Intergenerational Lizard Lounges do not Explain Variation in the Gut Microbiomes of Green Iguanas

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by

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ABSTRACT

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Herbivory is a rare diet specialization in lizards that requires numerous specializations including hindgut microsymbionts to aid in the digestion of plant meals. Researchers first attempted to identify the sources of the gut microbes hosted by herbivorous reptiles more than 30 years ago. These endosymbionts are needed to digest plant fiber and their fermentation products contribute substantially to their host’s energy budget, yet this symbiosis is poorly understood. Green Iguanas (*Iguana iguana*) are herbivorous throughout life, yet emerge from their eggs with sterile guts. Although rare in lizards, social interactions are a hypothesized route of microbe transfer via direct contact and/or eating feces of older conspecifics or previously inoculated peers. This study is the first to characterize the spatial, temporal, and social variation of these vital microbial communities using modern genomic techniques. I hypothesized that microbial communities will be more similar within than between sites, increase in diversity over
time, and vary with lounge size and proximity. I observed and individually marked juvenile, subadult, and adult iguanas alone and in social lounges at 11 sites on and around Barro Colorado Island, Panamá over two hatching seasons. Of the 540 focal observations of hatchlings, 38% were of social lounges (mean = 2.9 lizards/lounge). Only 0.7% of observations were of social lounges containing hatchlings and subadults/adults. I also collected microbe samples from the hindguts of adult and subadult iguanas to compare these communities to those from neonates that were sampled during the first 60 days posthatching. The 16S rDNA region was amplified from 100 samples and high-throughput sequenced via the Illumina HiSeq platform. Using the QIIME workflow, OTUs were identified by sequences with ≥ 97% similarity and matched to lowest taxonomic level via the Greengenes database. Proportions of OTUs in each sample were calculated, as were Chao1 alpha diversity and Bray–Curtis beta diversity differences. The hindgut bacterial communities were dominated by Firmicutes (43%), Bacteroidetes (27%), and Proteobacteria (20%). I found differences in the abundances of bacterial taxa by iguana age class, lounge size, and site. Alpha diversity of microbes was higher in adults/subadults than hatchlings. There were significant differences in the beta diversity of samples by individual, age, site, lounge size, and year. I found high variation in microbial community composition overall and by each of these categories, including within recaptured individuals over time. Intergenerational microbial community transmission is unlikely to be as important as previously hypothesized. This finding is based on the near absence of intergenerational associations and the dissimilarity in microbial community assemblages between hatchlings and adults/subadults. However,
within-hatchling microbial admixing was common, as evidenced by similarities in microbial community composition among hatchlings. Although microbial communities of iguanas differed among sites and lounge sizes, there were few clear patterns, with the exception that these differences likely resulted from ecological factors including microbial community succession within iguana guts. These findings lay the groundwork for understanding the patterns of gut microbial diversity in herbivorous lizards. Future research should focus on testing the role of soil on microbe acquisition, gene annotation of microbial communities to determine the OTUs contributing to digesting plant-fiber or acquiring nutrients, and to determine the effects of microbial community composition on aspects of host lizard biology.
Introduction

An animal’s diet affects most aspects of its biology. Diet specialists, for example, have morphological, physiological, and behavioral adaptations to aid in foraging, procuring, and assimilating their food. Feeding on specific plant tissues or their products (e.g., roots, leaves, flowers, pollen, nectar) is a common form of diet specialization. In vertebrates, plant eating is the derived condition and the proportion of herbivorous species in each of the major vertebrate lineages varies considerably. For example, more than 25% of mammal species are herbivores (Choat and Clemmens 1998), but herbivory is a rare diet strategy for non-avian reptiles. In fact, less than 1% of extant lizard species is primarily or strictly herbivorous (Cooper and Vitt 2002; Espinoza et al. 2004). Most herbivorous lizards have a suite of adaptations to aid in the cropping, digestion, and assimilation of plant tissues. These specializations include modified teeth, large bodies, voluminous and partitioned guts, and microbial fermentation systems (Pough 1973; Nagy 1977; Troyer 1982; Iverson 1982; McBee and McBee 1982; Bjorndal 1997; Cooper and Vitt 2002; Espinoza 2002; Espinoza et al. 2004; Mackie et al. 2004; O’Grady et al. 2005). Most of these traits are considered essential for herbivorous lizards because, bite for bite, plant tissues are lower in energy and nutrients and plant cell walls are made of layers of cellulose and hemicellulose—carbohydrates that cannot be cleaved by the endogenous enzymes produced by vertebrates.

Microbial Symbioses—Although not widely studied, herbivorous lizards likely receive a substantial proportion of their daily energy needs from hindgut microbial fermentation
(e.g., ~30% in *Iguana iguana*; McBee and McBee 1982). Communities of microbial endosymbionts (primarily bacteria, but also Archaea, protozoans, and fungi), as well as nematodes live in the anaerobic hindguts (and less commonly midguts) of herbivorous reptiles. There, these microbes hydrolyze cellulose and hemicellulose into sugars (e.g., β-glucose), which are fermented into short-chain volatile fatty acids (e.g., acetic and butyric acid) that can be passively absorbed by the host’s gut (Bjorndal 1997).

These digestive microbial communities vary with respect to host species (Hong et al. 2011), diet (Andres 2005), spatially (Lankau et al. 2012), and among individuals (Andres 2005; Lankau et al. 2012). Yet, there are few studies on the microbiomes of herbivorous lizards and little is known about the diversity, variation, turnover, and acquisition of these vital microbial communities. Because of recent technological advances (Lozupone and Knight 2005; Marguiles et al. 2005; Hamady et al. 2008; Caporaso et al. 2012), it is now possible to identify, or at least characterize, members of these microbial communities. This progress has allowed investigators to begin to address previously unanswerable ecological questions concerning these communities and their reptile hosts (e.g., Costello et al. 2010; Hong et al. 2011; Lankau et al. 2012).

*Acquisition of Microbes*—The guts of neonatal vertebrates are initially sterile. Many herbivorous vertebrates are thought to acquire their gut microbes soon after birth through social interactions with their parents like mutual grooming and licking, food sharing, or coprophagy (Minchin 1937; Hungate 1966; Francis-Smith and Wood-Gush 1977; Leon 1978; Jarvis 1981; Troyer 1982, 1984a). However, most reptiles lack parental care (Shine 1988), making this pathway for acquiring fiber-fermenting microbes in neonate
herbivorous lizards unlikely. Interestingly, herbivorous lizards are among the most social of all reptiles (Parker 1983; Troyer 1984b; Bull et al. 2000; Duckett et al. 2012). Thus, the question stands: how do neonate herbivorous lizards acquire their digestive microbes? It has been hypothesized that these microbial communities could be acquired by neonates from three non-mutually exclusive sources: (1) eating soil or contact with the environment (Sokol 1967; McBee and McBee 1982; Troyer 1982, 1984a); (2) intergenerational social interactions (direct contact, licking, coprophagy); (3) and/or through interactions with young already inoculated with the microbial communities (Troyer 1982, 1984a,b; Bjorndal 1997). In this study I focused on the latter two hypotheses.

*Natural History of Green Iguanas*—Green Iguanas (*Iguana iguana*) are ideal candidates for identifying the source(s) of symbiotic microbes of neonate herbivorous lizards. First, they are strictly herbivorous throughout life (Rand et al. 1990). Second, they are relatively common throughout the Neotropics and there is a wealth of information on their physiology, behavior, and ecology. This information was largely gleaned from studies of an insular population of iguanas in the Panama Canal Zone (e.g., Burghardt and Rand 1982; Troyer 1982, 1984a). Third, hatchling iguanas form social aggregations or “lounges” (Rand 1968; Montgomery et al. 1973; Burghardt et al. 1977; Burghardt and Rand 1985). Fourth, and most importantly, the original work hypothesizing and initially testing the potential sources of hindgut microbes in herbivorous lizards was conducted with this species on Barro Colorado Island (BCI), Panamá (Troyer 1982, 1984a).
Iguana iguana is a large-bodied Neotropical lizard with adults achieving body sizes up to 50 cm (snout–vent length) and 6 kg (Swanson 1950). Green Iguanas living on BCI are active year-round with peaks in activity corresponding to reproduction in January and February (Montgomery et al. 1973; Dugan 1982; Rand and Greene 1982; Rand 1984; Bock et al. 1985) and juvenile dispersal in May (Burghardt et al. 1977; Burghardt and Rand 1985). Data on breeding populations of I. iguana on BCI, Panamá have been collected since the 1960s until the early 2000s (Rand 1968; Burghardt et al. 1977; Burghardt and Rand 1985; M. Ryan and O. Acedevo, pers. comm.). Gravid females dig 1–2 m burrows and deposit their eggs, often at communal nesting sites, from late January to mid-March (Rand 1968). In late April to early May, neonates hatch and emerge synchronously from their underground nests and leave the nesting site in lounges (Burghardt et al. 1977; Bock 1989). At hatching, iguana hindguts are thought to be sterile (Troyer 1984a). Hatchlings spend 4–7 d digging to the surface, during which they eat soil and subsist off yolk reserves (Troyer 1982, 1987). Upon dispersing from the nesting site, hatchlings aggregate with other neonates, eat soil and plants, and have been observed licking and rubbing against each other (Troyer 1982, 1984a). It is unknown whether natural hatchling lounges are with siblings or whether they form mixed nest lounges, although hatchlings preferentially aggregated with kin in lab experiments (Werner et al. 1987). Troyer (1982) examined (600 × magnification) diluted hindgut contents of hatchling iguanas and detected the presence of bacteria. In their study of aggregating hatchling iguanas, Burghardt and Rand (1985) reported higher growth rates in lounges with three or more lizards compared to single iguanas or pairs of hatchlings. During the first three weeks after emergence, Troyer (1982) observed hatchlings closer to adult.
iguanas than expected for 83% of her observations \( (n = 25) \); during weeks 3–5, only 19% of observations \( (n = 16) \) were classified as intergenerational associations. Troyer (1984a) described the hindgut microflora of 2–3-wk old hatchlings as more complex based on microscopic examination, but because she was unable to identify most of the microbes, her designation of increased “complexity” was based on the novel acquisition of a protozoan.

