Assessing welfare of armadillos using hormonal & foraging indicators, and patch use in Argentinean birds

BY

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THESIS

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LIST OF ABBREVIATIONS

LPZ	Lincoln Park Zoo
SAZ	San Antonio Zoological Garden
ECL	Estrous cycle length
FPM	Fecal progestagen metabolite
FAM	Fecal androgen metabolite
FGM	Fecal glucocorticoid metabolite
SLQ	Sierra de las Quijadas
GUD	Giving-up Density
C	Metabolic Cost
P	Predation Cost
MOC	Missed Opportunity Cost

SUMMARY

This dissertation investigates the gonadal and adrenocortical activity of three-banded armadillos (*Tolypeutes matacus*). It combines adrenocortical activity with foraging behavior to assess the well-being of this armadillo. Patch use of a bird species in Argentina was also explored in this dissertation. Chapter 1 describes my journey to and through the dissertation, as I tackled my lab work, and broke free from my fear of field work. It also reflects my beliefs and personal goals for my post-doctorate career.

Chapter 2 describes the characterization of the gonadal hormone activity of the zoo-housed three-banded armadillo (*Tolypeutes matacus*). Although some anatomical and observational work had been done on the three-banded armadillo, no research had been conducted to evaluate its reproductive hormonal characteristics. Armadillos from Lincoln Park Zoo and San Antonio Zoological Garden were included in this study, where armadillos were maintained under similar conditions. Non-invasive fecal hormone analysis was used to evaluate the fecal progestagen metabolites (FPM) in females' samples and fecal androgen metabolites (FAM) in males' samples using enzyme immunoassays. Fecal samples were collected from 13 (7 males, 6 females) individuals for 1 year. For the females, the mean estrous cycle length (ECL), follicular phase length and luteal phase length was calculated. Females exhibited ECLs of 26 days which were similar across age, while the birth location (wild-born; zoo-born) and facility had no affect on ECL or mean FPM concentrations. Three pregnancies allowed for the measurement of gestation length, 114 days, and also established that FPM were greater during the second half of pregnancy than the first half and FPM concentrations in non-pregnant females. For males, mean FAM concentrations were similar across age and birth location with males reaching sexual maturity as early as 5 months of age. Seasons did not affect gonadal hormone

activity in female or male armadillos. The lack of seasonality was expected as the armadillos were housed under conditions that provided them with a constant temperature and resources throughout the year. North American armadillo birth history supported the lack of seasonality.

Chapter 3 describes the characterization of the adrenocortical activity of the zoo-housed three-banded armadillo. An adrenocorticotropic hormone (ACTH) challenge was conducted (1 male, 1 female) to validate the physiological response of elevated adrenocortical activity expected from an increase in ACTH. Biological events (aggressive pairing, copulation, pregnancy, veterinary procedures) validated the biological elevation of adrenocortical activity in response to a stressor. A longitudinal study of the adrenocortical activity of male and female armadillos was conducted. The biological events occurred during the longitudinal study. Non-invasive fecal hormone analysis was used to evaluate the fecal glucocorticoid metabolites (FGM) in male and female samples using enzyme immunoassays. Fecal samples were collected from 11 (5 males, 6 females) individuals for 1 year. The ACTH challenges induced elevated adrenocortical activity in both the male and female armadillo, which physiologically validated the non-invasive methods. The biological events also induced elevated adrenocortical activity in armadillos. Sex, age and birth location did not affect mean FGM concentrations. However, individuals did exhibit different FGM concentrations. Fecal glucocorticoid metabolites did not change over seasons, as expected by the constant housing conditions (consistent photoperiod, temperature and resources over the year).

Chapter 4 examines the integration of evaluating the adrenocortical activity and foraging behavior in zoo-housed armadillos to determine how they perceive their environment to assess their well-being. Foraging patches were used to quantify foraging intensity (giving-up densities, GUDs) of 7 (3 male, 4 female) armadillos within three patch treatments (varying substrate

quantity, patch quality and bedding amount). The food patch consisted of wax worm larvae (*Galleria mellonella)* mixed into topsoil in a black rubber tub. Fecal samples were collected before, during and after foraging treatments. Non-invasive fecal hormone analysis was used to evaluate the fecal glucocorticoid metabolites (FGM) in male and female samples using enzyme immunoassays. Four state categories (ideal, neutral, negative, positive) were identified, combining FGM and GUD measures, to score the overall well-being of the armadillos using mean FGM values and GUD measures. Individual mean FGM concentrations varied, but overall mean FGM concentrations for all armadillos were similar across all time periods (pre-sampling, foraging treatments, post-sampling). Fecal glucocorticoid metabolites varied across time periods for two individual armadillos. Giving-up density measures for individuals varied with all foraging treatments. In the substrate quantity treatment, armadillos did not bias foraging towards more shallow patches. Armadillos did not respond to patch quality, but instead used a fixed time strategy, harvesting the same amount of food items from rich and poor patches. However, bedding amount did affect the GUD measures of armadillos. As bedding amount increased, armadillos were more willing to forage in patches, demonstrating the importance of cover and the role of perceived predation risk in foraging decisions. Armadillos were ranked (1-7) according to their GUD measures for each patch treatment. Individual GUD ranks were similar across foraging treatments, suggesting that armadillos may have behavioral types. Correlations of FGM concentrations and GUDs showed no relation between the measures, making it necessary to conduct both measures to accurately assess well-being for these armadillos. The combination of FGM concentrations and GUDs resulted in three armadillos shifting to different state categories. The first individual moved from a negative to a neutral state after the substrate treatment where adrenocortical activity shifted from high to low. The next individual shifted

from a neutral to an ideal state after the bedding treatment, exhibiting an increase in foraging intensity with greater amounts of bedding. Another armadillo moved from a positive state to a neutral state after the bedding treatment with its foraging intensity decreasing as the bedding amount decreased. In all cases, armadillos finished in either a positive or neutral state with four armadillos remaining in the same ideal or neutral state categories throughout the treatments. This work has integrated hormone activity and foraging behavior thereby expanding the GUD methodology and creating new opportunities for improving husbandry and assessing the overall welfare of animals housed in zoos.

Chapter 5 addresses the foraging intensity of a seed-eating bird community in Argentina. The work supports the idea that birds are major seed consumers in South America and may out-compete rodents for seeds in some communities. Giving-up densities were measured to evaluate the bird's preferences for habitats (creosote, mesquite, sierra), microhabitat and time of day. Camera traps allowed for the identification of species that foraged from food patches, along with their spatial and temporal patterns of foraging. Five bird species (Brushland tinamou, *Nothoprocta cinerascens*; Crested gallito, *Rhinocrypta lanceolata*; Gray-hooded sierra finch, *Phrygilus gayi*; Golden-billed saltator, *Saltator aurantiirostris*; Rufous-collared sparrow, *Zonotrichia capensis*) contributed the majority (99%) of foraging. Overall, as exhibited by GUDs and picture data, the most foraging activity occurred in the sierra. However, only the tinamou and finch exhibited habitat preferences. As a community, birds biased their foraging activity towards patches in closed microhabitats. For the community as a whole, picture data revealed that foraging took place equally during the first half of the day (6 AM – noon) and the second half of the day (noon- 6 PM), with the tinamou showing time preferences between 08:00-10:00 and 16:00-18:00. Patch use methods of seed-eating bird studies were compared,

highlighting the need for standardized methods to make studies directly comparable across locations. When GUDs were scaled to the same volume, the bird community of SLL had lower GUDs than desert bird communities of the African savannah and Sonoran Desert, but not the Negev Desert.

I. INTRODUCTION

A. Overview

My plan is to use what I have learned to enrich the education, expand thought and re-engage science in the minds of minority Americans. My generation and those to follow have sadly lost their way in making a true connection with the natural world. For generations, science and nature was a necessity for our existence. We looked to science and nature as a source of food and medicine among other things. Now, most harbor a deep and unexplained fear for the outdoors, as described by David Sobel (1996). I can understand why this separation has happened. In the spirit of progress, our ancestors migrated from their indigenous lands to the big cities and technical industries abroad. As technology advanced from generation to generation, the awareness of nature and its benefits gradually dissolved into outdated practices of the past. It's time for people of color to come back to nature and reconnect with many of the very principles of stewardship of the land that was once a way of life for us. I want to use my experiences and education to begin giving nature back to my people one step at a time.

B. My journey to dissertation

As a child, my parents ensured that I was exposed to the arts, culture, traveling and much more. However, being the last child of three, with a 10 year gap between my sister and me, I did not experience the days of family camping trips and outdoor fun. Those days for me were comprised of resort based vacations. Although we spent time growing a garden every year, there was very little time spent in nature. My parents realized that I had a gift for music and that I loved math and science so most of my early life was spent playing music (piano and viola) and in science camps. As the time came to make a decision about what types of programs to apply for in college (music, biology, engineering), my parents believed that the most logical choice for me was to pursue engineering. In their minds, this path would ensure that I could be self-sufficient

and able to support myself monetarily. So, I started my undergraduate career in the computer engineering program at the University of Illinois at Urbana-Champaign. Throughout those first 2 years, I struggled to make a true connection to my engineering coursework, but loved the math and general science classes. In my junior year, I landed an internship with the Intel Corporation and I headed to California to work for one semester. My parents were so proud to tell everyone that I was working for Intel. After completing the internship, I was presented with the opportunity to finish my engineering degree and return to Intel for full-time employment. For many, this would have been a "Golden" opportunity. However, I did not experience that feeling of excitement and fulfillment. A nagging feeling that started at the beginning of my undergraduate career began to grow into a loud voice in my head. I had always heard this voice, but chose to ignore it for fear of disappointing my family. I knew that engineering was not for me because I had no passion for what I could potentially be doing for the rest of my life. Well, without consulting with my parents, I courageously made the decision to abruptly switch my major to animal sciences upon returning to school. My parents were good sports about the situation, but I believe they were a little skeptical about what I could do with a bachelor's of science in animal sciences. The rest of my undergraduate career was a breeze, as I thoroughly enjoyed my coursework and was always thirsty to learn more. Learning about reproduction and how hormones controlled much of the body's functions became a strong focus for the remainder of my undergraduate education. I was fascinated by the complex interplay between hormones and behavior. I had not been exposed explicitly to endocrinology or foraging ecology, but I knew that I had to pursue a higher degree to find a field that best suited me. Applying to the post-baccalaureate research education program (PREP) at the University of Chicago proved to be an extremely beneficial decision. I spent the next year in the PREP program conducting a hormonal and behavioral analysis of a pair of emperor tamarins (*Saguinus imperator*) maintained

at Lincoln Park Zoo (LPZ). During this study, I learned the basics of studying endocrinology and behavior while making important connections at LPZ. Rachel Santymire was the brand new endocrinologist at LPZ and I began helping out in her new lab. I knew that I wanted to continue my research at LPZ, as I had access to study the animals without being in the field. In my mind, being forced to go into the field for research was the last thing that I wanted to do. However, the lab felt like a safe and controlled environment where I could happily conduct my research. Around the same time, I had contacted Dr. Henry Howe about the ecology and evolution program at University of Illinois at Chicago. Not knowing that I was actually interviewing for a graduate position in the program, I met with Hank for lunch one day to discuss my interests and possible lab matches. Hank had invited Joel Brown and we had a spirited lunch, covering more topics than I thought possible in an hour. About a week later, I received an email from Joel expressing that he would love to welcome me into his lab and that I would be contacted by the first year graduate coordinator soon to fill out paperwork for a fellowship. Rachel had agreed to co-advise me with Joel and we set off on my journey towards my dissertation. I was extremely excited and a little scared of the possibilities. I was not sure if I would be required to head into the field with other lab members, but I was elated to be successfully admitted into this graduate program.

C. Gonadal hormones of the three-banded armadillo

Most research conducted in a zoo setting is done to solve specific problems within those communities. Though the problems may or may not be linked to the animals being maintained in a zoo setting, these studies generally allow for the collection of highly detailed data. In return, these data can be utilized to describe the biology of animals that may be difficult to study in natural habitats. Being the first endocrinologist at LPZ, Rachel was receiving all kinds of requests from curators across the zoo, asking her to take on projects to explain different issues or

concerns relating to their animals. One issue in particular sparked my interests and I saw it as an opportunity to get my own research started. Many species maintained in zoos are managed through the use of population management plans (PMPs). The goal of these plans are to keep track of the population numbers, breeding success and general well-being of a particular species within institutes belonging to the Association of Zoos and Aquariums (AZA). The general curator and manager of the PMP for the zoo-housed southern three-banded armadillo (*Tolypeutes matacus*) had expressed concern about the high infant mortality (47%) experienced by these armadillos within the first year of life (Bernier 2010). This armadillo is endemic to semi-arid regions of Argentina, Bolivia, Brazil and Paraguay and is unique because it is the only armadillo that has 2-4 moveable bands in its carapace allowing it to roll completely into a ball (Nowak 1999). Rachel explained to me that although the infant mortality was the big issue, there was no literature on the hormone activity of this species. After searching for literature on this armadillo, I found that there were some reports of genital tract morphology (Cetica et al. 2005), and spermatology (Cetica et al. 1997; Herrick et al. 2002). We saw a need to characterize the gonadal hormone activity of the three-banded armadillo to gain an understanding of its reproductive biology in the zoo. I wanted to gain basic information of the estrous cycle, the gonadal hormone range during pregnancies, gestation length (Meritt 1976), age at sexual maturity and how season affected reproduction of this armadillo. I had already learned the importance of conducting studies on animals in the least invasive manner possible, from my first study at LPZ (Monfort 2003). We decided that it was necessary to collect 1 year of fecal samples to properly characterize the gonadal activity of this armadillo. The lab work began, as an unbelievable amount of armadillo fecal samples began to fill up my shelf in the lab freezer. From then on, I was busy bouncing back and forth from school to LPZ. It is amazing how one decision could yield so much work. Once samples collection started, we set our sights on the

next logical step. Consequently, these data allowed us to be the first to characterize the gonadal hormone patterns of the three-banded armadillo. We were able to identify hormone ranges for non-pregnant and pregnant females, which can now be used to help diagnose pregnancy in zoo-housed three-banded armadillos. We also identified androgen hormone ranges for male armadillos, finding that immature males may be sexually mature as early as 5 months of age. Sexual maturity of immature males is very important husbandry management as they are most often separated from their dams at 5 months of age. This research also revealed that zoo-housed armadillos were no longer seasonal breeders as they would be in situ as a result of the constant temperature and resource availability they were exposed to throughout the year. Overall, we were able to learn about the reproductive biology of the three-banded armadillo in high detail, using a fair amount of individuals, which would be highly difficult to do in the wild.

D. <u>Adrenocortical activity of the armadillo</u>

The planned sample collection allowed us to also characterize the adrenocortical activity of the three-banded armadillo. After reading Touma and Palme (2005), I realized the importance of understanding the stress biology of this armadillo in the zoo. Making the connection that all stress is not bad was big for me. There are two types of stress, distress and eustress. Distress is "negative" stress that occurs when an animal's adrenocortical activity (stress hormones) increases as a result of a stressor such as the perception of danger. Eustress is "positive" stress that occurs when an animal's adrenocortical activity increases as a result of a positive interaction or perception such as playing with a new toy. At short intervals, both distress and eustress can have an overall positive effect on that animal's hypothalamic-pituitary-adrenal (HPA) axis by exercising its appropriate responses to stress. Distress occurring over a long period of time becomes chronic stress which is generally debilitating for an animal. Along with other negative effects, we understood that chronic stress could have been affecting the reproductive success of

the armadillos (Mostl and Palme 2002). Validations were necessary to make sure that the adrenocortical activity being measured was both physiologically and biologically relevant (Touma and Palme 2005). I was lucky enough to conduct two adrenocorticotropic (ACTH) challenges during the course of my study. We were the first to conduct a biologically relevant ACTH challenge on the zoo-housed three-banded armadillo. We also found several biological events in the animal records during the time of sample collection. Specifically, armadillos did exhibit elevated adrenocortical activity as a result of biological events such as mating, pairing and veterinary procedures. Adrenocortical activity was also not influenced by season due to the armadillos being exposed to constant temperatures and resources over all seasons. These results are very important for the management of this armadillo species in zoos. Animal care staff can better understand what stresses their animals while working to minimize possible causes of chronic stress in these animals.

E. <u>Patch use with zoo-housed armadillos</u>

At this point in my graduate career, I was learning about foraging ecology and how patch use could be used to determine how an animal perceives its environment (Brown 1988). After having many meetings with Joel, we decided to measure giving-up densities (GUDs, the amount of food left in a patch after the forager leaves the patch) in three-banded armadillos. Having a good understanding of the three-banded armadillos stress biology, I realized that their perception of safety or danger and whether they experienced eustress or distress ultimately controlled their adrenocortical activity levels. Those same perceptions would also control their willingness to forage in novel food patches in their enclosures. With these two measures, I could get information about the physiological and psychological state of the animal at any given time. This would allow me to identify situations or enclosure characteristics that allowed the three-banded armadillos to have the best overall well-being. Upon selling the idea to Rachel, she

informed me that I had the job of selling it to the LPZ research committee, the small mammal reptile house curator and the keepers that worked with the armadillos. The project was going to be a group effort and we needed to make sure that the patches would be safe for the three-banded armadillos. Finally, after several semesters and meetings, I got the okay from all important parties at LPZ to evaluate patch use of the armadillos. Once the big hurdle was over, we realized that fecal samples should be collected concurrently with the foraging patch treatments. The combination of measuring GUDs and adrenocortical activity would allow us have psychological and physiological snapshots of the armadillos' state. This had been used to describe the well-being of an animal (Hill and Broom 2009). From there, we developed a method to combine two very different tools to assess the well-being of the zoo-housed armadillo. Although I did not get to put our plans into effect until a year later, I was excited about the chance to incorporate patch use theory into my work with the armadillos. The combination of the two approaches was very successful in that I was able to get physiological and psychological feedback from the armadillos as it related to environment medications in resource availability and cover. I was the first to use a combination of hormone activity and foraging responses to reveal the well-being of a zoo-housed animal. I found that overall the armadillos were in good states of well-being but that armadillos responded differently to the same stimuli. This was an interesting result because it shows that armadillos may have behavioral types by which they could be managed more efficiently.

F. Patch use in a bird community of Argentina

As I listened to plans and watched the other Brown lab students prepare for their field seasons, it finally hit me that field work could not be all that bad. The other students would always come back with these amazing stories of all the animals and experiences they had out in the field. I, on the other hand, always had lots of lab work to do. I began to yearn for an

opportunity to see what it was like to go into the field. I had taken a sand ecology course with Dr. Dennis Nyberg a year earlier and I loved our field trips. It seems almost shameful that I was getting course credit for going out to sand dunes and taking nature walks while learning about grasses, trees, and the animals that lived there. I had so much fun in my landscape, ecological and anthropogenic processes (LEAP) courses and field trips. I knew that I wanted to try my hand at field work, but I had no idea how to make it happen. In a pivotal conversation with Joel, he suggested that I speak with Moira about her field work. After speaking with her and finding out that there may be armadillos in the areas that she was conducting her research (Argentina), I was all in for the adventure. Early research of granivorous animals over the world generalized that birds were not major seed-consumers anywhere (Mares and Rosenzweig 1978). Later, expanded research found that birds where actually a part of the major seed-eating communities in the deserts of South America and Australia (Monfort 1985). I set out to see if this trend held true for Sierra de las Quijadas (SLQ), Argentina. The plan was to opportunistically measure patch use of the armadillos and then measure the foraging intensity of the seed-eating birds in SLQ. As I prepared for the trip, Dr. Chris Whelan happily took me out on several birding trips to provide me with preliminary insight on birds. It was time for me to depart for Argentina! Although there was lots of hard work, lots of plants with spikes and stickers, no consistent running water, no real toilet, limited electricity in the evenings and no cell phone service… I loved it! We cooked our meals outside, which tasted great. I slept in a tent on a small self-inflating mattress and got the best sleep of my life. I washed my face and brushed my teeth by a picnic table every morning (how refreshing). And at night, the stars shined brighter and were closer than I had ever seen them. Tobias even came to Argentina for a week to experience a new country with me. In all honesty, my Argentina experience requires a whole other five pages for more elaboration. My one and only field season was over and I could tell people, much to their

amazement, that I camped in a desert for several weeks. The birds cooperated so well that I had a fan club waiting for me every morning at my patches. I also had some pretty cool data to analyze when I got back to the states. After working in the field and looking at camera data and foraging data, several trends emerged. It became clear that the birds had microhabitat, habitat and time preferences for foraging. These birds were definitely major seed consumers for this desert community of South America (Saba and Toyos 2003) and granivorous rodents were an imperceptible factor. I was even able to locate three-banded armadillos in SLQ, which I reported to the International Union of Conservation of Nature (IUCN) Xenarthra group, effectively increasing the range of the southern three-banded armadillo (Abba and Superina 2010).

G. Concluding remarks

It took me some time, but I finally finished the lab work and got some interesting results. I found that armadillos were reproductively active throughout the year due to zoo housing conditions. The armadillos were exposed to constant temperatures and resource availability throughout the year. All females showed discernible estrous cycle lengths (ECL) and males were sexually active as early as 5 months of age. Pregnancy resulted in significantly higher fecal progestagen metabolite (FGM) concentrations in the second half of pregnancy. The reproductive data made it clear that the three-banded armadillos should be able to reproduce successfully in a zoo setting. The ACTH challenges resulted in elevated adrenocortical activity, thereby physiologically validating the non-invasive fecal hormone analysis to evaluate adrenocortical activity. The reproductive events and veterinary procedures resulted in elevated adrenocortical activity, serving as biological validations for our non-invasive methods. We found that adrenocortical activity did not change with season in the zoo-housed armadillo. The stress data allowed us to see that though the armadillos experience forms of stress throughout the year, they are not experiencing so much stress that it is debilitating and that it is not affecting their ability to

reproduce in zoos. Combining adrenocortical activity with GUD measures allowed us to apply and develop a new framework for assessing the well-being of the zoo-housed armadillo. Specifically, we tracked the armadillos' state of well-being as a result of the foraging treatments, which could also be thought of as enclosure characteristics. Positively, all armadillos ended up in ideal or neutral states of well-being. The lack of correlation between the FGM measures and the GUD measures proved that both tools were necessary to accurately assess well-being. The birds of SLQ preferred foraging in closed microhabitat, foraging intensity and activity was much greater in the sierra habitat while some species had habitat and time preferences for foraging. The GUD measures of these birds revealed that the birds in were intense foragers and seed-consumers in the SLQ community, supporting the idea of birds being one of the major granivores in deserts of South America. All of these studies gave me great experience in the fields of endocrinology and foraging ecology.

The gap between minorities and nature desperately needs to be closed. It seems that the most effective way to tackle the problem is to begin with the children and their parents. Special programming needs to be developed and/or expanded in a manner that is hands-on, culturally sensitive and relevant to these communities. My research, especially the foraging ecology bits can really lend to that hands-on learning experience that I want to bring to my community. Once the ideas of perception, stress and well-being are changed, I believe that I could even work in learning experiences focusing on hormones and how they work in the body and control all of its functions. Making these important connections will not only help educate the community but will re-instill a love for the natural world around us. Childrens' science education needs to be supplemented as they make the association between classroom education and real-world experiences. While volunteering at the Charlotte Nature Museum for the past 7 months, I have had the pleasure of seeing such programs in action. From this experience, I realize that helping

the minority community make a reconnection to nature through programming is not an easy, quick task. However, I do know that embarking on this new adventure will be one of the most rewarding paths that I could ever take in life. There is nothing like seeing a child's eyes light up after an exciting experience in nature. I look forward to the many learning experiences that I am sure to experience along the way.

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II. USING NON-INVASIVE METHODS TO CHARACTERIZE GONADAL HORMONAL PATTERNS OF SOUTHERN THREE-BANDED ARMADILLOS (*TOLYPEUTES MATACUS*) HOUSED IN NORTH AMERICAN ZOOS

A. Abstract

Understanding the basic reproductive biology and limitations to successful breeding of the southern three-banded armadillo (*Tolypeutes matacus*) is necessary to maintain viable zoo populations. Our objectives were to: 1) describe the reproductive biology using non-invasive, fecal hormone analysis; 2) assess influence of season on gonadal hormonal patterns in both the sexes; 3) characterize reproductive cyclicity and pregnancy in the female; and 4) characterize the onset of sexual maturity in males. Nineteen armadillos were monitored including: 13 (7 males, 6 females) from Lincoln Park Zoo and six (3 males, 3 females) from San Antonio Zoological Garden. Fecal samples (n=5220; 275/animal/yr) were collected 5 to 7 times a week for 1 year. Hormones were extracted from feces and analyzed for progestagen (females) and androgen (males) metabolite concentrations using enzyme immunoassays. Mean estrous cycle length (26.4 ± 1.3 days) did not vary ($P < 0.05$) among individuals (n=9). Mean gestation length (n=3) was114.0 ± 0.6 days long with mean fecal progestagen metabolites increasing 10-fold during pregnancy. Seasons did not influence ($P < 0.05$) fecal androgen or progestagen metabolites. These data can assist with management decisions, which will directly affect the success of this species in zoos.

B. Introduction

The role of zoological institutions is becoming increasingly important for the management and study of threatened species. Because zoos provide excellent opportunities for longitudinal studies with species that would otherwise have low accessibility in the wild, information on the basic biology of wildlife species can be gathered. Additionally, understanding the fundamental reproductive biology and factors that may limit reproductive success of some species is necessary to maintain zoo populations. The southern three-banded armadillo (*Tolypeutes matacus;* Xenarthra, Dasypodidae) is a near threatened omnivore (Wilson and Reeder, 2005; Wetzel et al., 2007; Abba and Superina, 2010) that has been bred in zoos for over 40 years. In the wild, the southern three-banded armadillo inhabits parts of Argentina, Bolivia, Brazil and Paraguay (Arita et al., 1990; Noss et al., 2003; Meritt, 2008; Noss et al., 2008; Abba and Superina, 2010). This armadillo is in need of conservation due to the continual conversion of its natural habitat into agricultural and human dominated landscapes and is frequently exploited for food and arts (Noss et al., 2003; Meritt, 2008; Noss et al., 2008; Abba and Superina, 2010). The southern three-banded armadillo is a solitary animal for most of the year, but is known to nest communally during the breeding season (Meritt, 1973; Meritt, 2008). Though their reproductive biology is largely unknown, observations in the wild report a seasonal reproductive pattern (Noss et al., 2003; Cuéllar, 2008) with two possible peak rates of gestation (July-September and December-February) and birth of litters (ranging from 1 to 2 pups) occurring in the early spring and summer.

Zoo-housed southern three-banded armadillos exhibit single births that occur throughout the year (Meritt, 1976). Early breeding protocols in zoological institutions included sex ratios of two males and four to eight females housed within a single enclosure (Meritt, 1976). Currently, only a breeding pair, consisting of one male and one female, is kept together in an enclosure with

males being removed well before parturition since males are aggressive towards infants (Superina et al., 2008). Pups are generally weaned at 3 to 5 months and believed to be sexually mature at 12 months. In North American zoos, this species can live ~ 30 years and has an inter-birth interval of 1.5 years based on a pairing, full gestation, successful weaning of offspring and pairing again (Bernier, 2010). Historically, 525 armadillos have been born in North American zoological institutions (Bernier, 2010). Over 140 births have occurred at Lincoln Park Zoo (LPZ; Chicago, IL, USA) within the last 47 years. Despite these numbers, the reproductive biology of the southern three-banded armadillo remains poorly understood. Gaining knowledge of the reproductive biology of this species of armadillo is vital to its management.

The overall goal of this study was to advance our knowledge of the reproductive biology of male and female zoo-housed southern three-banded armadillos. Specifically, our objectives were to: 1) describe the reproductive biology of this species of armadillo using non-invasive, fecal hormone analysis; 2) assess the influence of season on gonadal hormone metabolite concentrations in both the males and females; and 3) characterize reproductive cyclicity and pregnancy in the female and 4) characterize the onset of sexual maturity in males. With these data, we will provide information to assist with management and husbandry decisions that directly affect the success of this species housed in zoos.

