2018

Adiposity and health in zoo African and Asian elephants

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ADIPOSITY AND HEALTH IN ZOO AFRICAN AND ASIAN ELEPHANTS

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2018

ADIPOSITY AND HEALTH IN AFRICAN AND ASIAN ZOO ELEPHANTS

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ABSTRACT

African (*Loxodonta africana)* and Asian (*Elephas maximus)* elephant populations are rapidly declining due to poaching, and habitat fragmentation and loss. Due to the precarious situation elephants are facing worldwide, captive elephant populations are being viewed as an insurance policy against extinction. Unfortunately, captive elephant populations are not self-sustaining, in part because of reproductive and health issues. One hypothesis for the observed reproductive and health issues is zoo elephants are obese. The current method of assessing obesity in elephants is based on a qualitative visual tool, the body condition score, which does not quantify fat mass. The purpose of this dissertation was, for the first time, to quantify fat mass in both zoo African and Asian elephants, and characterize the relationship between fat mass and reproductive and metabolic health. Based on deuterium dilution, body fat percentages ranged between approximately 5% and 16%, and 3.5% and 25% in our sample population of African and Asian elephants, respectively. We did not observe a relationship between higher fat mass levels and abnormal reproductive cycling status in either African or Asian elephants; however, we did observe a positive association between fat mass and metabolic biomarkers. In addition, in Asian elephants, older elephants walk less compared to younger elephants, with greater activity levels associated with lower fat mass and serum glucose levels. Finally, based on four different body condition score systems, we found that body condition scoring systems that use a wider range of numerical scores predict

absolute fat mass in Asian elephants, while three out of the four systems examined do not predict relative fat mass. Further, it appears that there is an inherent sex bias within the body condition score systems, with males receiving higher scores, speculatively because of their increased fat free mass even though they have less relative fat mass. Although the current method for assessing obesity in an elephant, the BCS, can predict absolute fat mass, it is not sensitive enough to predict relative fat mass, which may be more important for defining obesity. Overall, these data do not support the hypothesis that elephants with greater adiposity are more likely to exhibit abnormal reproductive cycling, but greater relative fat mas may contribute to other metabolic health issues.

Keywords: elephant, body composition, fat, reproductive cycling, metabolic, activity

DEDICATION

This work is dedicated to my mother, who taught me from a young age that whatever I dream, I can achieve.

“I don’t want to live a narrow life, I want to live a big, expansive one.”

-Hillary Rodham Clinton

ACKNOWLEDGMENTS

There are so many people I need to acknowledge and say thank you to that have supported me and guided me through my PhD career. First and foremost, my family. Mom and Larry, you have never waivered in your support of me and my aspirations. Mom, you always taught me to be strong, independent, and pursue my dreams. Larry, you provided me with an environment that fostered those principles, and whenever I needed it, you offered sage wisdom and advice. I am so grateful for loving and encouraging parents. My best friend and sister, Susan, what would I do or be without you. You have been a tremendous role model for me since we were children. Always had my back, always so proud, and always there for me when things were tough. I appreciate and hold your opinion in such high esteem, and without your help, my grammar would be way below par. Ayala, regardless of circumstance, you are with me and support me like no one else. When I waivered you were my rock, and you were always eager and excited to discuss my research and future projects.

To my dissertation committee: Drs. Tim Nagy, David Allison, Steve Austad, Mark Bolding, Janine Brown, and Daniel Smith Jr., a huge thank you. My committee members are all rock stars, contributing to their respective fields, but most importantly, are sound, remarkable scientists. You all invested me in, above and beyond, and for that I will forever be grateful. Dr. Nagy, I could not have asked for a better mentor. You provided me with autonomy and room to grow, but when I needed help and guidance, you were always there. You had confidence in me, and provided me with a chance to pursue an unorthodox project. This project would not have happened without you. Dr.

Allison, from the first day I entered your office, you backed me and my desire to study elephants. You didn't waiver even though it was outside of the norm of our department, but embraced it. So many times along the way you have put me in positions to succeed, helping me to grow as a scientist and to build my confidence. I look forward to future hikes and Seders. Dr. Austad, thank you for your continued mentorship. I enjoyed our talks, the new directions you offered, and appreciated your help in networking. Mark, thank you for your patience, listening, and countless encouragements along the way. Janine, what you have meant to me and this project cannot be put into words. You brought me into the secret world of elephants, opened up doors that would otherwise have been closed off, and allowed me to experience things people only dream of. I only hope I can help elephants a fraction of what you have done for them. I owe you many thanks. Lastly, I owe a big thank you to Dr. Smith. I cannot express how much I enjoyed our conversations. Your passion for science is unbeatable and contagious. I always left your office invigorated and excited about future projects.

Thank you to the Nutrition Obesity Research Center for supporting and expanding my training. The T-32 fellowship provided opportunities to learn what it means to be a scientist and to network with some of the nation's best. Dr. Jose Fernandez, thank you for helping me find the right path, and your continued guidance and advice over the years.

Dr. Barbara Gower, you and your core, have been instrumental. Dr. Gower, I am not sure you realize how much I enjoyed our conversations. I learned a great deal from you. Your knowledge is impressive, and you are always ready to share it. Maryellen, Heather, and Cindy, a huge thank you for all the hours you put in to make sure we were able to run our analyses. Dr. Gary Hunter, thank you for your guidance. A big thank you

to David Bryan. We spent many hours validating accelerometers, and I appreciate your time and patience.

A special thank you to the Birmingham Zoo, Dr. Stephanie McCain, Pat Flora, Laura Schillinger, their team and special elephants. The Birmingham Zoo allowed me to continuously workout and validate our methods prior to going to other zoos. Pat you brought me into your barn and made me feel like part of your team; taught me how to approach and read elephants, and I am forever grateful for what you and your team have done for me. I will cherish all our long elephant talks and brainstorming. Laura, thank you for always helping me get whatever it is I needed done, a listening ear, and a great friend.

A big thank you to Zoo Atlanta, Dallas Zoo, Jacksonville Zoo and Gardens, Maryland Zoo in Baltimore, Memphis Zoo, Zoo Miami, Nashville Zoo at Grassmere, North Carolina Zoo, Pittsburgh Zoo and PPG Aquarium, Riverbanks Zoo and Botanical Garden, San Diego Zoo Global, Wildlife Safari, African Lion Safari, Cincinnati Zoo, Columbus Zoo, Fort Worth Zoo, Little Rock Zoo, Oklahoma City Zoo, Oregon Zoo, Santa Barbara Zoo, Saint Louis Zoo, Thailand Elephant Conservation Center, their veterinarians, elephant keepers, and elephants. Zoo days were my favorite days. Not only did I get to work with and meet so many elephants, but I was able to learn about different programs and see different exhibits and barns. The elephant keepers always made me feel welcomed, made a concerted effort to collect all requested data, and provided me with so many special experiences and memories. I am truly lucky.

My experience wouldn’t have been as great as it was without my friends and peers. Rachel, Keith, Nate, Kat, Camille, Annie and Christian, we went through classes

and growing pains of what it means to be a graduate students together. Thank you for all the laughs and advice. I’m excited where life takes us all.

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ADIPOSITY, REPRODUCTIVE CYCLING STATUS AND ACTIVITY IN ZOO ASIAN ELEPHANTS (*ELEPHAS MAXIMUS)*

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ADIPOSITY AND HEALTH IN AFRICAN AND ASIAN ZOO ELEPHANTS

INTRODUCTION

Wild African (*Loxodonta africana)* and Asian (*Elephas maximus)* elephant populations are rapidly declining, primarily due to poaching, and habitat loss and fragmentation (1-3). Based on the Great Elephant Census, the first continent-wide population survey of African-savannah elephants, there are approximately 375,000 savanna elephants, and it is projected that half of this population will be lost every nine years (1). The Asian elephant population fairs worse, with an estimated population of 50,000 and decreasing (3). Although the plight of elephants has gained international attention, experts fear elephants could go extinct within the next few decades.

Because of the precarious situation facing free-ranging elephants, zoo elephant populations are increasingly viewed as a means of insurance against elephant extinction (4, 5). However, captive elephant populations are currently not self-sustaining as mortality rates exceed birth rates (6, 7). This is in large part due to a high prevalence of reproductive and health issues, the majority of which appear to be associated with obesity based on body indices and associations in other species (8-10). Therefore, this dissertation will focus on determining adiposity in zoo African and Asian elephants and its association with reproductive and metabolic health, in addition to how adiposity relates to current methods of categorizing elephants as obese.

Elephant reproduction and related health concerns in zoos

The elephant has the longest, spontaneous reproductive cycle of any terrestrial mammal, lasting 13-18 weeks, and consisting of a 4-6 week follicular and a 6-12 week luteal phase (8, 11). The follicular phase, like in other mammals, is characterized by elevated prolactin and follicle stimulating hormone (FSH) levels (12). FSH recruits follicles and initiates luteinizing hormone (LH) surges (8, 13). Whereas other mammals require only one LH surge to initiate ovulation, elephants require two distinct LH surges, with estradiol levels typically peaking prior to each LH surge (14, 15). The two LH surges are approximately 19-22 days apart, with only the second LH surge leading to ovulation and corpus luteum (CL) formation (8), transitioning to the luteal phase. Corpus luteum maturation occurs concurrently with a dramatic increase in progestogen levels, followed by a gradual rise in FSH a week later (8). In most mammals the dominant circulating form of progestogen is progesterone. In the elephant, however, the dominant forms are 5α-pregnane-3,20-dione and 5α-pregnane-3-ol-20-one, collectively termed progestagens. Progestagen concentrations are used to determine the reproductive status (i.e. cycling or non-cycling) of elephants. Cycling elephants are characterized with progestagen levels spiking at ovulation, whereas in non-cycling elephants progestagen levels remain at baseline levels (8).

Reproductive acyclicity (i.e., non-cycling) occurs in both zoo African and Asian elephants, but is more prevalent in zoo African elephants. Based on 95 African and 75 Asian elephants housed in Association of Zoos and Aquariums (AZA) accredited zoos, 51.6% and 26.7%, respectively, exhibit abnormal (i.e., irregular or non-cycling)

reproductive cycles (16). Reproductive acyclicity appears to be unique to zoo populations as wild populations do not experience prolonged acyclicity (17-19).

Why acyclicity occurs in zoo elephants remains unclear. Factors that have contributed to reproductive impairments in other species, such as stress, characterized by increased cortisol (20), hyperestrogenism (21), hyperandrogenism (22), and thyroid dysfunction (20) are not associated with acyclicity in elephants. Dow and Brown (2012) showed almost three-fourths of non-cycling African elephants had elevated prolactin concentrations compared to cycling elephants. In other species, hyperprolactinemia is associated with infertility (24, 25). However, when non-cycling elephants received a dopamine agonist, cabergoline, for up to 12 months, cycling status did not change even though prolactin concentrations were reduced (26).

Although it remains unclear why acyclicity occurs, some factors have been shown to be associated with this phenomenon, including age (16). It was initially theorized that non-cycling elephants experience premature 'menopause' due to constant cycling (27). Similar to other mammals, ovarian reserves are high at birth but naturally decrease over time through ovulation (28). While there is a decline in follicle reserves with age in elephants, reserves are still apparent in the 7^{th} decade of life in wild African elephants, suggesting elephants can ovulate up until death (29). Further support that elephants do not experience premature menopause came from a study conducted by Dow and colleagues (2011b). Anti-müllerian hormone (AMH) is a marker for the number of healthy oocytes within the follicular reserve (31, 32). Low AMH concentrations are used to clinically confirm menopause in women. When AMH concentrations were compared between cycling and non-cycling elephants, no significant concentration differences were

observed between the groups (30). Lastly, elephants switch between cycling and non-cycling status, which would not support the premature menopause theory. Therefore, it is unlikely premature menopause is the reason for acyclicity in elephants.

The majority of zoos house both cycling and non-cycling elephants; therefore, it is unlikely that there is just one contributing factor to acyclicity (33). Nevertheless, excess fat mass may be a contributor. Recent studies demonstrated that elephants that appear obese, based on body indices, are more likely to be acyclic (33, 34). This is supported by evidence in other species demonstrating obesity and related metabolic perturbations negatively influence fertility (35, 36). For example, people with obesity and *ob/ob* female mice fail to ovulate (37, 38), obese mares have a prolonged intervals between ovulations (35), and obese minipigs display abnormal reproductive function (39). In other species, obesity is linked to a higher incidence of stillbirths and dystocia (40, 41), which are primary contributors to elephant calf mortality (42). Therefore, it is not unreasonable to posit that excess fat mass may be contributing to fertility and health issues in zoo elephants.

Obesity in elephants

Concern regarding elephants with obesity in zoos has been acknowledged for over a decade (9). In a recent study conducted in North American AZA zoos, 78.3% of female and 57.7% of male African elephants and 75.3% of female and 65.2% of male Asian elephants were classified as overweight or obese, respectively, based on a body condition score (BCS) (43). Although the combined percentages of being overweight and obese are

similar between African and Asian elephants, comparatively, when those percentages are broken down, more Asian elephants are classified as obese.

Body condition score (BCS)

Rather than measuring body fat, the assessment of overweight and obesity in elephants is traditionally based upon a qualitative BCS. There are several different BCS systems for elephants that commonly use a photograph and rely on the qualitative scoring of the individual based on a range of key skeletal descriptors (e.g., ribs, pelvic bone, backbone) (43-45). Based on the appearance of these anatomical regions, the elephant is given a numerical score, with lower numbers implying less and higher numbers implying more amounts of fat. However, all these systems are based on the observable appearance of the individual with no quantitative assessment of the actual amount of fat (43-47).

Although BCS is the currently accepted method to assess adiposity (48-50), BCS was originally developed to assess the soft tissue (i.e., fat plus muscle) of livestock to evaluate their nutrition and economic efficiency (51). Therefore, the tendency to extrapolate BCS for the measurement of adiposity, rather than soft tissue, deviates from its original purpose without any supporting evidence. Further, the reliability of BCS is contingent on the reproducibility within and between assessors scoring the elephant (52, 53). Therefore, prior to accepting BCS as a reliable means of estimating adiposity in elephants, it is critical to validate BCS systems against a measurement of adiposity.

