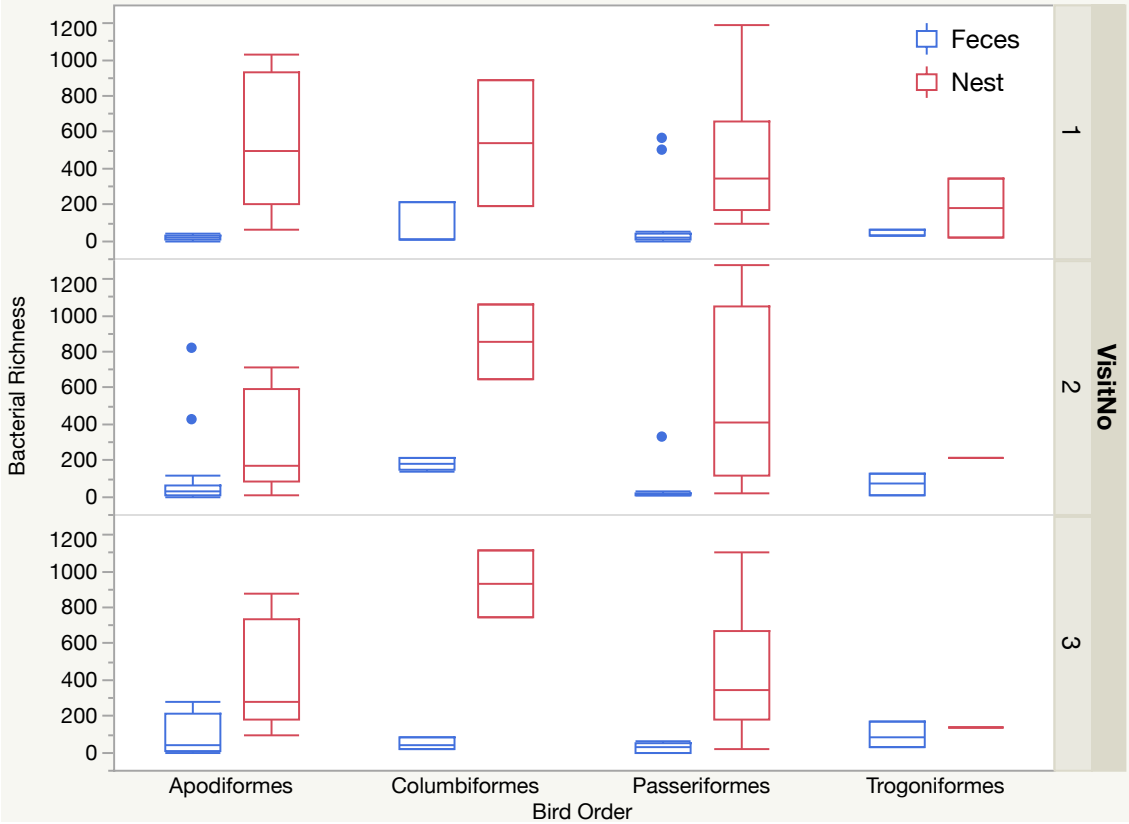

Figure 3. Bacterial Richness across bird orders in fecal and nest samples during the three visits. Nest samples showed higher bacterial richness than fecal samples.