After this short intergenerational association, hatchling iguanas on BCI tend to remain near other juveniles until they mature. Lounge size also increased during 3–5 wk post emergence, with lizards maintaining statistically higher than expected rates of lounging (Burghardt and Rand 1985). Troyer (1984a) showed that microbes can be behaviorally passed from an older, microbe-inoculated hatchling to a sterile-gut neonate. Juveniles may eat the feces of other hatchlings and continue direct contact with each other during this period, but it is not known whether hindgut microbe communities continue to change after their initial appearance. As juvenile iguanas mature, the number of plant species they consume increases (Troyer 1984c). However, it is not known whether there is a concomitant increase in the diversity of their hindgut microbial communities.

Although appropriate for the time, Troyer’s (1984a) characterization of these microbial communities would be considered imprecise and incomplete by today’s standards. For example, her lab experiments showed hatchlings grew faster when given access to soil during their first 3 wk, and also grew faster when fed the feces produced by an adult iguana from 3–5 wk (Troyer 1984a); however, these findings do not isolate the source(s) of the microbes, nor are they necessarily indicative of microbial activity.
**Objectives and Hypotheses**—The goal of my study was to attempt to characterize the variation in microbial communities of Green Iguanas across age classes (hatchling, subadult, and adult), temporally (within individuals), spatially, and in the context of hatchling social behaviors. Although Troyer (1984a) found that the gut microbial communities (broadly defined) of iguanas change over time ("sterile" → "simple" → "complex"), my study seeks to identify the members of these communities (given current knowledge and technology) and quantify temporal changes in microbial community composition.

In Galápagos iguanas (*Amblyrhynchus* and *Conolophus*), microbial communities are more similar in conspecifics from proximate sites than those from more distant islands (Lankau et al. 2012). I expected to see similar, yet subtler patterns for Green Iguanas, as my study focused on a single, primarily insular population at a finer spatial scale.

I predicted that both intergenerational lounging and hatchling sociality would best explain the community composition of gut microbes in hatchling Green Iguanas. Essentially, this expectation follows from the sociality hypotheses of Troyer (1982, 1984a) for the initial acquisition of these microbes. I further postulated that hatchlings found near adults/subadults would have microbial communities that are more similar to their older conspecifics than to hatchlings not found in intergenerational lounges. I generally expected sociality in hatchling iguanas to be the primary mechanism for transferring fiber-fermenting microbes and anticipated that this study would shed light on why hatchlings aggregate. This could manifest, for example, as more similarity in
microbial communities of hatchlings collected at the same site or from neonates observed in the same lounge. Hatchlings from larger lounges might be expected to have more diverse microbial communities compared to their less social conspecifics as more unique microbes could be shared among larger lounges. Conversely, per the intermediate disturbance hypothesis (Horn 1973; Connell 1978; Wilkinson 1999), high changes in an environment can lead to lower taxonomic diversity. Thus, hatchlings in larger lounges may have less diverse hindgut microbial communities because they may experience higher microbial community disturbance resulting from interactions with more conspecifics. If the latter is the case, I expected to observe greater microbial turnover over time.

I predicted that microbial communities of hatchling Green Iguanas would continue to change until the time they dispersed (~2 mo) and no longer had social contact with conspecifics, at which point their microbial communities would be compositionally similar to those of adults. Overlying these predictions, I expected to generally find high individual variation in microbial communities, as has been reported for other taxa (Andres 2005; Caporaso et al. 2011b).
Materials and Methods

Study Sites—The field component of this study was conducted on Barro Colorado Island (BCI) and the surrounding Barro Colorado Natural Monument (BCNM), Panamá (9.17 °N, 79.83 °W). BCI is a 1500-ha island located in Lake Gatún and administered by the Smithsonian Tropical Research Institute (STRI). The climate and habitat of BCI have been well characterized (Windsor 1990; Leigh 1999), and thus are summarized only briefly here. The island’s climate is typical of a Neotropical lowland, moist forest. There is virtually no seasonal, and only modest diel, variation in air temperature (Leigh 1999). Annual precipitation on Barro Colorado averages 2600 mm (range 1679–4133 mm; Windsor 1990), but a severe dry season typically extends from December through the end of April or early May (Windsor 1990; Leigh 1999).

Adjacent sites were considered different if divided by a physical barrier such as water (i.e., islands) or >500 m. Ten of the 11 sites where iguanas were observed and sampled are shown in Fig. 1. Barro Colorado Point (BCP) is an exposed flat, rocky beach extending ~2–10 m out from dense forest and walls of leafy vines. BCP has one ~3-m tall clump of Canal Grass (Saccharum spontaneum) in an otherwise open area. Sheltered within a cove on the north side of the island, Bat Cove (BCO) encompasses a ~3 × 4 patch of <1-m high grass on a sandy beach, a ~5 × 4-m nesting clearing, logs, a variety of trees and bushes ~4–8-m high on a moderate incline. One of the flat peninsulas in the North BCNM, Bohio Reach (BOH) has several small beaches (6–20 m from shore to forest) with clay, sand, or cobble substratum jutting out of dense forest. These BOH beaches have stands of ~3-m high Canal Grass, contrasting with the lianas and tall trees.
starting at the water’s edge in the rest of the BOH area. Bohio Sur (SUR) is a small (3-ha) island with a prominent peak near the center (~20 m above lake level), making most portions of this landmass relatively steep. Approximately 5% of the island has been cleared and is this area is cut monthly, leaving a short lawn around the iguana rookery at the rocky shore. The rest of SUR is covered in forest and bushes (trees up to ~25-m tall), a few tree-fall gaps, and some patches of dense vegetation, with little to no beach. Buena Vista Reach (BVP) in the northeastern portion of BCNM is a flat, tree-covered, 4-ha island nearly in contact with the larger peninsula. Plants extend to the edges of the island, branches hang low over the water, and thick, thorny vegetation and grasses cover the east side. The southern tip of Harvard Point (HPT) is also dense with thorny vegetation and tall grasses. Mangroves dominate the west stretch of HPT, whereas the rest of this flat peninsula is covered with trees and lianas shading narrow sand beaches. The laboratory clearing (LAB) is a frequently disturbed area on a mild slope with multiple-story stucco buildings, concrete paths, close-cropped grass lawns, and a sandy beach. Stands of forest surround the clearing and patches of pruned vegetation border the buildings. Iguana nests are found along the banks of the LAB clearing. Peña Blanca Point (PBP) is an open, sandy beach against eroded cliffs with two stands of ~3-m tall Canal Grass at the water’s edge. Stanley Cove (STY) is an area of narrow, densely forested peninsulas where the >10-m trees grow over the water, producing considerable shade. Van Tyne trail (VTN) ends at the mouth of a stream in sulfur-smelling marsh with tall grasses leading into the forested, sloped banks of the island. Gamboa (GAM, 9.12 °N, 79.70 °W), not shown on the map, is a suburb bordered by the Chagras River, the Panamá Canal, and forested land, approximately 13 km southeast of Barro Colorado Island. Iguanas in Gamboa were found
on manicured grass patches within 10 m of more overgrown trees/shrubs, on asphalt roads, and in Canal Grass along the Panamá Canal.

Temporal Sampling, Capture, and Marking—I made behavioral observations and sampled Green Iguanas for microbes during the first 2–3 mo of the rainy season over two field seasons (May–June 2010 and April–June 2011) as hatchlings were emerging and dispersing from rookeries. I captured 66 hatchlings from eight sites in 2010 (2 May to 29 June) and 242 hatchling iguanas from six sites in 2011 (19 April to 14 June). Adult iguanas were captured at two additional sites (Stanley Cove and Van Tyne Trail; Fig. 1) where other age classes were not observed.

In 2010, hatchlings had started emerging from their nests before I arrived (2 May), so the date of first emergence was unknown, but likely not more than ~3 wk based on the size of the neonates collected that year compared to those in 2011. In 2011, I sighted and captured the first hatchling on 19 April. During the “dry” season that year, BCI received the second highest single-month rainfall on record (1183 mm in December; S. Dos Santos, pers. comm.). Consequently, four sites where I found hatchling iguanas in 2010 (Barro Colorado Point, Bat Cove, Harvard Point, and Peña Blanca Point; Fig. 1), were flooded in 2011. Because these sites were not available in the second season, I collected iguanas from an additional site (Buena Vista Reach) and were able to more extensively survey the remaining sites.