Isotope Dilution

Isotope dilution appears to be a tenable solution for assessing adiposity in the largest terrestrial mammal, the elephant. The key to assessing body composition by isotope dilution relies on water being the major constituent of the body, but which is not distributed uniformly (54). Fat contains substantially less water than fat free mass (FFM). Further, the fraction of FFM as water, termed the hydration constant (total body water (TBW)/FFM), is remarkably stable in healthy adult mammals (55). Based on six mammalian species (rat, guinea pig, rabbit, cat, dog, and monkey), Rathburn and Pace found the hydration constant to be similar at ~73% (56). The hydration constant has subsequently been investigated, and confirmed (55), although some deviations have been noted. For instance, infants have a higher hydration constant (~0.81) (57), and the hydration constant may also be higher in elderly and people with obesity (58-60). Moreover, Pitts and Bullard showed a slight decrease in TBW/FFM with increasing body size ranging from 78% in small mammals, like the vole, to 71% in cattle. The hydration constant is required in the estimation of total body fat through the following equation: fat mass = body mass – TBW/hydration constant (62).

TBW can be estimated based on the dilution principle, which states that "the extent to which a substance is diluted in a solvent constitutes a measure of the volume of the solvent (63)." For example, if 1 g of sugar is added to a beaker of distilled water, and then after the sugar is mixed thoroughly with the water, the concentration is 0.01 g/mL, the volume of the beaker is calculated to be 100 mL (Equation 1).

$$V_2 = C_1V_1/C_2 \qquad \text{Equation (1)}$$

C_1 and V_1 are the concentration and volume of the solute prior to dilution, respectively, and C_2 and V_2 are the concentration and volume of the solute after dilution. The solute's concentration and volume prior to dilution is known, whereas the concentration after dilution is quantified experimentally (63). Using this example, the animal's body represents the beaker, while an ideal biological tracer is the solute. The extent to which the biological tracer becomes diluted measures TBW volume (63).

In the early 20th century, suitable biological tracers were investigated. Both isotopes of hydrogen, tritium (having two neutrons) and deuterium (having one neutron), demonstrated to be reliable for TBW measurement. Deuterium, discovered in 1932 by Urey and colleagues, ultimately proved more favorable than tritium. Although tritium oxide analyses are typically less expensive than deuterium oxide, and the equipment is more common (65), tritium is a radioactive isotope. As such, there are safety, legal, and ethical considerations when administering tritium oxide to an animal. Therefore, because deuterium being a stable isotope, deuterium oxide has been used more widely to assess the body composition of a variety of animals, including the bumble bee (66), dog (67), wolf (68), pony (69), polar bear (70), and walrus, the largest animal with body composition data using isotope dilution to date (71).

Physically and chemically similar to hydrogen, deuterium is able to directly label body water and subsequently becoming diluted (72). The extent deuterium is diluted can be determined by measuring the deuterium enrichment (i.e., proportion of deuterium to hydrogen) in a sample of body water (e.g., urine, blood, saliva) above background enrichment levels (65) either by the plateau or intercept method. While both the plateau and intercept methods require obtaining a biological sample prior to dosing to determine

background enrichment, the total number of samples required and when the samples are collected differ between the methods. The plateau method requires one sample collected during equilibrium (i.e., when deuterium has mixed completely with the body's water pools). Comparatively, the intercept method requires a minimum of three samples collected only after equilibrium has occurred and spanning several days.

The intercept method is likely more appropriate for large animals because of assumptions associated with the plateau method. The plateau method assumes rapid equilibrium and that neither the deuterium nor water are metabolized during the equilibrium period (63). Deuterium is mixed with the body water pools largely through diffusion and the cardiovascular system; therefore, it takes longer to reach equilibrium in larger animals due to their larger body water pools and slower heart rates (65). The aforementioned assumptions may be violated in the elephant due to their intuitively massive body water pools and comparatively slow heart rate, averaging 30 beats per minute (73).

There are additional assumptions that are required when applying deuterium dilution to the elephant. As previously stated, the hydration constant is critical in calculating body composition. The hydration constant is not known for elephants. To determine the hydration constant for the elephant, although theoretically possible, is not feasible as carcass analysis of multiple elephants is required (56). Therefore, rather than determining the exact hydration constant for the elephant, we can assume it to be that of the mammalian average (56). If the hydration constant for the elephant differs from the mammalian average (0.73), any error will result in a linear transformation of the body composition values across the population. Thus, deuterium dilution by the intercept

method is likely a technique that can be applied to both African and Asian elephants to compare relative body fatness.

Metabolic biomarkers: A potential link between acylicity and adiposity

In humans and other animals, the link between obesity and infertility has been well established (38, 74). Women with obesity have reduced rates of successful pregnancies, a higher prevalence of anovulation, and disruption in menstrual cyclicity (75-78), with weight loss improving fertility outcomes (79). Obesity increases the rates of miscarriage and stillbirths (41, 80), all of which have been documented in the zoo elephant population (8, 9, 12, 81). The mechanism(s) by which obesity impacts reproduction are likely complex and not entirely clear. In humans, elevated leptin levels, insulin resistance, and inflammation have all been implicated in the link between obesity and reduced reproductive success (38). Further investigations of metabolic health in zoo elephants should allow a clearer understanding as to how overall adiposity impacts impaired reproduction.

Leptin

Leptin is a polypeptide hormone predominantly produced by adipocytes and influences multiple neuroendocrine systems (e.g., puberty, fertility, and energy homeostasis) (82). Because leptin is predominantly secreted by adipocytes, serum leptin levels are associated with fat mass (83). Leptin regulates fat mass in part through its ability to adjust food intake and energy expenditure. Leptin suppresses food intake by suppressing appetite stimulating pathways (e.g., NPY and AgRP) and promoting appetite

reducing pathways (e.g., POMC, CART) (82, 84-86). As such, leptin plays a major role in responding to and defending against reductions in fat mass that may impair survival and reproductive fitness (87).

Under obese conditions, however, circulating leptin concentrations increase and energy balance regulation becomes impaired (88, 89). Specifically, leptin is unable to effectively induce fat loss through its appetite suppressing effects (90), which may indicate that the leptin signal to the brain is not properly received. Ultimately, leptin resistance can occur leading to increased food intake and reduced energy expenditure disrupting energy homeostasis (87).

Elevated leptin levels may partially contribute to obesity-related reproductive complications through its effect on the hypothalamic-pituitary-ovarian (HPO) axis (38). Leptin has been associated with altered ovarian steroidogenesis (91), and dysregulation of gonadotropin-releasing hormone (GnRH) secretion (92) and folliculogenesis (93). Indeed, normal-weight rats treated with exogenous leptin had significantly reduced ovulation rates compared to controls (93), suggesting elevated leptin levels inhibit fecundity (94). Recently it was reported that non-cycling African elephants have higher leptin levels compared to cycling elephants (34). Although the exact pathophysiological mechanism through which obesity may impact cycling status in elephants is unclear, data from humans and other animals suggests elevated leptin levels may play a role.

Insulin and glucose

Insulin is a peptide hormone that is critical in energy balance. Insulin regulates blood glucose levels predominantly by balancing the output of hepatic glucose with the

uptake of glucose by the skeletal muscle, adipose tissue, and liver (95). Blood glucose concentrations are tightly regulated, and for elephants typically are between 60 to 116 mg/dL (96). However, in a state of insulin resistance, with impaired skeletal muscle insulin signaling, glucose storage and metabolism are compromised. Concomitantly, there is an increase in blood glucose, and decrease in glucose storage, which can result in hyperglycemia. To prevent hyperglycemia and maintain normal glucose tolerance, compensatory hyperinsulinemia ensues. Reported insulin ranges for the Asian elephant are 0.11 to 6.24 ng/mL (units based on correspondence with authors) and 0.10 to 3.07 ng/mL for African elephants (units based on correspondence with authors) (34, 97). Hyperinsulinemia for the elephant is not known, but for the horse, an often used comparative model, hyperinsulinemia is defined when serum inulin concentrations are above ~1.41 ng/mL (98). Collectively these data suggest zoo elephants may be experiencing metabolic dysfunction, which may be associated with observed obesity and reproductive impairments in this population.

The link between obesity and insulin resistance has been well documented in humans (99) and in other animals, such as the horse (100), pig (101), and dog (102); therefore, it is reasonable to posit that this relationship may be present in elephants. Morfeld and Brown reported elephants categorized as obese based on BCS had greater insulin concentrations compared to elephants with a lower BCS (34). Obese elephants had significantly lower glucose to insulin ratios, indicating greater insulin resistance compared to elephants with a lower BCS. This was the first evidence indicating elephants may experience insulin resistance and warrants further research examining the relationship with fat mass.

Insulin plays a key role in integrating energy homeostasis with reproductive function through a variety of mechanisms (103-106). Brain insulin signaling has been linked to reproduction (107). For example, female mice with neuron-specific disruption of the insulin receptor gene had a 90% reduction in circulating LH concentrations (107). LH secretion is controlled by GnRH, and insulin improves the sensitivity of gonadotroph cells to GnRH (108). While insulin stimulates LH, it decreases plasma concentrations of sex hormone-binding globulin (SHBG) (108). Sex hormone-binding globulin binds sex steroids; therefore, by inhibiting SHBG synthesis, insulin changes sex steroid bioavailability (109). Although the mechanisms are not well understood, it is clear that proper insulin signaling is required for successful reproduction (110).

Inflammation

Insulin resistance is promoted by a state of chronic low-grade inflammation associated with obesity. With weight gain, adipose inflammation occurs in part by macrophage infiltration (111). The substantial increase in macrophage content observed in an obese state is correlated with age, being female, and the expression of various inflammatory markers (112, 113).

TNF-α and IL-6 are pro-inflammatory cytokines expressed by adipose tissue and are positively associated with obesity (114, 115). Inflammatory stimuli also promote acute phase protein (APP) synthesis, including serum amyloid A (SAA) (116-118). In humans, SAA is positively associated with body mass index (BMI) (119). Human adipocytes display greater SAA mRNA expression compared to liver tissue, although the reverse is true in the mouse model (120). SAA has yet to be evaluated in elephants in

conjunction with obesity, but has been demonstrated to be the most responsive major APP in Asian elephants when compared to C-reactive protein and haptoglobin (121).

TNF-α, IL-6, and SAA are all directly or indirectly involved in a cycle of macrophage recruitment, production of inflammatory cytokines, and impairment of adipocyte function (122). These factors have been shown to culminate in a chronic inflammatory state (122). Chronic inflammation in relation to obesity and its implications on reproductive function have yet to be investigated in the elephant.

Physical Activity

Independent of weight loss, it is acknowledged that physical activity can improve health. Physical activity can help prevent weight gain (123), while improving insulin sensitivity (124). Aerobic exercise improves insulin sensitivity in part by increasing glucose uptake by the skeletal muscle (62, 63). Therefore, it is not surprising that physical inactivity is a risk factor for obesity and diabetes (49). Many have suggested that elephants in zoos, because of spatial constraints, do not walk enough (125, 126).

In the wild, elephants move for a variety of reasons, including resource acquisition and mate searching, with total distances traversed dependent on the geography and season (i.e., wet versus dry season). Free-ranging African and Asian elephants have been observed to walk between 0.01 to 1.15 km on an hourly basis (127-130). Comparatively, zoo elephant populations have been reported to walk 0.05 to 1.29 km on an hourly basis (131, 132). Irrespective of why elephants walk (i.e., to secure resources, adapted to cover long distances, curiosity), the simple act of walking may exert metabolic benefits in elephants. For example, a recent study in zoo elephants showed

leptin concentrations and the ratio of glucose to insulin are negatively associated with time spent walking (97). Overall, there is a paucity of data examining the relationship between physical activity, body composition, and metabolic health in zoo African and Asian elephants.

Summary

The purpose of this dissertation is to 1) quantify adiposity and characterize its relationship with metabolic, inflammatory, and reproductive health in zoo African elephants; 2) quantify adiposity and characterize its relationship with metabolic, inflammatory, activity levels, and reproductive health in zoo Asian elephants; and 3) validate multiple BCS systems against adiposity in zoo Asian elephants. These studies were developed to determine, for the first time, the range of adiposity observed in zoo elephants and comprehensively investigate its role in overall health in these populations. These data will guide future research to examine how obesity is related to specific morbidity and mortality outcomes in zoo elephants, and potentially those in the wild.

ADIPOSITY AND REPRODUCTIVE CYCLING IN ZOO AFRICAN ELEPHANTS

by

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Abstract

The majority of zoo African elephants exhibits abnormal reproductive cycles, but it is unclear why. Acyclicity has been positively associated with body condition scores. Our objective was to measure body composition and examine the relationship between adiposity and cyclicity status, mediated by glucose, insulin, leptin and inflammation. Body composition was assessed by deuterium dilution in 22 African elephants. Each elephant was weighed, given deuterated water orally (0.05 mL/kg), and blood was collected from the ear prior to and five times after deuterium administration. Glucose, insulin, leptin and pro-inflammatory biomarker concentrations in serum were determined. Body fat percentage ranged from 5.24% to 15.97%. Fat adjusted for fat free mass (FFM) and age was not significantly associated with cyclicity status (P=0.332). Age was the strongest predictor of cyclicity status (P=0.040). Fat was correlated with weight (ρ=0.455, P=0.044) and when adjusted for FFM with circulating glucose (ρ=0.520, P=0.022), and showed a trend for association with leptin (unadjusted: ρ=0.384, P=0.095; adjusted for FFM: ρ=0.403, P=0.087). Deuterium dilution appears to be an available technique to measure body composition in African elephants. In this sample, fat was not associated with cyclicity status and age may be more important to cyclicity status.