Microbiome beta diversity

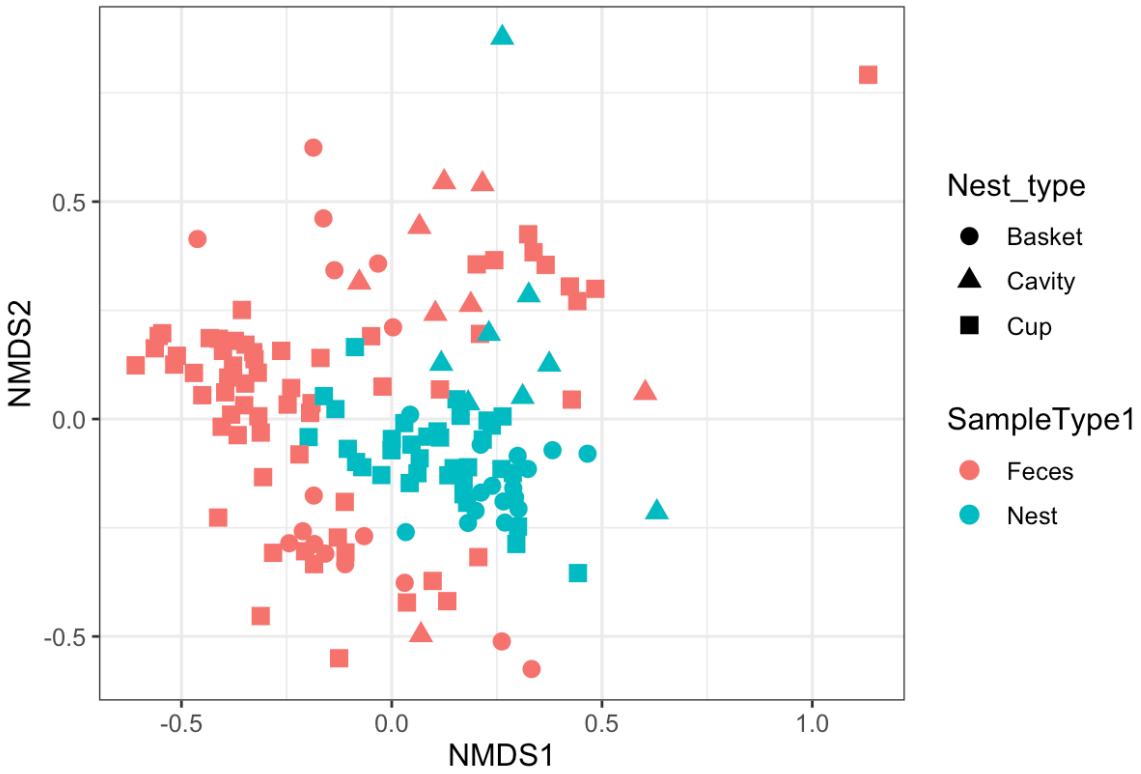

Figure 4. NMDS ordination of fecal and nest samples using Bray-Curtis distance (2 dimensions, stress = 0.237).