Nesting sites were identified by boating or walking around the perimeter of BCI and the peninsulas north of the island by day looking for signs of disturbed soil or leaf litter, loosely compacted soil, and/or iguana eggshells. I found hatchling lounge sites by
looking for Canal Grass and open, sun-exposed areas near shore. Lizards were captured by day (0530–1820) using a dental floss or braided fishing-line noose attached to 2- or 3-m collapsible poles. Some hatchlings were found while the lizards were sleeping at night (1940–2300) by spotlighting vegetation overhanging the lake from a canoe or small motorboat and capturing them by hand. Similarly, adults were spotlighted at night while boating around BCI and the adjacent Bohio Reach and startling them out of tree roosts using a 3-m pole or a partially filled 1.5-l plastic water bottle as a projectile. Startled iguanas were caught by hand as they fell or scooped out of the water with a net or by hand.

At the time of capture, the following data were recorded for each iguana: snout–vent length, body mass, location, height off ground, and behavior. Snout–vent length was measured with a measuring tape or plastic ruler (± 1 mm). Hatchlings were placed in tared bags and weighed with a 30-g spring balance (± 0.5 g) and adults and subadults were weighed with a 5-kg spring balance (± 10 g) (Pesola, Zug, Switzerland). Height off the ground was estimated to the nearest 0.5 m or more precisely (± 0.05 m) if the lizard was <0.5 m above the ground. Feeding, licking, movement, and social behaviors were recorded.

Prior to release, iguanas were permanently marked with a unique combination of colored beads (modified from Rodda et al. 1988) to facilitate subsequent identification at a distance. Six 3-mm plastic beads were threaded onto 20-pound test nylon monofilament and pierced through the dorsal skin flap of the hatchling’s neck with an 18-ga needle. Eight 5-mm plastic beads threaded onto 30-pound test monofilament were used for marking adults and subadults following the same protocol. The region of the dorsal skin
flap is not vascularized, so there was no loss of blood or need for anesthesia or antibiotics to insert the beads (Rodda et al. 1988). Half the beads were threaded onto each side (in a mirror-image pattern), facilitating identification from multiple directions (Fig. 2). The fishing line was melted onto a smaller bead, which served as a stopper. The beads did not exceed 7% of an iguana’s mass, and thus are not expected to hinder locomotion (Knapp and Abarca 2009). Beads were not removed at the end of the study, as Rodda and coworkers (1988) found iguanas with them suffer no long-term consequences.

Hatchlings were released within ~3 m of capture immediately after processing (~15 min). (However, in one notable case, a hatchling was plucked from the hand by a Double-toothed Kite [Harpagus bidentatus] after sampling but before release; Wehrle and Guzman 2012.) Special effort was made to recapture marked hatchlings, so long as 48 h had elapsed since the last capture. Upon detection, I recorded observations of each iguana as described below.

Lounging Observations—Once sighted, I recorded each iguana’s proximity to conspecifics as an index of lounging. An iguana lounge was defined as a focal individual and its conspecific neighbors within 6 m, following parameters described in Burghardt and Rand (1985). If an iguana was >6 m from the focal individual, but <6 m from another individual within the lounge, the more-distant iguana was included in the count for the lounge. I also estimated the distance from each focal iguana to its neighbors within the lounge. If lounge members had been previously marked, I recorded their identities. I calculated the proportion of iguanas in lounges, the mean and mode of lounge sizes, and the proportion of lounges containing both hatchlings and adults/subadults. I determined
the frequencies of lounge sizes and tested those against a Poisson distribution using a $\chi^2$ test in Excel (Microsoft, Redmond, WA).

For individuals observed more than once, I determined whether their lounging behavior was “high fidelity” (consistently observed alone or in lounges of similar size, i.e., pairs, or in larger lounges) or “rogue” (observed in lounges that fluctuated widely in size). I calculated the coefficient of variation (CV) for lounge sizes for each resighted iguana and divided the CV range into equal-sized quartiles. To test whether the CV quartile assigned was an artifact of the number of times an individual iguana was resighted and/or and affected by its lounge size, the number of observations per individual and average lounge size per individual were each compared by quartile using a Kruskal–Wallis rank sum test in R (R Development Core Team 2011).

As it was possible to observe an individual between capture events, I used mean lounge size when analyzing the microbial communities of iguanas so as to incorporate the social conditions an iguana experienced between microbial samplings. I calculated the mean lounge size an iguana experienced as it related to its microbial community, using the sightings from the period after the previous capture to the lounge size at the current capture. To allow for discrete, categorical analyses of lounge size in the case of non-whole number mean values, I binned the mean number of iguanas in a lounge in the following manner: 1 individual, >1 and ≤2 individuals, >2 and ≤3, etc.

Data for distance to nearest neighbor were visualized by site using box-plots of quartiles, minimum, and maximum. I estimated the local density ($\hat{N}_2$) of hatchlings for each site where lounges were observed using the formula:

$$\hat{N}_2 = \frac{n}{\pi \Sigma(r_i^2)}$$
where \( n \) = sample size (total number of iguanas observed in lounges) and \( r_i \) = the distance (m) of a random hatchling in the lounge to its nearest neighbor (Krebs 1998).

**Microbe Sampling**—I sampled microbes occurring on iguana skin and in their mouths and hindguts with sterile cotton swabs. Skin microbes were sampled immediately after capture by swabbing the scales on the back and sides of the lizards. Skin swabs were taken only if I was certain that neither my skin nor instruments had come into contact with the lizard prior to sampling. I sampled the oral cavities of iguanas by inserting swabs ~1 cm into each lizard’s mouth and rotating the cotton tip to achieve contact with the tongue and gums. Hindgut microbes were sampled by inserting swabs into the cloaca. Because the cloacal apertures of hatchlings were too small to accommodate the swab, I first removed ~50–80% of the cotton tip with sterilized forceps (dipped in 70% ethanol and flamed). The swab was then inserted 1–2 cm into the hindgut and rotated in an attempt to sample microbes from the intestinal walls and digesta. Tips of the cotton swabs containing the microbe samples were broken off into 1.8-ml cryovials that contained 0.15–0.35 ml of buffered glycerol solution (65% glycerol, 0.1 M MgSO\(_4\), 25 mM tris-HCl, pH 8; Ausubel et al. 2002). Microbe samples were stored at -20 °C within 4 h of collection or kept on ice until they could be frozen. While kept frozen at -20 °C to the extent possible, samples collected in 2011 were shipped to the U.S. on dry ice, kept frozen (-20 °C), then shipped from across the country (~7 h) at room temperature.

**Characterization of Microbial Communities**—DNA was extracted from individual microbe samples (1 microbe sample = the swab from one region of a single iguana at one
point in time, representing a single microbial community) using the MoBio UltraClean Soil DNA Kit (Carlsbad, CA) following the manufacturer instructions with the following modifications (Andres 2005; M. Haynes, pers. comm.): (step 6) vortexed 20 min; (step 19) 20 µl solution 5, let rest 1 min, centrifuged at 14,000 rpm for 30 s, added 20 µl solution 5, let rest 1 min. Using the whole sample extracted from the swab, this method produced ~40 µl of genomic prokaryotic DNA.

A subset (n = 100) of all hindgut samples (n = 384) from which DNA was extracted was selected for amplification. Samples were selected to represent all nine sites where hatchlings were observed (including both years), varying social lounge sizes, and all adults and subadults. Most samples (65%) were selected from the latter half of the collecting season (~1 mo after hatchling emergence), but multiple samples from 12 individuals collected over time were also included to test for temporal variation in the microbial community. Extracted samples were quality checked via PCR amplification using the following conditions: 25 µl reaction: 12.5 µl GoTaq Green Master Mix, Promega (Madison, WI); 2.5 µl Bact27F forward primer (5’–AGAGTTTGATCMTGGCTCAG–3’), 2.5 µl Bact1492R reverse primer (5’–TACGGYTACCTTGTTACGACTT–3’), 1 µl template. Thermal cycler conditions for PCR were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 65 °C (subtracting 0.5 °C/cycle) for 1 min, and 72 °C for 3 min, then reactions were kept at 72 °C for 10 min and held at 4 °C until moved to a −20 °C freezer (M. Furlan, M. Haynes, and F. Rohwer, pers. comm.). Because 16S rDNA sequences are highly conserved in prokaryotes (Weisburg et al. 1991), it is possible to amplify just the microbial 16S subunits, excluding host genetic material. Amplified samples were run on 2.5% agarose gels, stained with
ethidium bromide (12 µl 10 mg/ml stock EtBr for a 250-ml gel), and visualized under UV. Bands were scored as absent, weak, or good. Samples that did not form bands or only weak bands were excluded to insure high sample quality for sequencing.