C. Methods

Animals

Nineteen armadillos were monitored in this study including: 13 (7 males, 6 females) from LPZ and six (3 males, 3 females) from San Antonio Zoological Garden (SAZ). Both facilities fed Mazuri insectivore® diet (PMI Nutrition International) plus chopped vegetables and fruit. Information on the individual characteristics of each armadillo, including: age, birth location and facility location, is summarized in Table 1 and Table 2. Mean (± s.e.m.) age of females was 7.6

± 1.5 years (range, 1– 14 years) and 9.2 ± 2.5 years (range, 0.4 – 29 years) for males. Animals were housed individually and exposed to natural and artificial light throughout the year at both facilities. Artificial lights were on during the zoo's operational hours when staff were present. Temperature within the facilities was controlled to maintain consistent conditions throughout the year. Breeding pairs were placed in a neutral enclosure space during the day and separated in their own enclosures at night. Pairings were monitored and observations of copulation were recorded by zoo animal care staff. Three females (# 20727, 21310, 21732) at LPZ experienced pregnancies during the study, of which two male pups survived. These immature males were included in the study after they were weaned and separated from the dams at approximately 5 months of age. Samples were not collected from pregnant females after parturition or until weaning to minimize disturbance of mother and infant, which can greatly increase infant mortality in this species (Superina et al., 2008).

Fecal sample processing

Fecal samples (*n*= 5220; 275/animal/yr) were collected 5 to 7 times a week during routine enclosure cleaning procedures from November 2007 through November 2008. Two individuals were relocated to another zoological institution during the course of the study, resulting in fewer sample collections for these individuals (# 20204 and 21732). Fecal samples were collected from the two male juveniles (# 21858, # 21888) from 5 to 15 months of age from June 2007 through May 2008, and September 2007 through May 2008, respectively. Samples were stored in sealed bags at -20°C prior to processing. All fecal samples were processed at the LPZ Endocrinology Laboratory. First, fecal samples were lyophilized (Labconco Lyophilizer, Kansas City, MO, USA) and steroids were extracted using modified methods previously described for small mammals (Keay et al., 2006). Dried samples were pulverized and 0.02g (± 0.002g) of fecal powder was weighed, then 0.5 ml of 90% ethanol: distilled water was added to the samples

and they were briefly vortexed. After vortexing, samples were shaken (Glas-col mixer, Terre Haute, IN, USA; setting 60, 30 min) and then centrifuged (1500 rpm, 20 min). Extracts were poured off and the fecal pellets were re-suspended in 0.5 ml of 90% ethanol:distilled water and vortexed for 30 seconds. After centrifugation (1500 rpm, 15 min) extracts were combined and dried down under air. Samples were reconstituted in 0.2 ml of phosphate-buffered saline (PBS; 0.01 M PO_4, 0.14 M NaCl, 0.05% BSA, 0.01% NaN_3, pH 7), vortexed briefly, sonicated for 20 min and shaken (20 min), and then diluted in PBS prior to analysis. Extracted samples were analyzed for progestagen (females) or androgen (males) metabolite concentrations using enzyme immunoassays (EIA).

Enzyme immunoassays

Fecal progestagen metabolites (FPM) were analyzed using a progesterone EIA. Progesterone monoclonal antiserum (CL425; provided by C. Munro, University of California, Davis, CA) and horseradish peroxidase (HRP) was used at a dilution of 1:10,000 and 1:40,000, respectively (Loeding et al., 2011). Cross-reactivities of the progesterone antibody were previously published (Graham et al., 2001). The progesterone EIA was validated by demonstrating: 1) parallelism between binding inhibition curves of fecal extract dilutions (1:8-1:8192); and 2) significant recovery (> 90%) of exogenous progestagen added to fecal extracts (1:10,000; $\hat{y}= 0.883 +5.76$, $R^2= 0.98$). From the parallelism data, the appropriate dilution at 50% binding was prepared for progesterone (1:1000). Assay sensitivity was 0.78 pg/well for progesterone and intra- and inter-assay coefficients of variation were < 10% and 15%, respectively.

Fecal androgen metabolites (FAM) were analyzed using a testosterone EIA. The testosterone (HRP) ligands and polyclonal antiserum (R156/7; provided by C. Munro) were used at dilutions of 1:30,000 and 1:10,000, respectively (Santymire and Armstrong, 2010). Cross-

reactivities of the testosterone antibody were previously published (Santymire and Armstrong, 2010). The testosterone EIA was validated by demonstrating: 1) parallelism between binding inhibition curves of fecal extract dilutions (1:16 - 1:4096) and 2) significant recovery (> 90%) of exogenous testosterone added to fecal extracts (1:2,500; $\hat{y} = 0.99x - 5.65$, $R^2 = 0.919$). From the parallelism data, the appropriate dilution at 50% binding was prepared for testosterone (1:250). Assay sensitivity was 2.3 pg/well for testosterone and intra- and inter-assay coefficients of variation were < 10% and 15%, respectively.

Statistical analyses

Assumptions of normality for all data were checked using the Kolmogorov-Smirnov statistic. If data were not normal, a Kruskal- Wallis test was used to analyze data. For all analyses, $P < 0.05$ was considered significant. All statistical analyses were performed using Microsoft *Excel* (MS Office 2007), *SYSTAT* Version 10 (SPSS Inc. 2000) and *Sigma Stat* Version 3.0 (SPSS Inc., Chicago, IL, USA).

Female reproductive traits

For analysis of female reproductive traits, hormonal baseline values for FPM concentrations were determined by an iterative process in which high values exceeding the mean plus 1.5 standard deviations (SD) were excluded (Brown et al., 1994; Moreira et al., 2001). For each iteration, the mean was recalculated and the elimination process was repeated until no values exceeded the mean plus 1.5 SD, thus, representing baseline hormone values. Post-ovulatory increases in FPM concentrations were considered significant if values exceeded the baseline by 1.5 SD. Values were presented as mean ± s.e.m.

The estrous cycle length (ECL) was calculated as the number of days between the first elevated FPM concentrations of a luteal phase to the first elevated FPM concentration of the next cycle. To be considered the end of a cycle, FPM concentrations needed to be below baseline for

at least 3 days. The luteal phase was calculated by counting the number of days that FPM concentrations were continuously elevated within a cycle, excluding any 1- to 2- day drops in hormone concentrations. The follicular phase was calculated by counting the number of days that FPM concentrations were not elevated. The days from first daily pairing and observed copulation were counted to calculate the time elapsed from initial introduction to copulation in a breeding pair. Gestation length was calculated by counting the days from observed copulation in a breeding pair to parturition. Although pairings were monitored, there is a chance that copulations were missed. We assume that observed copulations resulted in the following births observed in paired females. Animal care staff determined birth upon observation of neonate.

We tested for the effect of age, birth location (zoo versus wild), facility and reproductive status on FPM concentrations using general linear models. We also tested for a difference between individual ECLs and how the above factors affected ECL and baseline FPM concentrations. To test for the effect of age, we used mean FPM, and ECL concentrations as the dependent variables and age as the independent in a regression, using all females. To test for birth location, we used FPM concentrations and ECLs as the dependent variables and birth location as the independent variable in an ANOVA, using only LPZ females as there were not sufficient wild-born SAZ females for analysis. To test for facility (LPZ, SAZ), we used FPM concentrations as the dependent variable and facility as the independent variable in an ANOVA, using all females. We tested the effect of the interactions between age and birth, and age and facility on baseline FPM concentrations and ECLs. To test for individual differences in ECL, we used ECLs as the dependent variable and individuals as the independent variable, using all females.

To determine if pregnancy influenced FPM concentrations, data from 60 days before copulation to parturition were divided into three timeframes (pre-pregnancy, 1st half of

pregnancy, 2nd half of pregnancy). A Kruskal-Wallis with a Dwass Steel-Chritchlow-Fligner test was performed on these data.

Male reproductive traits

For analysis of male reproductive traits, we tested for the effect of age, birth location and facility on FAM concentrations using general linear models using the same methods used for the females. In testing for birth location, only LPZ males were used as there were no zoo-born SAZ males for analysis.

Seasonal influences on reproduction

To test for seasonality in female reproduction throughout the year, we used the Rayleigh test (Zar, 1999; Bitetti and Janson, 2001) to determine the distribution of the first day of luteal phases for all females. The first days of luteal phases were converted to days of the year. These days were then converted to radians, which allowed for the calculation of the cosine and sine of these angles. These angles were then used to visualize the dates on a uniform circle to determine if any clusters or patterns existed using the Rayleigh test, representing seasonality in reproduction. Additionally, using general linear models, we evaluated the effect of season on FPM and FAM concentrations. The FPM or FAM concentrations of samples from the 10th through 20th of each month were averaged together, to ensure independence, for a monthly mean for each individual included in the analysis. To evaluate the effect of season on gonadal hormones in both female and male southern three-banded armadillos, monthly means were separated into seasons: Spring (March, April, May), Summer (June, July, August), Fall (September, October, November) and Winter (December, January, February). Then, we used FPM concentrations (or FAM values for males) as the dependent variable and season as the independent variable. Only females with data for at least 2 months of each season were included in this analysis. Females that experienced pregnancies were excluded from this analysis. For

males, two armadillos were excluded from this analysis due to the lack of data for 2 months of any particular season.

North American zoo armadillo births

Birth information for armadillos born in North America was retrieved from the three-banded armadillo studbook (Bernier, 2010) using Poplink version 2.1 (Chicago, IL). North American armadillo birth dates were converted to days of the year. Circular statistics were used to determine if historic three-banded armadillo births had a seasonal pattern, using the same four seasons, and this distribution was tested using the Rayleigh test.

D. Results

Female reproductive traits

All females (*n*=9) demonstrated discernible estrous cycles (Table 2.1). A representative FPM profile for a cycling female from LPZ and SAZ is shown in Fig. 2.1A and B, respectively. The ECLs were similar ($F_{8, 40} = 0.2$, $P > 0.05$) across individuals with a mean ECL of 26.4 ± 1.3 days (range, 18.0 – 29.4 days; Table 2.1). Overall mean FPM concentrations were 33.8 ± 7.6 µg/g dry feces (range, 15.6 – 82.8 µg/g dry feces; Table 2.1). Overall elevated FPM concentration (54.5 ± 7.2 µg/g dry feces; range, 30.4 – 103.3 µg/g dry feces) was approximately 3.5-fold higher than the mean baseline (15.6 ± 2.7 µg/g dry feces; range, 6.0 - 30.5 µg/g dry feces; Table 2.1). Age had no effect on mean FPM concentrations ($r^2 = 0.1$, $P > 0.05$) or on ECL ($r^2 = 0.1$, $P > 0.05$; Table 2.1). Mean FPM ($F_{1, 4} = 1.2$, $P > 0.05$) concentrations and ECL ($F_{1,4} = 0.9$, $P > 0.05$) of LPZ females were not affected by birth location (Table 2.1). There was no interaction between birth location and age for baseline FPM concentrations ($F_{1,7} = 2.7$, $P > 0.05$) or ECL ($F_{1,7} = 4.3$, $P > 0.05$; Table 2.1). Facility also did not influence mean FPM concentrations ($F_{1, 7} = 1.2$, $P > 0.05$) with no interaction between facility and age for baseline FPM concentrations ($F_{1,7} = 2.0$, $P > 0.05$) or ECL ($F_{1,7} = 0.3$, $P > 0.05$) (Table 2.1).

Table 2.1 Mean (± s.e.m.) reproductive traits of female southern three-banded armadillos evaluated for 1 year using non-invasive fecal hormone analysis. These data exclude samples taken during pregnancies experienced by individuals (# 20727, 21310 and 21732).

Female	Age (y)	Birth Location	Facility Location *	Duration of follicular phase (d)	Estrous cycle length (d)	Luteal phase length (d)	Baseline progestagen (µg/g dry feces)	Overall mean progestagen (µg/g dry feces)	Mean progestagen-luteal phase (µg/g dry feces)	Mean elevated progestagen-luteal phase (µg/g dry feces)
9717	13.8	Zoo	LPZ	5.8 ± 1.1 (n=6)	28.5 ± 4.4 (n=4)	27.0 ± 4.9 (n=7)	7.7 ± 0.2	15.6 ± 0.5	16.0 ± 0.7	30.4 ± 1.9
20198	9.2	Wild	LPZ	6.1 ± 4.1 (n=7)	29.4 ± 1.0 (n=5)	17.3 ± 9.3 (n=6)	6.0 ± 0.2	17.3 ± 0.9	18.1 ± 1.0	45.7 ± 24.0
20441	5.4	Zoo	LPZ	4.6 ± 0.6 (n=9)	24.5 ± 6.7 (n=4)	13.6 ± 3.9 (n=7)	16.9 ± 0.4	33.8 ± 1.0	31.3 ± 2.0	52.3 ± 9.9
20727**	4.0	Zoo	LPZ	9.3 ± 0.9 (n=3)	28.0 ± 7.0 (n=2)	18.7 ± 4.4 (n=3)	14.9 ± 0.5	25.0 ± 1.3	25.5 ± 1.6	44.5 ± 13.2
21310**	2.4	Zoo	LPZ	8.3 ± 3.2 (n=7)	28.5 ± 5.5 (n=6)	21.4 ± 5.2 (n=7)	7.6 ± 0.3	16.4 ± 0.9	19.9 ± 1.1	41.0 ± 10.4
21732**	13.2	Wild	LPZ	5.0 ± n/a (n=1)	18.0 ± n/a (n=1)	12.0 ± n/a (n=1)	30.5 ± 1.5	59.7 ± 6.3	63.7 ± 4.8	63.7 ± 4.8
970643	9.7	Zoo	SAZ	8.5 ± 1.4 (n=11)	29.3 ± 4.3 (n=8)	18.5 ± 2.2 (n=11)	15.9 ± 0.4	30.5 ± 1.2	37.9 ± 1.6	66.7 ± 7.3
990485	9.2	Wild	SAZ	15.3 ± 2.2 (n=11)	23.3 ± 2.7 (n=4)	10.2 ± 1.9 (n=9)	25.4 ± 0.6	82.8 ± 7.0	112.9 ± 17.1	103.3 ± 12.2
D05001	1.2	Zoo	SAZ	7.8 ± 1.5 (n=11)	27.7 ± 2.6 (n=10)	16.4 ± 2.8 (n=10)	15.7 ± 0.4	23.5 ± 0.7	28.6 ± 0.9	42.7 ± 3.4
			Overall mean	**7.9 ± 1.1 (n= 66)**	**26.4 ± 1.3 (n=44)**	**17.2 ± 1.7 (n=61)**	**15.6 ± 2.7**	**33.8 ± 7.6**	**39.3 ± 10.4**	**54.5 ± 7.2**

Values in parenthesis represent numbers of follicular phases, estrous cycles or luteal phases in each female.
***** Lincoln Park Zoo, Chicago, IL, USA (LPZ) and San Antonio Zoological Garden , San Antonio, TX, USA (SAZ)
****** Females that exhibited a pregnancy during the study.

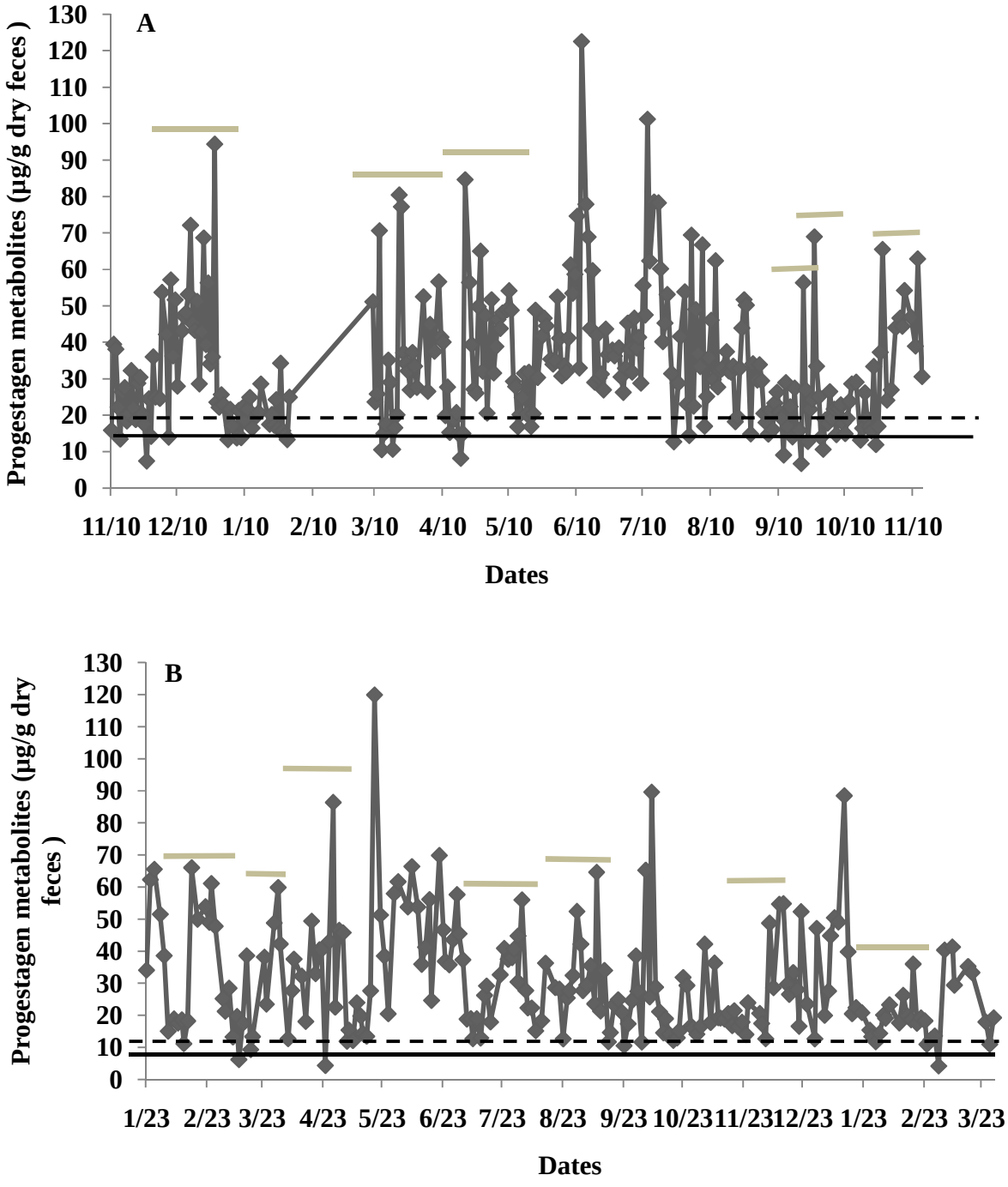

Fig. 2.1 Yearly profile for fecal progestagen metabolite (FPM) concentrations in a representative cycling female southern three-banded armadillo housed at A) Lincoln Park Zoo and B) San Antonio Zoological Garden. The solid horizontal line indicates the baseline value for FPM, and the dashed horizontal line indicates elevated FPM values. The horizontal solid bars above the profile indicate the estrous cycles.

A representative FPM profile for a pregnant female is shown in Figure 2.2A depicting the date of observed copulation, separation from the male and the entire duration of gestation until parturition. Copulation was observed on average (n=3) 17.3 ± 6.9 days (5-29 days) after initial introductions for daily pairing. Mean gestation length (n=3) was 114.0 ± 0.6 days (113 – 115 days). Mean FPM concentration for pregnant females was 184.2 ± 50.3 µg/g dry feces (range, 85.5 – 250.8 µg/g dry feces) and was approximately five-fold higher than mean FPM concentration for non-pregnant females (33.8 ± 7.6 µg/g dry feces; range, 15.6 – 82.8 µg/g dry feces; Table 2.1). Mean elevated FPM concentration for pregnant females was 680.3 ± 229.6 µg/g dry feces (range, 287.7 – 1083.0 µg/g dry feces; Fig. 2.2A). Pregnancy periods were significantly different (H_2= 209.5, $P < 0.05$; Fig. 2.2B), with the 2nd half of pregnancy period (265.6 ± 16.8 µg/g dry feces) having higher FGM concentrations than the 1st half of pregnancy (34.5 ± 4.2 µg/g dry feces) and the pre-pregnancy period (24.9 ± 1.6 µg/g dry feces) which were similar.

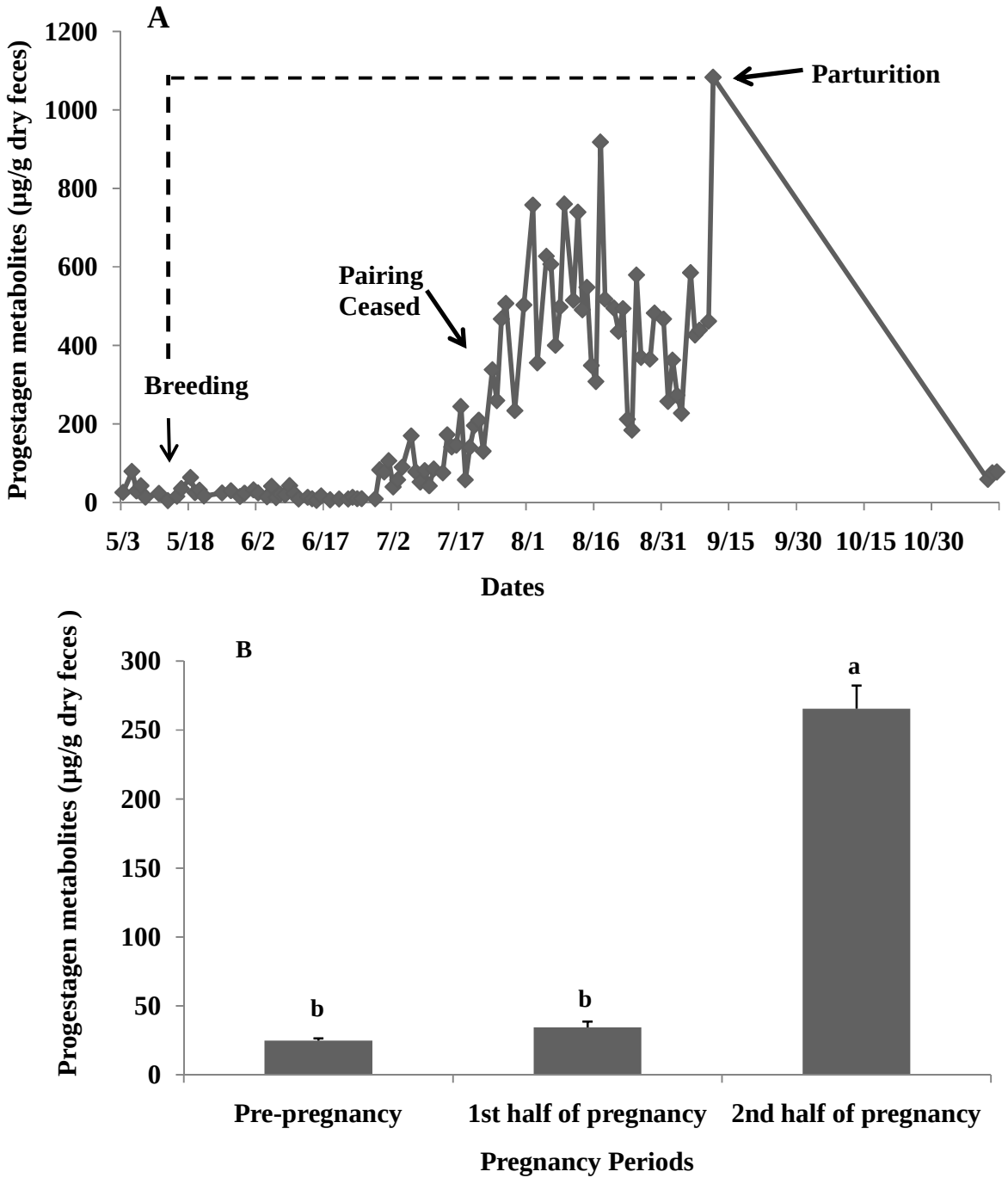

Fig. 2.2 A pregnancy profile in a southern three-banded armadillo demonstrating elevations in fecal progestagen metabolite (FPM) concentrations during gestation. Parturition of a pup was observed 38 days after male was separated from the female. Gestation length (113 days) calculated as the number of days from observed breeding to parturition (A). Mean (± s.e.m.) FPM concentrations for pregnant female southern three-banded armadillos (n=3) separated into three periods (pre-pregnancy, 1st half of pregnancy, 2nd half of pregnancy, B). ^{a,b}Superscripts represent significant differences ($P < 0.05$) among periods.

Male reproductive traits

Overall mean FAM concentration for males was 4.6 ± 0.3 µg/g dry feces (range, 3.3 – 6.7 µg/g dry feces, Table 2.2). Age did not affect (r^2=0.1, $P > 0.05$) FAM concentrations among male armadillos (Table 2.2). Birth location did not influence ($F_{1,5} = 0.2$, $P > 0.05$) FAM concentrations (wild: mean, 4.0 ± 0.3 µg/g dry feces; range, 3.3 – 4.4 µg/g dry feces; zoo: mean, 4.1 ± 0.3 µg/g dry feces; range, 3.6 – 4.5 µg/g dry feces; Table 2.2). No interaction was observed ($F_{1,3} = 4.7$, $P > 0.05$) between age and birth location and facility on mean FAM concentrations (Note: # 6474 was taken out of this analysis because he was older than the other male armadillos and was driving a false significant interaction; Table 2.2). However, there was an interaction between age and facility with SAZ armadillos having higher ($F_{1,7} = 31.8$, $P < 0.05$) mean FAM concentrations (5.9 ± 0.4 µg/g dry feces; range, 5.4 – 6.7 µg/g dry feces) than LPZ (4.0 ± 0.2 µg/g dry feces; range, 3.3 – 4.5 µg/g dry feces; Table 3.2).

Table 2.2 Mean (± s.e.m.) androgen concentrations (µg/g dry feces) of male southern three-banded armadillos evaluated for 1 year using non-invasive fecal hormone analysis.

Males	Age (y)	Birth Location	Facility*	Overall mean androgen
6474	29.2	Wild	LPZ	3.3 ± 0.1
20200	9.2	Wild	LPZ	4.4 ± 0.1
20202	9.2	Wild	LPZ	3.8 ± 0.2
20204	9.2	Wild	LPZ	4.3 ± 0.1
20243	6.8	Zoo	LPZ	3.6 ± 0.1
950435	9.2	Wild	SAZ	5.5 ± 0.2
990483	9.2	Wild	SAZ	5.4 ± 0.2
990484	9.2	Wild	SAZ	6.7 ± 0.2
21858	0.4	Zoo	LPZ	4.2 ± 0.3
21888	0.4	Zoo	LPZ	4.5 ± 0.2
			Overall mean	**4.6 ± 0.3**

***** Lincoln Park Zoo, Chicago, IL, USA (LPZ) and San Antonio Zoological Garden , San Antonio, TX, USA (SAZ)

Seasonal influences on reproduction

Season did not influence ($F_{3, 61} = 2.8$, $P > 0.05$) FPM values. During this study, cycling female southern three-banded armadillos (n=6) exhibited 48 luteal phases. Results from the circular statistics demonstrated a random pattern of luteal phases among females ($P > 0.05$), suggesting that female reproductive cyclicity did not vary across seasons (Fig. 2.1A and B). The number of luteal phases across the seasons was similar ($P > 0.05$): Fall (n=14, 23.7%), Winter (n=14, 23.7%), Spring (n=12, 20.3%) and Summer (n=8, 13.6%). Additionally, fecal androgen metabolite concentrations did not vary among seasons ($F_{3,70} = 2.2$, $P > 0.05$), but individuals' FAM concentrations varied ($F_{6,70} = 2.4$, $P < 0.05$).

North American zoo armadillo births

From 1969 through 2010, 367 births have occurred in the southern three-banded armadillo population housed in North America. Results from the Rayleigh test demonstrated a random pattern of birth occurrence ($P > 0.05$; Fig. 2.3). Furthermore, separating births into seasons confirmed that there was no seasonality ($P > 0.05$) to the birth pattern (Summer: n=100, 27.2%; Winter: n=93, 25.3%: Spring: n=92, 25.1%; Fall: n=82, 22.3%).