Introduction

Obesity is an epidemic not only affecting humans, but animals associated with humans, including companion and domestic animals (1), and possibly, zoo animals. Approximately 33% of North American female zoo African elephants (*Loxodonta africana)* are classified as obese, based on the body condition score (BCS) (2). BCS is a visual assessment of key skeletal structures and is, therefore, a subjective assessment of subcutaneous body fat stores (3). Although BCS provides an overall assessment of elephant body condition, it does not quantify body composition (fat [FM] or fat free mass [FFM]).

Over 50% of zoo African elephants in the United States exhibit irregular reproductive cycles or are acyclic (4), yet the causes of acyclicity remains unknown. Previous studies have demonstrated a positive association between condition indices (i.e., BCS and body mass index) with rates of reproductive acyclicity in zoo African elephants (5, 6) and there is evidence in other species linking body composition to reproductive impairments (7-9). Thus, it is not unreasonable to posit that zoo elephants with increased FM may be more likely to exhibit abnormal reproductive cycles than are elephants with lower FM.

Assessing elephant adiposity is a necessary next step in evaluating this species' reproductive and overall health. Similar reproductive impairments to those observed in zoo elephants have been noted in other species and shown to be associated with excess FM (9, 10). The relationship between excess FM and reproductive problems may be mediated through the animal's metabolic health. For instance, leptin and insulin are positively associated with FM (11, 12). Excess fat is also mechanistically linked with

inflammation (13). Leptin, hyperinsulinemia, and chronic inflammation have all been shown to play a role in reproduction and related dysfunction (14, 15).

Given the elephant's size, deuterium dilution offers a tenable solution to estimate body composition *in vivo*. Deuterated water has been used to measure total body water (TBW) (i.e., the combination of intra- and extracellular water) in animals ranging in body size from the bumblebee to the Atlantic Walrus (16, 17). Yet, to our knowledge, no published studies have been conducted in the largest terrestrial mammal, the elephant. Deuterium (^{2}H), a non-radioactive isotope of hydrogen (^{1}H), when administered to animals, is diluted by the ^{1}H in water molecules, providing an estimate of TBW (18). TBW is assumed to be restricted to the animal's FFM compartment, thereby, using the standard mammalian hydration constant, based on TBW of 0.73, FFM can be calculated (19). FM is then inferred by subtracting FFM from weight.

Thus, the purpose of the present study is to measure body composition via deuterium dilution and then to evaluate the relationship between body composition and reproductive cyclicity status in zoo African elephants and to investigate the relationship between body composition, circulating glucose, insulin, leptin and inflammatory markers.

Materials and Methods

Animals

This study was approved by the Institution Animal Care and Use Committee of the University of Alabama, Birmingham (UAB), and the participating zoos. Of 19 zoos contacted, 13 zoos participated and were visited between November 2014 and June 2016. Data from five of those zoos were omitted because the plateau method for determining

TBW was found to be invalid (Table 1). A total of 22 female African elephants of reproductive-age (≥16 years of age; Table 2) housed in eight accredited U.S. institutions were studied. Elephants were not pregnant. All isotopes were analyzed blind to the animal status.

Body Composition

Plateau method. From October-December 2014, elephants (N=4) were weighed to the nearest pound on the institution's scale. Venous blood was collected from an ear vein by zoo personnel. Blood was collected prior to deuterated water administration to determine the elephant's background isotope concentration. An oral dose of deuterated water was administered to the elephant (see intercept method below for details). Subsequently, blood (~9 mL) was sampled at regular intervals. For elephant 1 blood was collected at ~5, 7, 9 h from an ear vein post deuterium administration. The repeated blood samples over the 9h time period was conducted to determine the equilibrium time period, when the deuterated water mixed completely with total body water, for the African elephant. For elephants 2, 3, and 4 blood was collected ~6 h from an ear vein post deuterium administration. All samples sat up to 30 minutes in an airtight container to allow for coagulation. Whole blood was centrifuged and serum was collected, aliquoted, and frozen at a minimum of -20° C until driven to UAB on dry ice. Samples were then kept in a frost-free -20° C freezer until analysis.

Intercept method. Elephants were weighed to the nearest five pounds, pound, or kilogram, depending on the institutions' scale. Venous blood was collected from an ear vein by zoo personnel prior to deuterated water administration to determine background

isotope enrichment. Subsequently, an oral dose of (99.9% APE) deuterium oxide (0.05 mL D_2O/kg of weight; DLM-4-1000, Cambridge Isotopes, Tewksbury, MA) was administered using bread (Publix®, Birmingham, AL) as a vehicle. Bread was weighed (to the closest 0.01 g, Pioneer, Ohaus, Pine Brook, NJ), deuterated water was carefully added, and then reweighed. The difference in weight represented the dose of deuterated water. Each elephant received four to five pieces of bread with approximately 40-50 g of deuterated water per piece. Blood (~9 mL) was sampled at regular intervals (~24, 120, 240, 360, and 480 h) post deuterium administration. All samples sat up to 30 minutes in an airtight container to allow for coagulation. Whole blood was centrifuged and serum was collected, aliquoted, and frozen at a minimum of -20° C until shipped on dry ice overnight to UAB. Samples were then kept in a frost-free -80° C freezer until analysis.

Isotope ratio mass spectroscopy (Finningan Delta V Advantage, Thermo Fisher Scientific, USA) analysis was carried out by UAB's Nutrition Obesity Research Center's Metabolism Core with guidance and support from the Energetics Research Group at the University of Aberdeen, Aberdeen, Scotland. In duplicate, samples and standards (200 µL; SMOW and four in-house laboratory standards ranging from 149 to 383 parts per million [ppm]) were placed in a vial along with a Hokko bead. ^{2}H and ^{1}H isotope concentrations were measured by thermal ionization and the different masses were collected by magnetic sector separation. The $^{2}H/^{1}H$ delta value was converted to ppm, and used to calculate deuterium dilution (18). The dilution space is considered to reflect the TBW content. TBW was estimated by first calculating the dilution space (Nd, Equation 2), then converting it to grams (Equation 3), and subsequently to TBW, assuming ^{2}H exchanged with non-aqueous molecules (18) (Equation 4).

$$Nd_{(mol)} = Mol_{inj} * (E_i - E_{inj}) / (E_{bg} - E_i) \quad (2)$$

$$Nd_{(grams)} = Nd_{(mol)} * 18.020 \quad (3)$$

$$TBW = Nd_{(grams)} / 1.04 \quad (4)$$

Mol_{inj} was mols of the deuterated water administered orally, E_i was the elephant's initial ^2H enrichment, E_{inj} was the ^2H enrichment of the deuterated water administered, and E_{bg} was the elephant's background ^2H enrichment. Mol_{inj} was calculated by dividing the amount of deuterated water administered by the molecular mass of deuterated water. E_i was calculated by the back extrapolation method as described by Coward (20). TBW was subsequently used to estimate body composition based on the general allometric equation for mammals (21) (Equation 5).

$$FFM = TBW / 0.73 \quad (5)$$

To determine blood sampling intervals, in one elephant, blood was collected from an ear vein prior to deuterated water administration and then daily up to 391h post administration (Figure 1). These samples were analyzed by liquid water isotope analysis (Los Gatos Research, San Jose, CA, USA) at the Energetics Research Group (The University of Aberdeen, Scotland).

Body water turnover is the replacement of body water lost over a certain time and indicates water homeostasis (22). The water turnover rate was calculated from the washout of the isotope over the period of sampling time (~480 h) based on the following equations:

$$WTR_{(mol/min)} = 1.1016 * K_d * Nd_{(mol)} \quad (6)$$

$$WTR_{(g/min)} = WTR_{(mol/min)} * \text{molecular mass of } D_2O \quad (7)$$

WTR was water turnover, K_d is the elimination rate of the ^2H enrichment in the samples collected over time, Nd was dilution space obtained from equation 1, and 1.1016 represents a normalizing constant accounting for isotopic fractionation.

Determination of reproductive cyclicity status

Reproductive cyclicity status was provided by participating zoos, and was based on progestogen analyses of longitudinal serum samples (23). When possible, cycling status was confirmed through longitudinal samples (45% of samples) at the Smithsonian Conservation Biology Institute Endocrinology Laboratory.

Body condition score (BCS)

BCS was based on standardized photographs and taken as previously described (3). Briefly, a set of photographs of each elephant was taken from three angles (side view, rear view, and rear-angle view). Two assessors (DEC and KM) visually scored the elephant on a 5-point scale based on key skeletal regions (ribs, pelvic bone, and backbone), where a score of 1 represents least amount of fat and 5 represents the most amount of fat. Assessors followed the BCS Flow Chart developed and validated by Morfeld and Brown (3). One assessor (DEC) scored each elephant three times, with photographs blinded to the assessor on each occasion. Scores for elephants were averaged and rounded to the nearest 0.5 score to determine final BCS. Using R package, interclass correlations (ICC) was done to evaluate the correlation between assessors' scores, ICC(1,2) =0.77 (24). There were no significant effects on the results if the BCSs were used exclusively from DEC or KM scoring.

Serum analyses

Assays were conducted on the blood samples taken prior to administration of deuterium. The blood was collected in the morning before elephants received their first meal of the day. Elephants' last meals were given during the late afternoon, evening the day before blood collection. Samples were typically run neat (i.e., not diluted), but if above the assay range, they were diluted in reagent diluent until they fell within the detectable range of the assay.

Serum glucose was measured by an automated glucose analyzer (Stanbio Sirrus, Stanbio Laboratories, Boerne, TX, USA). Samples (3 µL) were done in singlicate and analyzed at the same time.

Serum insulin concentrations were determined with one assay using a solid-phase, two-site bovine insulin enzyme immunoassay (EIA; 10-1201-01; Mercodia, Uppsala, Sweden) validated for use in African elephants (5). As we have described elsewhere, bovine insulin standards, controls, and serum samples (25 µL) were incubated in duplicate in a 96-well anti-bovine insulin antibody-coated plate after immediate addition of 100 µL enzyme conjugate solution containing peroxidase-conjugated anti-bovine insulin antibodies. Plates were incubated for 2 h on a plate shaker at room temperature. To remove unbound enzyme-labeled antibody, plates were washed six times by hand with wash buffer. Bound conjugate was detected by reaction with 200 µL of 3,3'5,5'-tetramethylbenzidine (TMB) added to each well. After 15 min incubation at room temperature, 50µL of 0.5 M h_2SO_4 was added to stop the enzymatic reaction and the

optical density was determined spectrophotometrically (450 nm filter) with a Synergy HT Microplate Reader (BIO-TEK, Winooski, VT, USA) (5). The intra-assay CV was 5.2%.

Serum leptin concentrations were measured with one assay using a multi-species double-antibody RIA (XL-85K; Millipore, Billerica, MA, USA) validated for African elephants (5), which utilized a ^{125}I-human leptin tracer and a guinea-pig anti-human leptin antiserum. As we have previously described, serum samples (100 µL), analyzed in duplicate, were incubated with 100 µL antiserum at 4° C overnight; then, 100 µL of ^{125}I-leptin was added to each tube and samples were incubated at 4° C for 18-24h. The following day, 1.0mL cold precipitating reagent (goat anti-guinea-pig IgG serum, 3% polyethylene glycol and 0.05% Triton X-100 in 0.05 M phosphate-buffered saline) was added to all tubes except those for measurements of total counts. Tubes were vortexed, incubated at 4° C for 20 min, centrifuged at 4° C for 20 min at 2500g and the resulting supernatant decanted and counted for 1 min in a gamma counter (Perkin-Elmer, Shelton, CT, USA) (5). The intra-assay CV was 4.29%.

Serum Amyloid A (SAA) was determined using a RX Daytona automated clinical chemistry analyzer (Randox Industries-US Ltd., Kearneysville, WV, USA) and commercially available reagents (150 µL), calibrators (0.1-500 mg/L), with two-level controls (Eiken Chemical Co. Ltd, Tokyo, Japan). Samples (4 µL) were run in singlicate and analyzed at the same time.

Serum tumor necrosis factor alpha (TNF-α) concentrations were measured using an equine TNF-α sandwich enzyme immunoassay kit (EIA; ESS0017; Thermo Fisher Scientific, Frederick, MD; Edwards et al., unpublished data) according to manufacturer's instructions. A 96-well microtitre plate (Nunc-Immuno MaxiSorp®, Thermo-Fisher

Scientific, Rochester, NY, USA) was coated with 100 µl anti-equine TNF-α coating antibody diluted 1:100 in carbonate-bicarbonate buffer (0.2 M, pH 9.4, 0.22 µm filtered) and incubated overnight at room temperature. After aspirating the coating antibody, blocking buffer (4% BSA, 5% sucrose, Dulbecco's phosphate buffered saline (D-PBS) solution) was added and incubated for 1 h. Blocking buffer was aspirated, and standards, controls or samples (50 µL) were added in duplicate to each well before incubation for 1 h at room temperature on a plate shaker set to 500 RPM. Recombinant equine TNF-α standards ranged from 1000 to 15.6 pg/mL, serially diluted in reagent diluent (4% BSA in D-PBS, pH 7.4, 0.2 µm filtered). Serum samples were typically run neat, or diluted 1:5 in reagent diluent. The plate was then washed three times by hand using wash buffer (0.05% Tween ^{TM}-20 in D-PBS, pH 7.4). Next, 100 µL of anti-equine TNF-α detection antibody (diluted 1:100 in reagent diluent) was added to each well and incubated for 1 h at room temperature on a plate shaker set to 500 RPM. The plate was then washed three times by hand with wash buffer, and 100 µL of streptavidin-horseradish peroxidase (diluted 1:400 in reagent diluent) was added to each well followed by incubation at room temperature for 30 min on a plate shaker set to 500 RPM. The plate was then washed three times by hand with wash buffer. The TMB substrate solution (100 µL) was added per well and incubated in the dark for 20 min at room temperature. The enzymatic reaction was stopped by adding 100 µL of the stop solution to each well. The absorbance at 450 nm with a reference of 570 nm was measured using the Filtermax F5 plate reader (Molecular Devices LLC, Sunnyvale, CA, USA). The equine TNF-α EIA was validated for African elephant use by parallelism, recovery and linearity assessment. Parallelism was observed between the TNF-α standard curve and serum (50 µl: 1:2; 1:4; 1:8; 1:16; and 1:32)

serially diluted with reagent diluent ($y = 0.233x - 0.046$, $R^2 = 0.986$, $F_{1,4} = 281.928$, $P < 0.001$). There was significant recovery ($y = 0.961x - 2.934$, $R^2 = 0.999$, $F_{1,5} = 3762.296$, $P < 0.001$) of TNF-α standard following the addition of an equal volume of low TNF-α concentration serum. Linearity of 2-fold dilutions of serum spiked with recombinant TNF-α standard (hormone concentrations that varied no more than 80–120% between doubling dilutions) was achieved within the dilution range utilized for samples. Inter-assay CVs were 6.4% and 2.6% for low and high concentration controls, respectively. CVs for all duplicates were below 10%.