Associated metadata were submitted to the Earth Microbiome QIIME databank (http://www.microbio.me/qiime/). Samples were shipped on dry ice to the laboratory of Rob Knight at the University of Colorado, Boulder for 16S amplicon pyrosequencing. There, members of the Knight Lab amplified the V4 region of the 16S rDNA using the following protocol (Caporaso et al. 2011a, 2012): three 25-µl PCR reactions were performed per sample with the following conditions: 13 µl MoBio PCR water (Carlsbad, CA), 10 µl 5 Prime Hot Master Mix (Hamburg, Germany), 10 µM of primers comprised of 0.5 µl of forward primer 515F (5′–AATGATACGGCGACCACCAGATCTACACTATGGTAATTGT GTGCCAGCMGCGCCGCTAA–3′) and 0.5 µl of reverse primer 806R (5′–CAAGCAGAAGACGGCATACGAGATNNNNNNNNNAGTCAGTCAGCCGGA CTACHVGGGTWTCTAAT–3′), where “N” is a unique error-correcting 12-base Golay barcode for individual sample identification (Hamady et al. 2008), and 1.0 µl of template. The samples were placed in the following thermal cycler conditions for PCR amplification: 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1.5 min, then reactions were kept at 72 °C for 10 min. The resulting PCR products were cleaned using MO BIO 96 htp PCR clean up kit (Carlsbad, CA) and quantified using Picogreen dsDNA reagent (Invitrogen, Grand Island, NY) in 10 mM Tris buffer (pH 8.0). The 16S rDNA amplicons were then sequenced on an Illumina HiSeq2000 using the previously described 515F and 806R primers (Caporaso et al.)
Bioinformatics—The metadata mapping files, generated sequence files, and quality scores were analyzed using the QIIME bioinformatics pipeline (Caporaso et al. 2010). Sequencing reads having two consecutive bases with quality scores <10^5 were trimmed at that point. Reads that were ambiguous, did not match the expected PCR barcode, or were <75 bases long were removed. Sequences were then lumped by sample using their barcodes.

I assigned sequences to operational taxonomic units (OTU) based clustering at a 97%-similarity threshold (Stackebrandt and Goebel 1994; e.g., Caporaso et al. 2011a). The most common read in an OTU cluster was assigned as the “representative sequence” for analyses hereafter. Sequences were aligned and assigned to their lowest taxonomic units based on comparisons with sequences in the Greengenes reference library (DeSantis et al. 2006; McDonald et al. 2011; Werner et al. 2012). I filtered the reads for phylogenetically informative sequences and constructed a phylogenetic tree, OTU table, and taxon summary plots (at phylum, class, order, family, and generic levels). Differences in the presence of OTUs were determined for the 10 most common phyla and the 50 most common genera with respect to iguana age, lounge size, and site.

To characterize the number of unique OTUs in each microbial community, I calculated alpha diversity of each sample. In QIIME, I used Chao1 with bias corrections to assess the true OTU diversity by combining the number of observed OTUs with an estimation of unobserved taxa (Chao 1984, 1987; Colwell and Coddington 1994). This
estimator assumes that if there are many singly observed OTUs in a sample, there are likely more that were not observed. This is calculated as:

$$Chao \ 1 = S_{obs} + \frac{F_1^2 - 1}{2F_2 + 1},$$

where $S_{obs}$ is the number of OTUs observed in the sample, $F_1$ is the number of OTUs with only one observed occurrence, and $F_2$ is the number of OTUs occurring only twice in the sample. Samples with an unexpectedly low alpha diversity score (lower than two standard deviations from the mean) were excluded from further analyses as these results likely represent sampling or sequencing errors (see alpha diversity results for more information). Differences in Chao1 score of each sample were determined for the following categories using Kruskal–Wallis tests in R: age class, site, lounge size, and year. Microbe samples from adult and subadult iguanas were excluded for all analyses except age class. Recaptures were excluded from all alpha diversity analyses. When significant differences were detected in models for Kruskal–Wallis tests, I used the Multiple Comparisons for Kruskal test (Kruskalmc), a non-parametric posthoc test for multiple comparisons in R (Siegel and Castellan 1988; Giraudoux 2012) to determine which categorical groups differed in alpha diversity. To test whether alpha diversity of microbes increased over time, I regressed Chao1 of all hatchlings (minus recaptures) with date of sampling for 2010 and 2011 (and both years pooled), Chao1 of all iguanas (minus recaptures) vs. iguana body mass, and Chao1 of all iguanas (minus recaptures) vs. snout–vent length.

To contrast differences OTU composition between microbial communities (or beta diversity), I computed Bray–Curtis dissimilarity through QIIME. Bray–Curtis dissimilarity was calculated using the equation:
\[
\overline{BC} = 1 - \frac{2C_{ij}}{S_i + S_j},
\]
where \(C_{ij}\) is the number of OTUs common to both samples, \(S_i\) and \(S_j\) are the total number of OTUs found in samples \(i\) and \(j\), respectively (Legendre and Legendre 1998). A Bray–Curtis dissimilarity score of 0 represents complete taxonomic similarity between samples; a score of 1 represents no OTU overlap between samples. I calculated the mean beta diversity between captures of individual recaptured hatchlings to produce a pooled “within recapture” Bray–Curtis dissimilarity score. Using the same samples, I also calculated the mean beta diversity between samples from different individuals as “among recapture” dissimilarity. I compared “within” and the mean of the pooled “among” recapture Bray–Curtis scores using a Mann–Whitney \(U\) test in R to determine whether the microbial communities of hatchlings were more similar within individuals over time vs. among other hatchlings. Using the same principle, I tested whether there were differences in the beta diversity of hatchlings found in the same lounge vs. the same samples compared among lounges, pooling the mean within lounge Bray–Curtis dissimilarity score and comparing it to the mean score among lounges using a Mann–Whitney \(U\) test in R.

To determine whether beta diversity was different within versus among the following categories: age class, site, lounge size, capture week, and year, I used Kruskal–Wallis tests. For example, I compared the grand mean of the within BCO site beta diversity (the Bray–Curtis differences between each sample from the BCO site pairwise with every other sample from the BCO site) with the mean of the beta diversity of each sample from the BCO site, to each LAB site sample and so forth. To test whether the microbial communities of hatchling iguanas differed from older iguanas over time, I
compared the Bray–Curtis beta diversity values of adults and subadults to samples collected from hatchlings over each capture week using a Kruskal–Wallis test. For recaptured hatchlings sampled multiple times in a week, only the first sample in a capture week period was used for this analysis. The Multiple Comparisons for Kruskal test was used to determine which pairs of comparisons were different. Phylogenetic similarities among microbial communities were compared for iguana of different ages (hatchling, subadult, and adult) and from different sites using principal coordinates analyses (PCoA).
Results

Lounging—I marked and sampled 308 hatchlings (2010: \( n = 66 \); 2011: \( n = 242 \)), three subadults (all 2011), and six adults (2010: \( n = 2 \); 2011: \( n = 4 \)). In 2011, I observed many more hatchlings than the previous season and at higher densities at the sites where I found them. In total I made 540 observations of iguanas and collected 904 microbe samples (Table 1). I collected hindgut microbe samples from all captured iguanas. I did not sample the oral cavities of a small number of iguanas (\( n = 7 \)) because they licked my equipment or me before I could sample them. Likewise, I only took skin samples of 317 (36%) of the lizards because of the likelihood of contact with my skin during capture. Oral and skin microbial communities were not sequenced for this study.

Most iguanas were caught once, but 13.6% of hatchlings were recaptured and sampled multiple times (mean = 1.5 times over the season, recaptured at a mean interval of 6.2 d; Fig. 3). Whether resampled or not, 21.7% (\( n = 67 \)) of hatchlings were resighted an average of 3.6 times over the season, and over a mean sighting interval of 3.4 d. Of the resighted hatchlings, 27% were observed in the same size lounge each time (\( CV = 0 \)), whereas 73% were seen in a different-sized lounge each time (up to \( CV = 120 \); Table 2). High fidelity individuals made up 35.8% of resighted iguanas, 37.3% were low fidelity, 20.9% were low rogue, and 6% high rogue. The consistency of resighted iguana lounge size was significantly affected by lounge size (Kruskal–Wallis \( \chi^2 = 11.28 \), \( P = 0.010 \)) and by number of observations of an individual (\( \chi^2 = 14.53 \), \( P = 0.002 \)). The high-fidelity, low-fidelity, and low-rogue designations did not significantly differ with observed lounge sizes, but the high-rogue lizards were seen in larger lounges on average than the high-
fidelity individuals. Low-fidelity and low-rogue lounging lizards were observed more often than high-fidelity lounging individuals.

Of the 540 total observations of iguanas, 202 (37.4%) of the lizards were in lounges (≥2 individuals). Very few of the total observations (n = 4; 0.7%) were of intergenerational loungings (hatchlings with adults/subadults). Mean lounge size was 2.9 ± 1.7 individuals with a mode of two individuals. Figure 4 shows a frequency distribution of the number of iguanas per encounter or lounge size. These data are neither normally nor Poisson distributed (Shapiro–Wilk test, P < 0.05, P << 0.001, respectively). Though the majority (62.6%) of observations were of lone individuals, all individuals were found at sites were other iguanas were found and not randomly dispersed throughout the environment.