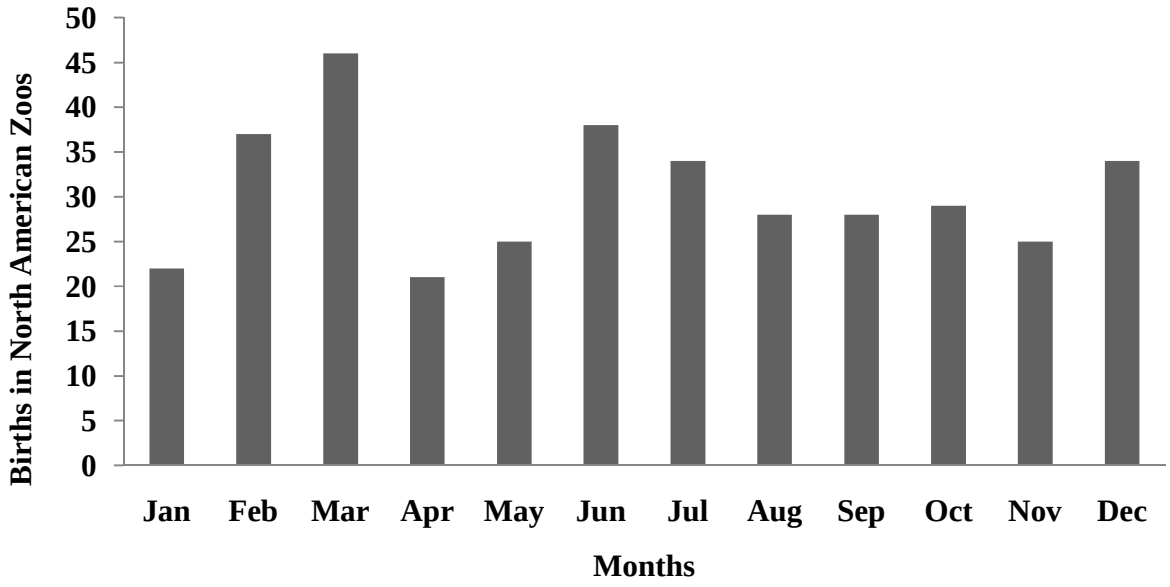

Fig. 2.3 Number of monthly southern three-banded armadillo births in North American zoological institutions based on Studbook data from 1969 to 2010.

E. Discussion

This study was the first to use non-invasive fecal hormone analysis to determine the reproductive characteristics of both female and male zoo-housed southern three-banded armadillos. Females had an approximately 26 day ECL and were reproductively active throughout the year. Pregnancy resulted in elevated FPM concentrations, starting with lower concentrations during the time of copulation and increasing throughout the pregnancy, which was 114 days long. The FAM and FPM concentrations did not fluctuate over the seasons.

Literature investigating the basic reproductive biology of the Family Dasypodidae has gradually increased over the years. Cetica et al. (2005) evaluated the morphology of the female armadillo genital tract of seven armadillo species (*Dasypus hybridus, Tolypeutes matacus, Chaetophractus villosus, Chaetophractus vellerosus, Zaedyus pichiy, Cabassous chacoensis* and *Clamyphorus truncatus*). Electroejaculation and other methods have been used to collect and study the semen characteristics of armadillo species in several studies (*Chaetophractus nationi,* Bermúdez et al., 2010; *Tolypeutes matacus,* Herrick et al., 2002; *Euphractus sexcinctus,* Serafim

et al., 2010; Santos et al., 2011; *Cabassous unicinctus*, Heath et al., 1987; *Dasypus novemcinctus*, Nagy and Edmonds, 1973a; Nagy and Edmonds, 1973b; Torres et al., 1981; Torres et al., 1983; Peppler, 2008). Cetica et al. (1993; 1997; 1998) and Cetica and Merani (2008) also did comparative spermatology work and evaluated the shape of sperm heads in various armadillo species (*Chaetophractus vellerosus, Chaetophractus villosus, Dasypus novemcinctus, Dasypus hybridus, Dasypus septemcinctus, Euphractus sexcinctus, Priodontes maximus, Tolypeutes matacus, Zaedyus pichiy*). The reproductive hormone profiles of the pichi armadillo (*Zaedyus pichiy*, Superina et al., 2009; Superina and Jahn, 2009), nine-banded armadillo (*Dasypus novemcinctus*, Peppler and Stone, 1980; Peppler and Stone, 1981; Peppler et al., 1986; Peppler, 2008), large hairy armadillo (*Chaetophractus villosus*) and the screaming hairy armadillo (*Chaetophractus vellerosus*; Luaces et al., 2011) have been previously described. The general biology of the southern three-banded armadillo is based on data collected from both zoo-managed (Meritt, 1976; Meritt, 2008) and wild populations (Cuéllar, 2002; 2008; Deem et al., 2009), including studies that investigated basic reproductive biology (Herrick et al., 2002; Noss et al., 2003; Cetica et al., 2005; Noss et al., 2008).

In monitoring gonadal hormone patterns of the zoo-housed southern three-banded armadillo, we observed an ECL of ~26 days, which is the first reported ECL of an armadillo. For example, Superina et al. (2009) did not report an ECL for pichi armadillos as all but 1 female in this study were paired for breeding. There was no clear pattern found in fecal progestagen and estrogen metabolites of females that were not pregnant or lactating during active reproductive season, suggesting that pichi armadillos may be induced ovulators (Superina et al. 2009). Luaces et al. (2011) also did not report an ECL for the large hairy or screaming hairy armadillo, though progestagen and estradiol profiles were characterized for a whole year. Though the reproductive

biology of the nine-banded armadillo has been described in depth (Peppler 2008), there was no ECL stated.

In addition to information on the reproductive cycle, knowledge of the gestation length of a species is crucial to ensuring reproductive success of this species due to high sensitivity to disturbance and resultant neonate mortality after giving birth (Superina et al., 2008). Here, we determined that the southern three-banded armadillo had a gestation length of approximately 114 days, which is similar to the 120 days previously reported (Meritt, 1976). The reported gestation length of related species ranged from ~60 days in the pichi armadillo (Eisenberg and Redford, 1992; Nowak et al., 1999; Superina et al., 2009) to 60-65 days in the six-banded armadillo (Gucwinska, 1971; Redford and Wetzel, 1985) and 60-75 days in the large hairy armadillo (Nowak et al., 1999). The nine-banded armadillo has a different strategy as it exhibits a 100 day delayed implantation followed by 135 days of gestation, and gives birth to four genetically identical offspring (Labhsetwar and Enders, 1968; Peppler and Stone ,1980; Loughry et al., 1998; Peppler, 2008). Progestagens in the nine-banded armadillo increase directly after delayed implantation and stay elevated throughout gestation (Labhsetwar and Enders, 1968; Peppler and Stone, 1980; Peppler et al., 1986). Mean fecal progestagen metabolite concentrations in pregnant southern three-banded armadillo females increased up to ten-fold higher than baseline FPM concentrations at approximately 50 days into the pregnancy. A similar pattern was found in the pichi armadillo with FPM concentrations increasing up to 10-fold (Superina et al., 2009) greater than baselines in the second half of pregnancy. This increase in progestagens is necessary for the maintenance of the pregnancy in mammals (Senger, 2003).

Reports of the age of sexual maturity are available for several armadillo species. In pichi armadillos, males were sexually mature at 9 months (Superina and Jahn, 2009). By 5 to 10 months of age, nine-banded armadillos had plasma androgen concentrations comparable to those

of mature males (Czekala et al., 1980; Peppler, 2008). Here, 5 month old males exhibited comparable FAM concentrations to adult male three-banded armadillos and males in general had no consistent pattern in fecal androgen metabolites with respect to age of the individual; however, we did not confirm testes size and spermatozoa production.

Reproduction in many armadillos is influenced by season; however, we did not observe seasonality in our zoo-housed southern three-banded armadillos with estrous cycles observed across the seasons. Noss et al. (2003) and Cuéllar (2008) used hunter self-monitoring records from the Bolivian Chaco to determine that the five (*Dasypus novemcinctus, Tolypeutes matacus, Euphractus sexcinctus, Chaetophractus villosus and Chaetophractus vellerosus*) armadillo species were likely to exhibit seasonal reproduction by evaluating when pregnant/lactating females were present and absent from wild captures. Male pichi armadillos were reproductively active during only 3-5 months of the year showing elevated fecal androgen concentrations, enlarged testes and increased aggressive behavior (Superina and Jahn, 2009). Captive male Andean hairy armadillos (*Chaetophractus nationi*) presented drastically dissimilar sperm concentrations in warm and cold seasons, proving that they are seasonally reproductive as well (Bermúdez et al., 2009).

Because photoperiod can be one of the driving factors in reproduction, as observed in the nine-banded armadillo and pichi armadillos (Torres et al., 1981; 1983; Superina and Jahn, 2009), birth location may influence reproduction. For example, Czekala et al. (1980) found that captive nine-banded armadillos exhibited higher plasma testosterone concentrations than wild-caught armadillos throughout the year, but both exhibited seasonal changes in androgen levels corresponding with reproductive months. Additionally, Peppler and Stone (1981) found little variation in plasma testosterone throughout the year in captive, wild-caught nine-banded armadillos. Similarly, we observed no differences in FAM concentrations across seasons in the

zoo-housed males. Additionally, using the North American southern three-banded armadillo birth data (Bernier, 2010), we determined that armadillo births of the North American zoo-housed population were evenly distributed across the year. The lack in seasonality of reproduction in both our study and the Peppler and Stone (1981) study could be a result of consistent housing conditions (i.e. controlled temperatures, lack of environmental changes and consistent nutritional access) throughout the year, that do not match the conditions in which these armadillos evolved.

Overall, these data add great value to the knowledge of Dasypodidae species. Our study was able to provide the first characterizations of the gonadal hormone activity of the zoo-housed three-banded armadillo. There is still more work required to obtain a better understanding of the southern three-banded armadillo's reproductive biology. Future work should include the analysis of fecal estrogen metabolites and determining the age of sexual maturity of female southern three-banded armadillos. Field work that would characterize reproductive biology of the southern three-banded armadillo in its endemic habitats would allow it to serve as a reproductive model for the vulnerable Brazilian three-banded armadillo (*Tolypeutes tricinctus*), which is the only armadillo species endemic to Brazil (Superina and Abba, 2010). This species is at extreme risk of local extinction, requiring further research and protection to conserve this species (Marinho-Filho et al., 1997). These data generated from this study could be used to help propagate the Brazilian three-banded armadillo in a zoo-housed environment in the future.

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III. CHARACTERIZING ADRENOCORTICAL ACTIVITY IN ZOO-HOUSED SOUTHERN THREE-BANDED ARMADILLOS (*TOLYPEUTES MATACUS*)

A. Abstract

Improving the husbandry in the southern three-banded armadillo (*Tolypeutes matacus*) through gaining knowledge of its stress physiology is imperative to maintaining a healthy, zoo-housed population. Our objectives were to: 1) validate the use of fecal hormone analysis for monitoring adrenocortical activity using both an adrenocorticotropic hormone (ACTH) challenge and biological events; and 2) characterize longitudinal adrenocortical activity in male and female southern three-banded armadillos. An ACTH injection was given intra-muscularly to one male (4 IU/kg; 5.6 IU total) and one female (5.5 IU/kg; 8 IU total) southern three-banded armadillo. Fecal samples were collected 1 day pre- and continued 5 days post-ACTH to capture the physiological response measured by elevated fecal glucocorticoid metabolites (FGM) to validate these techniques. Additionally, natural and routine events, including pairing individuals for breeding and veterinary procedures/handling, were used to biologically validate these techniques. To characterize adrenocortical activity, fecal samples (~3025 total; n= 275/animal/yr) were collected from 11 (5 males; 6 females) southern three-banded armadillos 5 – 7 times a week for 1 year at Lincoln Park Zoo (Chicago, IL). A cortisol enzyme immunoassay was used for FGM analysis. The ACTH challenge in the male resulted in a twofold increase of FGM (1123.2 ± 36.2 ng/g dry feces) above baseline (675.7 ± 10.0 ng/g dry feces) at approximately 54 – 94 h post-injection. The female exhibited a twofold increase (1635.4 ng/g dry feces) over baseline FGMs (608.5 ± 12.3 ng/g dry feces) approximately 30 h post-injection. Reproductive behaviors and veterinary procedures resulted in elevated FGM concentrations from all individuals except for one male. The longitudinal characterization demonstrated that sex and season did not influence ($P < 0.05$) FGM concentrations. Individuals were highly variable with mean FGM concentration

of 2010.1 ± 862.4 ng/g dry feces (range, 816.3 – 7,889.1 ng/g dry feces). Mean FGM baseline concentration was 878.5 ± 201.8 ng/g dry feces (range, 475.2 – 1955.5 ng/g dry feces) with a mean elevated FGM concentrations of 2694.3 ± 1111.4 ng/g dry feces (range, 1110.3 – 10,683.3 ng/g dry feces). This study provides the foundation for future research on how the environment directly affects the adrenocortical activity in this species of armadillo.

B. Introduction

Due to continual habitat loss and degradation, zoological institutions have become a safe haven for many of the 21 extant armadillo species [1,15,52], including the southern three-banded armadillo (*Tolypeutes matacus*), which has been housed in zoos for over four decades. The three-banded armadillo is a near threatened insectivore of the Family Dasypodidae, Order Cingulata, which inhabits parts of Argentina, Bolivia, Brazil and Paraguay [1,3]. In the wild, this species is in need of conservation and management due to the conversion of its natural habitat into agricultural lands and its frequent exploitation for food and the arts [29,32,39]. Although this species has been maintained in North American zoos for over 40 years, it still experiences limited reproductive success. The three-banded armadillo Population Management Plan® reports an offspring mortality rate of 47% (54% of males, 38% of females) across all North American institutions [5].

Improving the health and reproduction of *ex situ* populations is the goal of zoological institutions and wildlife managers [30,49]. To determine the factors that are limiting their success, stress physiology research is needed to understand how individuals respond to the environment [27,59]. In a stress response, a perceived threat to homeostasis triggers a neural signal that activates hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus releases corticotrophin releasing hormone (CRH), which stimulates the anterior pituitary to produce adrenocorticotropic hormone (ACTH). Then, ACTH acts on the adrenal cortex and causes the release of glucocorticoids which help the individual cope with the stressor [35,47]. Once the stressor is absent, the glucocorticoids are metabolized by the liver and kidneys. These steroid metabolites are excreted in urine and feces [35,36]. This response to minimal stressors can be healthy and positive for zoo-housed animals. However, chronic stress can lead to debilitating and negative results including reproductive failure, muscle wasting, decreased body condition and decreased immune system function [35,37].

Glucocorticoids and other steroid hormones can be directly measured in saliva and blood or their conjugates can be quantified in urine and feces [7,36]. Although feces is easy to collect, fecal hormone analysis requires validation to ensure that the hormonal metabolites are biologically relevant [42,55]. For example, an ACTH challenge can be used to stimulate the HPA axis, which replicates the physiological response that occurs in an individual. In this procedure, the animal is given an ACTH injection which stimulates the HPA axis causing the production of glucocorticoids. These are eventually metabolized and excreted in the feces. The ability of the hormonal assay to determine the increase of the FGMs in response to the ACTH validates the method of analysis. Biological events, including parturition, veterinary visits, handling and medical procedures, can also be used to validate the hormonal analysis procedure [55].

The aim of this study was to increase our knowledge of the three-banded armadillo stress physiology and gain a better understanding of how the environment is influencing their biology. Our specific objectives were to: 1) validate the use of fecal hormone analysis for monitoring adrenocortical activity using both an ACTH challenge and biological events; and 2) characterize longitudinal adrenocortical activity in the three-banded armadillo to lay the foundation for future research on how environmental factors influence their success.

C. Materials and methods

Adrenocortical activity validations

Physiological validation

For the ACTH challenge, a 10 year old male and 6 year old female three-banded armadillo from Lincoln Park Zoo (LPZ) were used. The ACTH (corticotrophin; Monument Pharmacy, Monument, CO) dosages were: 4 IU/kg (5.6 IU total) for the male (# 20243) and 5.5

IU/kg (8 IU total) for the female (# 21310), which was determined by the veterinarian. Dosage amounts varied because the female was slightly larger and could be given an increased amount of ACTH to ensure a physiological response. This species' carapace makes access to its limbs difficult; therefore the ACTH injections were given intramuscularly into the neck muscles. For the ACTH challenge, fresh fecal samples were collected once a day for 1 day prior to ACTH injection, twice (pre- and post-injection) on the injection day and twice a day for 5 days post-injection. This procedure was approved through LPZ's Research Committee.

Sample collections

From the ACTH challenge procedure, it was determined that the three-banded armadillo defecates approximately twice daily. However, for both the biological event validations and the adrenocortical activity characterizations, fecal samples (approximately 3025 total; 275/animal/yr) were collected once a day in the morning for 5 – 7 days per week during routine enclosure cleaning procedures. Samples were collected for 1 year from November 2007 through November 2008.

Biological validation

Five instances of biological events were used to validate the FGM analysis. The first three events were reproductive events including pairing, mating and pregnancy. The first reproductive event was a copulation observed between a male (# 6474) and female (# 20727) three-banded armadillo. These individuals underwent daily introductions and were separated at night, starting on December 23. On January 2, copulation was observed in this pair. The second reproductive occurrence was a pairing for mating (on April 2), which was halted due to aggression exhibited by the male (# 20202) towards the female (# 21310). The three-banded armadillos were separated to prevent injury of the female. The last reproductive event used to biologically validate our methods was a pregnancy (female #21310 from May 22 – Sept 12).

The two non-reproductive events used for validation involved veterinary procedures, which require inhalant isoflurane anesthesia delivered by chamber induction method. The animals are then maintained by maintained under anesthesia by facemask until recovery.

The first veterinary procedure (February 3rd) involved a female (# 9338) which had been under treatment for ocular irritation for 4 days prior to the procedure. Following anesthesia, the animal received a full physical examination, had blood collected from the ventral tail vein and was examined by radiography to assess the severity of dental disease, which was suspected to be associated with the ocular presentation. Severe dental disease was identified and seven teeth were extracted. The female then was given oral anti-inflammatory for 72 h, eye drops for 4 days and oral antibiotics for 10 days post-procedurally. The second veterinary procedure (March 22nd) involved a male (# 20243) with a mandibular abscess. Following anesthesia, the animal received a full physical examination, had blood collected from the ventral tail vein and was examined by radiography and ultrasound to drain the mandibular abscess. During this procedure, the affected area was incised surgically and debrided. The individual then was given antibiotics parenterally or orally once daily for 10 days following the procedure.

Adrenocortical activity characterization

Eleven three-banded armadillos (5 males, 6 females) housed at LPZ were included in this portion of our study. Mean (± SEM) age was 11.0 ± 4.4 years old (range, 3– 31 years old) for females and 12.7 ± 4.1 years old (range, 7– 29 years old) for males. Animals were housed individually and exposed to natural and artificial light throughout the year. Individuals were fed Mazuri insectivore® diet (PMI Nutrition International) plus chopped vegetables and/or fruit. During the course of the study, two females experienced pregnancies (# 20727 and 21310). Samples were not collected from these individuals directly following parturition and throughout the weaning of their offspring to limit disturbance of mother and infant. One individual (#

20204) was also relocated to another zoological institute during the course of the study, resulting in fewer samples collected.

Fecal sample processing

Samples were stored in sealed bags at –20°C prior to processing and analysis. All fecal samples were processed at the LPZ Endocrinology Laboratory. Fecal samples were lyophilized (Labconco Lyophilizer, Kansas City, MO) and steroids extracted using methods modified from previously described procedures [43]. Dried samples were pulverized and 0.02g (±0.002g) of fecal powder was briefly vortexed with 0.5 ml of 90% ethanol:distilled water. After vortexing, samples were shaken (Glas-col mixer, Terre Haute, IN, setting 60, 30 min) and then centrifuged (1500 rpm, 20 min). Extracts were poured off into a clean test tube. Fecal pellets were re-suspended in 0.5 ml of 90% ethanol:distilled water and vortexed for 30 s. After re-centrifugation (1500 rpm, 15 min) extracts were combined with the first extracts and dried down under air. Samples were then reconstituted in 0.2 ml of phosphate-buffered saline (PBS; 0.01 M PO_4, 0.14 M NaCl, 0.05% BSA, 0.01% NaN_3, pH 7), vortexed briefly, sonicated for 20 min and shaken (Glas-col mixer, setting 60, 20 min). Extracted samples were diluted (range, 1:4 –1:40) using PBS for hormonal analysis.

Enzyme immunoassay (EIA)

Fecal glucocorticoid metabolites concentrations were analyzed using a cortisol enzyme immunoassay (EIA). Cortisol polyclonal antiserum and HRP (R4866; provided by C. Munro, Davis, CA) were used at a 1:8500 and 1:20,000 dilution, respectively [28]. Cross-reactivity to the cortisol antiserum were: cortisol, 100%; prednisolone, 9.9%; prednisone, 6.3%; cortisone, 5%; corticosterone, 0.7%; deoxycorticosterone, 0.3%; 21-deoxycortisone, 0.5%; 11-deoxycortisol, 0.2%; progesterone, 0.2%; 17α-hydroxyprogesterone, 0.2%; pregnenolone, 17α-hydroxypregnenolone, anderostenedione, testosterone, androsterone, dehydroepiandrosterone,

dehydroisoandrosterone-3-sulfate, aldosterone, estradiol-17β, estrone, estriol, spironolactone and cholesterol, 0.1% [28]. The cortisol EIA was validated biochemically in the laboratory by demonstrating: 1) parallelism between binding inhibition curves of fecal extract dilutions (1:2 – 1:256); and 2) significant recovery (>90%) of exogenous cortisol added to fecal extracts (1:200; $\hat{y} = 1.035x - 9.44$, $R^2 = 0.9786$). From the parallelism results, the appropriate dilution (1:20) at 50% binding was prepared for cortisol EIA. Assay sensitivity was 3.9 pg/well and intra- and inter-assay coefficients of variation were <10% and 15%, respectively. In comparison, the ACTH challenge samples also were analyzed using a corticosterone EIA (CJM006, provided by C. Munro, Davis, CA) that has been used in the LPZ endocrinology laboratory previously [46]. After conducting the same biochemical validations, we determined that the corticosterone EIA provided similar results (1:20 at 50% binding); however, we chose to use the cortisol EIA.

Data analyses

For statistical analysis, hormonal baseline values of FGM concentrations were determined using an iterative process in which high values exceeding the mean +1.5 standard deviations (SD) were excluded [21,62] for each individual separately. All FGM values from the year's sampling were averaged and values that were greater than the mean +1.5SD were removed. The mean was recalculated for each iteration and the process repeated until no values exceeded the mean plus 1.5 SD, thus, representing baseline hormone values. Increases in glucocorticoids were considered significant if values exceeded the baseline by 1.5 SD. The overall mean FGM concentration was the mean of all the samples from the 1 year of sampling for each, individual three-banded armadillo. Mean elevated FGM concentration was the mean with all of high samples excluded (during the iterative process) for each, individual armadillo (range, 93 –189 samples). Baseline values calculated during this process, for each armadillo, were used as the baseline values in the physiological validations (ACTH challenges), biological

validations and adrenocortical characterization analyses. Values were presented as mean ± standard error (SEM).

We tested the longitudinal data for the effect of sex, age, birth location (wild-born, zoo-born) and season on FGM concentrations. Assumptions of normality for all data were checked using the Kolmogorov – Smirnov statistic. If data were not normal, a natural log transformation was used. To test for the effect of sex on FGM concentrations, we used FGM concentrations as the dependent variable and sex with individuals nested within sex as the independent variables in a *t*-test. To test for the effect of age on FGM concentrations, we used baseline and elevated FGM concentrations as the dependent variable and sex as the independent variable and age as a covariate in an ANCOVA. To test for homogeneity of slopes we initially included the age by sex interaction term and later dropped the interaction from the analysis, as it was not significant. To test for the effect of birth location, we used baseline and mean FGM concentrations as the dependent variable and birth location as the independent variable in an ANOVA. A comparison of the effect of birth location on FGM concentrations could not be performed on males because of the lack of zoo-born individuals (1 zoo-born, 4 wild-born). To evaluate the effect of season on adrenocortical activity in both female and male three-banded armadillos, FGM concentrations of samples from the 10^{th} through 20^{th} of each month were averaged together to represent the monthly mean. To determine if there was a seasonal influence on FGM concentrations, we used a two-way ANOVA with a Student – Newman – Keuls post – hoc test to compare Fall (September, October, November), Winter (December, January, February), Spring (March, April, May), Summer (June, July, August) and individuals. Three individuals (two pregnant females: # 20727 and # 21310 and one male # 20204) were not included in the season analysis, because not all of the seasons were represented due to the lack of sample collection.

To determine if pregnancy influenced FGM values, data were divided into three timeframes (pre-pregnancy, first half and second half of pregnancy) and a one-way ANOVA with the Student – Newman – Keuls post – hoc test was used. For all analyses, significance was assessed at the $P < 0.05$ level. All statistical analyses were performed using Microsoft Excel (MS Office 2007) and SYSTAT 10 (SPSS Inc. 2000) general linear models.

D. Results

Adrenocortical activity validations

Physiological validation

In the male (# 20243), the mean baseline FGM concentration was 675.7 ± 10.0 ng/g dry feces with an increase of FGM concentrations (1123.2 ± 36.2 ng/g dry feces) from 54– 94 hours post-ACTH injection (Fig. 3.1A). For the female (# 21310), the mean baseline FGM concentration was 608.5 ± 12.3 ng/g dry feces with an increase of FGM concentrations (1635.4 ng/g dry feces) approximately 30 h post-ACTH injection (Fig. 3.1B).

Biological validation

Copulation resulted in a stress response from both the male (# 6474) and female (# 20727) three-banded armadillos (Fig. 3.2A and B, respectively). In the male, reproductive behavior resulted in an increase in FGM concentrations (5602.6 ± 2211.4 ng/g dry feces) above the baseline (1955.5 ± 55.8 ng/g dry feces) that peaked at day 2 and remained elevated (above baseline) for 6 days after the observed copulation (Fig. 3.2A). The female exhibited elevated FGM concentrations (2045.2 ± 1267.7 ng/g dry feces) above baseline values (475.2 ± 18.7 ng/g dry feces) on day 1, and 4–6 after the observed copulation (Fig. 3.2B). The aggressive pairing of a male (# 20202) and female (# 21310) three-banded armadillo also resulted in a stress response from only the female (Fig. 3.3A) with an increase in FGM of 1229.8 ± 223.6 ng/g dry feces compared to baseline values (608.5 ± 12.3 ng/g dry feces) and remained elevated (above

baseline) for 2 days (Fig. 3.3A). The aggressive male only exhibited a small increase resulting in an elevated FGM value of 1607.6 ng/g dry feces compared to baseline (1217.51 ± 25.5 ng/g dry feces) on day 3 after the pairing (Fig. 3.3B).

The pregnancy (female # 21310) resulted in a mean FGM of 1496.3 ± 90.0 ng/g dry feces above mean baseline (608.5 ± 12.3 ng/g dry feces) and remained elevated from day 60 through parturition (Fig. 3.4). Female # 21310 had higher ($F_{1,226}$= 18.6, $P < 0.001$) FGM concentrations than female # 20727 (1295.5 ± 109.4 ng/g dry feces). Overall, mean FGM concentrations prior to pregnancy (978.2 ± 63.3 ng/g dry feces) and 1st half pregnancy (1058.1 ± 86.4 ng/g dry feces) were similar (P > 0.05) to each other, but both were lower ($F_{2,226}$=46.5, $P < 0.001$) than FGM concentrations during the 2nd half of pregnancy (1950.0 ±103.1 ng/g dry feces, Fig. 3.5).

The veterinary procedure resulted in a stress response from a female (# 9338) three-banded armadillo (Fig. 3.6A). Mean baseline FGM concentration was 753.7 ± 14.1 ng/g dry feces compared to the mean FGM during the veterinary procedure (1945.4 ± 448.8 ng/g dry feces: Fig. 3.6A). Elevated FGM concentrations were observed 1 day after the veterinary procedure and peaked at day 7 (Fig. 3.6A). The male three-banded armadillo (# 20243) exhibited a stress response following the veterinary procedure with a FGM baseline concentration of 675.7 ± 10.0 ng/g dry feces compared to elevated FGM concentration of 1165.3 ± 98.5 ng/g dry feces (Fig. 3.6B). Elevated values were exhibited 3 days after the procedures with peaks FGM concentrations on days 4 and 6 day (Fig. 3. 6B).