Statistical analyses

All statistical analyses were performed using SAS 9.4 statistical software (SAS Institute, Cary, NC, USA) and specified prior to examining data, unless otherwise stated. Although 22 elephants were included in this study, two elephants were excluded from body composition statistical analyses because blood sample time points were unknown and therefore accurately calculating body composition was not possible.

The primary model to address our main hypothesis was a generalized estimating equation (GEE), regressing cyclicity status on FM adjusting for FFM and age to the power lambda (age^{λ}). The lambda value for age was calculated by fitting a non-linear model based on data previously collected on 95 female African elephants, where age and cyclicity status were known. The best estimate of lambda was 1.62. FM, FFM, and $age^{1.62}$ were included as continuous variables. To adjust for factors related to residing in the same zoo, the zoo ID was treated as random effect in all the models. The second logistic model added FM^2 and FFM^2 to the primary model as the relationship between body

composition and cyclicity status may be nonlinear. Secondary sensitivity analyses were conducted on the primary logistic model after looking at the data. Theses analyses included the addition of nulliparous status, dominance status, and whether the elephants were housed with male elephants. Nulliparous and dominance status were included as dichotomized variables, while elephants were characterized as either not housed with male elephants, housed with males with direct contact, or housed with males without direct contact.

Regardless of the treatment effect (i.e., association) of the primary model, it is still useful to conduct mediation analyses to further examine whether a treatment is affecting the outcome through any mediator (25). Because approximately half of the TNF-α and SAA samples were below detection, these variables were analyzed two ways: (1) dichotomized: above and below detection; and (2) as continuous variables, whereby the lowest detectable value was used if the sample was below detection, effectively winsorizing the data (26).

Pearson correlations between FM and weight, BCS, leptin, glucose, insulin, and Spearman correlations were conducted between FM and SAA and TNF-α because of the inflammatory biomarkers' non-normal distributions, were calculated. Correlations between body weight and water turnover, in addition to BCS and weight, FFM, FM, age$^{1.62}$ and percent body fat were determined. Partial correlations between FM and leptin, glucose, and insulin, adjusted for FFM, were conducted. Relative fat was determined by the residual for each elephant when FM was regressed on weight.

Descriptive statistics between cycling and non-cycling groups were assessed. *T-tests* were used to compare the means of age, body weight, FM, FFM, height, body

length, BCS, glucose, insulin and leptin by cyclicity status. Wilcoxon test was used to compare the means of SAA and TNF-α by cyclicity status because of their non-normal distributions. Fisher's exact test was used to compare the proportion of nulliparous elephants by cyclicity status. Significance level was accepted at $P<0.05$ (2-tailed).

Results

Fifty-nine percent of the elephants exhibited normal reproductive cycles at the time of body composition assessment (*n*=13 from 8 zoos; mean age 31.3 ± 2.1 years; age range 16-48 years), while the remaining 41% exhibited abnormal reproductive cycles (*n*=9 from 6 zoos; mean age 39.9 ± 1.9 years; age range 33-51 years). Descriptive statistics by cyclicity status are in Table 3.

Body composition and reproductive cyclicity status

Body composition was estimated by deuterium dilution (Table 2) based on the washout curve for each elephant (Figure 2). Body fat percentage averaged 9.69% (SD: 3.18, range: 5.24 -15.97%; N=20). GEE models analyzing predictors of cyclicity status are in Table 4. FM was not shown to be associated with cyclicity status, either unadjusted (P=0.131) or adjusted for FFM and age$^{1.62}$ (P= 0.332; Figure 3). When FFM^2 and FM^2 were included in the model, FM was not significantly associated with cyclicity status (P=0.350). When nulliparous status was included, the primary model improved but remained non-significant (P=0.158). The addition of male interaction to the primary model did not improve significance (P=0.337), but did so when included with nulliparous status (P=0.075). When nulliparous and dominance status were included in the primary

model, FM was not associated with cyclicity status (P=0.172). BCS did not predict cyclicity status, either unadjusted (P=0.234) or adjusted for age$^{1.62}$ (P=0.750). Age$^{1.62}$ was a significant predictor of cyclicity status (P=0.040). Weight, unadjusted, was a significant predictor of cyclicity status (P=0.038), but was not significant when adjusted for age$^{1.62}$ (P=0.647).

BCS was strongly correlated with age (ρ=0.603; P=0.003), weight (ρ=0.759; P<0.0001; Figure 4A), FFM (ρ=0.702, P=0.001; Figure 4B) and FM (ρ=0.583; P=0.007; Figure 4C), but not with relative fat (ρ=0.256; P=0.276; Figure 4D) or percent body fat (ρ=0.337; P=0.146).

Body composition and glucose, insulin, leptin and inflammatory biomarkers

The correlation between FM and relative fat with glucose (ρ=0.379; P=0.100; ρ=0.555; P=0.011, respectively; Figure 5A&B), insulin (ρ=0.369, P=0.110; ρ=0.352; P=0.128, respectively; Figure 5C&D), and leptin (ρ=0.384; P=0.095; ρ=0.399; P=0.081, respectively; Figure 5E&F) nearly reached significance. FM, adjusted for FFM, was correlated with glucose (ρ=0.520; P=0.022), and trended towards significance with insulin (ρ=0.371; P=0.118) and leptin (ρ=0.403; P=0.087). FM was not correlated with SAA (ρ=0.007; P=0.979; Figure 5G) or TNF-α (ρ=-0.0353; P=0.883; Figure 5H). Weight was not correlated with water turnover rate (ρ=0.357; P=0.123). Glucose was correlated with insulin (ρ= 0.430; P=0.046).

Cyclicity status and glucose, insulin, leptin and inflammatory biomarkers

Glucose, (P=0.366), insulin (P=0.406), leptin (P=0.991), SAA (P=0.095) and TNF-α (P=0.349) did not significantly differ by cyclicity status (Table 3). Although there was no overall association between FM and cyclicity status, mediation analyses were still conducted to better understand why the variables did not relate. Glucose (P=0.276) and insulin (P=0.220) were not mediators between FM and cyclicity status. SAA, analyzed as a dichotomous (P=0.564) and continuous variable (P=0.477), and TNF-α, analyzed as a dichotomous (P=0.841) and continuous variable (P=0.432) were not mediators between FM and cyclicity status.

Discussion

This study investigated the association between adiposity with reproductive cyclicity status, mediated through glucose, insulin, leptin and pro-inflammatory biomarkers. We did not find that FM was significantly associated with cyclicity status in zoo African elephants, nor was the relationship mediated through inflammation, glucose, or insulin. FM was correlated with glucose, and nearly with leptin levels. Non-cycling elephants were more likely to be older.

Deuterium dilution by the intercept method appears to be a useful non-invasive technique that can be used to estimate body composition of African elephants. Deuterium dilution measures TBW, by using either the plateau, or the intercept method (27). The plateau method assumes equilibrium is reached rapidly and neither deuterium nor body water are metabolized during equilibrium (18). These assumptions may be violated in elephants because of their large body volume and slow heart rate (28) **(**Table 1). The

intercept method does not rely on a measurement during equilibrium and therefore allows for a longer equilibrium period and continuous water turnover over an extended period of time by collecting several samples over time and back extrapolating to when deuterated water was administered (27). The intercept method was therefore used in the present study, where we observed a log-linear elimination in deuterium enrichment over time in the elephants.

The derivation of FM from TBW estimated by deuterium dilution is based on the ratio of TBW to FFM, termed the hydration constant (19), which to our knowledge, has not been determined in elephants. Therefore, we used the most regularly used hydration constant of ~0.73 (29). Pace and Rathbun (21) first recommended this hydration constant based on several species of mammals, and since then it has been reinvestigated and confirmed (29). However, the hydration constant may vary by body size. Pitts and Bullard (30) demonstrated that as body size increases, there is a slight decrease in the TBW/FFM. This may be related to larger mammals having a larger proportion of their body mass comprised of bone, as skeletal tissue is comparatively “dry” (30). This could certainly pertain to the African elephant, which may have a hydration constant different from 0.73. For instance, if the hydration constant was 0.71 body fat for this sample would be ~3-14%. If the hydration constant was 0.75 body fat for this sample would be ~8-18%. Even though the exact hydration constant of the elephant is unknown, it should not affect the primary results, as any change in the hydration constant would result in a linear transformation of the FM values.

Body fat percentages in this study sample ranged from approximately 5% to 16%. Most African ungulates have little subcutaneous fat, which may help facilitate heat

dissipation (31). Most published studies examine only small areas of the body, which show limited quantities of fat, and may be inappropriate to extrapolate to total body fat. For example, female giraffe are found to have 0.52-1.39% rump fat (31). Similarly, the rump fat percentage of male kudu and blesbok is 1.3% and 1.4%, respectively (32). Comparatively more work has been done with domesticated animals. For instance, horses have on average 5.1-22.3% body fat, which is correlated with rump fat (33). Elephants have a low surface area to volume ratio, so it would be expected elephants may have little subcutaneous fat, however the study sample is from zoos where food is plentiful and of high quality. To determine the accuracy of our body composition measures would require comparing our technique to carcass analysis, something that is not feasible.

Obesity has been associated with anovulation in other mammalian species (7, 34, 35), and in previous studies a relationship between body condition indices (e.g., BMI and BCS) and cyclicity status was observed in zoo African elephants (5, 6). However, the current study did not find such a relationship between adiposity and cyclicity status, which could be due to a variety of factors. For instance, our sample size (n = 20) was smaller than our previous work (9), which examined 50 elephants (half cycling, half not). Based on our data, to have 0.80 power to detect an association between FM and cyclicity status would require approximately 170 elephants. Differences in results may be age related, which is supported by a recent study demonstrating age is positively associated with acyclicity (4). We found non-cycling elephants were significantly older and heavier than those that were cycling, with age and weight strongly positively correlated. In our previous study (9), we did not adjust for age, which might explain the discordance in results. Further, BCS correlated most strongly with weight. Therefore, it appears BCS

may be capturing the overall weight, and not the amount of fat of the elephant. As age and weight are highly correlated (ρ=0.679), age may be driving the relationship between BCS and cyclicity status, and should be considered in future studies.

The elephant's metabolic state, rather than absolute FM, may be more important to consider regarding reproductive and overall health. FM, adjusted for FFM, was positively correlated with glucose levels, and FM, adjusted and unadjusted, almost reached significance with insulin levels. There was one insulin outlier, and although there was no reason to exclude it, running the analyses without this data point resulted in a significant correlation between unadjusted and adjusted FM and insulin (ρ= 0.479, P= 0.038; ρ=0.516, P=0.028, respectively). In a post-prandial state, elevated blood glucose levels stimulate insulin secretion (36), in turn promoting glucose uptake and utilization, and suppressing gluconeogenesis (36); therefore, abnormal insulin secretion, in addition to insulin resistance, can lead to hyperglycemia (36). Hyperglycemia and hyperinsulinemia are both associated with obesity and comorbidities (36, 37). Although FM may not be associated with acyclicity, it may impact health in other ways, such as increasing the risk of arthritis and calving problems (38).

Hyperglycemia and hyperinsulinemia stimulate an inflammatory state (39, 40) and in other species, inflammation is associated with reproductive impairments and obesity (15). However, our results do not indicate an inflammatory state. The SAA reference interval for clinically healthy Asian elephants is 0-47.5mg/L (41). No elephant, cycling or non-cycling, had levels greater than 3.5mg/L, suggesting all the elephants in this study had low levels of inflammation. Further, SAA levels were not correlated with FM, adjusted or unadjusted, even though SAA is the most sensitive acute phase protein in

elephants (41). TNF-α was not associated with FM, adjusted or unadjusted. Taken together, results suggest these elephants did not have chronic inflammation, as observed in other species that exhibit obesity and reproductive impairments (15, 42), at least measured by these inflammatory factors, thus these elephants may not be obese.

FM should be monitored as it is an active endocrine organ. In addition to other adipokines, white adipose tissue produces leptin (43). Leptin plays a permissive role in activating the reproductive axis, and when levels are abnormally high, as observed in an obese state (43), prevents ovarian steroidogenesis, inhibiting proper follicle development (44). This is likely not a reason for acyclicity in these elephants, as leptin levels were similar between the two groups; however, as leptin nearly correlated significantly with FM, there may be other related health issues to consider. For example, similar to girls with obesity (43), zoo elephants reach puberty at earlier ages than their wild counterparts (10). Earlier puberty will likely expose the elephants to more reproductive cycles and associated endogenous hormones over their lifetime, which may lead to the development of reproductive tract pathologies (10). With known endocrine function, continued fat accrual may possibly lead to a point where elephants show similar metabolic dysfunction exhibited by humans and domestic animals with obesity.