The density of lounging hatchlings (\( \hat{N}_2 \)) differed at some sites (Fig. 5), perhaps because of variation in vegetation and habitat structure. For example, iguana density was low at LAB, a largely open grassy site with buildings, yet high at HPT, where the vegetation was dense. Although the criteria I used to define lounging allowed iguanas to be up to 6 m from their nearest neighbor (Burghardt and Rand 1985), lounging individuals tended to be much closer to each other (Fig. 6). Overall, lounging hatchlings averaged 1.2 (± 0.07) m from their nearest neighbors.

Although the Kruskal–Wallis test indicated significant differences in hatchling lounge size by site (\( \chi^2 = 20.93, P = 0.007; \) Fig. 7), the Multiple Comparisons for Kruskal test did not indicate any differences at an \( \alpha = 0.05 \) level. Apparently, this finding was not the result of a low number of observations from some sites (and thus artificially low or
high variance) as the same results were observed when the low-sampled sites were removed from the analysis.

**Taxonomic Composition**—One hundred microbial community samples were sequenced from hindgut swabs of 77 iguanas (67 hatchlings, 3 subadults, and 7 adults) resulting in 13,423,506 sequences read. Summaries of microbial taxa (Archaea and Bacteria) were generated for each sample, resulting in an estimated total of 37 phyla, 75 classes, 145 orders, 304 families, and 790 genera. Although I identified a total of 790 putatively unique OTUs, the true generic diversity is certainly higher than reported here as 30% of the ordinal and 32% of familial-level taxonomy were undescribed. The microbial communities were dominated (90%) by three bacterial lineages: Firmicutes (43%), Bacteroidetes (27%), and Proteobacteria (20%). The most common taxa and their relative (%) occurrence are listed in Tables 3–4.

The mean percentage of each of the 10 most common phyla and the 50 most common genera in each microbial community was compared across iguana age classes. Microbe samples taken from subadult and adult iguanas were pooled because there were no significant differences between those age classes at either the phylum or generic levels. However, the sixth most common phylum, Tenericutes (Table 3), was significantly higher in subadults and adults than in hatchlings (Kruskal–Wallis $\chi^2 = 8.40$, $P = 0.004$). No other common phyla differed among age classes (Table 4; Fig. 8). There were significantly higher proportions of nine genera in adults/subadults compared to hatchlings (Fig. 9): Unknown Genus 1 (Family: Ruminococcaceae) ($\chi^2 = 9.62$, $P = 0.002$), Unknown Genus 2 (Family unknown, Order: Clostridiales) ($\chi^2 = 8.79$, $P = 0.002$),
0.003), Unknown Genus 4 (Family: Catabacteriaceae) ($\chi^2 = 9.92, P = 0.002$), Roseburia ($\chi^2 = 6.20, P = 0.013$), Staphylococcus ($\chi^2 = 14.57, P << 0.001$), Epulopiscium ($\chi^2 = 5.43, P = 0.020$), Acidovorax ($\chi^2 = 14.81, P < 0.001$), Unknown Genus 7 (Family: Rhodocyclaceae) ($\chi^2 = 7.096, P = 0.077$), Unknown Genus 8 (Family: unknown, Order: unknown, Class: YS2) ($\chi^2 = 11.69, P < 0.001$). The genus Sarcina was present in significantly lower proportion ($\chi^2 = 5.89, P = 0.015$) in the hindguts of adult/subadult iguanas than in hatchlings. There were no significant differences between iguana age classes in the proportion of the additional 40 most common genera.

The Kruskal–Wallis test detected significant differences in the proportions of three phyla (Fig. 10) and 11 genera of hindgut microbes as a function of the sites where hatchling iguanas were observed. Although the proportions of the phyla Euryarchaeota, Fusobacteria, and Lentisphaerae were different among iguanas from different sites based on the Kruskal–Wallis analyses of variance full models ($\chi^2 = 15.55, P = 0.049$; $\chi^2 = 18.17, P = 0.020$; $\chi^2 = 16.15, P = 0.040$, respectively), the Multiple Comparisons for Kruskal test did not detect any pairwise differences as a function of site. The genus Sarcina was present in greater proportions ($\chi^2 = 16.32, P = 0.038$) in iguanas at the BCO site than the LAB site. Parabacteroides was more prevalent ($\chi^2 = 16.91, P = 0.031$) in iguanas at BCO compared to those from SUR. Conversely, Sphingobacterium percentages were elevated ($\chi^2 = 21.00, P = 0.005$) in iguanas at SUR compared to BCO. The following eight genera had significant differences according to the Kruskal–Wallis analyses of variance full models, but there were no pairwise differences detected among sites: Unknown Genus 1 (Family: Ruminococcaceae) ($\chi^2 = 17.54, P = 0.025$), Unknown Genus 2 (Family: unknown, Order: Clostridiales) ($\chi^2 = 15.84, P = 0.045$), Unknown
Genus 6 (Family unknown, Order: RF39, Class: Mollicutes) ($\chi^2 = 24.43$, $P = 0.002$), *Adlercreutzia* ($\chi^2 = 16.61$, $P = 0.034$), *Fusobacterium* ($\chi^2 = 16.03$, $P = 0.042$), Unknown Genus 9 (Family: Enterobacteriaceae) ($\chi^2 = 16.69$, $P = 0.034$), *Lachnospira* ($\chi^2 = 16.10$, $P = 0.041$), Unknown Genus 10 (Family: Clostridiales Family XIII *incertae sedis*) ($\chi^2 = 17.36$, $P = 0.027$).

In the majority of the 50 most common genera, the mean percentage of each in the microbial community was not different in different sized lounges. Bacterial species in the Phylum Actinobacteria trended towards being most prevalent in lounges of three iguanas (Fig. 11) and were significantly more common in trios of iguanas than in lounges of four ($\chi^2 = 17.20$, $P = 0.016$). A Kruskal–Wallis analysis of variance identified a significant difference in the proportion of Proteobacteria by lounge size ($\chi^2 = 14.44$, $P = 0.044$), but there were no clear differences among groups indicated by the post hoc analysis. Two genera in the family Enterobacteriaceae, *Klebsiella* and Unknown Genus 9, were both significantly more prevalent in lounges of seven iguanas than lounges of four ($\chi^2 = 16.10$, $P = 0.024$ and $\chi^2 = 14.43$, $P = 0.044$, respectively). *Lachnospira* is more prevalent in lounges of four iguanas than in lone individuals ($\chi^2 = 17.42$, $P = 0.015$). No other significant differences in taxonomic proportions were detected with respect to lounge size.

*Alpha Diversity*—Chao1 alpha diversity scores were not normally distributed (Shapiro–Wilk normality test, $W = 0.94$, $P << 0.001$), with a relatively large distributional break between Chao1 scores under two standard deviations below the mean and Chao1 scores above that threshold. Nine samples were outliers, with very low Chao1 alpha diversity.
indices. These outliers were from hatchling iguanas, but shared no other characteristics (e.g., individual, date of collection, site, lounge size). Indeed, some of these samples were recaptured individuals for which the Chao1 scores of the prior and subsequent samples fell within the typical alpha diversity range. Such a rapid plummet in OTU diversity is not biologically likely. Thus, I interpreted these low diversity samples to be the result of sampling or sequencing errors rather than biological phenomenon and they were excluded from subsequent analyses.

The microbial communities from the hindguts of adult and subadult iguanas had higher alpha diversity than those of hatchling iguanas ($\chi^2 = 14.92, P = 0.001$), but were not significantly different from each other (Fig. 12). Although there were differences by age class, there was no predicted increase in alpha diversity over the course of weeks in hatchlings captured for the first time ($n = 60, r^2 = 0.007, P = 0.507$, Fig. 13). Alpha diversity increased linearly with iguana mass ($n = 70, r^2 = 0.078, P = 0.022$, Fig. 14) and snout–vent length ($n = 70, r^2 = 0.156, P << 0.001$, Fig. 15), but these relationships were weak. Chao1 scores did not differ among hatchlings by site ($\chi^2 = 2.088, P = 0.837$), nor were there alpha diversity differences by lounge size ($\chi^2 = 11.61, P = 0.114$) or year ($\chi^2 = 1.067, P = 0.302$).

**Beta Diversity**—The Bray–Curtis beta diversity of microbial communities differed among age classes ($\chi^2 = 60.20, P << 0.001$). The beta diversity dissimilarity (where a Bray–Curtis of 0 indicates no differences in community composition and a value of 1 is no similarities) was higher among hatchlings (0.85 ± 0.10) than among adults (0.78 ± 0.08) or between adults and hatchlings (0.83 ± 0.09) or adults and subadults (0.76 ±
0.08), indicating higher variation among hatchlings than any other age class. There were also higher microbial community dissimilarities between adults and hatchlings (0.83 ± 0.09) and subadults and hatchlings (0.84 ± 0.10) than between adults and subadults. Thus, there were no significant differences between adults and subadults, but significant differences between adults and hatchlings. This is visualized as a principal coordinate analysis (PCoA) in Fig. 16. Although the Bray–Curtis values were significantly different, the first three principal coordinates only explained 7.2, 5.5, and 5.0% of the variation in the data, respectively.