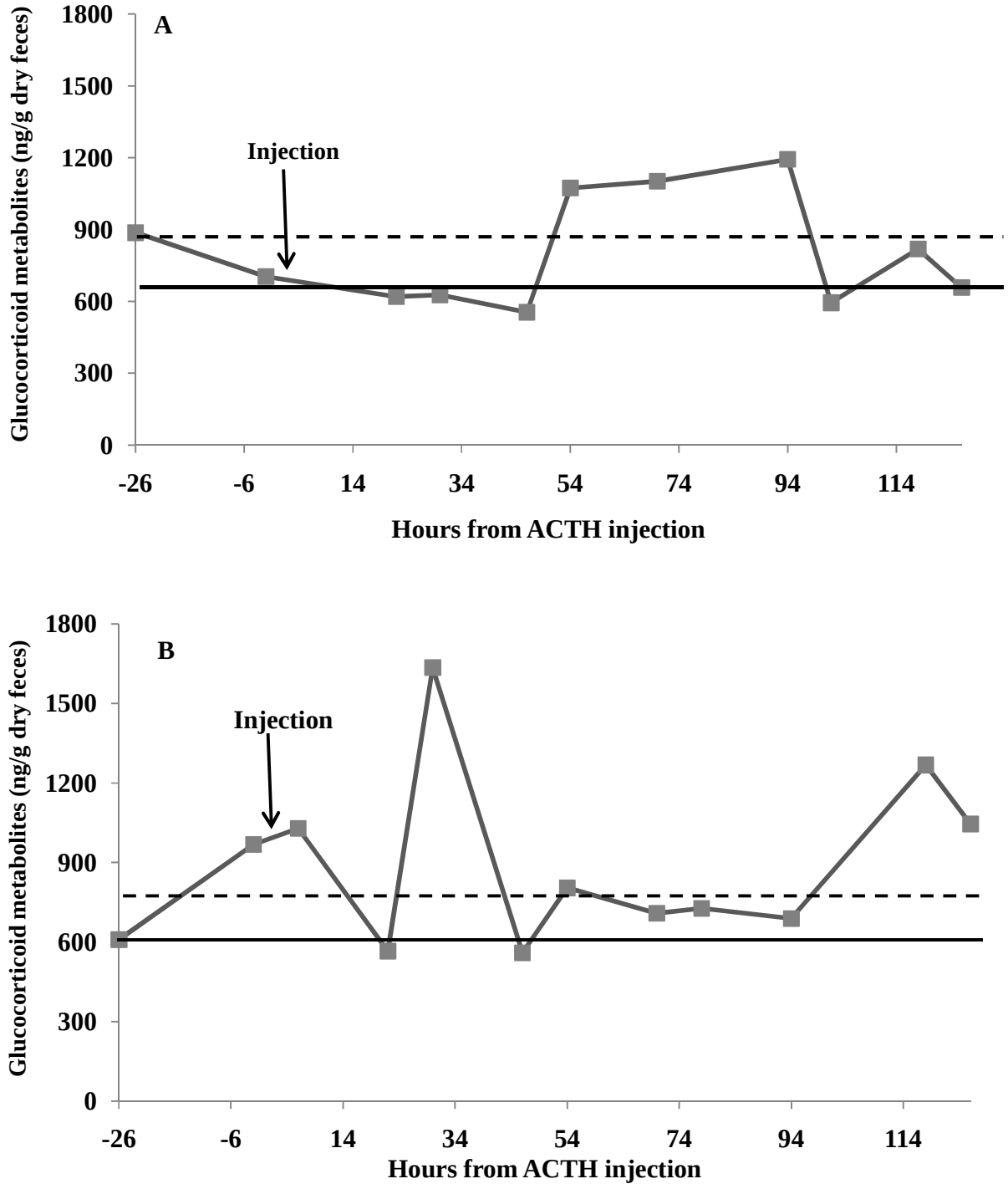


Figure 3.1 Fecal glucocorticoid metabolite (FGM) concentration (ng/g dry feces) profile from an adrenocorticotropic hormone (ACTH) challenge in a male (A, # 20243) and female (B, # 21310) three-banded armadillo. The ACTH injections for the male (4 IU/kg; 5.6 IU total) and female (5.5 IU/kg; 8 IU total) were administered at hour 0 with sample collection beginning 26 hours prior to the injection through 126 hours post- injection. The solid line represents the baseline and the dashed line represents the elevated FGM concentrations.

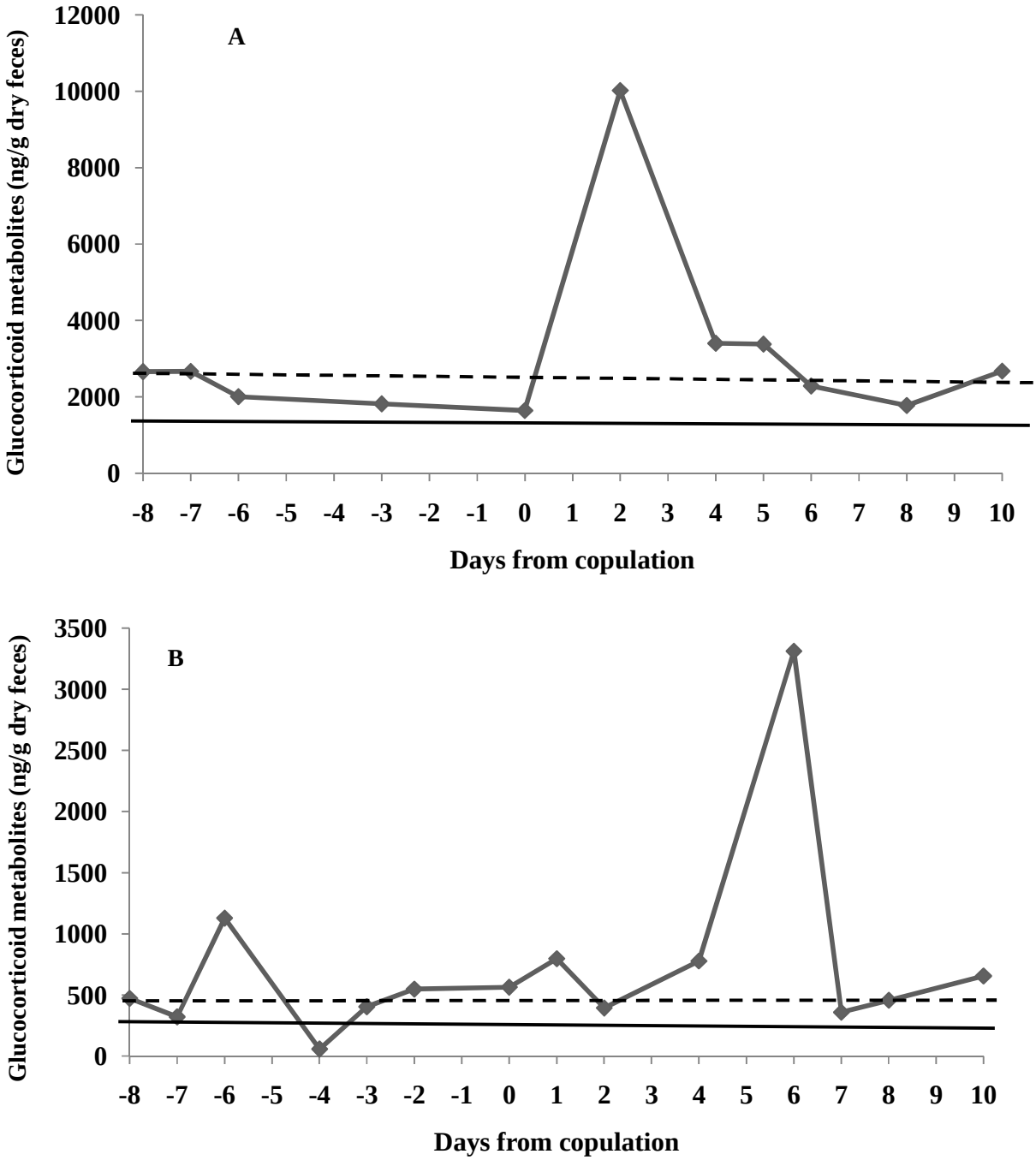


Fig. 3.2 Fecal glucocorticoid metabolite (FGM) concentration (ng/g dry feces) profile from a male (A, # 6474) and female (B, # 20727) three-banded armadillo observed copulating. Copulation occurred on day 0. These data are used for a biological validation of adrenocortical activity analysis. The solid line represents the baseline and the dashed line represents the elevated FGM concentrations.

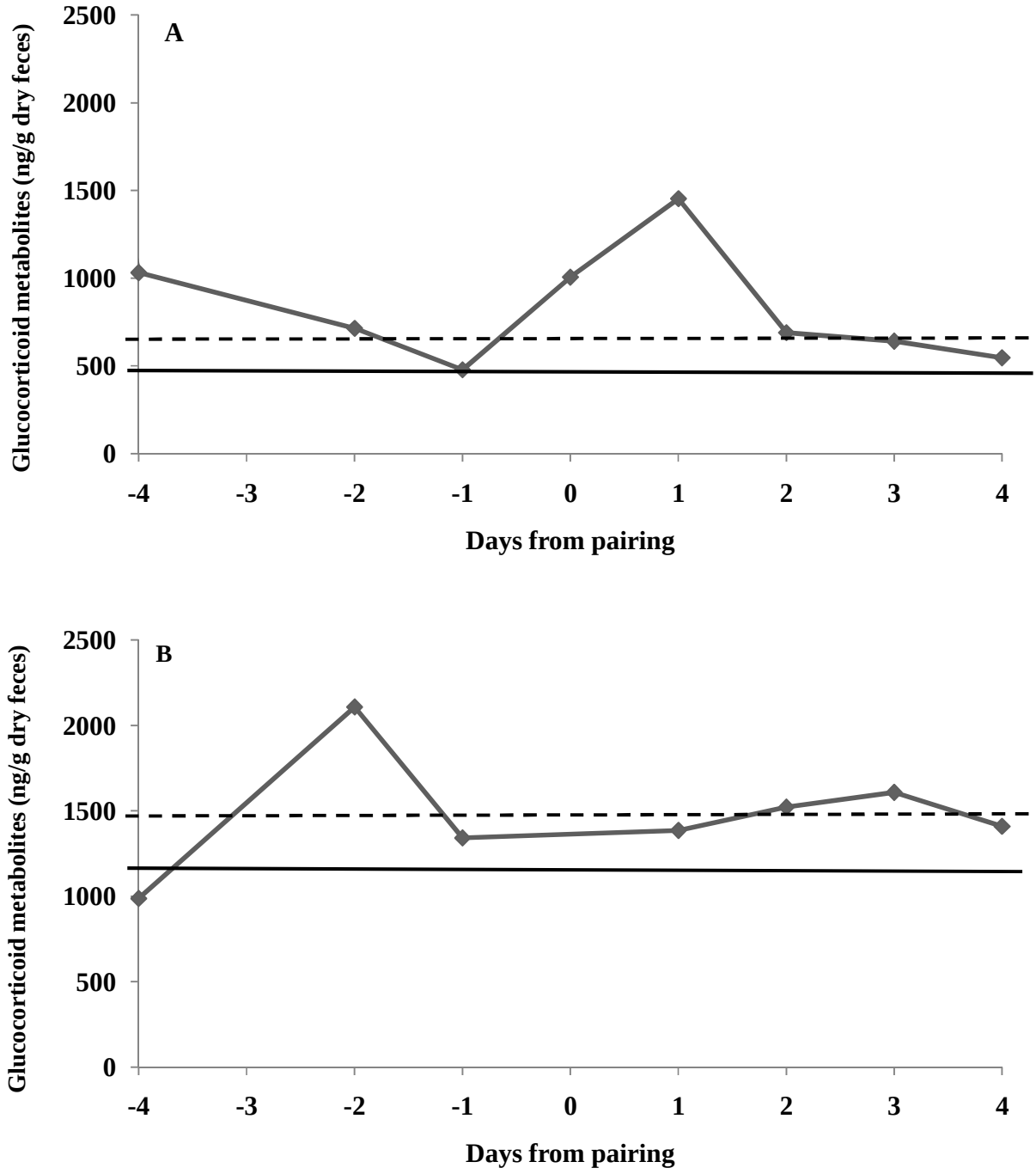


Fig. 3.3 Fecal glucocorticoid metabolite (FGM) concentration (ng/g dry feces) profile of a female (A, # 21310) that was introduced to a male three-banded armadillo for mating (B, # 20202). The introduction on day 0, which was halted due to the aggressive nature of the male (# 20202), was a biological event used to validate the adrenocortical activity analysis. The solid line represents the baseline and the dashed line represents the elevated FGM concentrations.

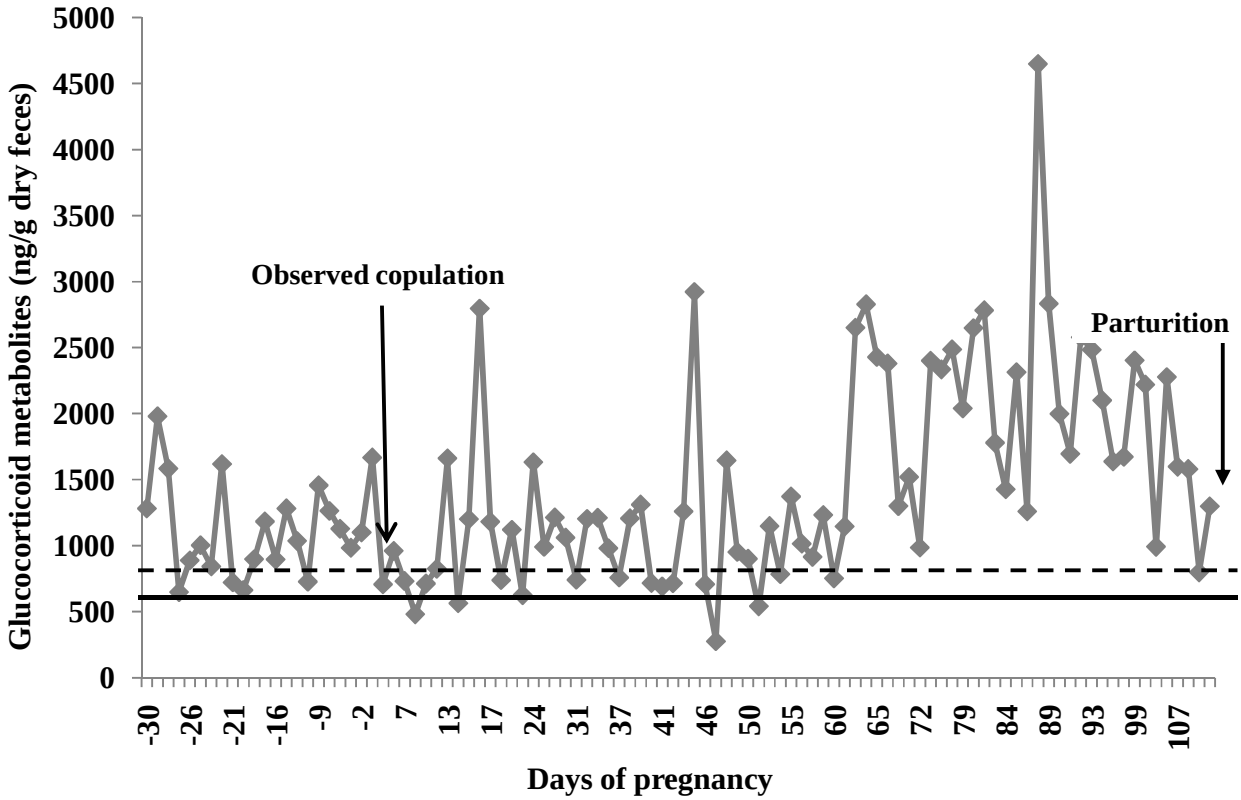


Fig. 3.4 Fecal glucocorticoid metabolite (FGM) concentration (ng/g dry feces) profile from a representative pregnant female (# 21310) three-banded armadillo from 1 month prior to copulation through parturition. The solid horizontal line represents the baseline and the dashed horizontal line represents the elevated FGM concentrations.

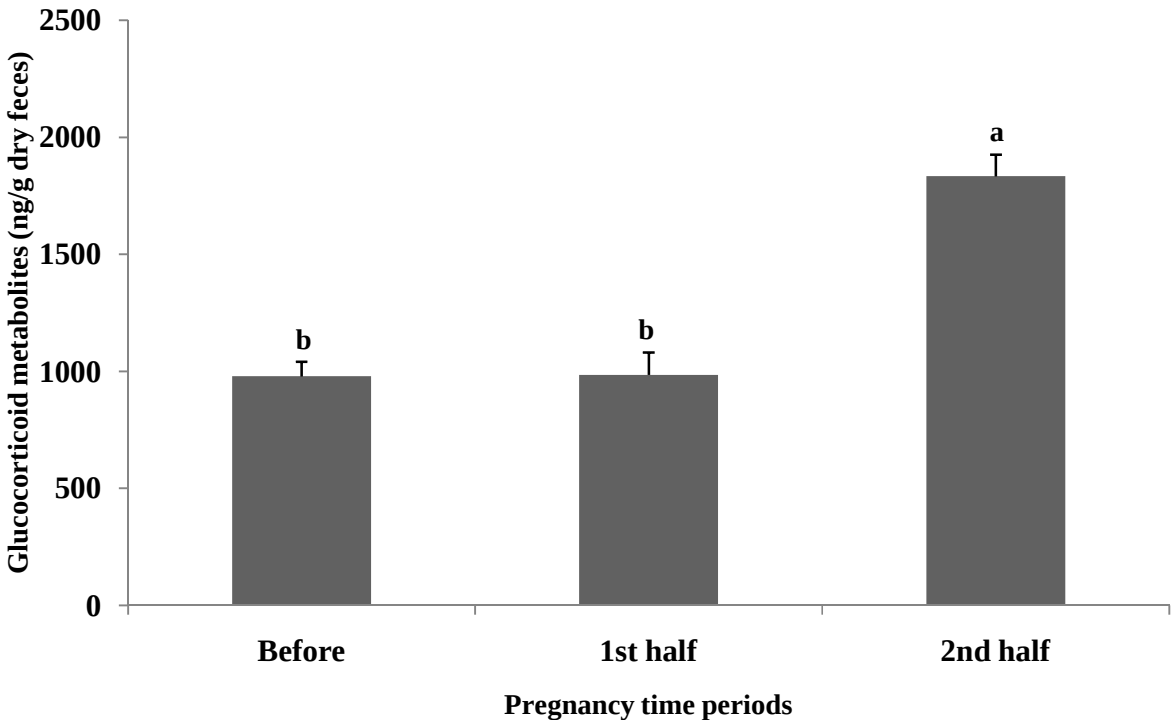


Fig. 3.5 Mean (± SEM) fecal glucocorticoid metabolite (FGM) concentrations (ng/g dry feces) of female three-banded armadillos (n=2) during pregnancy broken into time periods: 30 days before pregnancy, during the 1st half and 2nd half of pregnancy. [a,b]Superscripts represent significant differences ($P < 0.05$, B) in time periods.

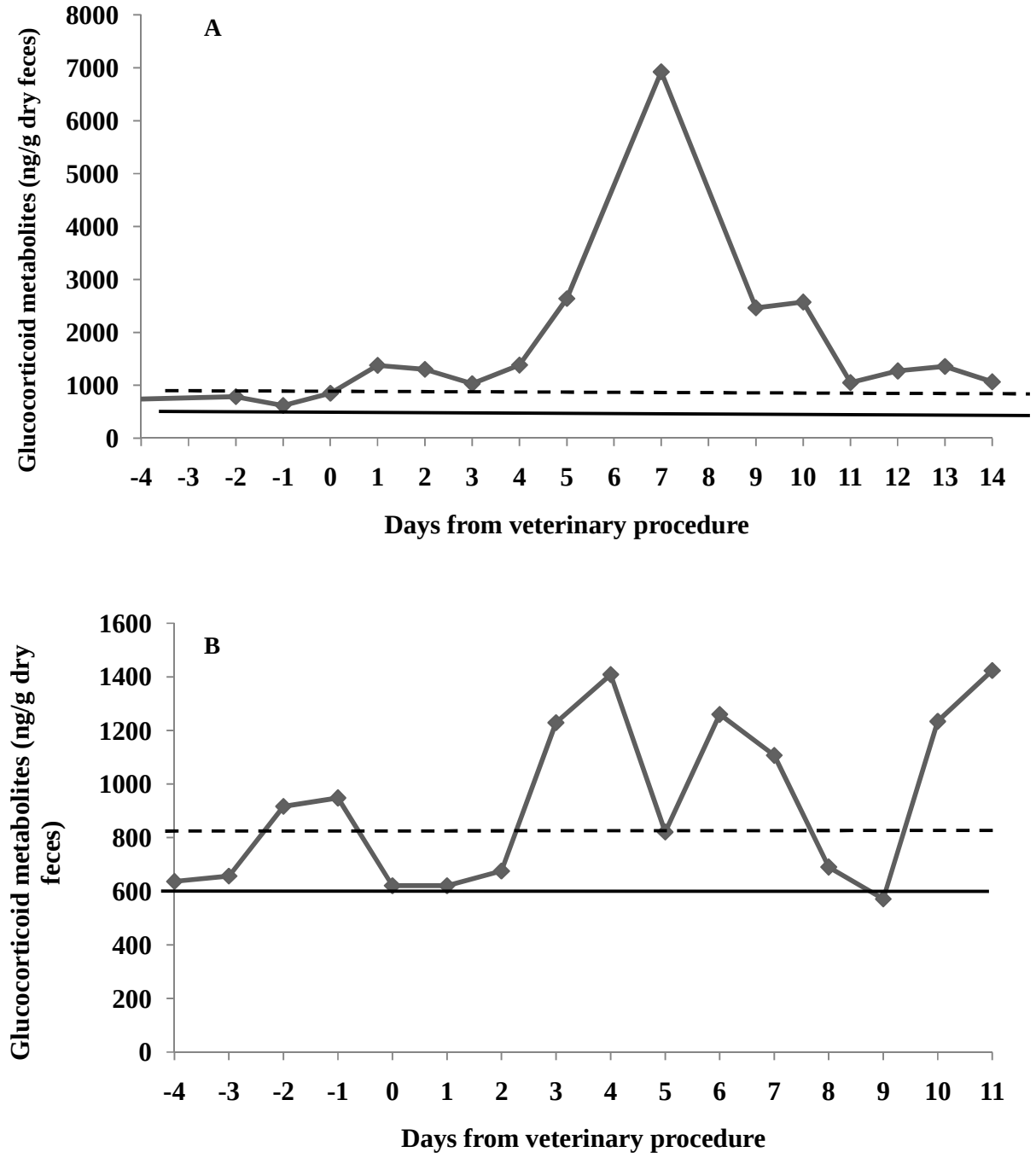


Fig. 3.6 Fecal glucocorticoid metabolite (FGM) concentration (ng/g dry feces) profile from a female (A, # 9338) and a male (B, # 20243) three-banded armadillo that experienced elevated adrenocortical activity resulting from veterinary procedures (day 0), including anesthetization and medical treatment. The solid line represents the baseline and the dashed line represents the elevated FGM concentrations.

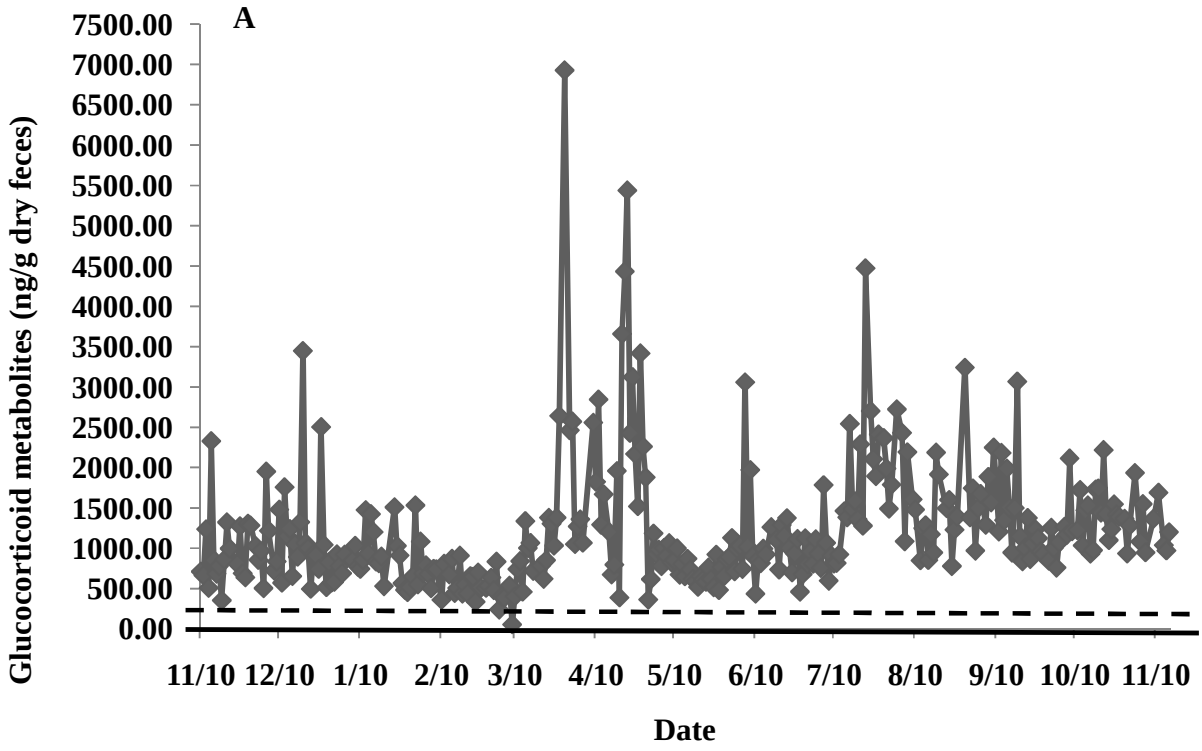
Adrenocortical activity characterization

Sex did not influence ($T_{1, 9}$= -1.416, $P > 0.05$) mean FGM concentration, but values varied ($P < 0.001$) among individuals. Neither sex ($F_{1,8} = 1.4$, $P > 0.05$) nor age ($F_{1,8} = 0.8$, $P > 0.05$) had an effect on baseline or elevated FGM concentrations. The overall mean, baseline and elevated FGM concentrations for all males and females are shown in Table 1. Females exhibited a mean FGM baseline of 676.7 ± 52.7 ng/g dry feces (range, 475.2– 822.3 ng/g dry feces) and elevated FGM concentration of 1582.9 ± 96.4 ng/g dry feces (range, 1243.4– 1857.6 ng/g dry feces). Figure 3.7A and B depicts two representative females' longitudinal adrenocortical activity profile over the year. Males displayed a mean FGM baseline of 1080.2 ± 240.5 ng/g dry feces (range, 667.2– 1955.5 ng/g dry feces) and elevated FGM concentration of 3805.5 ± 1809.1 ng/g dry feces (range, 1110.3 – 10,683.3 ng/g dry feces; Table 1). Figure 3.7C and D depicts two representative males' daily adrenocortical activity over 1 year (exception: # 20204, daily adrenocortical activity for 9 months). In females, birth location had no effect ($F_{1,5} = 3.58$, $P > 0.05$) on baseline or mean FGM concentrations ($F_{1,5} = 0.83$, $P > 0.05$). The FGM concentrations were similar across seasons ($F_{3,68} = 1.9$, $P > 0.05$), but varied across individual ($F_{7,68} = 1.9$, $P < 0.05$, Table 1) armadillos.

Table 3.1 Male and female fecal glucocorticoid metabolite (FGM) concentrations (ng/g dry feces) assessed by longitudinal analysis using a cortisol enzyme immunoassay. Age is reported in years of age.

Male	**Age**	**Overall Mean FGM***	**Baseline FGM****	**Mean Peak FGM*****
6474[a]	29.2	7889.1 ± 490.6	1955.5 ± 55.8	10683.3 ± 625.6
20200[c]	9.2	843.8 ± 21.4	667.2 ± 8.4	1110.3 ± 40.4
20202[b]	9.2	3458.3 ± 285.1	1217.5 ± 25.5	4215.1 ± 363.8
20204	9.2	1207.4 ± 39.2	885.3 ± 15.4	1813.6 ± 76.7
20243[c]	6.8	963.8 ± 20.6	675.7 ± 10.0	1206.3 ± 25.8
Female				
9338[c]	31.2	1226.3 ± 53.9	753.7 ± 14.1	1857.6 ± 101.6
9717[c]	13.8	816.3 ± 22.3	630.5 ± 10.4	1243.4 ± 46.1
20198[c]	9.2	1284.9 ± 51.4	769.8 ± 18.9	1851.9 ± 78.0
20441[c]	5.4	1128.3 ± 31.3	822.3 ± 11.8	1524.9 ± 52.0
20727	4.0	1282.8 ± 67.4	475.2 ± 18.7	1539.6 ± 77.8
21310	2.4	1177.7 ± 47.3	608.5 ± 12.3	1480.1 ± 59.6
Overall mean	**11.9 ± 0.9**	**2010.1 ± 862.4**	**878.5 ± 201.8**	**2694.3 ± 1111.4**

Values are means ± SEM in samples collected over 1 year (5 samples/week; total sample =3025; ~275 per individual). * Overall mean FGM value is the average of all values across the year. **Baseline was calculated using an iterative process that eliminates all of the values that are 1.5 SD above the mean. ***Mean peak FGM is the average of the values removed during the iterative process. [a,b,c]Superscripts represent differences ($P < 0.05$) within individuals across seasons. Individuals (#20204, 20727 and 21310) were not included in the seasonal analysis due to the lack of data.