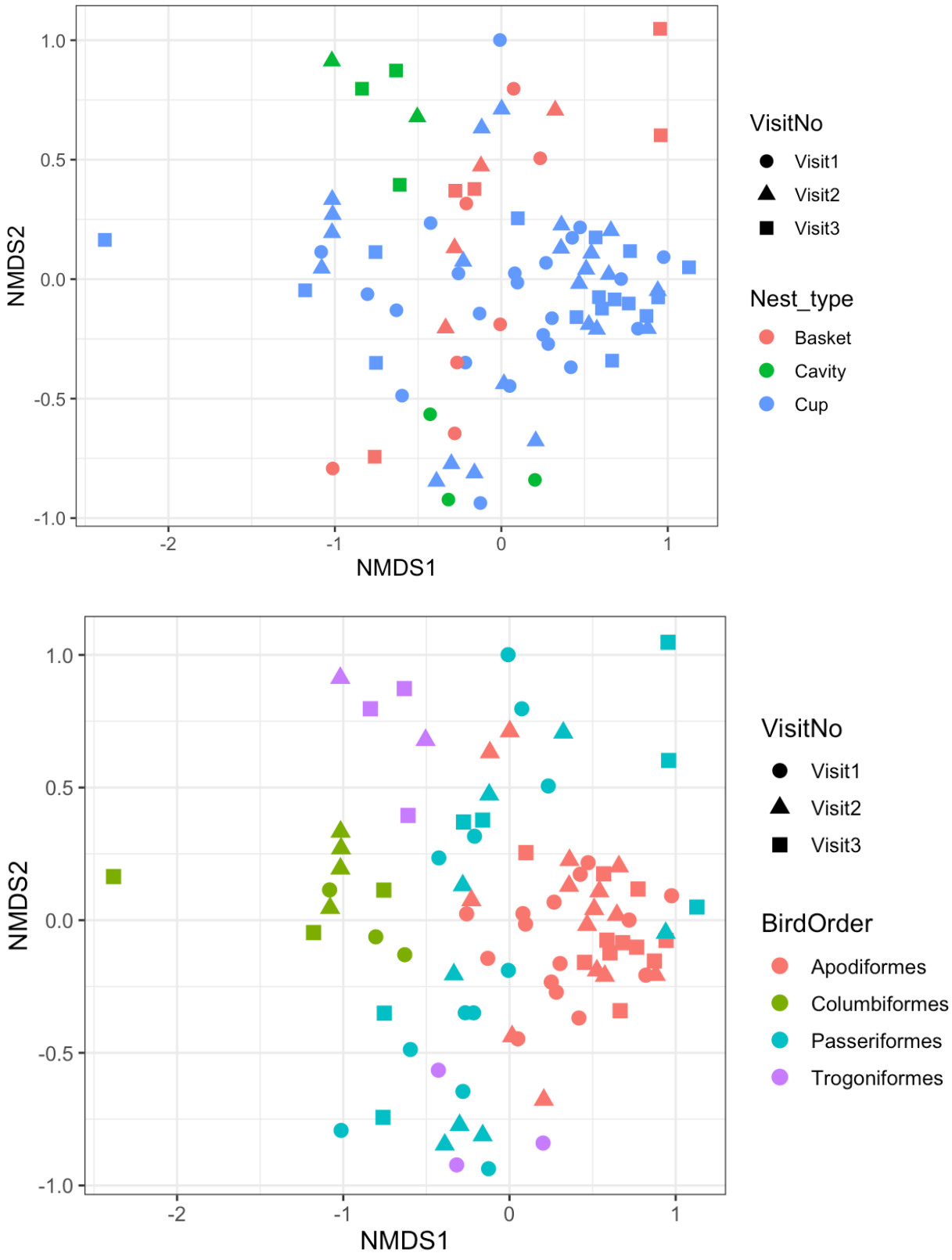

Figure 5. NMDS ordination of fecal samples using Bray-Curtis distance (2 dimensions, stress = 0.212). A) upper – by nest type, B) lower – by bird order.