The mean Bray–Curtis differences within sites ranged from 0.71–0.97, indicating high variation in iguana microbial communities from the same site. There were significant differences in beta diversity of microbial communities of iguanas compared between and within sites ($\chi^2 = 231.81, P << 0.001$). The microbial communities of iguanas from the SUR and BCO sites and the BOH and BCO sites were more divergent than to comparable communities within the BCO site. The amount of divergence between sites was not equal among comparisons, but no other combinations were significantly different between vs. within a single site. The differences in the hindgut microbial communities of iguanas among more proximate sites were not less than those from sites that were farther from each other. For example, the Bray–Curtis value between the microbial communities of iguanas from adjacent sites BOH and SUR, was larger than the value for iguanas from BCO and HPT, which were on opposite sides of the island. A PCoA of microbial community phylogenetic distance by site of iguana collection (Fig. 17) supported BCO iguanas as distinct from those found at SUR and BOH, but no other obvious patterns emerged.
I found larger beta diversity differences in microbial communities overall in pairs of hatchlings than in hatchlings found alone ($\chi^2 = 90.72, P << 0.001$). This trend did not follow for larger lounges and no other informative trends were found between beta diversity and lounge size.

The compositional differences of microbial communities were significantly larger between years than within each year based on comparisons of their Bray–Curtis distances with a Kruskal–Wallis test ($\chi^2 = 110.62, P << 0.001$), suggesting considerable annual variation. When broken down by week, I continued to find significant differences ($\chi^2 = 400.35, P << 0.001$). There were larger differences in the microbial community composition for the first three weeks of sampling in 2010, compared to weeks four and six, yet a progressive increase in dissimilarity within weeks four, five, and six. In 2011, the second and fourth weeks showed greater phylogenetic dissimilarity than week five, as did week four from week six. The early weeks of the two years were not dissimilar from each other, nor were the latter weeks. These patterns correspond somewhat with the comparisons of hatchling microbial community similarity to that of adults/subadults by week ($\chi^2 = 141.14, P << 0.001$). Hatchling microbial communities from the first sampling week in 2010 had a higher beta diversity difference compared to adults/subadults than week four of 2010 and week five of 2011. Surprisingly, though all subadult and nearly all adult iguanas were sampled in 2011, hatchlings sampled in week four of 2010 were more similar to adults/subadults than those hatchlings sampled in week five of 2010 and weeks two and four of 2011.

The Bray–Curtis beta diversity of microbial communities was more similar within recaptured individuals than between them ($n = 11, W = 20.00, P = 0.007$), although
differences from one capture to the next were still very high at 0.70 ± 0.04. Hatchlings from the same lounges had more similar Bray–Curtis values to each other than among lounges (n = 9, W = 5595, P = 0.002). Still, the microbial community diversity within individuals in a lounge was slightly higher (0.72 ± 0.12) than the mean beta diversity for recaptures of the same individual. The magnitude of difference within an individual was not different from that within a lounge (W = 41.00, P = 0.552). To ensure that this was not an artifact of variation, I determined that these beta diversity values are homoscedastic using a Bartlett test (Bartlett’s $K^2 = 0.375$, $P = 0.540$). Although I intended to analyze beta diversity within intergenerational lounges, they were far less common than anticipated (or observed by researchers studying iguanas in the same area in the 1980s) and I was unable to sample any of the adults/subadults observed near hatchlings.
Discussion

Lounging—Both the observational and genomic results of this two-year study suggest that it is unlikely that hatchling Green Iguanas acquire their digestive microbes via intergenerational social interactions. Indeed, I observed only four lounges (of 202) with both hatchlings and adults/subadults. In contrast, Troyer (1982) observed hatchlings high in the canopy close to adults, but I neither commonly observed hatchlings very high in the foliage nor in similar areas as adults and subadults. In fact, many of the larger iguanas I observed were at sites where hatchlings were not found. In the past 30 years, many of the sites on BCI where Green Iguanas had previously nested have been changed through anthropogenic interference (e.g., efforts to widen the Panama Canal, increased erosion from ships), perhaps forcing adult iguanas to change their dispersal behaviors. Poaching of many commercially valuable animals, including iguanas, spiked during this period (R. Moreno, pers. comm.; Wright et al. 2001), affecting species abundances at multiple trophic levels.

Hatchling lounging was very common, but individual iguanas did not exhibit preferred lounge sizes. Although other studies have observed social and asocial behavioral syndromes in lizards (e.g., Cote et al. 2008), in my study there was a tendency for lizards sighted more times to be observed in different lounge sizes from one sighting to the next. Thus, single observations of lounge size in Green Iguanas may not be the most informative trait. Yet in some cases, I observed marked hatchlings at the same location over several days and lounging with a new set of previously unsampled iguanas each time. These established hatchlings may function as way stations for microbial
transfer during hatchling dispersal. I did not observe this often enough to test whether the microbial communities of established hatchlings were more similar to those of adults.

*Iguana Age and Microbial Communities*—I predicted that the number of microbial OTUs present in an iguana’s hindgut would vary spatially, temporally, and with respect to social interactions, but this was not the case. Overall, I only observed differences in the number of OTUs among age classes. This may be because microbial communities are constrained by the morphology and physiology of the gut as well as ecological interactions within the microbial community (Arrhenius 1921; MacArthur and Wilson 1967; Andres 2005). Yet, because I found the number of OTUs in iguana guts scaled only weakly with size, the increase in adults/subadults is not likely attributable solely to gut size, but also to other ontogenetic, morphological, physiological, or behavioral differences. I acknowledge that because I used the same sampling methods in all age classes of iguanas it is possible that I may not have sampled the microbes from the same gut region. The greater number of OTUs in adults/subadults is consistent with increases in the diversity of plant material eaten by iguanas as they age (Troyer 1984c). Thus, older iguanas may acquire more microbe species, including specialists, to facilitate the digestion of diverse plant species. However, my data indicate that this increase in OTUs does not occur over the first 2 mo posthatching. This may be consistent with ontogenetic changes in the gut milieu, such as pH or hindgut size and niche complexity, and these changes in the microbial habitat can serve as filters determining the presence of certain kinds of microbes (Keddy 1992; Andres 2005). The overall microbial communities of adults and subadults were not more different between the two age classes than within them, but both were less variable than
the microbial communities found in hatchlings. This suggests a highly variable acquisition source, such as soil that is essentially filtered through the digestive tract, yet ultimately leads to a similar microbial community over time.

The microbial taxa found to be the most common in adult/subadult iguanas have all been reported from the gastrointestinal tracts of other vertebrates (e.g., Clements 1997; Huws et al. 2011; Barry et al. 2012; Hooda et al. 2012), some of which are known fiber-fermenting organisms (although some members of the Class Mollicutes have been identified as pathogens; Barré et al. 2004). The genus Sarcina was more prevalent in hatchlings than in adults/subadults. This genus is a known cellulose synthesizer (Ross et al. 1991), making it both very hardy as well as able to bind other microbes together, facilitating symbiotic interactions. It is interesting to consider that the cellulose produced by Sarcina could provide substrate for fiber-fermenting microbes in the hindguts of hatchlings iguanas, hindering the digestion of plant fiber due to increased cellulose substrate competition.

Spatial Variation in Microbial Communities—I was surprised to find that the microbial communities of hatchling iguanas did not show evidence of similarity among spatially proximate sites. Given that I expected to find evidence of social transmission of microbial communities among iguanas from proximate sites, I hypothesized that iguanas within a site would be have the most similar microbial communities, followed by iguanas from proximate sites that hatchlings had dispersed to, and microbial communities of iguanas from distant sites (i.e., those beyond the typical dispersal distance of 300 m; B.C. Bock, unpubl. data) would be the least similar. Yet, the only significant differences were
between the iguanas from BCO and SUR sites and the iguanas from BCO and BOH sites (Fig. 1), with greater microbial community variance at SUR and BOH than BCO. These three sites had similar densities of hatchlings, distributions of distances of focal individuals to their neighbors, and lounge sizes (Figs 5, 6, 7). Although their microbial communities differed overall, iguanas from BCO also had more Sarcina bacteria than LAB iguanas, a trend also observed between hatchlings and adults. BCO is centrally located relative to the other sites and, although it is also a protected inlet and across the Panama Canal from SUR and BOH, it is closer to them than to the other sites (Fig. 1). GAM was >10 km from any other site and thus well outside the radius of dispersal for young iguanas, yet the microbial communities of iguanas from this site were not different from any other site. Additionally, the microbial communities of iguanas from the two proximate sites BOH and SUR were less similar to each other than were the microbial communities of iguanas from several pairs of sites that were across the island from each other. While overall spatial patterns of microbial community diversity were not evident, I note that iguanas were found at BCO only in 2010 and the majority of samples collected at SUR and BOH were from 2011. Yet, if year was an important predictor of community diversity, I would have expected to observe significant differences at other sites that were only sampled in 2010 (BCP, HPT, PBP), yet this was not the case. There was especially high microbial community variation at SUR, perhaps because of the large number of hatchlings emerging from nests and interacting at this site. Lounge sizes were not predictably variable by site and hatchlings from the sites with the greatest differences in hatchling density did not have evident patterns of differences in their microbial communities. There may have been differences in the soil microbiomes of each site that
were responsible for the differences in gut microbial communities that I detected. Future analyses of soil microbial communities in conjunction with host microbiomes may lend some clarity to my observations of this spatial variation.