This study demonstrated that deuterium dilution can be used to estimate body composition in African elephants. We used a method that has proven accurate over the decades in other species with a 10^5 times difference in body size. Thus, although assumptions were made (e.g., hydration constant) and there was an inability to validate the technique by total carcass analysis, the method appears to be robust over time and

species. Results open up a new avenue of research questioning for large herbivores and the effects of diet and exercise on adiposity and its relation to health and reproduction.

In conclusion, obesity and inflammation were not unequivocally found to be significant factors for this study sample regarding acyclicity status. Regardless, there does appear to be a relationship between FM and metabolic health in African elephants. The majority of these elephants appear to be metabolically healthy, but some could be categorized as overweight or at risk for metabolic dysfunction. This supports the need to individualize management strategies, as each elephant responds uniquely to the environment and diet. This is of paramount consideration, as the zoo elephant population is currently not self-sustaining.

Acknowledgements

The authors thank Dr. Barbara Gower, Maryellen Williams, Heather Hunter, and Cindy Zeng at the UAB NORC's Metabolism Core for their assistance with hormone assays and mass spectroscopy and Dr. Katie Edwards at SCBI for inflammatory analyses. The authors thank Zoo Atlanta, Dallas Zoo, Jacksonville Zoo and Gardens, Maryland Zoo in Baltimore, Memphis Zoo, Zoo Miami, Nashville Zoo at Grassmere, North Carolina Zoo, Pittsburgh Zoo and PPG Aquarium, Riverbanks Zoo and Botanical Garden, San Diego Zoo Global, and Wildlife Safari for their agreement to participate in this study. Specifically, thank you to the zoos' elephant keepers and elephants. They made this study possible. A special thank you to the Birmingham Zoo and Pat Flora and his elephant team for their continuous support, help and input with method improvement.

Funding

This work was supported in part by the Eppley Foundation for Research, the UAB Nutrition Obesity Research Center (P30DK056336), the Diabetes Research Center (P30DK079626), and the Nathan Shock Center on Aging (P30AG050886). DEC is supported by the National Heart, Lung, and Blood Institute (T32HL105349).

Disclosure

The opinions expressed herein are those of the authors and not necessarily those of any other organization with which the authors are affiliated. DBA reports grants from NIH/NIDDK, grants from NIH/NIA, grants from NIH/NHLBI, outside the submitted work.

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Table 1. Body composition of zoo African elephants using deuterium dilution by the plateau method.

ID	BW (kg)	TBW (kg)	TBW (%TBM)	FFM (kg)	FM (kg)	FM (%)
1	2254	1703.21	75.55	2333	-79	-3.50
2	3620	2926.01	80.84	4008	-388	-10.73
3	4488	3809.38	84.87	5218	-730	-16.27
4	3454	4108.27	118.94	5628	-2174	-62.93

BW: body weight; TBW: total body water in kg and the percent of total body mass (TBM); FFM: fat free mass; FM: absolute fat mass in kg and percent fat mass.

Table 2. Body composition of reproductive-aged female zoo African elephants using deuterium dilution by intercept method.

ID	Age	BW (kg)	Nd (kg)	TBW (kg)	TBW (%TBM	FFM (kg)	FM (kg)	WTR (L/d)
A	32	3436	2467.22	2372.32	69.04	3249.75	186.25	301.01
B	51	4125	2874.53	2763.97	67.00	3786.26	338.74	260.40
C	30	3311	2210.63	2125.61	64.19	2911.79	399.21	214.77
D	26	2850	1982.87	1906.61	66.90	2611.79	238.21	458.38
E	26	3080	2050.21	1971.35	64.00	2700.48	379.52	317.24
F	26	3112	2089.72	2009.35	64.57	2752.53	359.47	385.71
G	35	3833	2558.20	2459.80	64.18	3369.59	463.41	291.87
H	37	4090	2942.45	2829.28	69.17	3875.73	214.27	441.79
I	43	4010	2860.40	2750.39	68.59	3767.66	242.34	489.45
L	33	2979	2068.54	1988.99	66.76	2724.64	254.36	341.28
M	33	3538	2263.92	2176.84	61.53	2981.98	556.02	234.60
N	37	3613	2534.21	2436.74	67.45	3337.99	275.01	262.13
P	33	3973	2835.94	2726.87	68.63	3735.43	237.57	285.99
R	32	3703	2514.20	2417.50	65.29	3311.64	391.36	338.25
S	34	3029	2056.79	1977.68	65.29	2709.15	319.85	215.63
T	16	2211	1575.02	1514.44	68.50	2074.58	136.42	169.45
U	43	4375	3020.52	2904.34	66.38	3978.55	396.45	319.15
W	48	3160	2120.27	2038.72	64.52	2792.77	367.23	297.92
X	40	3920	2654.58	2552.48	65.11	3496.54	423.46	366.13
Y	40	4465	2848.31	2738.76	61.34	3751.72	713.28	399.08

BW, body weight; Nd: dilution space; TBW, total body water in kilograms and the percent of total body mass; FFM, fat free mass; FM, fat mass; WTR, water turnover rate.

Table 3. Sample characteristics of the study sample.

	Cycling (n=13)	**Non-cycling (n=9)**
Age (years)	31.3 ± 2.1	39.9 ± 1.9[a]
Body Weight (kg)	3321 ± 137	3818 ± 176[b]
Fat Mass (kg)	312 ± 34	394 ± 53
Fat Free Mass (kg)	3028 ± 145	3448 ± 173
Height (in)	99 ± 1	101 ± 2
Length (in)	88 ± 2	96 ± 1[b]
Nulliparous	8/13	7/9
BCS	3.6 ± 0.2	4.3 ± 0.3
Glucose (mg/dL)	78.62 ± 4.52	83.44 ± 2.60
Insulin (μg/L)	0.309 ± 0.043	0.490 ± 0.202
Leptin (ng/mL)	8.53 ± 0.61	8.51 ± 1.12
SAA (mg/L)	0.2 ± 0.1	0.9 ± 0.4
TNF-α (pg/mL)	36.4 ± 9.1	20.6 ± 3.5

Data, except for nulliparous, presented as mean ± SE. Nulliparous data presented as number of elephants that were nulliparous out of total number of elephants. BCS: body condition score.

[a]$P<0.01$ significance between cycling and non-cycling elephants.

[b]$P<0.05$ significance between cycling and non-cycling elephants.

Table 4. Estimates for FM in statistical models to predict cycling status.

Model	Estimate	SE	95% CI		P
Cycling = FM	0.005	0.003	-0.002	0.012	0.131
Cycling = FFM FM	0.005	0.004	-0.004	0.014	0.249
Cycling = age$^{1.62}$ FFM FM	0.004	0.004	-0.004	0.013	0.332
Cycling = age$^{1.62}$ FFM^2 FM^2	0.000	0.000	-0.000	0.000	0.350
Cycling = age$^{1.62}$ nulliparous FFM FM	0.016	0.011	-0.006	0.037	0.158
Cycling = age$^{1.62}$ dominant FFM FM	0.004	0.004	-0.004	0.012	0.359
Cycling = age$^{1.62}$ dominant nulliparous FFM FM	0.014	0.010	-0.006	0.034	0.172
Cycling = age$^{1.62}$ male FFM FM	0.004	0.004	-0.004	0.013	0.337
Cycling = age$^{1.62}$ male nulliparous FFM FM	0.046	0.026	-0.005	0.097	0.075

Cycling: cycling status; FFM: fat free mass; FM: fat mass; Dominant: dominance status; Nulliparous: nulliparous status; Male: housed with males with direct contact, housed with males with indirect contact, or not housed with males.

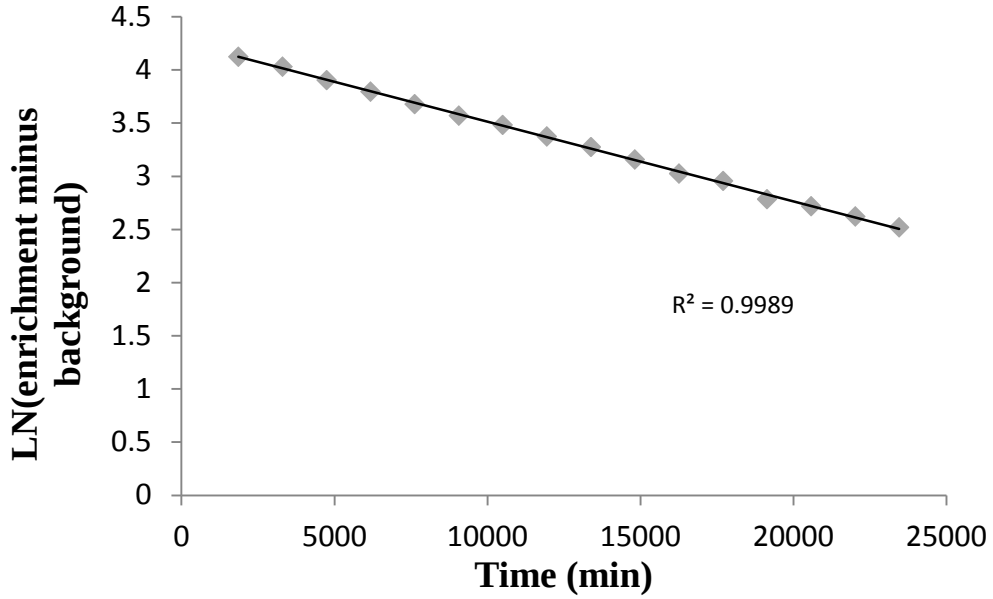

Figure 1. Natural log of deuterium concentration in venous blood in one reproductive-age female zoo African elephant after enriched orally with deuterated water at Time=0.

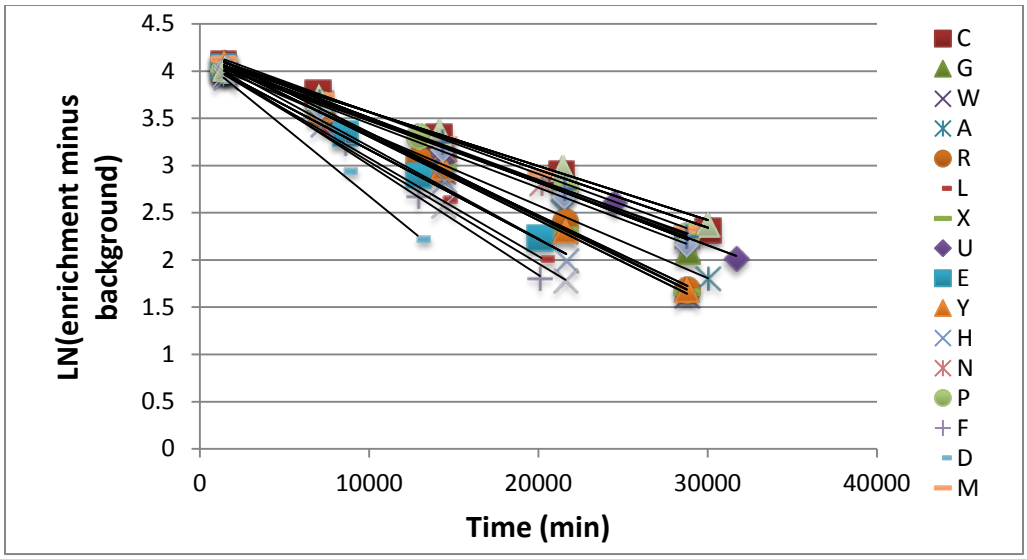

Figure 2. The washout curves for each elephant showing the decrease in deuterium enrichment over time.

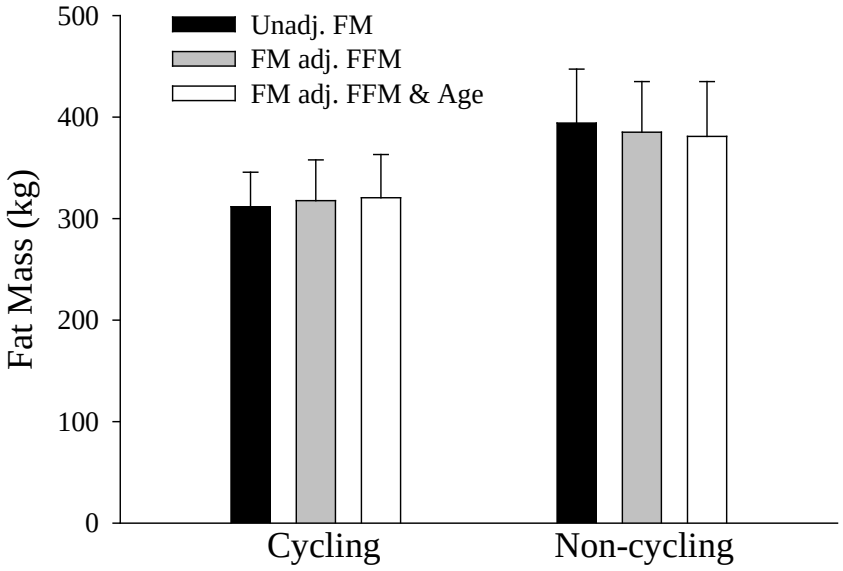

Figure 3. The difference between fat mass, fat mass adjusted by fat free mass, and fat mass adjusted by fat free mass and age with cyclicity status. FM: Fat mass; FFM: Fat free mass.