A
Glucocorticoid metabolites (ng/g dry feces)
7500.00
7000.00
6500.00
6000.00
5500.00
5000.00
4500.00
4000.00
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
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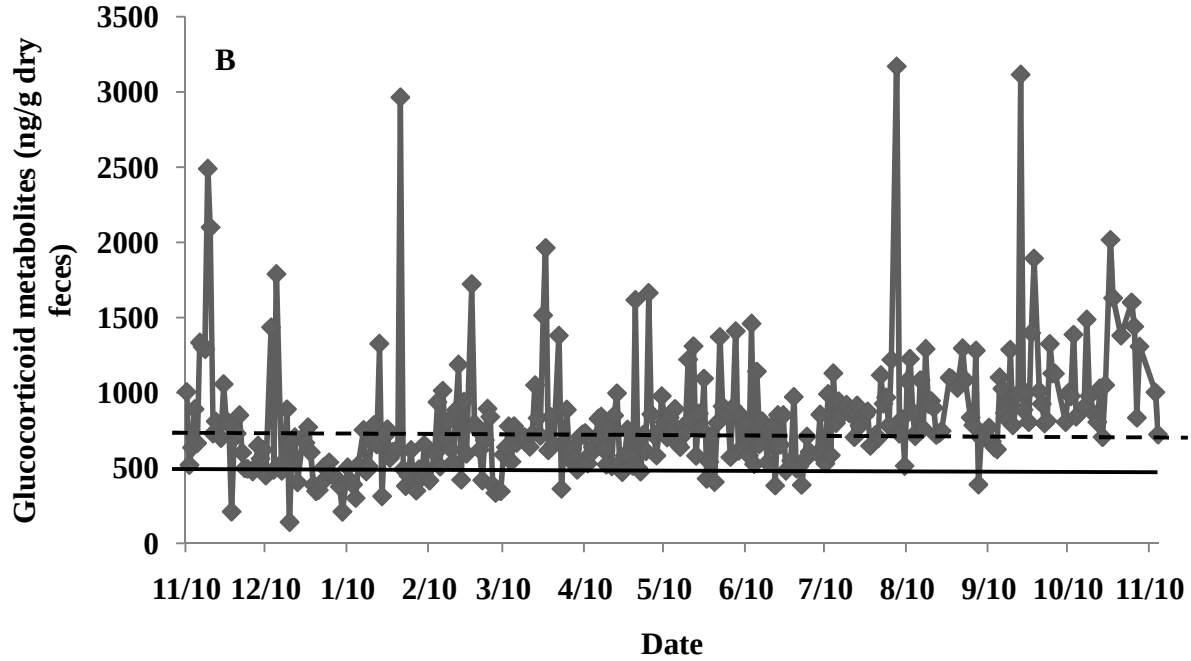
0.00
11/10 12/10 1/10 2/10 3/10 4/10 5/10 6/10 7/10 8/10 9/10 10/10 11/10
Date


B
Glucocorticoid metabolites (ng/g dry feces)
3500
3000
2500
2000
1500
1000
500
0
11/10 12/10 1/10 2/10 3/10 4/10 5/10 6/10 7/10 8/10 9/10 10/10 11/10
Date

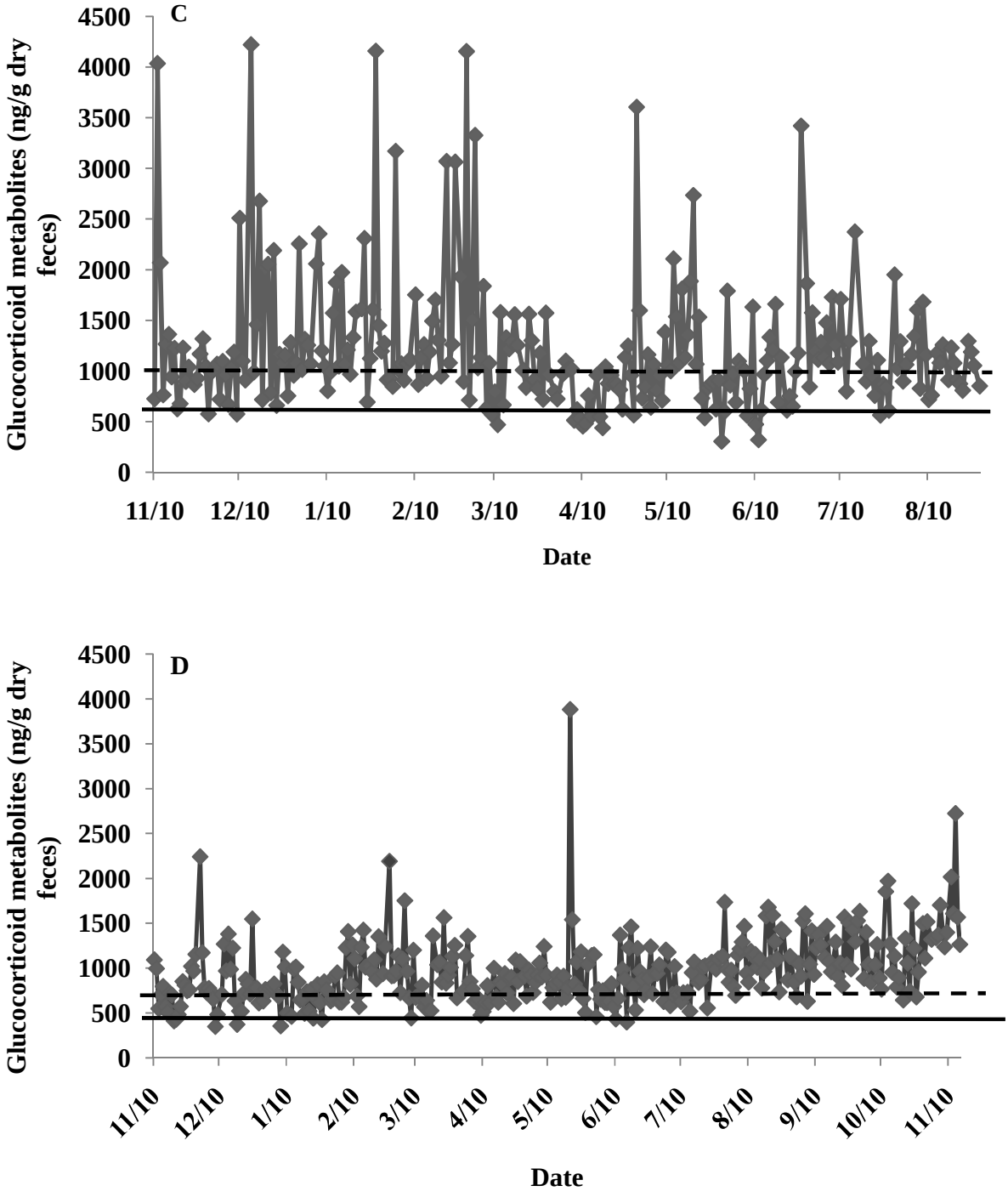


Fig. 3.7 Fecal glucocorticoid metabolite (FGM) concentration (ng/g dry feces) longitudinal profile of representative females (A, # 9338; B, # 9717) and males (C, # 20204; D, # 20243) three-banded armadillo from November 2007 – November 2008 (exception: #20204, November 2007–August 2008). The solid line represents the baseline and the dashed line represents the elevated FGM concentrations

E. Discussion

This study was the first to use fecal hormone analysis to monitor the adrenocortical activity of the three-banded armadillo. Here an ACTH challenge was used successfully to induce adrenocortical activity in both the male and female, which was reflected in the FGM analysis, thus, physiologically validating the non-invasive methods. Several events (reproductive behaviors and veterinary procedures), which elicited a stress response, were also successfully utilized as biological validations of these methods. Fecal hormone analysis from 11 three-banded armadillos provided baseline FGM data for this species. These data revealed that sex, age, birth location and season had no effect on FGM concentrations, but FGM concentrations were highly variable among individuals.

This was the first time an ACTH challenge was conducted in the three-banded armadillo. In the nine-banded armadillo (*Dasypus novemcinctus*), an ACTH challenge resulted in a 100-fold increase in plasma cortisol at 2.5 h post-injection [16]. In this study we did not collect blood, but used non-invasive fecal hormone analysis to monitor a stress response in both a male and female three-banded armadillo. The male experienced a two-fold increase in FGM, compared to baseline values, at 54– 94 hours post-ACTH. The female also exhibited an increase in FGM that was twofold greater than the baseline, at 30 h post ACTH injection. The difference in response time and the length of elevated FGM concentrations between individuals may be a result of a slightly higher dose in the female versus male. It may also demonstrate the variance that may be observed within a species and the importance of employing multiple forms of validation when using a technique on a species for the first time.

Naturally occurring events that may elicit a stress response have previously been used to validate the use of fecal hormone monitoring including: the translocation of animals [20], immobilization [54], mate introduction, exposure to construction, social tension and medical

treatment [60]. Several biological events that occurred during the longitudinal characterization of adrenocortical activity validated our FGM analysis in the three-banded armadillo. In this study, we demonstrated that certain reproductive events could elicit stress responses in both male and female three-banded armadillos. Copulation and sexual behavior have triggered increases in adrenocortical activity in other species including the boar (*Sus domestica,* [58]) and rat (*Rattus norvegicus*,[53]). Leuner et al. [26] demonstrated that stress from copulation was positive and activated neurogenesis in the brain of the rat. Here, the male three-banded armadillo exhibited elevated FGM concentrations up to fivefold greater than his baseline on day 2 after copulation. The female also exhibited elevated FGM concentrations, up to sevenfold greater than her baseline on day 6 after copulation. The difference in response time again could be attributed to individual differences in response to stressors; however, the two armadillos continued to be paired and the female may have also exhibited elevated adrenocortical activity due to another encounter with the male, no specific notes were recorded.

Stress hormones are known to increase throughout pregnancy to aid in parturition and due to physiological exertion of pregnancy [34]. As the fetus grows and reaches the capacity of the uterus near the end of pregnancy, the fetal HPA triggers the release of glucocorticoids which ultimately initiates the first stage of parturition [50]. Elevated glucocorticoids during pregnancy have been demonstrated in the Belding's ground squirrel (*Spermophilus beldingi* [41]), domestic cattle (*Bos taurus* [25]) and golden lion tamarins (*Leontopithecus rosalia* [4]). Although others species like the common hamster (*Cricetus cricetus* [19]), show no difference in FGM values during pregnancy. Our study demonstrated that pregnancy, in two females, resulted in elevated mean FGM concentrations up to fourfold greater than baseline FGM concentrations around day 60 post-copulation. Gestation lengths in this species have been observed to be a length of ~120 days [31]. This increase in adrenocortical activity was similar to the rise in progesterone during

pregnancy [23], which has also been observed in pygmy rabbits (*Brachylagus idahoensis* [48]), black-footed ferrets (*Mustela nigripes* [6]), felids [9] and guinea pigs (*Cavia aperea f. porcellus* [33]).

Social introductions for pairing and co-habitating can be stressful events because they may result in injury [20] and even mortality as observed in the clouded leopard (*Neofelis nebulosa* [10]). Species that have exhibited increased cortisol concentrations after aggressive social interactions include horses (*Equis caballus* [2]), African wild dogs (*Lycaon pictus*), gray wolves (*Canis lupus*) and dwarf mongoose (*Helogale parvula* [14]). In this study, a stress response was elicited in a female three-banded armadillo after being paired with a male three-banded armadillo that behaved aggressively towards her, resulting in their separation. She exhibited FGM concentrations twofold greater than her baseline FGM concentrations 1 day afterwards, which was similar to her response to the ACTH challenge. In contrast, the male armadillo in this aggressive pairing showed little FGM response, highlighting individual differences in response to stressors.

Various studies have demonstrated that veterinary exams can cause both physiological and behavioral stress in animals. Waiblinger et al. [57] determined that veterinary procedures resulted in quickened heartbeats and distressed dairy cattle. Pain and distress after veterinary procedures can also cause increased adrenocortical activity which was demonstrated in the horse post-surgery [44]. Sedation resulted in increased FGM concentrations of the cheetah (*Acinonyx jubatus* [54]) and the clouded leopard [60]. As demonstrated with laboratory cats (*Felis catus* [12]), handling, unpredictable husbandry and translocation of animals can also cause elevated adrenocortical activity. However, minimally invasive sedation methods can result in little to no adverse effects in some species, including the collared-anteater (*Tamandua tertradactyla* [18]), Hoffman's two-toed (*Choloepus hoffmanni*) and brown throated three-toed sloth (*Bradypus*

variegates [22]). In this study, the female three-banded armadillo submitted to sedation, surgery and handling for medication administration showed a response of elevated FGM concentrations on days 1 and 2 and then exhibited a peak ninefold greater than her mean baseline FGM concentrations on day 7 post-procedure. The male animal, that underwent medical treatment, experienced a stress response as demonstrated by FGM concentrations that were twofold greater than baseline FGMs on days 3, 4, 6 and 7 days after the dental procedures. Interestingly, this male is the same individual that was administered the ACTH challenge and both events resulted in similar elevated FGM concentrations excreted approximately 3 days after the stressor. Overall, although these individuals may have been in pain due to their various illnesses, it seems that the handling/veterinary procedures, translocation for procedures and administration of medications caused greater adrenocortical activity after the procedures compared to before the procedures, as shown by Fig. 6.

This long-term monitoring of the adrenocortical activity in the three-banded armadillo added to the knowledge of the basic physiology of this species, specifically providing data on fluctuations in FGM concentrations. From these data, ranges for mean, baseline and elevated FGM concentrations were identified within individual three-banded armadillos, making it possible to find trends within the population. We determined sex, age and birth location did not affect the FGM concentrations of these armadillos, but individuals varied greatly in their FGM concentrations. We also found that though season differed within individuals, season across all individuals did not affect the FGM concentrations of these armadillos. Even though these armadillos are exposed to some natural photoperiods, we did not expect FGM to be influenced by seasonal environment changes, because temperature, food availability and prolonged artificial lighting remain constant throughout the year for zoo-housed animals. In the wild, however, animals living in habitats that experience extreme changes in temperature and food availability

are expected to experience elevated adrenocortical activity as result of these external pressures, as exhibited by spider monkeys (*Ateles geoffroyi yucatanensi*, [45]) and Siberian tigers (*Panthera tigris altaica* [38]).

High variability in individuals makes it necessary to conduct a longitudinal study overtime to characterize adrenocortical activity for each individual. Differences in FGM concentrations within individuals has been documented in quite a few species, such as spiny mice (*Acomys cahirinu* [40]), Syrian hamster (*Mesocricetus auratus* [13]), black (*Diceros bicornis*) and white rhinoceros (*Ceratotherium sim* [8]) and red deer (*Cervus elaphus* [24]). The high variability in adrenocortical activity determined within individuals points to the possibility that husbandry and management may need to be customized for each individual to better maintain zoo-housed populations. Furthermore, previous studies have demonstrated that animals have behavioral types, which can affect how they react in various environments and stressful situations [11,17,56]. Here, there was one individual (male # 6474) in particular that varied greatly from the other armadillos. He had been identified by Animal Care staff as an aggressive individual, even with females during pairing for mating, which was reflected in his FGM concentrations. This male was an outlier among all individuals, exhibiting higher FGM concentrations than the other armadillos, though he exhibited lower fecal androgen metabolites than other males [23]. After considering other possible variables, we were unable to identify a cause for this male's elevated adrenocortical activity, but we consider this an example of just how great individual variability can be within a species. This individual confirms that further research into individual behavioral types of three-banded armadillos may also help management make more successful decisions on mate compatibility and decrease the chance of injury.

In conclusion, our results confirm that fecal hormone analysis can be used to monitor adrenocortical activity in the three-banded armadillo. We acknowledge that the work conducted

here has some limitations. For example, additional individuals for both ACTH challenges and hormone profile characterizations would assist with gathering more basic information on this species. Furthermore, conducting a radio-labeled metabolism study could be used to determine the major route and rate of excretion of FGMs in both males and females. Future adrenocortical activity studies on this species would also benefit from performing high-performance liquid chromatography to determine the exact fecal glucocorticoid metabolites and develop a specific hormonal assay for those metabolites. This would allow for clearer adrenocortical activity profiles. However, these data provide a foundation for further research into how changes in the environment directly affect the adrenocortical activity in this armadillo. These data may be useful for *in situ* populations, if individuals could be identified and sampled over time. The knowledge gained of the basic adrenocortical activity in the three-banded armadillo may allow it to serve as a model the closely related Brazilian three-banded armadillo (*Tolypeutes trincinctus*; categorized as vulnerable [51]), which is also affected greatly by human encroachment and deterioration of its natural habitat.

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III. INTEGRATING ADRENOCORTICAL ACTIVITY AND FORAGING BEHAVIOR AS AN APPLICATION FOR PROVIDING INSIGHT INTO THE WELFARE OF ZOO-HOUSED THREE-BANDED ARMADILLOS (*TOLYPEUTES MATACUS*)

A. ABSTRACT

Monitoring and improving the welfare of zoo-housed animals is of increasing importance. The goal of this study was to develop a conceptual model for integrating the use of adrenocortical activity and foraging behavior analyses to determine how an animal perceives its environment and develop a well-being metric using those analyses. Our specific objectives were to: 1) develop a food patch that could be used to measure the armadillo's foraging behavior, via giving-up density (GUD); 2) determine the effects of food patch characteristics (substrate quantity and patch quality) and enclosure characteristics (bedding amount) on individuals' GUDs and adrenocortical activity (via fecal glucocorticoid metabolite analysis); and 3) use these results to assign each armadillo a well-being category. To assess fecal glucocorticoid metabolites (FGM) concentrations, fecal samples (~630 total; *n*= 90/animal) were collected from 7 (3 males; 4 females) southern three-banded armadillos from 1 month preceding foraging treatments, throughout foraging treatments and 1 week post foraging treatments at Lincoln Park Zoo (Chicago, IL). A cortisol enzyme immunoassay was used for FGM analysis. A food patch was designed for zoo-housed armadillos consisting of a black rubber round pan, loose topsoil and wax worm larvae (*Galleria mellonella*). Armadillos were allowed to forage in food patches from 4:00 PM until 8:00 AM and were exposed to each foraging treatment and enclosure characteristic for 4 nights (substrate quantity: 4 nights, patch quality: 4 nights, bedding amount: 4 nights per bedding amount). Four state categories (neutral, ideal, negative and positive) were created to score the overall well-being of the armadillos using mean FGM values and GUD measures. Mean FGM concentrations were variable across individuals, but mean FGM concentrations for

all armadillos were similar across the pre-sampling, treatments and post-sampling. Within two individual armadillos, FGM concentrations varied (#9338, #20202) across pre-sampling, treatments and post-sampling. GUD measures for individuals varied greatly for all foraging treatments. Armadillos did not detect patch depth or quality, but bedding amount did influence foraging intensity. Ranked individual GUDs were similar across the treatments. Four armadillos remained in the same state category (3 in ideal state, 1 in neutral state) throughout the foraging treatments; while three armadillos transitioned to different states, with one shifting from a negative state to a neutral state after the substrate quantity treatment. The remaining two armadillos moved from an ideal to a neutral state and a neutral state to an ideal state, respectively, after the bedding treatment. All armadillos ended in either a neutral or positive state category. Measuring adrenocortical activity and foraging behavior provided insight into separate metrics, the physiological and psychological state of the animals, contributing to their welfare. These measurements were not correlated and were required to properly evaluate the welfare of each armadillo. This work has expanded the GUD methodology and created new opportunities for the improvement of husbandry and overall welfare of animals housed in zoos.

B. Introduction

Monitoring and improving the welfare of zoo-housed animals is of increasing importance; therefore, recent efforts into the development of methods to evaluate animal welfare are on the rise [1]. One increasingly prevalent method is the evaluation of an individual's adrenocortical activity (i.e. stress) because of its relationship to an animal's health and well-being. In understanding the stressors perceived by animals, it is valuable to differentiate between eustress and distress [2]. Minimal stressors, such as social introduction or a reproductive event, that occur for a short period of time, can have a positive effect on the physiological state of the animal (i.e. eustress) [3]. On the other hand, negative stressors (or distress), such as sharing an exhibit with an overly aggressive mate, when chronic, can be debilitating to the physiological state of the animal [3]. Prolonged elevated adrenocortical activity can result in a compromised immune system, consumption of more energy, increased self-destructive behaviors and reduced reproduction [3-5]. Monitoring the adrenocortical activity of animals through the use of non-invasive fecal hormone analysis is an effective method for gaining insight into how stressors affect an animal's physiological state [6-9]. These methods have been used to assess the adrenocortical activity of a wide variety of both wild and zoo-housed species, from birds to mice to elephants, for at least 20 years [8, 10-13]. Although this method requires proper validation of physiological and biological relevance [14], non-invasive fecal hormone analysis allows for longitudinal evaluation of pooled hormone concentrations [15, 16] through simple sample collection.

Although monitoring adrenocortical activity will evaluate the animal's physiology, it does not provide a complete understanding of the psychological state, which also contributes to an animal's overall welfare. Due to the unnatural environment, it is important to determine a zoo-housed animal's perception of its surroundings [17]. Using foraging behavior may provide

an effective method for gaining insights into an animal's perception of the environment and its well-being. Diverse forms of feeding enrichment have been used for zoo-housed animals including: buried food in outdoor enclosures for wombats (*Lasiorhinus latifrons*) [18], food filled bones for coyotes (*Canis latrans*) [19], and hiding dead mice in enclosures for maned wolves (*Chyrsocyon brachyurus*) [20].

Depletable food patches, with substrate, can also be used as a form of feeding enrichment. A food patch is depletable when the animal experiences diminishing yields from the effort of searching for food within the patch [21]. For example, the animal should leave a resource patch once its energetic gains (harvest rate, H) of foraging no longer exceed its costs of foraging (predation cost, P; metabolic cost, C; and missed opportunity cost, MOC) [21]. These benefits and costs form an equation: H= P + C + MOC [22, 23]. The giving-up density (GUD), which is the amount of food remaining in the patch once foraging has stopped, can be used to identify an animal's H [21]. Foraging efforts require the animal to make a decision about when to cease feeding, while balancing P, C and MOC. Patch use and GUDs have been used to evaluate foraging ecology in various wild species (e.g. birds [24], rodents [25,26] and primates [27]). Foraging decisions may be affected by location [28] (i.e. in the open, close to cover, or at different elevations), time of day [29] and food preferences (e.g. seed size, seed type, seed density, toxin contents) [30, 31]. These methods also have been used successfully in several zoo-housed populations (American bison, *Bison bison bison;* Grant's zebra, *Equus burchelli;* rock hyrax, *Procavia capensis*) demonstrating that animals from different source populations have varying expectations of their environment as evident by foraging decisions [33] and space usage [32].

In this study, our goal was to develop a conceptual model for integrating the use of adrenocortical activity and GUDs analyses to determine how an animal perceives its

environment. Combining these analyses will provide a method to evaluate an individual's physiological and psychological states, thus providing a well-being metric. We chose to apply this model to the southern three-banded armadillo (armadillo; *Tolypeutes matacus*) because of this species' limited breeding success despite its long history in zoos [34, 35]. Our specific objectives were to: 1) develop a food patch that could be used to determine the armadillo's foraging intensity measured by GUDs; 2) determine the effects of food patch characteristics (substrate quantity and patch quality) and enclosure characteristic (bedding amount) on individuals' GUDs and adrenocortical activity (via fecal glucocorticoid analysis); and 3) use these results to assign each armadillo a well-being state category (ideal, positive, negative, or neutral).

C. Methods

Animals

Seven (3 males, 4 females) armadillos, housed at Lincoln Park Zoo (LPZ), were included in this study. Mean (± SE) age of the armadillos was 16.2 ± 3.8 years old (range, 6.5 – 33.8 years). Animals were housed individually and exposed to natural and artificial light throughout the year. Enclosures were 112 cm x 58 cm x 61 cm with ~ 1.0 kg (i.e. ~2 flakes) of straw for bedding material. Individuals were fed Mazuri insectivore diet® (PMI Nutrition International) plus chopped vegetables and/or fruits and six wax worm larvae (*Galleria mellonella,* as treats). During the study, diets remained consistent, except larvae were excluded from the regular diet as armadillos were offered 36 worm larvae /week (maximum number that was approved by the resident nutritionist, Dr. Shana Lavin) during the foraging experiments.

Fecal sample collection and processing

Fecal samples were collected 5 to 7 times a week during routine cleaning of the armadillos' enclosures. Sample collection began 1 month prior to foraging patch treatments,

continued during foraging patch treatments, and ended 1 week after the completion of all foraging treatments (630 total samples; ~90 per animal). Samples were stored in sealed bags at -20°C until processing.

All fecal samples were processed at the LPZ Endocrinology Laboratory. Fecal samples were lyophilized (Labconco Lyophilizer, Kansas City, MO) and steroids extracted using methods modified from previously described procedures [36]. Briefly, dried samples were pulverized and 0.02g (± 0.002g) of fecal powder was briefly vortexed with 0.5 ml of 90% ethanol. Samples were shaken (Glas-col mixer, Terre Haute, IN, setting 60, 30 min) and then centrifuged (1500 rpm, 20 min). Extracts were poured off into clean test tubes and the fecal pellets then re-suspended in 0.5 ml of 90% ethanol and vortexed for 30 seconds. After centrifugation (1500 rpm, 15 min) extracts were combined with the first extracts and dried down under air. Samples were then reconstituted in 0.2 ml of phosphate-buffered saline (PBS; 0.01 M PO_4, 0.14 M NaCl, 0.05% BSA, 0.01% NaN_3, pH 7), vortexed briefly, sonicated for 20 min and shaken (Glas-col mixer, setting 60, 20 min). Extracted samples were diluted with PBS (1:20) for hormone analysis.

Enzyme immunoassay

Fecal glucocorticoid metabolites (FGM) concentrations were analyzed using a cortisol enzyme immunoassay (EIA). Cortisol polyclonal antiserum and HRP (R4866; provided by C. Munro, Davis, CA) were used at a 1:8,500 and 1:20,000 dilution, respectively [37]. Cross-reactivity to the cortisol antiserum were: cortisol, 100%, prednisolone, 9.9%; prednisone, 6.3%; cortisone, 5%; corticosterone, 0.7%; deoxycorticosterone, 0.3%; 21-deoxycortisone, 0.5%; 11-deoxycortisol, 0.2%; progesterone, 0.2%; 17α-hydroxyprogesterone, 0.2%; pregnenolone, 17α-hydroxypregnenolone, anderostenedione, testosterone, androsterone, dehydroepiandrosterone, dehydroisoandrosterone-3-sulfate, aldosterone, estradiol-17β, estrone, estriol, spironolactone and

cholesterol, 0.1% [8]. Assay sensitivity was 3.9 pg/well and intra- and inter-assay coefficients of variation were <10% and 15%, respectively. The cortisol EIA was previously validated for this species using biochemical, biological and physiological methods [49].

Foraging patch procedure and treatments

Foraging patches consisted of one (7.6 L) black rubber round pan (36.8 cm diameter, 10.2 cm deep) and 3.8 L of loose topsoil with larvae mixed randomly into the topsoil. Previously, we had confirmed that the larvae were a highly desirable food for the armadillos by placing them into the patch without the topsoil. Each armadillo readily consumed 30 or more worms. Hence, we concluded that the GUDs of armadillos within food patches with substrate represent a balance between the armadillos' perceived costs and benefits of foraging. Foraging patches were placed into the enclosures at the end of the day (~ 4:00 PM) and recovered the next morning (~ 8:00 AM). The remaining larvae were collected by sieving the entire patch and counted, thus providing the GUD. Prior to all treatments, armadillos were given the patch with nine larvae for 4 consecutive nights to eliminate any neo-phobic reactions to foraging patches [38].

Substrate Quantity

To determine if the quantity of patch substrate affected foraging patterns, each armadillo was given two foraging patches (each with 9 larvae) per night with varying amounts of topsoil, 3.8 and 5.7 L. This treatment was repeated 2 times a week for 2 weeks, for a total of 4 replicates. All armadillos were measured on the same nights.

Patch Quality

To determine if the initial food abundance affected armadillo foraging behavior, armadillos were offered two patches with the same amount of topsoil (3.8 L) but with varying initial prey densities (6 or 12 larvae). This treatment was repeated two times a week for 2 weeks, for a total of 4 replicates.

Bedding Amount

To determine if the amount of bedding (straw) affected armadillo foraging behavior, we varied the amount (1, 2 or 3 flakes of straw) of bedding armadillos received in enclosures. Bedding was placed at one end of enclosures, leaving clear room for feeding dishes and food patches. Armadillos were given two foraging patches with nine larvae and 3.8 L of topsoil for each treatment day. A three-sided die was rolled to determine the order in which each armadillo received the three bedding treatments. Armadillos received each treatment for 4 consecutive days, resulting in 12 total points of data for each armadillo. All armadillos were measured on the same nights.