Sociality and Microbial Communities—The fact that I detected nearly as much variation within the microbial community of an individual from one capture to the next as there was within individuals in a single lounge indicates that social transmission of microbial communities is likely important for shaping a hatchling iguana’s gut microbiome. Hatchlings observed together had similar OTUs in their microbial communities, supporting hatchling–hatchling social interactions as a route of microbial transmission. However, I did not test the role of iguana kinship in determining the gut microbial community. Werner and colleagues (1987) showed that hatchling Green Iguanas can identify and are more likely to lounge with close kin than less-closely related hatchlings. Thus, it is possible that the similarities in microbial communities that I detected are driven by genetic similarities more so than microbe sharing. (I also collected blood samples from most individuals and plan to test the role of kinship in determining the gut microbial community in a future study.)

While I have evidence of endosymbiont sharing among iguanas, the microbial communities did not differ with social behavior in the manner predicted. For example, I expected to find a positive relationship between lounge size and the number of OTUs in the community, but microbial diversity did not vary among hatchlings from different-sized lounges. I also expected greater differences in microbial communities among individuals found in larger lounges. This is because the more frequent exchanges of
microbes in larger lounges would be expected to yield microbial communities with higher OTU turnover (i.e., the intermediate disturbance hypothesis of Connell 1978). Yet I only found higher differences in microbial communities between pairs of hatchling iguanas. Rates of microbial transmission between pairs of iguanas may in fact be the intermediate level of disturbance that is sufficient to drive changes in the microbial community (Connell 1978). Under this scenario, iguanas from larger lounges would experience more opportunities for exchanging new microbes than establish residence in the gut and thus only the most hardy, competitive, and/or quickly proliferating microbes (e.g., Enterobactericeae) would persist.

Temporal Variation in Microbial Communities—While the number of OTUs in hatchling iguana guts did not increase during the first 2 mo of life, I generally observed more variation in the microbial communities during the first 3 wk posthatching than in the latter three. This finding is consistent with either socially driven microbe transfer (as later in the season, there would have been more opportunities for microbes to be shared, homogenizing the communities among individuals) or changes in the physiology of the gut (leading to the assembly of a similar microbial community from a once more diverse assemblage). The microbial communities of hatchling iguanas most closely resembled those of adults as the matured through their first season (although still not as similar as adults were to each other). Thus, in support of my hypothesis, the gut microbiomes of hatchlings are driven towards less variable, more adult-like assemblages over time. Greater sampling of adults and a longer sampling period (>2 mo) of hatchlings would
allow me to determine when the gut microbe community stabilizes to its adult assemblage, as it appeared to remain in a state of flux at the end of my sampling period.

As described above, there was a tendency towards the microbial communities of hatchling iguanas to resemble those of older iguanas as time progressed. Yet this itself is not indicative of intergenerational microbe transmission because the community could have achieved a near-stable state, and/or been driven by changes in diet, or physiological or morphological changes in the gut. The temporal difference in beta diversity between hatchlings and adults was not significantly different than that found among hatchlings in the same lounge, so I cannot completely rule out intergenerational lounging (although larger Bray–Curtis values earlier in the season and more consistent similarity later in the season would support this notion).

Given that the microbial communities of hatchling iguanas were significantly different between the two years of observation, I hypothesize that environmental factors affect gut microbiomes as well. Specifically, 2010 was a drier-than-usual year on Barro Colorado Island and 2011 was one of the wettest, likely leading to untold ecological differences between the two years. A long-term dataset (e.g., 5–10 yr) on the hindgut microbes of hatchling Green Iguana may provide insight into the environmental factors that drive microbial community variation and perhaps even population-wide variation in digestive efficiencies. Changes in nutrient acquisition and assimilation of Green Iguanas could have cascading effects across the ecosystem (Swierk and Langkilde 2009).

*Individual Variation in Microbial Communities*—Although I observed differences in the beta diversity of microbial communities by iguana age, site and lounge size, individual
variation in the microbial communities of Green Iguanas was still very high. Andres (2005) found greater microbial variation in the guts of Desert Iguanas (*Dipsosaurus dorsalis*) at the level of the individual despite dramatic differences in experimental diets.

In this study, I documented high variation in microbial communities of recaptured iguanas. Similarly, Caporaso and colleagues (2011b) reported high turnover between repeatedly sampled human-associated microbiota. In both Green Iguanas and humans, variation in the microbial communities within individuals was still smaller than variation among individuals. Thus, even if it is changing, there is still an inherent, individual microbial community. That the variation in microbial communities within an individual over time was smaller than among iguanas exchanging microbes suggests the importance of assembly rules (Diamond 1975a,b; Simberloff 1978; Keddy 1992) in shaping the endosymbiont communities of animal guts thereafter and/or the variation in the physiology of individual lizards for maintaining differences in microbiomes.

The gut microbiome of the Green Iguana is very diverse. As in other herbivorous vertebrates (Costello et al. 2010; Godoy-Vitorino et al. 2010; Caporaso et al. 2011b; Hong et al. 2011), the hindguts of Green Iguanas are dominated by Firmicutes and Bacteroidetes. This, however is contrary to the finding by Hong and colleagues (2011), who sequenced the hindgut microbes of two Green Iguanas from El Salvador, revealing Prevotellaceae as the dominant microbe. Curiously, Prevotellaceae were not detected in the iguanas from my study.

Interestingly, despite the high microbial variation and turnover, these communities still manage to serve the vital function of digesting plant fiber for their hosts. There are likely many alternative assemblages of microbial communities that can
hydrolyze the cellulose in an iguana’s diet. In fact, it seems likely that the genes that encode for these functions are more important than the microbial taxa. Future studies combining a characterization of the microbiome with an annotation of transcriptomes of these variable microbial communities and simultaneous measures of digestive efficiencies of the lizards would give great insight into how these communities function and affect the performance of their herbivorous hosts.

Conclusions — Although I did not find strong spatial patterns of microbial community diversity in my study of iguanas on and around Barro Colorado Island, Panamá, there are likely differences in Green Iguana microbiomes at larger spatial scales. For example, *I. iguana* is widely distributed throughout Central and South America and have been introduced into the Caribbean and to Florida and Hawai’i, (USA) as well (Kraus 2009). I predict that gut microbiomes exhibit geographic divergence. This is important not only in understanding the basic biology and physiology of this model herbivorous lizard, but also in determining their effects on their ecosystems. For example, Swierk and Langkilde (2009) found that invasive iguanas in Puerto Rico contribute substantial micronutrients into the ecosystem that may affect native plant communities, an influence that is at least partially determined by the digestive microbial community of the host.

Why do lizards lounge? In the case of hatchling Green Iguanas, one of the reasons is to transfer digestive microbes. The surprising lack of direct observation of intergenerational lounging leads me to believe this phenomenon is not an essential or even common route for the acquisition of gut microbes in hatchling Green Iguanas,
contrary to previous hypotheses (Troyer 1982, 1984a) and popular belief (e.g., Troyer 1984b; Wikelski et al. 1993; Main and Bull 1996; Cooper and Lemos-Espinal 2001; Gray 2001). Considering that my study shows little support for intergenerational microbe transfer, I suggest soil is necessary for initial microbe acquisition, with hatchling microbe transfer influencing the temporal assembly of the community. To fully understand how hatchling Green Iguanas acquire their gut microbes, it will be necessary to examine the role of soil, both by characterizing the soil microbiome in Green Iguana habitat and via laboratory experiments designed to determine how consuming microbiota in soil (and perhaps of other substrata) translates into the microbial community that eventually stabilizes in an iguana’s gut.

Green Iguanas are the most well-studied herbivorous lizard and because of this wealth of knowledge (e.g., Burghardt and Rand 1982), they are an important model organism. Yet, I are uncertain if Green Iguanas are even “typical” herbivorous reptiles because I do not know how varied the gut microbiomes are among herbivorous lizard species. Are the behavioral and microbial patterns that I observed in Green Iguanas shared among phylogenetically diverse plant-eating lizards? This question is stating to be answered (Hong et al. 2011; Lankau et al. 2012), yet future work characterizing gut microbiomes of additional herbivorous lizard species will bring much needed insight into the ecology and evolution of this vital symbiosis.
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<table>
<thead>
<tr>
<th></th>
<th>BCO</th>
<th>BOH</th>
<th>SUR</th>
<th>BVP</th>
<th>GAM</th>
<th>HPT</th>
<th>LAB</th>
<th>PBP</th>
<th>STY</th>
<th>VTN</th>
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<tbody>
<tr>
<td>Hindgut</td>
<td>5</td>
<td>56</td>
<td>79</td>
<td>97</td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>116</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Oral</td>
<td>5</td>
<td>53</td>
<td>77</td>
<td>97</td>
<td>9</td>
<td>7</td>
<td>9</td>
<td>116</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Skin</td>
<td>5</td>
<td>48</td>
<td>6</td>
<td>31</td>
<td>0</td>
<td>3</td>
<td>7</td>
<td>38</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2. Green Iguanas (*Iguana iguana*) resightings over two field seasons (2010–11) on Barro Colorado Island, Panamá divided into quartiles by coefficient of variation (CV) of lounge size. Data are means (± SE).