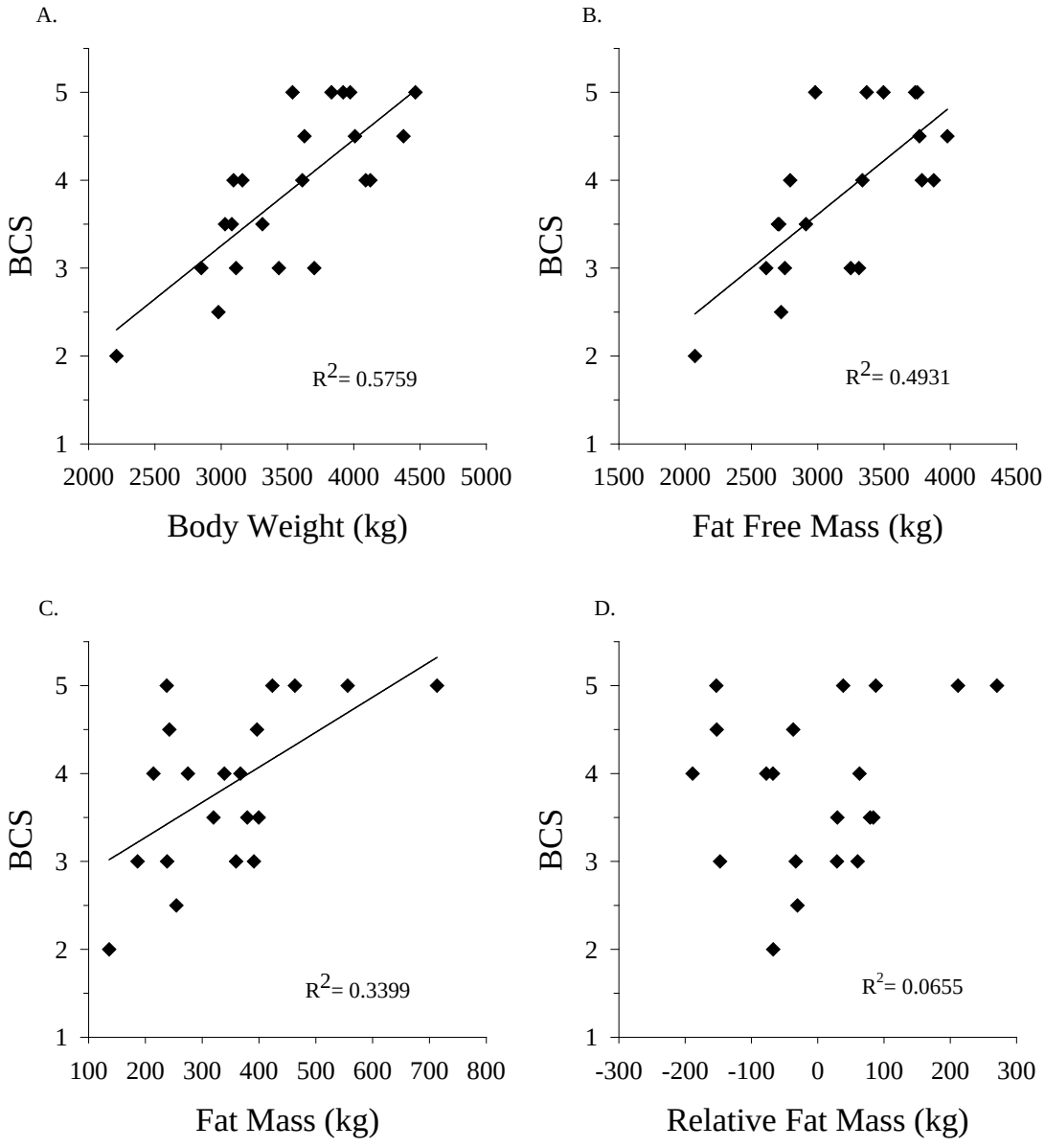

Figure 4.
A: The relationship between BCS and body weight; B: Relationship between BCS and FFM; C: Relationship between BCS and fat mass; D: Relationship between BCS and relative fat. Relative fat was determined by the residual for each elephant when fat mass was regressed on body weight.

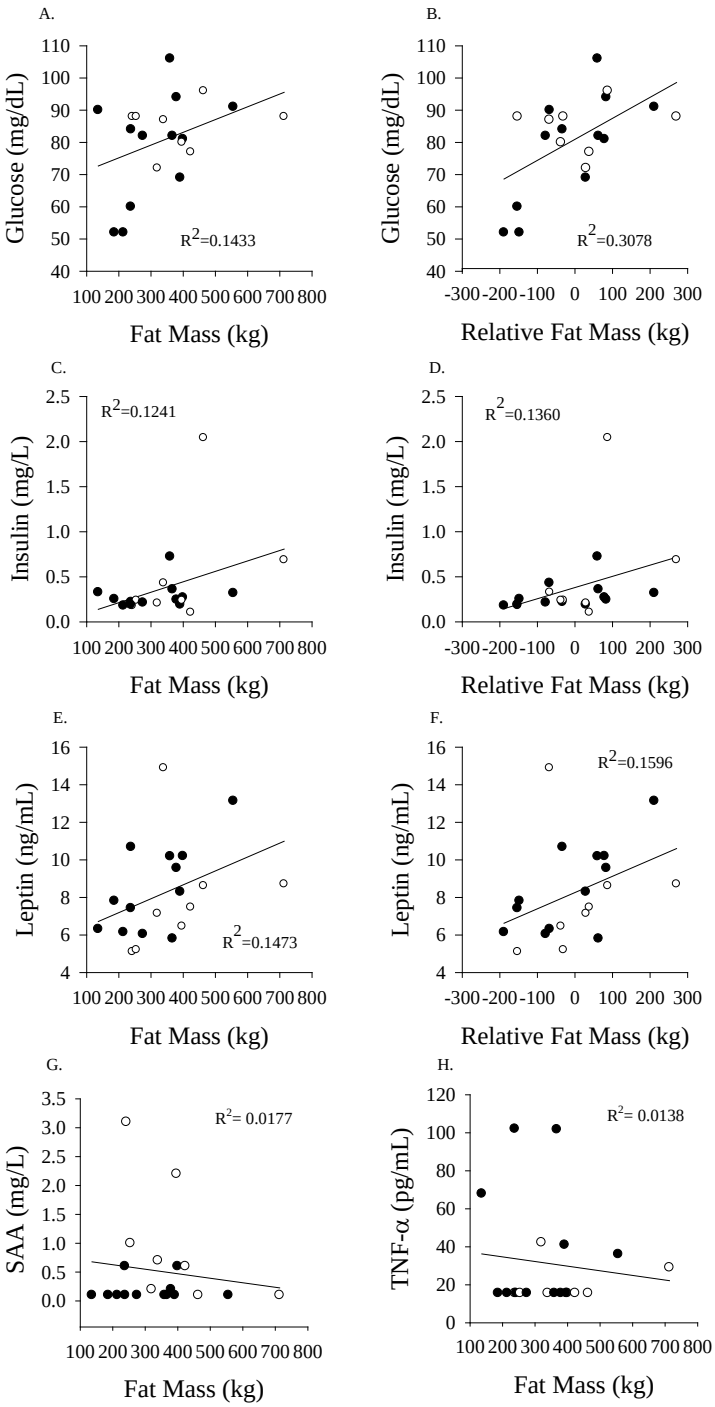

Figure 5. A: The relationship between fat mass and glucose; B: Relationship between relative fat mass and glucose; C: Relationship between fat mass and insulin; D: Relationship between relative fat mass and insulin; E: Relationship between fat mass and leptin; F: Relationship between relative fat mass and leptin; G: Relationship between fat mass and SAA; and H: Relationship between fat mass and TNF-α. Closed circles: cycling elephants; Open circles: non-cycling elephants. Relative fat was determined by the residual for each elephant when fat mass was regressed on body weight.

ADIPOSITY, REPRODUCTIVE CYCLING STATUS AND ACTIVITY LEVELS IN ZOO ASIAN ELEPHANTS (*Elephas maximus)*

by

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In preparation for *Experimental Biology*

Format adapted for dissertation

Abstract

With Asian elephants on the endangered species list, and population numbers continuing to decline, captive breeding is viewed as a means to protect the species from extinction. Unfortunately, captive populations are not self-sustaining, which may be due to obesity related health and reproductive issues. The objective of this study was to estimate body composition and investigate the relationship between fat mass with glucose, inflammation, and activity levels in male and female Asian elephants. In addition, to examine the association between cycling status and fat in females. Deuterium dilution was used to estimate body composition in 35 (n=28 females; n=7 males) Asian elephants. Each elephant was weighed, ingested deuterated water orally (0.05 mL/kg), and blood was collected from either an ear or leg vein prior to and five times after deuterium administration. The same vein location (i.e., ear or leg) was used consistently within an elephant for the sequential blood collections. Serum glucose, serum amyloid A, and activity levels were assessed. Activity levels were based on the elephant wearing an accelerometer on their front leg for a minimum of two days. For all elephants, body fat percentage ranged from 3.54% to 24.59%. Male elephants were significantly heavier (P=0.012), with significantly more fat free mass (FFM) (P=0.002), but not fat mass (P=0.915) compared to females. For all elephants, neither fat mass (ρ=0.054, P=0.773) nor relative fat mass (ρ=0.108, P=0.563) were correlated with serum glucose. Distance walked was negatively correlated with fat mass (ρ=-0.412, P=0.033), glucose (ρ=-0.476, P=0.008), and age (ρ=-0.374, P=0.041) for all elephants. Fat mass adjusted for FFM (P=0.031), in addition to adjusted for FFM and age (P=0.001), were significantly associated with cycling status in females. Deuterium dilution appears to be a tenable body

composition technique for Asian elephants. In this sample, fat mass predicted cycling status, and greater activity levels may contribute to lower glucose levels and fat mass.

Introduction

With Asian elephants (*Elephas maximus)* on the endangered species list, captive breeding is a means to protect the species from extinction (1, 2). Worldwide, there are approximately 16,000 Asian elephants under human-care (e.g., zoos, circuses, logging and tourist camps). However, the majority of captive elephant populations are not self-sustaining due to related reproductive and health issues (3-6). Therefore, emphasis has been placed on understanding the underpinnings of morbidity and poor reproduction (7).

The health and reproductive concerns observed in zoo elephants appear to be related to excess adiposity. Approximately 75% of females and 65% of male Asian elephants residing in American Zoo and Aquarium (AZA) accredited zoos are classified as either overweight or obese (8). In elephants, the typical obesity metric is a subjective body condition score (BCS) based on visual assessment of specific skeletal structures. Although BCS is a quick and inexpensive method, similar to body weight, any change in an individual's BCS or weight does not provide any information on the proportions of fat and fat free mass (FFM). Knowing the relative amounts of fat and FFM will allow for a clearer understanding of factors related to health and reproduction in elephants, as it does in other species (9).

In other species, obesity is associated with infertility and anovulation (10, 11). Further, in some instances, excess fat mass disrupts glucose regulation (12) and promotes a state of chronic low grade inflammation (13). Collectively, this has overall and reproductive pathophysiological ramifications, which may be ameliorated through higher levels of physical activity (14, 15). Physical activity is known to improve health by preventing weight gain (16), and improving insulin sensitivity (17), in part by increasing

skeletal muscle glucose uptake (62, 63). Because of the potential benefits of habitual physical activity, it is important to further understand its relationship with fat and metabolic health in elephants.

We have previously demonstrated that isotope dilution appears to be the most feasible *in vivo* body composition technique for an animal as large as the elephant (18). Isotope dilution relies on the use of the non-radioactive isotope deuterium to measure how much water is in the body (total body water: TBW) (19). Based on the assumption that TBW is a fixed proportion of FFM (i.e., hydration constant), knowing the amount of TBW ultimately allows for the calculation of FFM and fat mass (20).

The potential negative impact of excess fat on elephant health has just started to be acknowledged. To date, no studies have quantified fat mass in Asian elephants, females or males; therefore, the exact relationship between fat and reproduction or fat and metabolic health (i.e., glucose-insulin regulation, inflammation) is unknown. Therefore, the primary aim of this study is to quantify fat mass, and evaluate its relationship with reproductive cycling status, glucose, inflammation, and activity levels in male and female zoo Asian elephants.

Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of the National Zoo, the University of Alabama, Birmingham (UAB), and by participating zoos. Eight participating zoos were visited between June 2016 and January 2018. A total of 28 female and seven male Asian elephants (≥ 8 years of age; Table 1) were studied.

Female elephants were not pregnant, but four females had calves (calf age: 1.5 to 4.5 years). Males were not in full musth. All isotopes were analyzed blind to the animal's identification.

Body Composition

Body composition was determined by deuterium dilution as we previously described (18). In brief, elephants were weighed to the nearest pound or five pounds, depending on the institution's scale. To determine background isotope enrichment, venous blood was collected from an ear or leg vein prior to deuterated water administration. Blood collection location depended on the institution's preference; however, within elephant, each blood sample was collected only from one anatomical location (i.e., either always from the ear, or always from the leg). Thereafter, an oral dose of (99.9% APE) deuterium oxide (0.05 mL D2O/kg of weight; DLM-4-1000, Cambridge Isotopes, Tewksbury, Massachusetts) was orally administered using bread (Publix, Birmingham, Alabama) as a vehicle. Bread was weighed (to the closest 0.01 g, Pioneer, Ohaus, Pine Brook, New Jersey), deuterated water was carefully added, and the bread was then reweighed. The difference in weight represented the dose of deuterated water. Each elephant received four to six pieces of bread with approximately 40 to 50 g of deuterated water per piece. Blood (~9 mL) was sampled as regular intervals (~24, 120, 240, 360, and 480 hours) post deuterium administration. All samples sat at room temperature up to 30 minutes in an airtight container to allow for coagulation. Whole blood was centrifuged and serum was collected, aliquoted, and frozen at a minimum of -20°C until shipped on dry ice overnight to UAB. Samples were kept in a frost-free -80°C freezer until analysis.

Isotope ratio mass spectroscopy (Finningan Delta V Advantage, Thermo Fisher Scientific, Waltham, Massachusetts) analysis was conducted by the UAB Nutrition Obesity Research Center' Metabolism Core with guidance and support from the Energetics Research Group at the University of Aberdeen, Aberdeen, Scotland. As we previously described (18), the $^{2}H/^{1}H$ delta value was converted to parts per million and used to calculate deuterium dilution space size (18). The dilution space (N_d) is considered to reflect TBW content, which is then converted to FFM using the mammalian hydration constant (0.73).

The water turnover rate was calculated as previously described (18).

Determination of reproductive cyclicity status

For female elephants, reproductive cyclicity status was provided by participating zoos, and was based on progestogen analyses of longitudinal serum samples (21).