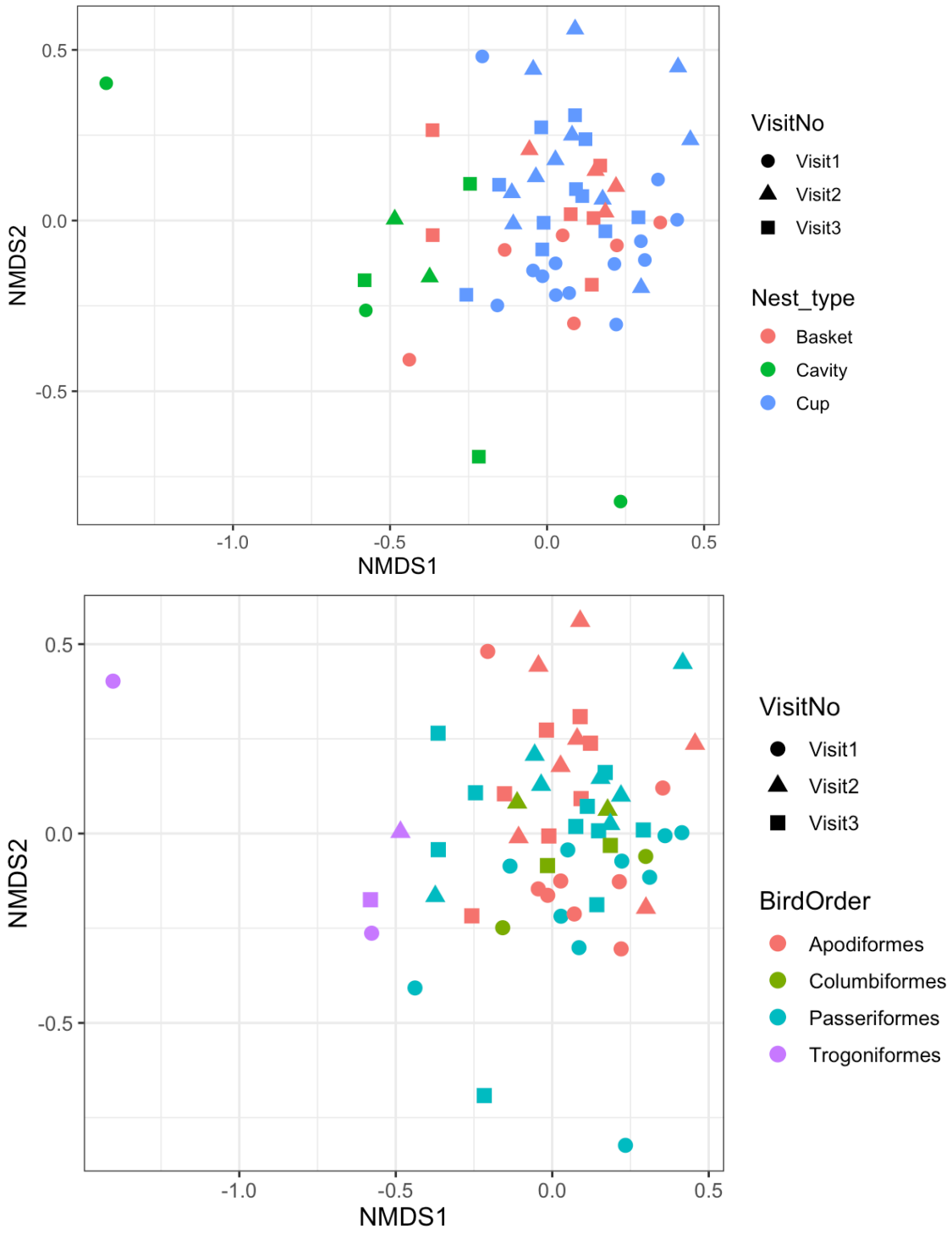

Figure 6. NMDS ordination of nest samples using Bray-Curtis distance (2 dimensions, stress = 0.197). A) upper – by nest type, B) lower – by bird order.

Table 1. Bird taxonomy and nesting details of sampled nests (April – August 2015, Palenque, Chiapas, Mexico).

Order	Family	Species (nests)	Nests	Nest type
Apodiformes	Trochilidae	*Amazilia candida*	1	Cup
		Amazilia tzacatl	3	Cup
		Campylopterus hemileucurus	1	Cup
		Phaethornis longirostris	6	Cup
Columbiformes	Columbidae	*Geotrygon montana*	2	Cup
		Leptotila verreauxi	1	Cup
Trogoniformes	Trogonidae	*Trogon massena*	1	Cavity
		Trogon melanocephalus	1	Cavity
Passeriformes	Cardinalidae	*Habia fuscicauda*	1	Cup
	Emberizidae	*Arremonops chloronotus*	2	Basket
	Formicariidae	*Formicarius analis*	1	Cavity
	Fringilidae	*Euphonia gouldi*	1	Basket
	Furnariidae	*Lepidocolaptes souleyetii*	1	Cavity
	Pipridae	*Ceratopipra mentalis*	2	Cup
	Thraupidae	*Saltator coerulescens*	1	Cup
	Trogloditidae	*Henicorhina leucosticta*	1	Basket
		Thryothorus maculipectus	2	Basket
	Turdidae	*Turdus grayi*	2	Cup
	Tyrannidae	*Mionectes oleaginous*	2	Basket
		Tolmomyias sulphurescens	3	Basket

CHAPTER V
CONCLUSION

Despite the evident importance of microbiomes to their host's fitness and development, the initial microbial colonization of a host remains obscure. Environmental exposure has been hypothesized as an important factor shaping the microbiome of newborn hosts, making the initial environment where a host is born or hatches a pivotal factor in microbiome assembly. In this dissertation, I present evidence that nests play a central role in early microbiome assembly. I present an integrative framework that invites researchers to go beyond recognizing individual microbial sources during microbiome assembly and to consider their emergent interactions, not only early after birth or hatching, but throughout important developmental stages. This framework provides a clear but flexible template that should allow the integration of multiple host species with different life histories into the general understanding of microbiome assembly.

Using tropical birds, I show that there are important differences in nesting environments between the traditional pattern observed in the nests of temperate bird species and the microclimate provided by tropical bird nests. My research suggests that researchers should avoid simple extrapolations from temperate to tropical ecosystems. The fact that tropical nests present a different microclimatic pattern than temperate nests opens the possibility of additional differences between tropical and temperate nesting biology, which could in turn affect the microbiome of nests and their relationship to microbiome assembly of nestlings in tropical ecosystems.

Finally, I provided the first evidence of nest architecture affecting not only the microbiome of the nest walls but also the microbiome of nestlings. The effect of nest architecture on the nestling's microbiome persisted across time and led to different trajectories in microbiome composition. Future research should explore the fitness implications of such effects and how parental inputs interact with nest architecture to influence the microbiome assembly of nestlings.

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CHAPTER III

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