Integrating hormones, foraging and state categories

To determine the overall well-being of the armadillos, adrenocortical activity and GUD measurements were separated into categories as low and high measures. Low GUD indicated the patch was foraged thoroughly versus high GUD which indicated limited foraging. For FGM, low represented reduced adrenocortical activity versus high indicated elevated adrenocortical activity. Determinations of high or low for FGMs were evaluated for individual armadillos, based on their own FGM measures. Category A (high GUD, low FGM) indicates that an animal is in a neutral state where limited foraging is not being driven by environmental stressors (Table 4.1). Category B (high GUD, high FGM concentrations) is a negative state that may indicate individuals are not foraging due to perceived stressors (Table 4.1). Category C (low GUD, low FGM concentrations) is a near ideal state because the individual is foraging thoroughly and is not experiencing elevated adrenocortical activity (Table 4.1). In category D (low GUD, high FGM concentrations), the individual is in a positive state where both foraging intensity and adrenocortical activity are elevated as possible signs of eustress.

Table 4.1 The conceptual model framework for combining adrenocortical activity (measured via fecal glucocorticoid metabolites, FGM) and foraging behaviors (Giving up densities, GUDs) into an overall assessment of the armadillo's well-being.

	Low FGM	High FGM
High GUD	**State category A: Neutral** High foraging costs but low adrenocortical activity	**State category B: Negative** High foraging costs and high adrenocortical activity
Low GUD	**State category C: Ideal** Low foraging costs; and low adrenocortical activity	**State category D: Positive** Low foraging costs and high adrenocortical activity

Statistical analysis

Adrenocortical activity

To determine the effect of foraging treatments on adrenocortical activity, data were natural log transformed to conform to the assumptions of normality. A Kruskal-Wallis with a Dunn's method post-hoc comparison was then performed to compare the overall mean (± SE) FGM concentrations across individuals and treatments (pre-sampling, each of the three treatments, post-sampling) where the FGM concentrations were considered as the dependent variable and individual and treatment were the independent variables.

Giving-up density

We analyzed GUD measures using general linear models. GUD measures were square root transformed to conform to the assumptions of normality. To test for the effect of patch substrate amount, initial prey density (IPD) and bedding amount on GUDs, we used GUD measures as the dependent variable, and date, individual, treatment and interaction between treatment and individual as independent variables in separate ANOVAs for each treatment. For the patch quality treatment, we measured the effect of IPD on proportion harvested and GUD

measures. For proportion harvested, GUD measures were transformed to determine the proportion of IPD harvested using the equation, 1- GUD/IPD. To test for the effect of IPD on portion harvested, we used proportion harvested as the dependent variable, and date, individual and IPD as the independent variables in an ANOVA. For the bedding sustrate amount treatment, GUD measures from within patches for each bedding amount were summed together to create a total GUD. To determine if GUDs remained consistent within an individual among all treatments, we assigned individual's mean GUD measures for each treatment a rank (range, 1-7), with the lowest GUD ranked as 1 and the highest GUD ranked as 7, and calculated Kendall's W coefficient of concordance for rank using Friedman's test.

State categories

State categories were assigned to each armadillo for each treatment using a combination of FGM concentrations and GUD measurements (Table 4.1). The adrenocortical activity of each consecutive treatment period (pre vs. substrate quantity, substrate quantity vs. patch quality, patch quality vs. bedding amount) was compared to determine how the previous treatment affected the individual's adrenocortical activity. To determine if FGM concentrations for each treatment were different, a repeated measures ANOVA was performed on the natural log of each individual's FGM concentrations for all treatment periods. If treatment FGMs were higher ($P<0.05$) than the previous treatment FGMs, then FGM values were considered 'high'. If treatment FGMs did not differ or were lower ($P<0.05$) than the previous treatment FGMs, then FGMs was considered 'low'. The GUDs were considered to be high if the mean GUD measurement was greater than half of the highest mean possible GUD measurements (GUD=4.5), otherwise the GUD measurement was low. To determine the correlation between FGM concentrations and GUD measures for each treatment, a Spearman rank correlation was performed using all individual's measurements for each treatment. A Spearman rank correlation

was used because the data did not meet the assumptions of a Pearson correlation. For all parametric statistical analysis: pair-wise comparisons for significant treatments effects were made using a Student-Newman-Keuls test. Normality assumptions were tested using a Kolmogorov-Smirnov test for each analysis. All statistical analyses were performed using Microsoft *Excel* (MS Office 2007), SYSTAT 10 (Systat Software, Inc, Chicago, IL), Sigma Stat Version 3.0 (SPSS Inc., Chicago, IL, USA) and StatTools.net was used to calculate Kendall's W.

D. Results

Adrenocortical Activity

Overall mean FGM concentrations of all armadillos were similar (H_4=2.5, P>0.05) across all periods (pre-sampling, 3195.3 ± 1951.4 ng/g dry feces; substrate quantity, 2967.3 ± 1587.9 ng/g dry feces; patch quality, 3703.4 ± 2252.8; bedding amount, 3107.7 ± 1996.3 ng/g dry feces; post-sampling (3345.7 ± 2317.9 ng/g dry feces; Table 4.2). Fecal glucocorticoid metabolites varied (H_6=253.5, P<0.05) across individuals and FGM concentrations across pre-sampling, treatments and post-sampling did vary (# 9338, $F_{4,66}$= 3.1, P< 0.05; # 20202, $F_{4,63}$=0.6, P<0.05; Table 4.2) for two individual armadillos.

Table 4.2 Mean (± SEM) fecal glucocorticoid metabolite (ng/g dry feces; FGM) values of samples collected pre-sampling, during each foraging treatments, and post-sampling for male and female armadillos housed at Lincoln Park Zoo.

		Foraging treatments			
Individuals	**Pre-sampling**	**Substrate quantity**	**Patch quality**	**Bedding amount**	**Post-sampling**
Male					
6474[a]	14824.9 ± 1396.7	12403.3 ± 1327.6	17040.0 ± 3480.7	15055.9 ± 3298.9	17243.6 ± 3729.8
20200[d]	679.0 ± 49.7	1004.2 ± 110.4	918.9 ± 77.5	857.5 ± 126.7	1022.5 ± 223.2
20202[b]	2530.7 ± 441.4	2529.2 ± 404.6	3625.1 ± 426.5	1897.8 ± 277.4*	1397.5 ± 155.8
Female					
9338[c]	1052.9 ± 96.7	1249.6 ± 100.8*	1001.0 ± 67.0	1027.3 ± 140.9	1181.3 ± 258.0
9717[c,d]	1254.6 ± 424.0	1057.2 ± 93.9	1294.3 ± 189.1	864.1 ± 124.6	1006.0 ± 182.0
20441[c]	1122.1 ± 107.1	1717.4 ± 484.1	1173.8 ± 187.7	1204.3 ± 165.3	893.0 ± 136.0
20727[c,d]	903.1 ± 48.2	810.4 ± 47.1	870.5 ± 73.1	847.2 ± 95.5	676.2 ± 100.5
Overall mean	**3195.3 ± 1951.4**	**2967.3 ± 1587.9**	**3703.4 ± 2252.8**	**3107.7 ± 1996.3**	**3345.7 ± 2317.9**

[a,b,c]Superscripts represent differences ($P<0.05$) among individual mean FGM concentrations. Asterisks represent differences ($P<0.05$) between the previous treatment and the current treatment within an individual.

Giving-up density measures

For low and high substrate quantity, GUDs measures were similar among dates ($F_{3,45}$ = 2.0; P>0.05) and substrate level ($F_{1,45}$ = 0.7; P>0.05); therefore, these variables were dropped from the analysis. The GUDs did vary ($F_{6,45}$= 28.4; P<0.05) among individual (Fig. 4.1). Date ($F_{3,45}$=0.2; P>0.05), IPD ($F_{1,45}$=0.2; P>0.05) and IPD within individual ($F_{6,39}$ = 0.1) did not influence proportion harvested, so these variables were dropped from the analysis. However, individual did influence ($F_{6,45}$=6.4; P<0.05) proportion harvested (Fig. 4.2A). Additionally, date ($F_{3,45}$ = 0.3; P>0.05), IPD ($F_{1,45}$ = 1.9; P>0.05) and IPD within individual ($F_{6,39}$= 0.4, P<0.05) did not affect the GUD measures in the patch quality treatment, but individuals did vary in overall GUD measures ($F_{6,45}$= 8.5; P<0.05; Fig. 4.2B). The GUD measure for the bedding amount treatment was affected by both individual ($F_{6,28}$ = 32.6; P<0.05, Fig. 4.3) and bedding substrate amounts ($F_{2,28}$ = 4.7; P<0.05, Fig. 4.4), but not date ($F_{5,28}$ = 2.4; P>0.05) or bedding amount by individuals ($F_{12,21}$= 1.5, P>0.05). Individual GUD ranks (range, 1-7) were similar (W=0.67, P<0.05) throughout treatments (Table 4.3).

Table 4.3 GUD ranks of armadillos for each foraging treatment.

	Treatments		
Individual	Substrate quantity	Patch quality	Bedding amount
6474	4	2	5
9338	5	5	7
9717	7	7	3
20200	3	1	4
20202	6	6	6
20441	2	4	2
20727	1	3	1

GUD rank (range, 1-7), with the lowest GUD=1 and the highest GUD=7.

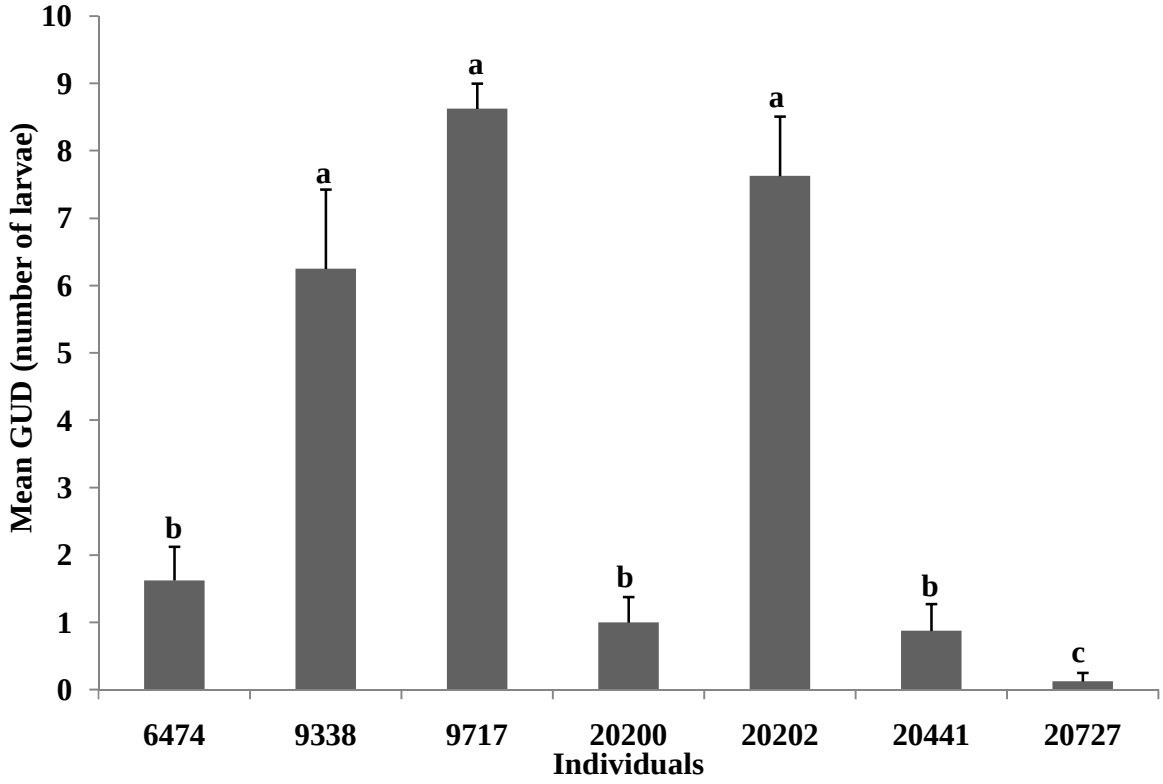

Fig. 4.1 Mean (± SE) Giving Up Density (GUD; number of larvae left after foraging overnight, 16 hours) measures of individual armadillos during the substrate quantity treatment. [a,b,c]Superscripts represent differences ($P<0.05$) among individuals.

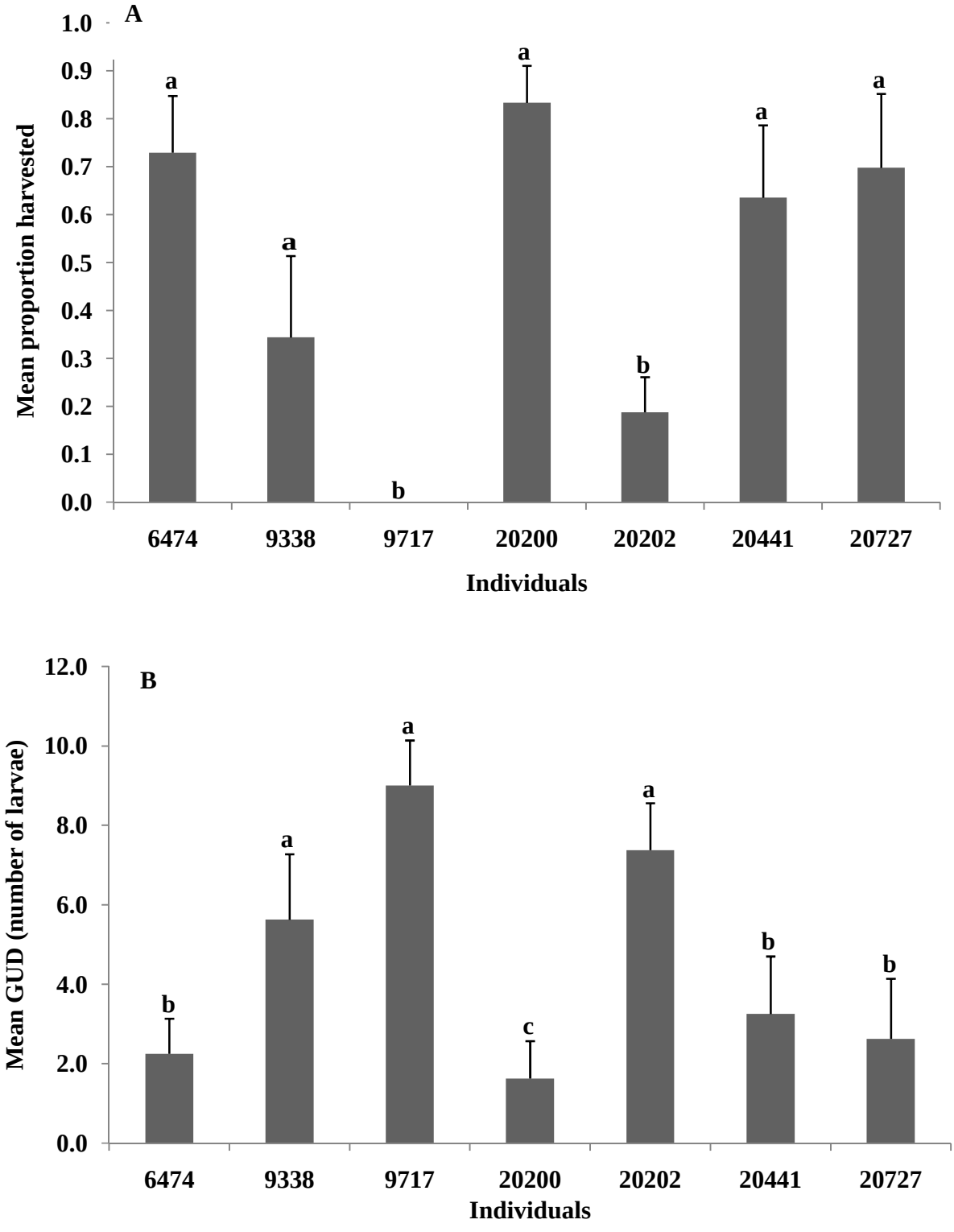

Fig. 4.2 Mean (± SE) proportion harvested by each individual armadillos during the (A) patch quality treatment; (B) Giving Up Density (GUD; number of larvae left after foraging) measures during the patch quality treatment. [a,b,c]Superscripts represent differences ($P<0.05$) among individuals.

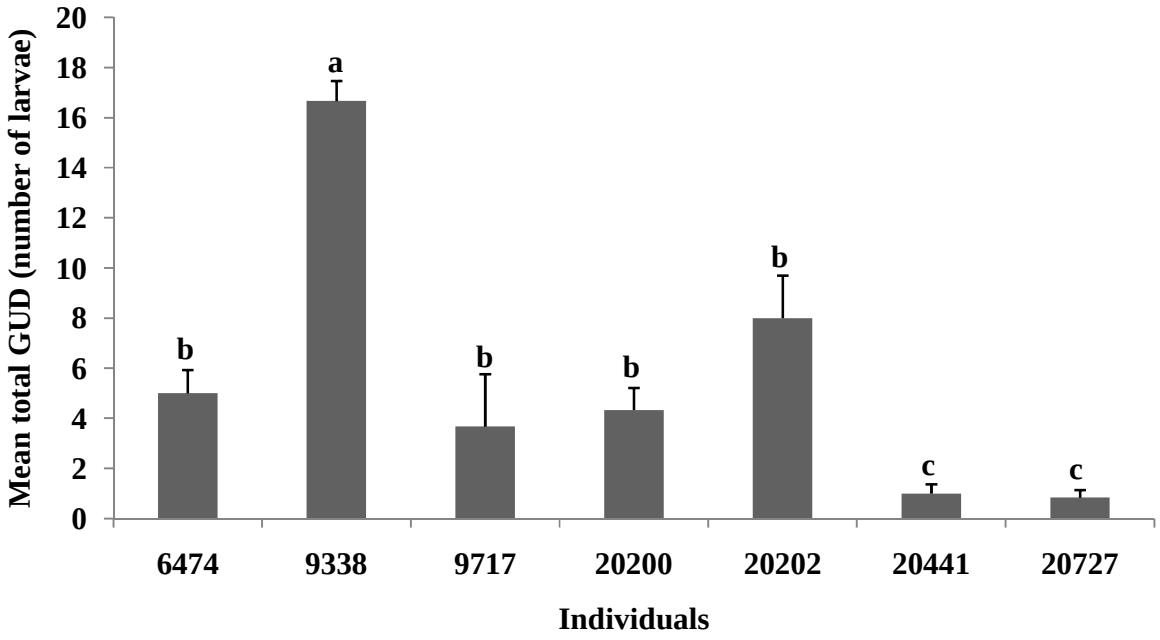

Fig. 4.3 Mean (± SE) total Giving Up Density (GUD; number of larvae left after foraging) measures of individual armadillos during the bedding amount treatment. [a,b,c]Superscripts represent differences ($P<0.05$) among individuals.

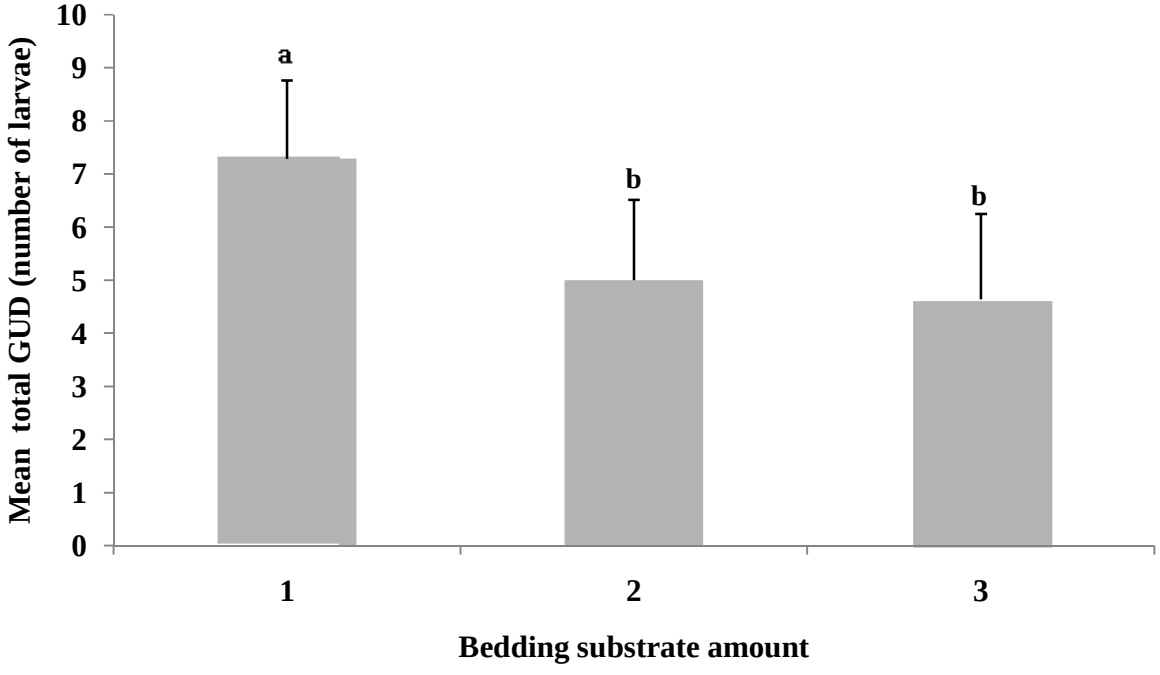

Fig. 4.4 Mean (± SE) total Giving Up Density (GUD; number of larvae left after foraging) measures of all armadillos (n=7) for each bedding amount treatment. [a,b]Superscripts represent differences ($P<0.05$) among bedding amounts.

State categories

The FGM concentrations and GUD measures were not correlated in the different treatments (substrate quality: $r_s = -0.06$, P= 0.8; patch quality: $r_s = -0.4$, P= 0.1; bedding amount: $r_s = 0.2$, P= 0.2). Using both the FGM values and GUDs, each individual was assigned to a well-being state category for each treatment (Table 4.4). Three armadillos' (male # 20200, female # 20441, female # 20727) states remained in the ideal category C throughout all treatments. One male (# 20202) remained in the neutral category A throughout all treatments. Male # 6474 remained in the ideal state category C until after the bedding amount treatment, transitioning to a neutral state, category A (Table 4.4). One female (# 9338) transitioned from the negative category B (substrate quantity) to neutral, category A (patch quality, bedding amount; Table 4.4). Female # 9717 remained in the neutral category A during the substrate quantity and patch quality treatments, shifting to the ideal category C after the bedding amount treatment (Table 4.4).

Table 4.4: Interpretation of state category (A, B, C, or D) for armadillos based on conceptual model of well-being in Table 1. Armadillos (n=4) that did not change categories with treatments were not included in this table.

	Treatments		
Individual	Substrate quantity	Patch quality	Bedding amount
6474	C →	C →	A
9338	B →	A →	A
9717	A →	A →	C

E. Discussion

This study was the first to determine the relationship between adrenocortical activity and foraging behavior and develop a well-being index using these non-invasive measures for zoo-housed armadillos. The armadillo was an excellent candidate for this non-invasive research because of its ecology. This species is a near-threatened omnivore [39] of the family Dasypodidae (Order Cingulata) that inhabits the xeric regions of Argentina, Bolivia, Brazil and Paraguay [40]. Both species of the three-banded armadillo, the Brazilian (*Tolypeutes tricinctus*) and southern, are declining in the wild due to conversion of their natural habitat into agricultural lands and exploitation for foods and arts [40-43]. Capture is easy due to predator avoidance strategy of rolling into a ball [42, 44]. Due to the uniqueness, this armadillo has been housed in zoos for over 40 years [45]. Currently it resides in 38 North American zoological institutions. Unfortunately, the armadillo's reproductive success is limited due to an offspring mortality rate of 47% (54% of males, 38% of females) across all North American institutions [35]. Therefore, it is important to evaluate how environmental changes affect the armadillo's adrenocortical activity [45], but to also formalize and extend the use of depletable food patches as a behavioral indicator.

Although captive environments present different costs of foraging, zoo-housed animals may still behave in ways shaped by evolution within their native habitats [17]. For instance, although costs of predation are negligible in zoos, the animal may still perceive a high predation risk due to sensory (i.e. visual, olfactory, auditory) stimuli of a predator species. The armadillos, in this study, were housed in a room adjacent to a sand cat (*Felis margarita*) enclosure. Elevated adrenocortical activity and a high GUD could be indicators of these perceived dangers. Therefore, zoological institutions could benefit from methodologies for evaluating how an animal perceives its environment. Institutions spend considerable money and resources to create

naturalistic enclosures aimed at promoting desirable species specific behavior. Recent studies have monitored how zoo-housed species use enclosure space and how husbandry practices affect animal behavior and welfare [46-48]. These studies found that animals, specifically chimpanzees (*Pan troglodytes*) and gorillas (*Gorilla gorilla gorilla*), that used more of available enclosure spaces exhibited decreases in inappropriate behavior, decreased inactivity and higher welfare indices.

Collecting GUDs can be a quick, simple and inexpensive tool for gathering data on an animal's perceptions of its environment. Food patches to collect GUDs can be simple to develop and include a natural food item. Abu Baker and Brown [24] found that the abundance of food, patch substrate volume and patch area affected the GUDs measured for mourning doves (*Zenaida macroura*) and cotton tailed rabbits (*Sylvilagus floridanus*). Several additional factors must be considered when developing food patches for zoo animals including safety for the animal and food preference. The chosen vessel must meet several criteria including that it must be short enough for the animal to be able to climb in and out for foraging, deep enough to hold appropriate amount of substrate to create a foraging challenge, an appropriate size for the animal's limited enclosure space, and not cause a neophobic reaction [38]. The substrate must be deemed safe for the animal (ingestion and allergies), not too messy, easily sifted and soft enough to not destroy the food item (to collect the GUD). Food items must be a highly favorable item so that animals will want to forage for them [21]; however, nutritional content should be taken into consideration. Here, a successful food patch was developed for the armadillo. In the field, Meritt [44] observed that this armadillo's primary food included worms, grubs, insects and carrion. Also, Bolkovic et al. [39] found that insect larvae made up 25% of the total weight of wild armadillo stomachs. Though the armadillo is the least fossorial of all extant armadillos

[34], the patch developed for this study may be used as a starting point for patch development when working with other armadillo species.

Assessing GUDs allowed for direct insight into the armadillo's perceptions of its environment. The amount of substrate within a patch was expected to decrease the quantity of food items harvested [24, 30] and increase GUDs; however, armadillos did not bias their foraging towards shallower patches, but had similar GUDs in both patches despite substrate amount. Using a fixed amount strategy, it would be expected to observe the proportion of food harvested declining with initial abundance. A fixed time patch use strategy predicts no effect of initial food abundance on the proportion of food harvested [60]. Here, armadillos were not able to detect the quality of a patch, so the initial abundance of food did not influence the proportion of food harvested or the GUD in the patch quality treatment. Despite reported good senses of smell and sight [34], armadillos used a fixed time foraging strategy. This could be the most optimal behavior for foragers that cannot assess IPD [61] that may risk over utilizing poor patches [60]. Individuals, however, influenced both the proportion of food harvested and the overall GUD. In a lab foraging experiment, [62] wild caught screaming armadillos (*Chaetophractus vellerosus*) took more food items from high compared to low IPD patches. However, these patches were small containers containing food and did not contain substrate and therefore did not pose as a foraging challenge for armadillos. It could be possible that if these experiments, in our study, had run for more days, the armadillos may have learned to detect patch depth or quality and begun to forage optimally, as described by Baker and Brown [24].

The bedding amount was expected to reduce predation risk by providing more cover. In nature, this armadillo can be found foraging in the open, but uses burrows for shelter and protection from predators [44]. Here we determined that bedding amount affected the foraging decisions of armadillos. One flake of straw resulted in the highest GUDs, however adding one or

two additional flakes resulted in armadillos giving similarly lower GUDs. Interestingly, GUDs varied across individuals in all treatments, but overall individual GUD ranks remained consistent across the treatments. More specifically, individual armadillos responded in a similar way to consecutive food patch characteristics and enclosure characteristics, retaining their foraging intensity. Again, this illustrates the need for behavioral type assessments to better understand varying responses of individuals to the environment.