Serum Analyses

Analyses were conducted on the blood samples collected prior to deuterated water administration. The blood was collected in the morning prior to the elephant receiving their first meal of the day. Elephants' last meals were given no later than 10 hours prior to blood collection.

Serum glucose was measured by an automated glucose analyzer (Stanbio Sirrus, Stanbio Laboratories, Boerne, Texas, USA). Samples (3 µL) were analyzed in singlicate.

Serum Amyloid A (SAA) was determined using a RX Daytona automated clinical chemistry analyzer (Randox Industries-US Ltd., Kearneysville, WV, USA) and

commercially available reagents (150 µL), calibrators (0.1-500 mg/L), with two-level controls (Eiken Chemical Co. Ltd, Tokyo, Japan). Samples (4 µL) were run in singlicate and analyzed at the same time.

Activity

An accelerometer (Actigraph wGT3X, Actigraph, Pensacola, Florida, USA) was placed in two industrial strength plastic bags, and then inside a waterproof protective case. The case was then inserted into a pouch on a customized bracelet (Delta Rigging, Hurst, Texas) placed on the front leg of each elephant. The accelerometer was oriented such that the y positive axis was oriented up, and the z positive axis was oriented in the direction the elephant walked forward. The bracelet was worn for a minimum of two days from the time the elephant left the barn in the morning until when they were brought in at the end of the day (~6-9 hours/day). Each elephant was directly observed wearing their bracelet for a minimum of 20 minutes, and any activity associated with the leg wearing the bracelet was documented.

Step counts were determined by graphing the raw z axis data and counting each peak, which corresponded to a step. This technique was validated prior to the start of the study and in a subsample (n=22) of the study population. Prior to the study, two elephants were directly observed and video recorded while wearing the accelerometers for two to three days, a minimum of seven hours/day. Direct observations of steps were compared to the graphed peaks and found to be in agreement based on a Bland Altman plot, both for the two validation elephants and for elephants in the study sample. In addition, for elephants in the study sample, intraclass correlations (ICC) were conducted to investigate

the agreement between direct observed steps to those steps counted by the accelerometer. ICC (2,1) = 0.987, P<0.0001.

A standardized walk test was implemented to determine average stride length on a per elephant basis. Each elephant walked a premeasured distance (58 – 200 feet depending on institution) three times, while starting/ending time and steps taken were documented. For the purpose of this study, a step equated to when the leg with the bracelet was lifted up, moved forward along the z axis, and then was placed down different from the starting position. The distance of the standardized walk test divided by the number of steps taken to traverse the premeasured distance represented the average stride length. The mean of the average stride length was calculated based on the three standardized walk tests, and represented the overall average stride length for that particular elephant. Overall average stride length was multiplied by step counts to determine distance traveled. Because the duration each elephant wore the accelerometer differed, distance traveled was ultimately examined at 60-minute intervals.

Statistical analyses

All statistical analyses were performed using SAS 9.4 statistical software (SAS Institute, Cary, NC, USA) and specified prior to examining data, unless otherwise stated. Although 35 elephants were included in this study, four elephants were excluded from body composition statistical analyses because the amount of deuterated water ingested could not be determine; therefore, it was not possible to accurately calculate body composition. Calves derive the majority of their nutrients from the mother's milk until three years of age (22). One female had a calf younger than three years of age; therefore,

statistical analyses on body composition outcomes were done with and without her as the impact of lactation on the hydration state of the elephant is not known.

The primary model to address our main hypothesis was a generalized estimating equation (GEE), regressing cyclicity status on FM adjusting for FFM and age to the power lambda (age^{λ}). The lambda value for age was calculated by fitting a non-linear model based on data previously collected on 80 female Asian elephants, where age and cyclicity status were known. The best estimate of lambda was 4.4. FM, FFM, and $age^{4.4}$ were included as continuous variables. To adjust for factors related to residing in the same zoo, the zoo ID was treated as random effect in all the models. After examining the data, secondary sensitivity analyses were conducted on the primary logistic model and included the addition of fixed and random effects. Fixed effects included the addition of dominance status and whether the elephants were housed with male elephants. Dominance status was included as a dichotomized variable, while elephants were characterized as either not housed with male elephants, housed with males with direct contact, or housed with males without direct contact. Random effect included the addition of familial relatedness to the primary model.

Pearson correlations between FM and FFM, $age^{4.4}$, weight, glucose, relative fat, and distance walked were conducted, in addition to correlations between body weight and water turnover. Spearman correlations were run between FM and SAA, because of the non-normal distribution of SAA. Partial correlations between FM and glucose, adjusted for FFM, were conducted, in addition to distance walked and glucose, adjusted for FFM. Relative fat was determined by the residual for each elephant when FM was regressed on

weight. Relative FM is an estimation of the amount of fat the animal has taking into consideration their body size.

Descriptive statistics between cycling and non-cycling groups were assessed. *T-tests* were used to compare the means of age, body weight, FM, FFM, height, body length, glucose, and distance walked by cyclicity status. Wilcoxon test was used to compare the means of SAA by cyclicity status because of their non-normal distributions. Fisher's exact test was used to compare the proportion of nulliparous elephants by cyclicity status.

Descriptive statistics by sex were assessed. *T-tests* were used to compare the means of age, height, and body length. Wilcoxon test was used to compare the means of body weight, FM, FFM, glucose, distance walked, and SAA because of their non-normal distributions. Significance level was accepted at $P<0.05$ (2-tailed).

Results

At the time of body composition measurement, approximately 64% of the elephants exhibited normal reproductive cycles, compared to the 36% of elephants that exhibited abnormal reproductive cycles (Table 1). Eighty percent of the sample population were females. Descriptive statistics by sex are in Table 2.

Body composition

Deuterium dilution was used to estimate body composition (Table 3). For the entire sample population body fat percentage averaged 10.40% (SD: 4.99, range: 3.54 – 24.59%; n = 31). For females only, body fat percentage averaged 11.06% (SD: 4.97,

range: 3.54 – 24.59%; n=26), while for males only, body fat percentage averaged 7.01% (SD: 3.88, range: 4.23 – 12.44%, n=5).

Males weighed significantly more (P = 0.012) and had significantly more FFM compared to females (P = 0.002). There were no significant differences in FM by sex (P=0.915).

Body composition and reproductive cycling status

Predictors of cycling status were analyzed using GEE models in females (n=28) (Table 4). Unadjusted FM was not significantly associated with cycling status (P=0.075; Figure 1), but was significantly associated with cycling status when adjusted for FFM (P=0.031) and FFM and age$^{4.4}$ (P=0.001). The inclusion of male interaction to the primary model was significant (P=0.004), as was when dominance status was included in the primary model (P=0.025). Weight, unadjusted or adjusted for age$^{4.4}$, was not significantly associated with cycling status (P=0.740; 0.351, respectively). The addition of family relatedness as a random effect to the primary model resulted in a non-significant model (P=0.117).

Body composition, glucose, SAA, and activity levels

The correlation between FM and relative fat with glucose was not significant (Figure 2A&B), nor was there a significant correlation between FM and SAA (Figure 3). Fat mass, but not FFM, was negatively correlated with distance walked (Figure 4&5, respectively). Distance walked was negatively correlated with glucose levels (Figure 6), but not after adjusting for FFM (ρ=-0.105, P=0.611). Distance walked was negatively

correlated with age (Figure 7). Weight was positively correlated with water turnover rate (ρ=0.647, P<0.001). There were no significant differences in glucose by sex (Table 2). Males tended to walk more than females (Table 2)

Discussion

This study examined the relationship between adiposity and cycling status, in addition to the relationship between adiposity, glucose, SAA, and activity levels in zoo Asian elephants. We found that FM, adjusted for FFM and age, was significantly associated with cycling status, such that non-cycling elephants had less FM compared to cycling elephants. Greater activity levels were correlated with lower glucose concentrations and FM.

Deuterium dilution is an available *in vivo* body composition technique that can be applied to the Asian elephant. Body composition by deuterium dilution hinges on the proportion of water in FFM. Because the exact relationship is not known for the Asian elephant, we used the average mammalian hydration constant, for females and males. Some have postulated that women may have greater variability in water and bone mineral content compared to males (23, 24), but literature reviews do not support this notion (20, 25). Regardless of the elephant's hydration constant, any disagreement would lead to a linear transformation of the FM result.

The use of the average mammalian hydration constant may not be appropriate in lactating elephants. Lactating mice have approximately 10% greater total body water than non-lactating mice (26); however, no differences were observed in total body water between lactating and non-lactating cows (27). The observed species differences are

likely attributed to differences in body size. As the animal gets bigger the effects of lactation get smaller due to the scaling of milk production and metabolic rate, and water turnover. Although the one female in our study with a calf younger than three years of age had a comparatively higher water turnover for body weight, it was still within two standard deviations of the mean. Ultimately the impact of lactation is not known, but the inclusion or exclusion of this female in the analyses did not alter the significance of the statistical models.

In this study sample, body fat percentages ranged from approximately 3.5% to 26%. The lower range of values are comparable to our observed results in African elephants (18); however, the Asian elephants in this study had comparatively higher body fat percentages. This may simply be attributed to the animals which participated in the study or Asian elephants may be more susceptible to fat accrual based on the zoo environment. In a recent survey of 240 elephants housed in North American zoos, based on a body condition score (scale from 1-5, higher numbers implying greater fat), the majority of female African elephants were scored as a four, whereas the majority of Asian elephants were scored as a five (8). In addition, body composition differences by sex were observed. Similar to other species (28), male elephants had significantly more FFM and less relative FM compared to the females. Future studies should investigate sex differences in fat deposition and related health outcomes, particularly with aging.

In other mammals, excessive and deficient levels in fat are associated with infertility (29-31). We did not find that greater amounts of fat to be associated with anovulation in the present study. Rather, non-cycling elephants, after adjustment, had less

fat mass compared to cycling elephants. There may be a couple explanations related to this finding.

As previously eluded to, evidence has indicated that too low of levels of fat are associated with reproductive cycle disorders and reproductive dysfunction (32, 33). In women, it has been postulated that body fat percentages lower than 17% may lead to amenorrhea (34). Whether the elephants in the present study have insufficient fat stores to exhibit a normal reproductive cycle is not likely. While we do not know the range of body fat percentages in wild elephants across the dry and wet seasons, wild elephants can undergo anestrous when food quality is low (35). This is likely not the case for the study population, as food and water are plentiful in zoos. In addition, in our study population, the female with the lowest body fat percentage (~3.5%) did exhibit a normal reproductive cycle. Taken together, this may indicate that insufficient fat stores are not contributing to abnormal reproductive cycling.

Another possible explanation for the observed relationship may be related to a disruption in the hormonal milieu in non-cycling elephants. Brown and colleagues demonstrated that non-cycling Asian elephants had approximately three times higher estradiol levels compared to cycling Asian elephants (36). Evidence has demonstrated that estradiol decreases fat accrual via uptake and storage of triglycerides in adipose tissue (37), increases in oxygen consumption (37), and decreases in food intake (38). Indeed, ovariectomized rats, mice, cats, and other species have greater overall FM compared to age-matched controls (39-41). Nevertheless, these results should be taken cautiously due to the small sample size. Further studies are warranted to determine if such a relationship persists.

Although excess FM may not negatively impact the reproductive cycle of Asian elephants, it may negatively influence other aspects of health. An obesogenic state can disrupt glucose homeostasis (42). In a healthy state, glucose concentrations are tightly regulated because of their physiological importance. Glucose is the preferential metabolic fuel for mammalian tissues and in part, regulates the metabolism of other metabolic substrates (43). In the elephant, glucose values often range between 60 and 116 mg/dL (44). The majority of the elephants in the present study were within this range, with no elephants appearing to be hyperglycemic. Further, we did not find a correlation between FM and glucose levels. However, two elephants had glucose values below 60 mg/dL. The lower glucose values may be related to greater activity levels. Activity levels were negatively correlated with glucose, FM, and age. Activity can help prevent weight gain (16) and improve insulin sensitivity (17) by increasing glucose uptake by skeletal muscle (45, 46). Such findings support the rationale for assessing physical activity interventions, and if benefits are continued to be observed, the implementation of activity programs for zoo elephants, particularly with aging elephants.

Chronic inflammation is associated with obesity and obesity-related infertility (47). However, our results do not support a relationship between relative fat mass and anovulation or a chronic low grade inflammatory state. There were no significant differences in SAA values between cycling and non-cycling elephants, nor was SAA correlated with FM. The SAA reference interval for clinically healthy Asian elephants is 0-47.5 mg/L (48). There were five elephants with elevated SAA levels, three females and two males, between eight and 47 years of age. At the time of blood sampling, one elephant had an abscess that was not life-threatening. This elephant had the second

highest SAA concentrations. Thus, the observed increased inflammatory concentrations likely reflect an injury or other underlying health concern rather than an obese state.

In conclusion, deuterium dilution is a tenable body composition technique for Asian elephants, and male Asian elephants have greater FFM, but less relative fat mass compared to females. Excess FM does not appear to be a factor contributing to abnormal reproductive cycles. The majority of the elephants in this study appear to have clinically healthy glucose and inflammatory concentrations, with greater activity levels associated with body composition. This supports the notion that understanding differences in body composition can help foster individualized welfare strategies on a per elephant basis.

Acknowledgements

The authors thank Dr. Barbara Gower, Maryellen Williams, Heather Hunter, and Cindy Zeng at the UAB NORC's Metabolism Core for their assistance with hormone assays and mass spectroscopy and Dr. Katie Edwards at SCBI for inflammatory analyses. The authors thank Cincinnati Zoo, Columbus Zoo, Fort Worth Zoo, Little Rock Zoo, Oklahoma City Zoo, Oregon Zoo, Santa Barbara Zoo, and Saint Louis Zoo for their agreement to participate in this study. Specifically, thank you to the zoos' elephant keepers and elephants. They made this study possible. A special thank you to the Birmingham Zoo and Pat Flora and his elephant team for their continuous support, help and input with method improvement.