<table>
<thead>
<tr>
<th>Lounge Category</th>
<th>n</th>
<th>Lounge Size</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High fidelity</strong></td>
<td></td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>(CV = 0–30)</td>
<td>24</td>
<td>(± 0.2)</td>
<td>(± 0.2)</td>
</tr>
<tr>
<td><strong>Low fidelity</strong></td>
<td></td>
<td>2.2</td>
<td>4.1</td>
</tr>
<tr>
<td>(CV = 31–60)</td>
<td>25</td>
<td>(± 0.5)</td>
<td>(± 0.2)</td>
</tr>
<tr>
<td><strong>Low rogue</strong></td>
<td></td>
<td>2.3</td>
<td>4.6</td>
</tr>
<tr>
<td>(CV = 61–90)</td>
<td>14</td>
<td>(± 1.0)</td>
<td>(± 0.2)</td>
</tr>
<tr>
<td><strong>High rogue</strong></td>
<td></td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>(CV = 91–120)</td>
<td>4</td>
<td>(± 0.6)</td>
<td>(± 1.1)</td>
</tr>
</tbody>
</table>
Table 3. Ten most common microbial phyla sampled from the hindguts of *Iguana iguana* over two field seasons (2010–11) on Barro Colorado Island, Panamá and corresponding average percentages of incidence per sample. Phyla are Bacteria unless otherwise noted.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Average %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>44.00</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>26.41</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>19.28</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>5.90</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>1.31</td>
</tr>
<tr>
<td>Tenericutes</td>
<td>1.35</td>
</tr>
<tr>
<td>Euryarchaeota (Domain: Archaea)</td>
<td>0.69</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.41</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>0.34</td>
</tr>
<tr>
<td>Lentisphaerae</td>
<td>0.21</td>
</tr>
</tbody>
</table>
Table 4. Ten most common microbial genera sampled from the hindguts of *Iguana iguana* over two field seasons (2010–11) on Barro Colorado Island, Panamá and corresponding average percentages of incidence per sample.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Average %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacteroides</em> (Family: Bacteroidaceae)</td>
<td>18.90</td>
</tr>
<tr>
<td>Unknown Genus 1 (Family: Ruminococcaceae)</td>
<td>7.81</td>
</tr>
<tr>
<td>Unknown Genus 2 (Family: unknown, Order: Clostridiales)</td>
<td>7.17</td>
</tr>
<tr>
<td>Unknown Genus 3 (Family: Lachnospiraceae)</td>
<td>6.23</td>
</tr>
<tr>
<td><em>Klebsiella</em> (Family: Enterobacteriaceae)</td>
<td>5.43</td>
</tr>
<tr>
<td><em>Clostridium</em> (Family: Lachnospiraceae)</td>
<td>4.33</td>
</tr>
<tr>
<td><em>Kocuria</em> (Family: Micrococcaceae)</td>
<td>4.00</td>
</tr>
<tr>
<td><em>Parabacteroides</em> (Family: Porphyromonadaceae)</td>
<td>3.08</td>
</tr>
<tr>
<td><em>Clostridium</em> (Family: Clostridiaceae)</td>
<td>2.69</td>
</tr>
<tr>
<td><em>Acinetobacter</em> (Family: Moraxellaceae)</td>
<td>2.08</td>
</tr>
</tbody>
</table>
APPENDIX B: FIGURES

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Figure 1. Sites where iguanas were sampled on Barro Colorado Island (BCI), Lake Gatún, Panamá (9.17 °N, 79.83 °W). Dots denote only adults observed at site: Barro Colorado Point (BCP), Bat Cove (BCO), Bohio Reach (BOH), Bohio Sur (SUR), Buena Vista Reach (BVP), Harvard Point (HPT), Laboratory Clearing (LAB), Peña Blanca Point (PBP), Stanley Cove (STY), and Van Tyne (VTN). The Gamboa (GAM) site is not shown, but lies ~13 km southeast of BCI.

Figure 2. Bead-marked juveniles (A, B) and an adult (C) Green Iguana (*Iguana iguana*) from Barro Colorado Island, Panamá.

Figure 3. Time in days between initial Green Iguana (*Iguana iguana*) capture and subsequent captures for recaptured hatchlings over two field seasons on Barro Colorado Island, Panamá. Different shades denote first recapture, second recapture, etc.

Figure 4. Frequency of Green Iguana (*Iguana iguana*) lounge sizes observed over two field seasons (2010–11) on Barro Colorado Island, Panamá.

Figure 5. Density (mean ± SE) of Green Iguanas (*Iguana iguana*) in lounges (*\( \hat{N}_2 \)) for each location where lounges were observed over two field seasons on Barro Colorado Island, Panamá.
Figure Legends (continued)

Figure 6. Boxplots of distance (m) of focal Green Iguana (*Iguana iguana*) to nearest conspecific neighbor by site with minimum, quartiles, and maximum.

Figure 7. Green Iguana (*Iguana iguana*) lounge sizes (mean ± SE) by site over two field seasons on Barro Colorado Island, Panamá.

Figure 8. Composition of microbial communities at phylum level in Green Iguana (*Iguana iguana*) adults, subadults, and hatchlings from Barro Colorado Island, Panamá.

Figure 9. Percentage (mean ± SE) of microbial taxa present in the microbial community of Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá by iguana age class. Only genera that have significant different proportions by age class are included: (A) Unknown Genus 1 (Family: Ruminococcaceae), (B) Unknown Genus 2 (Family unknown, Order: Clostridiales), (C) Unknown Genus 4 (Family: Catabacteriaceae), (D) *Sarcina*, (E) *Roseburia*, (F) *Staphylococcus*, (G) *Epulopiscium*, (H) *Acidovorax*, (I) Unknown Genus 7 (Family: Rhodocyclaceae), (J) Unknown Genus 8 (Family unknown, Order unknown, Class: YS2).

Figure 10. Phylum-level composition of hindgut microbial communities of hatchling Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá as a function of capture site.
Figure Legends (continued)

Figure 11. Phylum-level composition of hindgut microbial communities of hatchling Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá as a function of lounge size.

Figure 12. Estimation of OTU richness (Chao1 alpha diversity ± SD) of microbial communities of adult, subadult, and hatchling Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá.

Figure 13. Estimation of OTU richness (Chao1 alpha diversity) of hindgut microbial communities as a function of capture day ($r^2 = 0.007, P = 0.51$) for hatchling Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá.

Figure 14. Estimation of OTU richness (Chao1 alpha diversity) of hindgut microbial communities of Green Iguana (*Iguana iguana*) from Barro Colorado Island, Panamá as a function of lizard mass (g) ($r^2 = 0.078, P = 0.022$).

Figure 15. Estimation of OTU richness (Chao1 alpha diversity) of hindgut microbial communities of Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá as a function of lizard snout–vent length (mm) ($r^2 = 0.156, P << 0.001$).
**Figure Legends (continued)**

Figure 16. Principal coordinate analysis of phylogenetic similarity of microbial communities from the hindguts of Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá as a function of age class.

Figure 17. Principal coordinate analysis of phylogenetic similarity of microbial communities from the hindguts of Green Iguanas (*Iguana iguana*) from Barro Colorado Island, Panamá as a function of site.
Fig. 3

Days between captures

Iguana ID
Fig. 4

Number of observations

Number of iguanas in lounge

<table>
<thead>
<tr>
<th>Number of iguanas in lounge</th>
<th>Number of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>339</td>
</tr>
<tr>
<td>2</td>
<td>122</td>
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<tr>
<td>3</td>
<td>42</td>
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<tr>
<td>4</td>
<td>18</td>
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<tr>
<td>5</td>
<td>9</td>
</tr>
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<td>6, 7, 8, 9</td>
<td>1, 2, 2, 4</td>
</tr>
<tr>
<td>10, 11, 12</td>
<td>1, 1, 1</td>
</tr>
</tbody>
</table>
Fig. 5

Index of density

Site

BCP  LAB  PBP  BCO  BOH  SUR  BVA  HPT
Fig. 6

Distance to nearest neighbor (m)

Site

BCP  LAB  PBP  BCO  BOH  SUR  BVP  HPT
Fig. 7

Number of individuals in lounge

Site

GAM  PBP  LAB  BCO  BOH  BVP  SUR  BCP  HPT
Fig. 8

Cumulative abundance

- Adults
- Subadults
- Hatchlings

- other
- Verrucomicrobia
- Actinobacteria
- Proteobacteria
- Bacteroidetes
- Firmicutes
Fig. 9

Hatchlings
Adults/Subadults

Percentage of taxon present

Taxon
Fig. 10
Fig. 11

Cumulative abundance of bacterial classes in the number of iguanas in the lounge.

- Verrucomicrobia
- Actinobacteria
- Proteobacteria
- Bacteroidetes
- Firmicutes
- Other
Fig. 12

![Bar chart showing CHAO1 values for Adults, Subadults, and Hatchlings.](image)
Fig. 13

![Scatter plot showing CHAO1 Score vs. Days with data points for 2010 and 2011.](image)
Fig. 14

\[ r^2 = 0.078 \]
Fig. 15

$\hat{r}^2 = 0.156$
Fig. 17