This is the first study to use fecal hormone analysis and GUDs, together, to evaluate the well-being of an animal. Previous work on the adrenocortical activity of this armadillo demonstrated that FGM concentrations varied greatly among individuals, but were similar across other factors (e.g. sex, age and birth location) [49]. Similarly, we found that FGMs varied significantly among individuals; however, overall FGM concentrations were similar across the time periods (pre-sampling, treatments and post-sampling). Individual differences in adrenocortical activity may be attributed to differing behavioral types in the armadillos. These differences should be further investigated as individual armadillos may react differently in various environments and when exposed to stressors, requiring more customized husbandry and management [50-52]. The FGM values within each individual did not differ significantly across treatments for five of the seven armadillos. In the end, though not significant, enclosure characteristics and food patches affected the adrenocortical activity of the armadillos. Four of seven armadillos had mean post-sampling FGM concentrations 28% lower than their pre-sampling FGM concentrations; while the remaining three armadillos had post-sampling FGM concentrations that were 26% higher than their pre-sampling FGM concentrations. This could have been affected by the short post-sampling period, though keeper notes did not reflect any changes that could have caused these differences in FGM concentrations.

Behavioral enrichment is an important tool used to increase the well-being of zoo-housed animals [53]. Mellen and MacPhee [54] reported that providing species-appropriate behavioral opportunities based on an animal's natural and individual history is critical to obtaining optimal physiological and psychological well-being in zoo-housed species. Foraging accounts for the majority of activity budgets in wild animals and it is important to provide zoo-housed animals the opportunity to 'work' for food (ie. providing bamboo shoots for zoo-housed pandas) [55]. Feeding enrichment generally evokes a sensory experience by encouraging natural foraging behaviors to increase the length of foraging. Changing a small portion of African elephants (*Loxodonta africana)* diet to a food requiring greater handling time significantly decreased their time spent being inactive [56]. In large felids, providing live fish and large bones significantly increased the variety of feeding behaviors exhibited [57]. Customized food patches (food items distributed into a substrate) can be used to induce natural foraging behaviors [58] and gain insight into the animal's well-being. Here, foraging patches provided armadillos with the chance to dig and root in soil for insect larvae, allowing them to interact and work for their food in a natural way [59].

In this study, state categories were developed using all the adrenocortical activity and GUD possibilities. In category A, the animal was expected to have reduced energetic benefits of foraging and perceived stressors were minimal. For example, elevated ambient temperatures could reduce foraging as a result of high metabolic costs, but the individual is not physiologically stressed as described in the neutral state category A. Mogerman [32] showed that zoo-housed American bison (*Bison bison bison*) and Grant's zebras (*Equus burchelli*) exhibited high GUDs on days with extreme temperatures, showing that they perceived the cost of body temperature regulation (C) to be greater than the benefit that could have been gained from foraging. Perceived high predation cost due to olfactory or auditory cues received from other zoo-housed

animals, for an example, could cause an individual to exhibit the negative traits of category B. Mogerman [32] also showed that animals may perceive foraging (high GUDs) near exhibit borders (rock hyrax, *Procavia capensis*, and bison) or close to blocked sight lines (zebra) to be risky, making their perceived predation costs high. Interestingly, zebras also avoided foraging (high GUDs) near latrines since predators may lurk around latrines, as they know the prey visits this area. Zebras may also avoid foraging near latrines because the strong odor may inhibit the zebra from detecting an approaching predator in time to escape. Category C, the ideal state category, results from an animal that perceives its environment to be safe and can adapt to the environment modifications. An individual in category D could be experiencing eustress due to the sensory experience of foraging in a more natural context. Using these state categories, our assessment revealed three armadillos remained consistent in state category C, while another remained in state category A throughout the treatments. The remaining three armadillos shifted to different states, with two individuals moving to better states. For instance, one individual (#9338) moved from state B to A after the substrate treatment. Here, adrenocortical activity shifted from high to low which could be a result of foraging in the patches. This supports the idea of using these methods as feeding enrichment. Individual # 9717 shifted from state A to C after the bedding treatment. This female barely foraged in the patches until given more cover during the bedding treatment, highlighting the importance of cover for this armadillo and the species as previously described by Meritt [44] and [42]. The last armadillo (#6474) moved from a positive state to a neutral state (C to A) after the bedding treatment. Foraging intensity in this individual decreased as the bedding amount decreased, as observed in individual #9717. In all cases, armadillos finished in either a positive or neutral state.

We conclude that the zoo-housed armadillo could benefit from continuing the use of the food patches and fecal hormonal practices to determine how enclosure characteristics affect

individuals. This approach is applicable to assessing animal welfare of various other zoo-housed animals. Zoo institutions could use this tool to evaluate and prioritize planned exhibit and management changes and identify the most effective changes. For example, these techniques can be used to assess the effect of enclosure size on the welfare of the animal [45]. Comparing the results from other institutions could also provide a unique opportunity to assess the range of individual differences within a species, allow for direct management comparisons and facilitate the sharing of knowledge of improved husbandry.

F. Acknowledgments

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IV. PATCH USE OF A GRANIVOROUS BIRD COMMUNITY IN ARGENTINA

A. Abstract

Patch use was used to quantify the foraging intensity of a seed-eating bird community in Sierra de las Quijadas (SLQ), Argentina. Giving-up densities evaluated the birds' preferences for habitat, microhabitat and time of foraging. Camera traps identified species and revealed temporal and spatial patterns of foraging by birds in the patches. Five bird species (Darwin's tinamou, *Nothura darwinii*; Crested gallito, *Rhinocrypta lanceolata*; Gray-hooded sierra finch, *Phrygilus gayi*; Golden-billed saltator, *Saltator aurantiirostris*; Rufous-collared sparrow, *Zonotrichia capensis*) contributed the majority (99%) of foraging. Overall, birds biased their foraging efforts to closed microhabitats. Giving-up densities (GUDs) were the lowest in the sierra, which was supported by picture data. Two of the five species showed habitat preferences and one showed time preferences for foraging. Patch use methods of seed-eating bird studies were compared, highlighting the need for standardized methods to make studies directly comparable across locations. When GUDs were scaled to the same volume, the bird community of SLQ had lower GUDs than desert bird communities of the African savannah and Sonoran Desert, but not the Negev Desert. Our data adds evidence to support that birds fill the role of the major granivore in South America as rodents fill that role in South Africa.

B. INTRODUCTION

Deserts can seem barren and unproductive. In reality, they are full of life including annual plants and shrubs. These plants support communities of granivorous species that compete for seeds (Reichman 1979, Marone et al. 2000b). Studies of desert granivores have identified coexisting species (Blendinger 2005), species that facilitate granivory, and even competition between similar or very different seed-eating species (Reichman 1979, Brown et al. 1994a, Blendinger and Ojeda 2001). Studies consistently find three major groups of granivores in deserts: ants, birds, and rodents. While there has been debate over which taxa dominates foraging in the various deserts of the world (Mares and Rosenzweig 1978, Morton 1985, Lopez de Casenave et al. 1998), it seems that birds and ants tend to be the major granivores in the deserts of Australia (Morton 1985, Marone et al. 2000b) and South America (Lopez de Casenave et al. 1998, Marone et al. 2000b), while rodents and ants tend to dominate the deserts of South Africa (Kerley 1992) and North America (Mares and Rosenzweig 1978). The role of ants seems to increase with the increase average temperature in the desert (Lopez de Casenave et al. 1998, Marone et al. 2000b, Saba and Toyos 2003).

Granivorous birds were not considered as important seed consumers, like mammals and ants, in many deserts (Mares and Rosenzweig 1978). Although, Pulliam and Brand (1975) found birds to be diverse and abundant in the more grassland-type deserts between the Sonoran and Chihuahan deserts of North America. Avian species richness is generally lower in desert ecosystems due to harsh environmental conditions, large thermal ranges, and unpredictability of food resources (Blendinger 2005). However, abundance and diversity of granivorous avian species may be strongly correlated with vegetative productivity (seed density) and habitat structure (Lima and Valone 1991, Blendinger and Ojeda 2001). This suggests that bird communities may be limited by food availability, especially in the winter (Schluter and Repasky

1991, Marone 1992, Cueto et al. 2006, Tsurim et al. 2009). Birds have been found to be high impact seed consumers, especially in South American locations (Marone et al. 1998, Marone et al. 2000b). Due to their high mobility, birds are able to locate and exploit high density seed patches quickly, giving them an advantage over ants and rodents (Thompson et al. 1991). In many deserts, birds may be the "cream skimmers" and ants the "crumb pickers" (Brown 1989).

The Monte desert differs from other arid desert environments. The Monte has a predictable, diverse and abundant assemblage of birds. These include both resident and regular migrants. The Monte seems to offer a highly regular rainy season and, thus, seasonally predicable resources (Blendinger 2005). These characteristics of the Monte give granivorous birds the opportunity to be the most important seed consumers in the Central Monte desert (Saba and Toyos 2003). Furthermore, they seem unusually adept at quickly discovering and consuming seeds from bait experiments, allowing for more accurate estimates of their possible impact on overall seed density (Marone et al. 2000b). Soil seed banks in the Central Monte are similar to those of North American deserts (Marone et al. 2000a). This is not a surprise as North and South American deserts share many forb, shrub, and grass genera in common. Grass seeds have been shown to be the preferred food of granivorous birds (Schluter and Repasky 1991, Marone et al. 1998). Birds in the Central Monte are highly selective for grass seeds, with 83% of seeds found in bird stomachs being grass seeds (Marone et al. 2008). This is especially interesting since only 30% of seeds available are grass seeds (Marone et al. 2008). Overall, Saba and Toyos (2003) showed that birds were the main and most consistent seed consumers in the Monte throughout the year.

Until Brown's (1988) seminal paper introducing giving-up densities (GUDs, the amount of food remaining in a depletable food patch once foraging has ceased), the foraging intensity of seed-eating birds was measured through seed removal rates from artificial food patches only

containing seeds. These studies could not affectively measure all of the costs of foraging because seeds were not distributed in a substrate (Mares and Rosenzweig 1978, Morton 1985). Without a substrate, birds could simply consume seeds without little effort, no search time and without a declining harvest rate (Brown 1988). Today, some studies of patterns in granivory are still conducted with only seed removal experiments (Lopez de Casenave et al. 1998, Sassi et al. 2006); however, collecting GUDs has become a popular method for evaluating methods of coexistence (Kotler and Brown 1999), habitat selection (Tsurim et al. 2007), predation costs (Tsurim et al. 2010) and other factors in granivorous bird communities.

During this study, our aim was to apply optimal foraging theory and GUDs in depletable food patches to assess the foraging intensity of seed-eating birds in Sierra de las Quijadas desert (SLQ), Argentina. Measuring foraging intensity provided an inexpensive, non-invasive, and efficient method to gain insight of how the bird community perceives its environment. We used camera traps to identify the species and timing of visitors to food patches. Our objectives were to: 1) use food patches to measure the foraging intensity of granivorous birds; 2) use camera traps to identify bird species foraging in patches; and 3) to compare our results to other studies that have measured GUDs on granivorous bird species.

C. Methods

Foragers must make decisions about which patches to forage in and how long to forage in each patch (Charnov 1976). The longer a forager stays in a patch, the harvest rate decreases to the point that the benefits of foraging no longer exceed the costs of foraging (quitting harvest rate; Brown, 1998). The costs include the metabolic cost (C), the cost of predation (P), and the missed opportunity cost (MOC) of foraging in other patches and not engaging in other activities; $H = C + P + MOC$ (Brown 1988). Quitting harvest rate can be estimated by measuring the GUD

(Brown 1988, Brown et al. 1994a). These methods allow for the assessment of foraging preference, based on the foraging costs incurred by the granivorous bird community.

Patch use in bird community studies

Researchers using GUDs to study patch use characteristics of granivorous birds within deserts employ diverse methodologies to assay these communities. The differences can include, but are not limited to varying choices of substrate, patch vessel dimensions, substrate type (sand, soil, moist, dry), substrate volume and food items. All of these factors can affect foraging costs and therefore how birds utilize patches (Brown 1988, Abu Baker and Brown 2009). Table 5.1 highlights some differences in patch use studies of bird communities including: habitat type, volume of substrate, tray dimensions, food type and mean GUD. These variations are important to note, as comparisons made between different seed-eating bird communities assayed using different patch use methods is difficult and could lead to misleading conclusions.

Study site

We studied the foraging behavior of seed-eating birds during the dry season in SLQ (32°47’ S and 67°10' W, 800 m elevation) National Park of Argentina, a protected area located 120 km northwest of San Luis City in Central Argentina. As one of Argentina’s most important national parks, it contains over 150,000 ha of biotically and geologically diverse arid habitats including flatlands, alluvial planes, hillsides and rock formations. The Park can be described as a mixture between the Monte Desert (xerophytic, resinous and thorny shrubs) and the Chaco Desert (hardwood forests) (Fernández and Busso 1999).

The park consists of three clearly distinct vegetative habitats including: creosote bush, dense mesquite woods and a large expanse of sierra. The creosote bush flats, near the front of the park, are dominated by *Larrea cuneifolia* (10-20% shrub cover, grass, and firm loess soils). The dense mesquite woods (*Prosopis sp*.) are characterized by short, thorny trees and gravelly

soil along hillside washes and drainages in the middle of the park. At the back of the park, the large expanse of sierra (steep, bulky cliffs) is dominated by *Larrea divaricata* and *Bromelia* sp. Sierra de las Quijadas is one of the driest regions of the San Luis province, receiving 350 mm annual average rainfall (Fernández and Busso 1999, Sombra and Mangione 2005, Núñez and Mangione 2008). A dry season occurs in fall and winter (May to September) and a wet season in spring and summer (October to April). These habitats provided an unique opportunity to evaluate how the granivorous birds partitioned the park in terms of preferred foraging sites.

Bird community

Sierra de las Quijadas is home to over 180 avian species (UNESCO 2005). Seven of these bird species (Darwin's tinamou, *Nothura darwinii*; Crested gallito, *Rhinocrypta lanceolata*; Sandy gallito, *Teledromas fuscus*; Yellowish pipit, *Anthus chii*; Gray-hooded sierra finch, *Phrygilus gayi*; Golden-billed saltator, *Saltator aurantiirostris*; Rufous-collared sparrow, *Zonotrichia capensis*) participated in the foraging experiments. Five bird species contributed the bulk of this foraging (99%): Darwin's tinamou, 229 g; Crested gallito, 62 g; Gray-hooded sierra-finch, 24 g; Golden-billed saltator,42 g; Rufous-collared sparrow, 20 g (Dunning, 2008). We focus on the five species for our analyses in this study. Sherman traps were used to trap and identify small mammal species that might forage within the patches. Heavy use by small mammals of such food patches would be the norm for North American, Middle Eastern, and south African deserts (Brown et al. 1994a, Brown et al. 1994b, Perrin and Kotler 2005). We did not trap any small mammal species, and we saw no evidence of small mammal activity within our experimental patches. The total absence of small mammals in our study was perhaps a first for such a seed-tray experiment.

Food patch stations

Each habitat (creosote, mesquite and sierra) was assayed at three different sites. Each site, within a habitat, included four stations consisting of two food patches (open microhabitat, closed microhabitat). Patches were considered to be in an open microhabitat when 1 m from any woody cover. Closed microhabitat patches were near or directly under woody vegetation. This yielded a total of 36 stations over the course of the study. Sites within a habitat were located 200 m apart. At a site, stations were arranged at least 20 m apart. Within a station micro-habitat specific patches (closed and open) were 10 m apart. Giving-up densities were collected for three days at the first site, in each habitat, and four days at the second and third site locations.

The food patch consisted of a circular plastic pan (36 cm diameter x 7.6 cm deep), 1.8 L of sifted sand and 10 grams of American proso millet (*Panicum mileacium*). Stations for the first three sites were pre-baited from 16 July through 21 July 2009. Acclimatization to food patches took place from 22-23 July. After the acclimatization period, birds were foraging in the patches within all habitats. Giving-up densities were collected from 24 July through 8 August 2009. Patches were loaded each day with millet and the remaining millet was collected the next morning after birds were allowed to forage. The patches were then reloaded with millet. The GUD (grams of millet left in patch) was then recorded after the remaining millet, from the previous day, was cleaned and weighed.

Camera traps

Game camera traps were used to identify the bird species foraging within the experimental food patches. During the course of the study, six (2 per habitat) commercial game cameras (Moultrie Game Spy D-40 digital game camera) were randomly placed at stations in open or closed patches. Each station was monitored with camera traps for the same amount of days. The infrared motion sensor had a sensitivity range of 9 m. When an animal was within

that range, the camera captured up to three images every 15 seconds with a 1.5 min delay between signals sent to the camera. The cameras were set to record the time of day and date on each picture. Camera traps were set up and pictures were recorded from 16 July – 8 August.

Statistical analyses

We used mean GUD for each site to account for the different amount of days of data collected from each site (3 vs. 4 days). Giving-up density data were then natural log transformed for normalization. We used a general linear model to analyze transformed GUD data with microhabitat, habitat, the interaction between microhabitat and habitat and station nested within habitat as independent variables. Camera data were analyzed to determine if bird species favored particular habitats and times of day for foraging and if they were more often photographed in or out of patches. Habitat preferences and preferences for time of day were analyzed for each bird species using a chi-square test of goodness-of-fit. The difference in absence or presence of bird species within the patch was analyzed using a chi-square test of independence. Camera data were used to quantify characteristics of the granivorous bird community including the number of birds of the same species that aggregate together in a food patch, which species were the first to forage in food patches and which species were the last to forage (setting the GUD) and which bird species aggregate together near a food patch. A Scaled GUD (g/L) was calculated for the studies using patch use to evaluate the foraging intensity of specific bird communities. These Scaled GUDs are reported in Table 5.1. The Scaled GUD was calculated by dividing the mean GUD by the substrate volume. To determine the correlation between the seeds harvested and the number of pictures taken in each habitat, a Spearman rank correlation was performed on data from all 36 stations. A Spearman rank correlation was used because the data did not meet the assumptions of a Pearson correlation. The amount of seeds harvested was calculated by subtracting the GUD measure for a station from the initial amount of

food in the food patch. This correlation is graphically demonstrated as a cumulative harvest curve. Normality assumptions were tested using a Kolmogorov-Smirnov test for each analysis. All statistical analyses were carried out using SYSTAT version 10.0 (SPSS Inc. 2000). Means reported are ± standard error (SE), rejection levels were set at $P \leq 0.05$.

D. Results

Studies reporting GUDs for granivorous bird communities were collected (Table 5.1). The majority of these studies (except multiple studies by the same authors) employed different methods, making it difficult to put studies in the same context and make direct comparisons across locations. The Scaled GUD was reported to help make more direct comparisons of GUDs between these studies and bird communities, Table 5.1.

Foraging intensity varied significantly with microhabitat (Table 5.2, Fig.5.1). GUDs were higher in open microhabitats. Foraging intensity varied significantly with habitat (Table 5.2, Fig. 5.2A). Birds left the highest GUDs in the mesquite habitat and their lowest GUDs in the sierra habitat. Stations within habitats differed significantly from each other (Table 5.2).

Table 5.1. Studies using patch use to evaluate foraging intensity of bird communities by collecting giving up densities (GUDs). Scaled GUD was reported for studies conducted in the desert using a sand substrate and millet as a food item to compare foraging intensity of bird community in Sierra de las Quijadas (SLQ) to these studies.

Habitat Type	Species	Vlm of sand (liters)	Tray dimensions (cm)	Food type	Mean GUD (g)	Scaled GUD (g/L)
Desert[a]	*Nothura darwinii, Rhinocrypta lanceolata, Phyrygilus gayi, Saltator aurantiirostris, Zonotrichia capensis*	1.80	36 diameter x 2.5	10 g millet seed	closed (1.2), open (1.6)	0.67, 0.89
Desert[b]	*Galerida cristata*	5.00	45 x 60 x 2.5	3 g millet seed	bush (2.3), open (2.1)	0.46, 0.42
Desert[c]	*Galerida cristata*	5.00	45 x 60 x 2.5	3 g millet seed	sand (2.5), rock & loess (2.6)	0.50, 0.52
Desert[d]	*Passer domesticus, Carpodacus mexicanus, Toxostoma curvirostre, Zenaida asiatica, Zenaida macroura, Columbina inca*	4.80	36 diameter	20g millet seed	bush (5.6), open (11.9)	1.17, 2.48
Dry woodland savannah[e]	*Galerida modesta, Ploceus cucllatus, Lagonostica senegala, Emberiza tahapisi, Sporopipes frontalis, Euplectes franciscanus. Lagonostica sanguinodorsalis, Estrilda caerulescens, Estrilda melpoda, Uraeginthus bengalus*	1.00	25 diameter x 4	7g millet seed	cover (3.7), open (4.5)	3.70, 4.50

Habitat type	Species	Vlm of sand (liters)	Tray dimensions (cm)	Food type	Mean GUD (g)	Scaled GUD (g/L)
Dry woodland savannah[f]	*Ploceus cucullatus, Sporopipes frontalis, Euplectes franciscanus, Lagonosticta senegala, Lagonostitca sanguinodorsalis, Estrilda caerulescens, Estrilda melpoda, Uraeginthus bengalus*	2.00	25 diameter x 4	7g millet seed	Cover (1.2), Near (1.6), open (1.9)	0.60, 0.80, 0.95
Desert[g]	*Zenaida macroura, Callipepla gambelii*	3.00* (soil)	45 x 45 x 2	(LVE: 2.7, 3.0, 3.3), (HVE: 1.5, 3.0, 4.5) g millet seed	Mourning dove: 1.31, Gambel's quail: 0.16	0.44, 0.05
Desert[h]	*Corvus coronoides*	2.00	15 diameter x 4	2.6g peanuts	bush (10.1), open (6.75)	5.05, 3.38
Desert[i]	Galerida cristata	6.80	45 x 60 x 2.5	6g mixed small and large cracked wheat seed	2.3	0.34
Desert[j]	*Diuca diuca, Zonotrichia capensis*	0.63* (soil)	22 x 28 x 5	30g oat seed	24	38.10
Backyards[k]	*Zenaida macroura, Streptopelia decaocto, Passer montanus*	0.90, 2.40, 4.60	27 diameter x 1.5, 32 diameter x 3, 36 diameter x 4.5	initial prey density: 2, 4, 6g millet seed	1.1, 2.2, 3.1	1.22, 0.92, 0.67
Forb community[l]	*Colinus virginianus*	0.60	22 x 22 x 4	5g milo seed	Cover (0.2), Open (0.8)	0.33, 1.33
Grass patches in deciduous forest[m]	*Corvus brachyrhynchos*	1.20	24 x 30 x 7	15.4g husked sunflower seed	1.17	0.98
Outdoor aviaries[n]	*Sturnus vulgaris*	2.20	25 x 25 x 3.5	initial prey density = 1, 5, 10, 20g mealworms	no GUD reported	N/A
Outdoor aviaries with shrubs[n]	*Sturnus vulgaris*	2.20	25 x 25 x 3.5	36 & 24g meal worms	high food safe (16.75), high food risky (18), low food safe (6.75), low food risky (8.75)	7.61, 8.18, 3.07, 3.98

Habitat type	Species	Vlm of sand (liters)	Tray dimensions (cm)	Food type	Mean GUD (g)	Scaled GUD (g/L)
Outdoor aviaries[o]	*Passer domesticus, Passer hispaniolensis*	* (artificial grass)	40 x 55 x 2.5	10g millet seed	intact wings: 4.5, augmented wings: 5.8	N/A
Urban grassland[p]	*Turdus migratorius, Sturnus vulgaris*	0.70* (vermiculite)	53 x 27 x 6	1.9g meal worms	canopy cover (12.6), open canopy (14.1), shrub cover (5.6), open shrub (10.6)	18.00, 20.14, 8.00, 15.14

Scaled GUD (g/L) was calculated by dividing the mean GUD (g) by the substrate volume (L) for that study

* Food patch substrate was not sifted sand

[a,b,c, d, e, f, g, h, i, j, k, l, m, n, o, p]Superscripts represent different authors: [a]Howell-Stephens et al. 2012, [b]Brown et al.1 997, [c]Kotler and Brown 1999, [d]Shochat et al. 2004, [e]Molokwu et al. 2008, [f]Molokwu et al. 2012, [g]Valone and Brown 1989, [h]Kotler et al. 1998, [i]Garb et al. 2000, [j]Kelt et al. 2004, [k]Abu Baker and Brown 2009, [l]Kohlmann and Risenhoover 1996, [m]Kilpatrick 2003, [n]Olsson et al. 2001, [n]Olsson et al. 2002, [o]Tsurim et al. 2010, [p]Oyugi and Brown 2003

The cameras yielded 4249 pictures of birds over a period of 20 days. The majority of pictures (73%) showed birds inside food patches. The remaining pictures (26%) were of birds either surveying the patch from afar, looking at the camera, or simply nearby the patch. The sparrow was captured in 39% of the total pictures and the finch was captured in 35% of the total pictures. All pictures were of birds within 9 m of the patch. The presences of two bird species (Darwin's tinamou, Gray-hooded sierra finch; Table 5.3, Fig. 5.2B) were not equally distributed across the three habitats. All foraging activity occurred during the hours between 06:00 and 18:00. Only one species (Darwin's tinamou, Table 5.3) did not forage equally across the time slots. The majority of tinamou foraging took place during the 08:00-10:00 and 16:00-18:00 time blocks. Birds were much more likely to be photographed inside rather than outside of the patch ($X^2(4)= 149.8$, $P < 0.05$). The sparrow was the first species to forage in the creosote habitat for all sample days. The tinamou was the first to forage in the mesquite 63% of the time. The first species to forage in the sierra was the finch (50%). The sparrow was last to forage in patches within the creosote and sierra habitats 83% and 54% of the time, respectively. There were equal occurrences (43%) of the tinamou and the saltator being the last to forage in the mesquite habitat. The majority of pictures (94%) of any one bird species had only one bird of that particular species (Fig. 5.3). The remaining pictures were of two (6%) and three to five birds (<1%) of the same species. Pictures showed sparrows foraging in groups of two (*n*=64) and three to five ((*n*=3) birds of the same species. The saltator was captured foraging in groups of two (*n*=30). The finch also foraged in groups (two, *n*=125; three to five, *n*=25). There were a number of pictures (9%) that captured multiple bird species together. Only 3% of those pictures (*n*=145) captured multiple bird species in patches. Pictures with birds in the patch exhibited combinations of the finch and sparrow (*n*=113) and saltator and the sparrow (*n*=32).

The GUD measures and number pictures taken in each habitat were not correlated (creosote: r_s = -0.05; mesquite: r_s = -0.2; sierra: r_s = 0.3; Fig. 5.4).

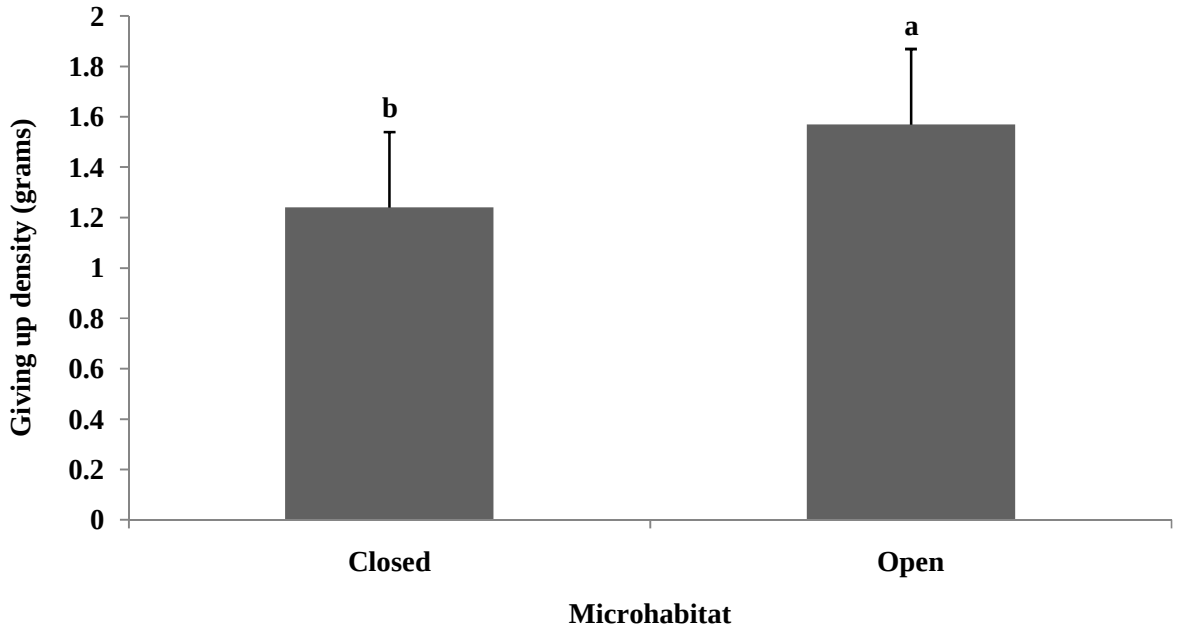

Figure 5.1 Mean (± SEM) giving up densities (GUDs, weight of seeds remaining in food patches following patch exploitation) of birds based on the microhabitat (closed, open). [a,b]Superscripts represent significant differences ($P < 0.05$) in microhabitat.

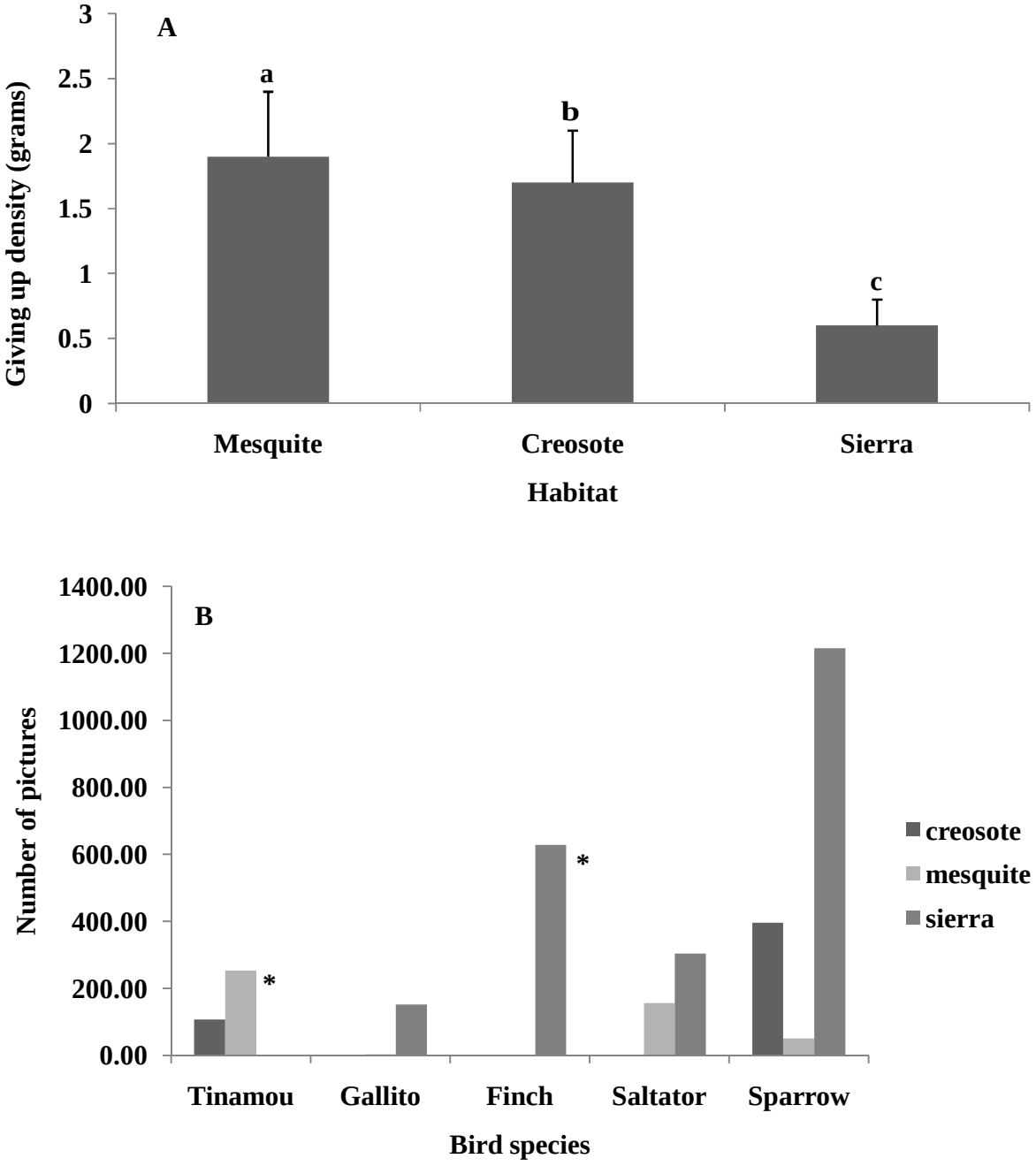

Figure 5.2 Mean (± SEM) giving up densities (GUDs, weight of seeds remaining in food patches following patch exploitation) of birds based on the habitat type (bajada, creosote, mesquite; A). Number of pictures of bird species at food patches in habitat types (B). [a,b]Superscripts represent significant differences ($P < 0.05$) in habitat type. Asterisks represent significant differences in the expected and observed number of pictures of each bird species within habitat types.

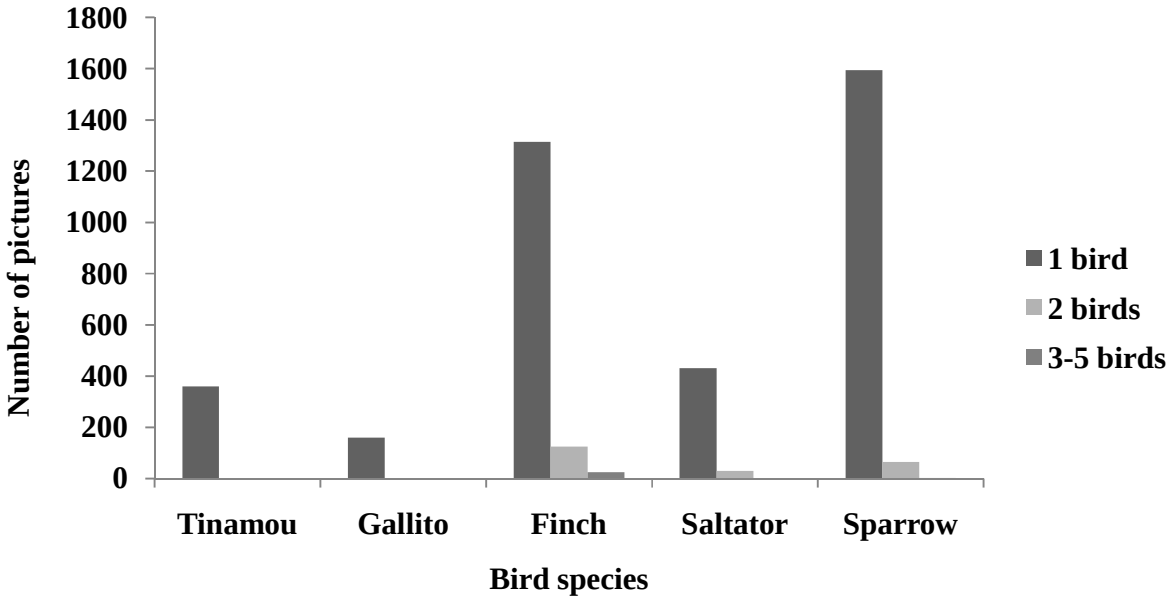

Figure 5.3 Number of pictures of each bird species with varying numbers (1, 2, 3-5) of birds of the same species in the picture.

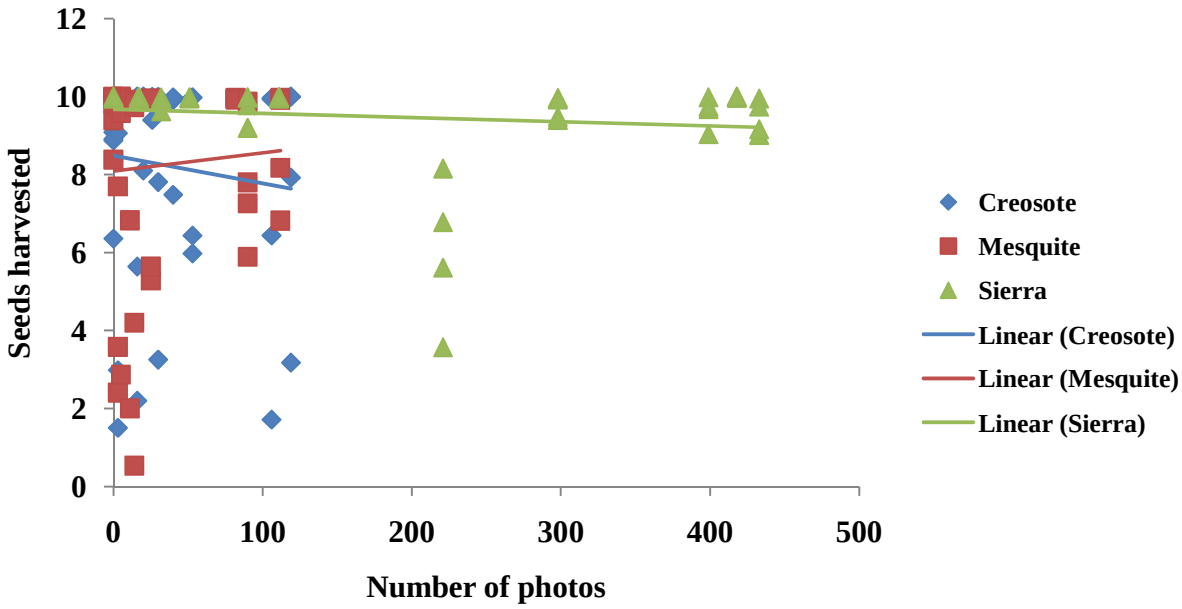

Fig 5.4 Cumulative harvest curves depicting the relationship between the seeds harvested and number of photos taken in each habitat (creosote, mesquite, sierra).

E. Discussion

Measuring patch use through GUDs allowed the seed-eating bird community of SLQ to reveal that they are major seed consumers during the dry season. The GUDs were significantly lower in closed than in open microhabitats. At a larger scale, GUDs were significantly lower in the sierra habitat. Picture data revealed strong habitat preferences of the tinamou (creosote and mesquite) and the finch (sierra). The tinamou also exhibited significant time preferences (08:00-10:00, 16:00-18:00) for foraging. Overall, it seems that the major foraging cost driving these preferences in this bird community is predation risk. Reviewing previous studies of granivorous bird studies made it clear that comparison across different deserts and communities within deserts would be difficult due to differing methodology. Scaled GUDs revealed that the bird community of SLQ had lower GUDs than desert bird communities of the African savannah and Sonoran Desert, but higher GUDs than birds in the Negev Desert. Cumulative harvest curves for each habitat showed that there was no relationship between seeds harvested and number of photos.

Granivorous desert taxa tend to shift in their importance, as seed consumers, with fluctuations in temperature and rainfall (Lopez de Casenave et al. 1998). Here, we expected for birds to dominate diurnal foraging and rodents the nocturnal foraging. Furthermore, birds and rodents should be successful as endotherms during the winter when cold temperatures may inhibit ant activity (Brown et al. 1997). Our study was conducted during the winter although temperatures during the day still reached 18°C. Though foraging activity was expected to originate from both small mammals and birds, our data showed that birds were the primary seed consumers in SLQ. We were not able to trap any small mammal species in the course of this study. In general, several previous studies also reported low occurrences of trapping small mammals in SLQ and the Monte desert. Núñez and Mangione (2008) trapped over 13 small

mammal species in SLQ, with no apparent change in abundance with seasons. Live trapping, in this previous study, took place within the same habitats in which our study was conducted. Over a total of 15 nights of trapping with 200 Sherman traps, small mammal abundance was estimated to be very low (creosote, *n*=19; sierra, *n*=39 individuals). In another study (Tabeni and Ojeda 2005), 40 nights of live trapping with 300 Sherman traps resulted in trapping 410 small mammals in the Monte Desert. Saba and Toyos (2003) conducted a seed removal study in the Monte using experimental seed trays to determine importance of granivorous taxa over a span of 2 years. Though seed removal by rodents (2 species) and ants (1 species) was detected in this study, birds (*Zonotrichia capensis*, *Duica duica*, *Phrygilus carbonarius* and *Mimus patagonicus*) were found to be responsible for the greatest seed removal and were the most consistent granivores in the Monte desert throughout the sampling period. In contrast, Brown et al. (1994a) reported that small mammals were abundant in the Negev Desert after 2 nights of live trapping with 162 Sherman traps and trapping over 650 small mammals. The lack of small mammal populations in the Monte desert adds evidence to the idea that birds fill the role top granivore role in South American deserts that rodents fill in North America and South Africa.

Though it is unclear why rodents occur in so few numbers in the Monte desert, we must consider that small mammals in South America may be out-competed by birds for grass seeds, which are an important food source for granivorous taxa (Marone et al. 1998). Marone et al. (1998) estimated soil seed reserves in the summer and compared them to reserves after granivorous bird consumption throughout the fall and winter seasons in the Monte desert. The differences in the soil seed reserves were compared to the contents of a sample of birds' (*Zonotrichia capensis*, *Diuca diuca*, *Phrygilus carbonarius* and *Saltatricula multicolor*) digestive tracts and droppings. Losses in the soil reserve were positively correlated to the mass and types of seeds found in the birds digestive tracts and droppings and that these losses resulted in

quantitative and qualitative impacts on the reserves. Results concluded that birds showed a high preference for grass seeds during the fall and winter seasons. Cueto et al. (2006) also studied seed preferences in common granivorous birds (*Zonotrichia capensis, Duica duica, Poospiza ornata* and *Saltatricula multicolor*) of the Monte desert. Birds were caged individually and given up to eight forb and grass seed options in seed choice experiments and a single seed species in non-choice experiments. Again, these birds showed that grass seeds were preferred over forb seeds, but that some bird species preferred certain grass species over others.

Predation costs could also explain low small mammal abundances in SLQ. Picture data may support this idea, as the South American gray fox (*Pseudalopex griseus*), was the first animal to investigate the food patches during the baiting period. The gray fox prefers to depredate small-medium rodents, with various species being found in fox scat during both the dry and wet season at SLQ (Núñez and Bozzolo 2006). Though birds may also be an easy target for the fox, only eggs shells of the tinamou have been found in fox scat (Núñez and Bozzolo 2006). There was however, what seemed to be a large population of the herbivorous southern mountain cavy (*Microcavia australis*) throughout SLQ, which would most certainly be a target for the fox (Tognelli et al. 2001). So, higher predation risk of small mammals may be an unlikely cause for the lack of seed-eating small mammal activity in SLQ. Though we are unsure of why the small mammal population in SLQ and the Monte is lacking, there are other possible causes, such as human encroachment and agricultural development, which require further research. However, our data show that the birds filled the top granivore spot in SLQ and adds support for birds holding that top spot throughout the deserts of South America. Holding the top granivore spot makes studying the patterns and intensity of seed consumption by birds in SLQ not only interesting but will add important information to the body of desert foraging ecology literature.

Differences in foraging within open and closed microhabitats could have emerged from the birds' perceptions of predation risk (Brown and Kotler 2004) and their anti-predator escape tactics (Lima and Valone 1991). Giving-up densities were lower in closed than in open microhabitats meaning that birds, overall, perceived food patches close to cover as safer than patches in the open. As a whole, most of the species were most likely cover-dependent species that dive into vegetation as an escape tactic (Lima and Valone 1991). Interestingly, the tinamou was the only species that would abruptly take flight when approached, possibly being a cover-independent species (Lima and Valone 1991). Another factor that could affect the choice between microhabitats is the metabolic cost of thermoregulation, where birds may seek to forage in the shade due to high temperatures (Molokwu et al. 2008) or in the sun during cold seasons (Kilpatrick 2003). Though this study was conducted during the cold season, temperatures during the daylight hours, when all foraging activity took place, were fairly neutral due to solar radiation. As a result, relative predation costs of microhabitats are believed to be the main factors driving GUDs low in closed microhabitats. In SLQ, small-medium sized birds may be depredated by the Peregrine falcon (*Falco peregrinus*, Narosky and Yzurieta 1993; Ellis et al. 2002) and the crowned solitary eagle (*Harpyhaliaetus coronatus*, Narosky and Yzurieta 1993; Lobos et al. 2011). The peregrine perches on high trees and rocks, swooping down to attack birds (Narosky and Yzurieta 1993). The eagle hunts primarily at dawn and dusk, directly attacking birds on the ground (Capadevielle et al. 2007). Similarly, house sparrows (*Passer domesticus*) and finches (*Carpodacus mexicanus*), in a desert habitat of Arizona, preferred to forage under cover, using vegetation as a shelter from predators (Shochat et al. 2004). Birds of the Simpson (Australian raven, *Corvus coronoides*; Kotler et al. 1998) and Negev Deserts (Crested lark, *Galerida cristata*; Brown et al. 1997) biased foraging efforts towards open microhabitat as a result of higher predation costs in closed microhabitat.

Camera trapping has been very popular in mammal foraging studies (Altendorf et al. 2001, Hernandez et al. 2005, Abu Baker and Brown 2010), but not very common in bird studies (von Post et al. 2012). This could partly be due to the ability of birds to move between habitats quickly and freely. As a result of their flexibility (Tsurim et al. 2007), birds were able to exploit food patches in all three habitats within SLQ. Habitat preferences revealed by the birds' patch use was supported by picture data. The majority of pictures, depicting foraging activity in food patches, were captured in the sierra habitat where GUDs were the lowest for this bird community. Picture data also revealed habitat preferences within species. The tinamou exhibited a preference for the creosote and mesquite habitats. This was expected as the tinamou was never observed in the sierra habitat during the course of the study. The finch showed a large habitat preference for the sierra and was never observed in the creosote or mesquite habitat. These differences in habitat preference could be due to how the species use the habitat spatially, as the creosote and mesquite habitats had more open space and the sierra had more ground cover, in general.

Picture data also revealed the relative activity and foraging of bird species participating in the study. Birds have the ability to find exploit rich food patches in space and time (Thompson et al. 1991). Crested larks (*Galerida cristata*), in the Negev desert, foraged when seeds were freshly uncovered by wind in the mornings and afternoons (Brown et al. 1997). Here, all foraging activity occurred during daylight hours. According to picture data, an equal amount of foraging took place during the first half (06:00-12:00) and the second half of the day (12:00-18:00) for all but one bird species. A bias for foraging in the morning is expected, as patches were replenished in the morning. However, foraging in the afternoons is significant as patches were not replenished during these hours and would generally have lower seed densities as a result of morning foraging. The tinamou foraged mostly in the early morning and late afternoon.

Though it is unclear what was driving these time preferences, it is be assumed that the missed opportunity costs or predation costs of foraging in food patches were high, making it necessary for the tinamou to decrease foraging activity during the late mornings and early afternoons. Other trends in bird foraging activity were revealed through the use of camera traps. Overall, the most common species captured in pictures were the sparrow and the finch. Similarly, Kelt et al. (2004) found that the sparrow (*Zonotrichia capensis*) and the common finch (*Diuca diuca*) foraged consistently from food patches and the sparrow was the most common forager in a bird community of Chile. "Cream skimmers" are generally the first species to forage, exploiting patches at higher seed densities (Brown 1989). Here, the sparrow was always the first to forage in the creosote habitat. The tinamou was the first to forage in the mesquite and the finch was the first forager in the sierra the majority of the time. When food patches are foraged by many individuals, the patch only measures the GUD of the last forager (Brown 1988). The species that gave the GUD varied with habitat. The sparrow gave the GUD the majority of the time in the creosote and sierra; while, the saltator and the tinamou gave the GUD in the mesquite. Another pattern exhibited by this community was the prevalence of birds foraging with more than one bird of the same species. This patch sharing behavior was only exhibited by the sparrow, finch and saltator. Though this represented a small number of instances for all species, the finch foraged in groups more than the sparrow and saltator combined. There were also a number of pictures that captured multiple bird species in patches foraging together. The number of pictures capturing the finch and sparrow greatly outnumbered the pictures of the saltator and sparrow together. The sparrow exploited patches in a greater portion of the park than the finch and saltator and was also the last to forage in both the creosote and sierra habitats. This may mean that the sparrow had a higher foraging efficiency than the other birds, allowing them to forage together in patches and habitats (Brown 1989).

Comparing the GUDs of birds in SLQ to birds of other desert bird communities required that GUD measures for those communities be scaled to the volume used within each study. The bird community of the African savannah (Molokwu et al. 2008) gave much higher scaled GUDs than the birds of the SLQ bird community. Adding water near patches decreased the cost of predation and the missed opportunity cost of these birds (Molokwu et al. 2010), making the GUDs of birds in Africa similar to those of the SLQ community. House sparrows and finches in the Sonoran desert exhibited lower foraging efficiencies, associated with higher GUDs, than the SLQ bird community. Shochat et al. (2004) evaluated the foraging intensity of birds in the Sonoran Desert during a time of very hot and dry conditions, resulting in higher metabolic costs and higher scaled GUDs. However, at an equal substrate volume, crested larks in the Negev Desert (Brown et al. 1997, Kotler and Brown 1999) gave lower scaled GUDs than birds in SLQ. Birds in the Negev may have foraged more intensively for seeds to compete with small mammals, whereas birds in SLQ did not have to deal with that constant pressure during this study.

This study again supports that birds are an important granivorous taxa (Marone et al. 1998, Saba and Toyos 2003, Kelt et al. 2004) that may dominate seed consumption in South America. Whether birds are able to be the top seed consumers due to a low of small mammal population or simply being able to out-compete, there is mounting evidence that birds hold the top granivore role in South America that is help by rodents in South Africa and North America. Here, we found that food patches and camera traps proved to be a successful combination for identifying and evaluating the foraging intensity of the seed-eating bird community of SLQ. Future work should be done to further assay the SLQ bird community during the wet season and by providing water near patches to evaluate how these factors affect foraging intensity and preferences of the bird community. It would also be advantageous to assay other taxa to identify

and evaluate foraging efforts of both rodents and ants in SLQ. Once data are gained, comparisons across the deserts of South America and worldwide deserts could be completed. However, as highlighted in this study, we recommend that methods (for all taxa) are standardized to put deserts within the same context and framework. Standardizing methods of measuring patch use would add great value to desert foraging ecology literature and allow for directly comparable and more complex studies.

F. Acknowledgements

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H. APPENDIX

Table 5.2 The ANOVA showing the effects of microhabitat (closed or open), habitat (bajada, creosote, mesquite), the interaction between microhabitat and habitat on giving-up densities. Station is a variable nested within habitat. The model yields $r^2 = 0.93$

Group variable	d.f.	MS	*F*	*P*-significance
Microhabitat	1	4.4	5.1	*
Habitat	2	18.3	21.1	***
Microhabitat x habitat	2	2.5	2.9	
Station nested in habitat	33	7.7	8.8	***
Error		0.9		

* $P < 0.05$; *** $P < 0.001$.

Table 5.3 The chi-square test of goodness-of-fit showing the habitat and time slot preferences of the bird species.

	Habitat preference			Time preference		
Bird Species	Chi-square statistic	df	P-value	Chi-square statistic	df	P-value
Darwin's tinamou	12.8	2	0.002	13.3	5	0.02
Crested gallito	2.4	2	0.3	3.5	5	0.6
Gray-hooded sierra-finch	13	2	0.001	1.2	5	0.9
Golden-billed saltator	5.6	2	0.06	5.68	5	0.3
Rufous-collared sparrow	1.8	2	0.4	1.7	5	0.9

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2008 - 2012	National Science Foundation Integrative Graduate Education & Research Traineeship Fellow, Chicago, IL
2007 - 2008	Teaching Assistant, University of Illinois at Chicago, Chicago, IL
2005 - 2006	Post-Baccalaureate Research Education Fellow, University of Chicago, Chicago, IL
2005 - 2006	Research Assistant, Lincoln Park Zoo, Chicago, IL
2005	Research Assistant, University of Illinois, Department of Entomology, Champaign, IL
2004 - 2005	Research Assistant, University of Illinois, College of Veterinary Sciences, Champaign, IL
2003 - 2004	Veterinary Technical Assistant, VCA Heritage Animal Hospital, Champaign, IL

Awards and Honors:

Chancellor Supplemental Research Fellowship	2010- 2011
NSF Integrative Graduate Education and Research Traineeship (Igert) Fellowship	2008 - 2012
Abraham Lincoln Fellowship	2006, 2011
Carman, O.S. Scholarship, University of Illinois at Urbana-Champaign	2005
National Dean's List	2005
Graduated, high honors from University of Illinois at Urbana-Champaign	2005

Publications, Abstracts and Manuscripts:

Howell-Stephens, J., J. Brown, D. Bernier, D. Mulkerin, R. Santymire, 2012. Characterizing adrenocortical activity in zoo-housed southern three-banded armadillos (*Tolypeutes matacus*). *General and Comparative Endocrinology* 178: 64-74.

Howell-Stephens, J., J. Brown, D. Bernier, D. Mulkerin, R. Santymire, In Review, Using non-invasive methods to characterize gonadal hormonal patterns of southern three-banded armadillos (*Tolypeutes matacus*) housed in North Americans zoo. *Animal Reproduction Science*.

Howell-Stephens, J., R. Santymire, J. Brown, 2010. Developing a Model to Assess the Influence of Environmental Factors on the Adrenocortical Activity of the Southern Three-Banded Armadillo (*Tolypeutes matacus*). *The Wildlife Society Conference*, Snowbird, UT.

Howell-Stephens, J., R. Santymire, J. Brown, 2010. Characterizing Gonadal and Adrenocortical Hormonal Patterns of Southern Three-Banded Armadillos (*Tolypeutes matacus*) house in North America. *Association of Zoos and Aquariums Conference*, Houtson, TX.

Gottschalk-Druschke, C., **J. Howell-Stephens**, E. Kuroiwa, C. Seltzer, C. Shierk, 2009. Bee species richness and abundance in an urban landscape: Chicago Area Pollinator study as a citizen science project. *Association of Environmental Studies and Sciences Conference*, Madison, WI.

Howell-Stephens, J., R. Santymire, J. Brown, D. Bernier, D. Mulkerin, 2009. Characterizing Gonadal and Adrenocortical Hormone Patterns of Southern Three-Banded Armadillos (*Tolypeutes matacus*) Housed in North America: Preliminary Results. *International Mammalogical Congress 10*, Mendoza, Argentina.

Howell, J., S. Margulis, J. Mateo, 2006. Reproduction in Emperor Tamarins (*Saguinus imperator*) at Lincoln Park Zoo: a Study of Behavioral and Hormonal Patterns. *Animal Behavior Society Conference*, Snowbird, UT.

Synergistic Activities:

1. Volunteered as a docent and public interpreter and assisted with program development at Charlotte, Nature Museum, Charlotte, NC.
2. Co-developed and executed a citizen science study to assay the richness and abundance of bees in the Chicago area, Chicago Area Pollinator Study, University of Illinois, Chicago, IL.
3. Completed three of the required Integrative Graduate Education & Research Traineeship (IGERT) National Science Foundation (NSF) Fellow Courses of Landscape, Ecological and Anthropogenic Processes, University of Illinois, Chicago, IL.
4. Participated in calculus club in conjunction with population ecology course, University of Illinois, Chicago, IL.
5. Teaching assistant, Intro to Ecology & Evolution and Life Evolving, University of Illinois, Chicago, IL.
6. Invited presentation: Reproduction in Emperor Tamarins (*Saguinus imperator)* at Lincoln Park Zoo: A Study of Behavioral and Hormonal Patterns, Lincoln Park Zoo, Chicago, IL.
7. Conducted an independent research project in conjunction with Lincoln Park Zoo and University of Chicago. The topic focuses on a breeding pair of Emperor Tamarins (*Saguinus imperator)* and will include behavioral and reproductive hormone monitoring."Chicago, IL.
8. Responsible for maintaining and independently created and facilitated biogenic amine bioassay protocol with all six ant colonies, University of Illinois, Champaign, IL.
9. Assisted in identifying and marking foragers and neonates in several bee colonies, University of Illinois, Champaign, IL.

Dissertation Chapters: Assessing welfare of armadillos using hormonal & foraging indicators, and patch use in Argentinean birds

1. Using Non-Invasive Methods to Characterize Gonadal Hormonal Patterns Of Southern Three-Banded Armadillos (*Tolypeutes Matacus*) Housed in North America Zoos
2. Characterizing Adrenocortical Activity in the Zoo-housed Southern Three-Banded Armadillos (*Tolypeutes matacus*)
3. Integrating Adrenocortical Activity and Foraging Behavior as an Application For Providing Insight into the Welfare of Zoo-Housed Three-Banded Armadillos (*Tolypeutes Matacus*)
4. Determining the Relationship between Adrenocortical Activity and Giving-Up Densities as an Application for Providing Insight into Welfare of Zoo-Housed Three-Banded Armadillo (*Tolypeutes Matacus*)
5. Patch use of a Granivorous Bird Community in Argentina

Graduate Advisors:

Dr. Joel Brown (University of Illinois at Chicago), Dr. Rachel Santymire (Lincoln Park Zoo), Dr. Christopher Whelan (Illinois Natural History Survey, UIUC), Dr. Miquel Gonzalez-Meler (University of Illinois at Chicago), Dr. Thomas Park (University of Illinois at Chicago), Dr. Michelle Rafacz (Columbia College at Chicago)