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Table 1. Sample characteristics of the female study sample.

	Cycling (n=18)	**Non-cycling (n=10)**
Age (years)	27.1 ± 3.0	43.4 ± 2.8[a]
Body Weight (kg)	3315 ± 167	3400 ± 218
Fat Mass (kg)	422 ± 61[+]	326 ± 50
Fat Free Mass (kg)	2894 ± 148[+]	3074 ± 176
Height (in)	94 ± 2	93 ± 2
Length (in)	85 ± 2	86 ± 3
Nulliparous	10/18	8/10
Glucose (mg/dL)	72.11 ± 2.26	71.50 ± 2.60
SAA (mg/L)	17.2 ± 9.3	0.9 ± 0.4
Distance (km)	0.55 ± 0.08*	0.47 ± 0.15[#]

Data, except for nulliparous, presented as mean ± SE. Nulliparous data presented as number of elephants that were nulliparous out of total number of elephants. Distance walked is depicted on 60-minute intervals for a minimum of six hours.
[a]$P<0.01$ significance between cycling and non-cycling elephants.
*n =17; [+]n=16; [#]n=8

Table 2. Sample characteristics of the study sample.

	Females (n=28)	**Males (n=7)**
Age (years)	32.9 ± 2.6	20.4 ± 3.9[a]
Body Weight (kg)	3345 ± 131	4342 ± 556 [a]
Fat Mass (kg)	385 ± 43[*]	372 ± 161[+]
Fat Free Mass (kg)	2963 ± 113[*]	4208 ± 616 [a+]
Height (in)	94 ± 1	105 ± 3[b]
Length (in)	85 ± 2	94 ± 4
Glucose (mg/dL)	71.89 ± 1.70	65.57 ± 4.62
SAA (mg/L)	11.4 ± 6.1	20.7 ± 13.3
Distance (km)	0.53 ± 0.07[#]	1.02± 0.46[+]

Data presented as mean ± SE. Distance walked is depicted on 60-minute intervals for a minimum of six hours.

[a]P<0.05 significance between female and male elephants.

[b]P<0.002 significance between female and male elephants.

[*]n =26; [+]n=5; [#]n=25

Table 3. Body composition of female and male Asian elephants using deuterium dilution by intercept method.

ID	Age	Sex	BW (kg)	Nd (kg)	TBW (kg)	TBW (%TBM)	FFM (kg)	FM (kg)	WTR (L/d)
A	50	F	3343	2369.10	2277.98	68.14	3120.53	222.47	269.66
B	19	F	3699	2117.59	2036.14	55.05	2789.23	909.77	245.67
C	21	F	3483	2257.94	2171.09	62.32	2974.10	509.90	351.32
D	45	F	4345	2872.12	2761.65	63.56	3783.08	561.92	238.80
E	45	F	3611	2563.00	2464.42	68.25	3375.92	235.08	170.66
F	41	F	4819	3039.75	2922.84	60.65	4003.89	815.11	313.59
G	56	F	2854	1944.17	1869.39	65.50	2560.81	293.19	192.02
H	22	F	3313	2368.40	2277.31	68.74	3119.61	193.39	236.34
I	34	F	3733	2519.95	2423.03	64.91	3319.22	413.78	232.10
L	24	F	2089	1492.91	1435.49	68.72	1966.43	122.57	264.59
M	8	M	3198	2314.28	2225.27	69.58	3048.32	149.68	204.46
N	15	F	3520	2338.17	2248.24	63.87	3079.78	440.22	189.51
P	8	F	2538	1636.88	1573.93	62.01	2156.06	381.94	119.00
R	42	F	4216	2753.52	2647.61	62.80	3626.87	589.13	227.22
S	8	M	3128	2274.39	2186.91	69.91	2995.77	132.23	290.46
T	29	M	7382	4850.40	4663.85	63.18	6388.83	993.17	534.87
U	29	F	3484	2551.41	2453.28	70.42	3360.66	123.34	274.61
W	18	F	2762	1971.29	1895.47	68.63	2596.54	165.46	244.29
X	28	F	3526	2234.32	2148.38	60.93	2942.99	583.01	241.45
Y	24	M	4740	3315.16	3187.65	67.25	4366.65	373.35	419.31
Z	11	F	2064	1441.06	1385.64	67.13	1898.13	165.87	174.12
AA	10	F	1823	1314.90	1264.33	69.35	1731.96	91.04	127.51
BB	46	F	3062	2178.23	2094.45	68.40	2869.11	192.89	261.10
CC	46	F	3329	2223.51	2137.99	64.22	2928.76	400.24	228.41
DD	21	F	3300	2314.23	2225.22	67.43	3048.25	251.75	217.45
EE	36	F	3146	2065.05	1985.62	63.12	2720.03	425.97	184.51
FF	46	F	3214	2268.40	2181.15	67.86	2987.88	226.12	202.95
GG	34	M	4454	3219.28	3095.46	69.50	4240.36	213.64	332.84
HH	44	F	3828	2459.96	2365.34	61.79	3240.20	587.80	129.21
II	42	F	4423	2907.73	2795.89	63.23	3829.99	592.01	216.27
JJ	31	F	3538	2291.55	2203.41	62.28	3018.37	519.63	157.31

BW, body weight; Nd: dilution space; TBW, total body water in kilograms and the percent of total body mass; FFM, fat free mass; FM, fat mass; WTR, water turnover rate.

Table 4. Estimates for FM in statistical models to predict cycling status.

Model	Estimate	SE	95% CI		P
Cycling = FM	0.002	0.001	-0.0002	0.005	0.075
Cycling = FFM FM	0.005	0.002	0.0005	0.010	0.031
Cycling = age$^{4.4}$ FFM FM	0.008	0.003	0.003	0.013	0.001
Cycling = age$^{4.4}$ dominant FFM FM	0.008	0.004	0.001	0.053	0.025
Cycling = age$^{4.4}$ male FFM FM	0.008	0.003	0.003	0.013	0.004

Cycling: cycling status; FFM: fat free mass; FM: fat mass; Dominant: dominance status; Nulliparous: nulliparous status; Male: housed with males with direct contact, housed with males with indirect contact, or not housed with males.

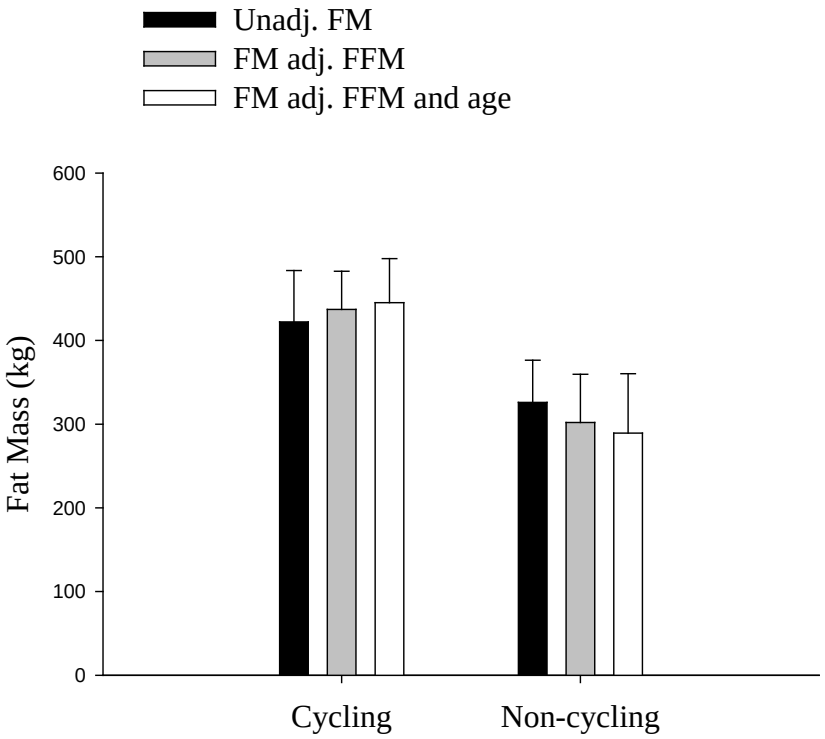

Figure 1. Mean differences in fat mass, fat mass adjusted by fat free mass, and fat mass adjusted by fat free mass and age by cycling status. FM: Fat mass; FFM: Fat free mass.

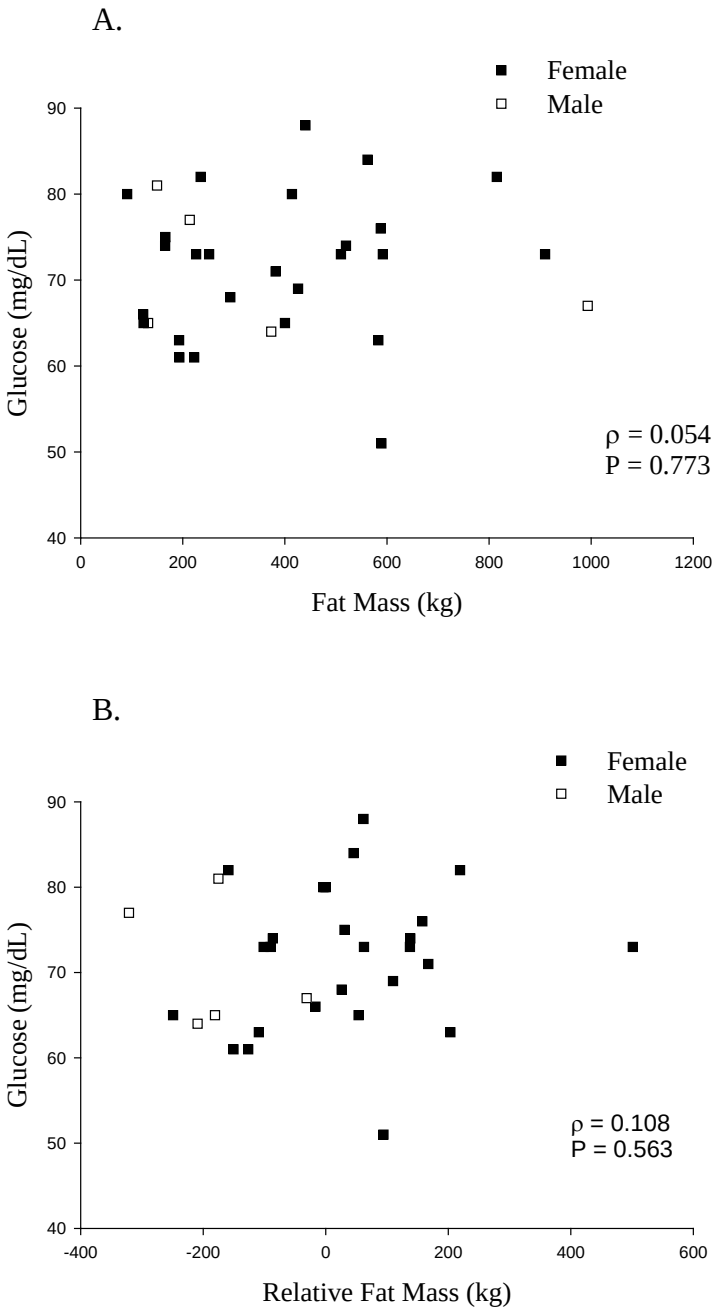

Figure 2. The relationship between body composition and glucose. A: The relationship between fat mass and glucose; B: Relationship between relative fat mass and glucose. Relative fat mass was determined by the residual for each elephant when fat mass was regressed on body weight. Analyses were done with females and males together. Different symbols are for representative purposes only.

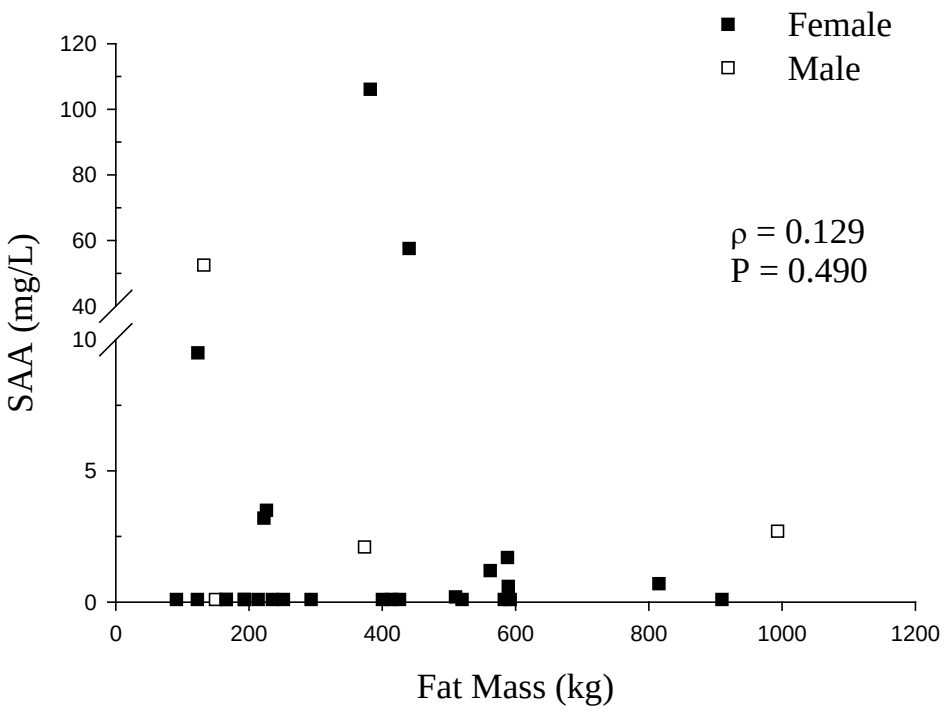

Figure 3. The relationship between fat mass and serum amyloid A (SAA). Analyses were done with females and males together. Different symbols are for representative purposes only.

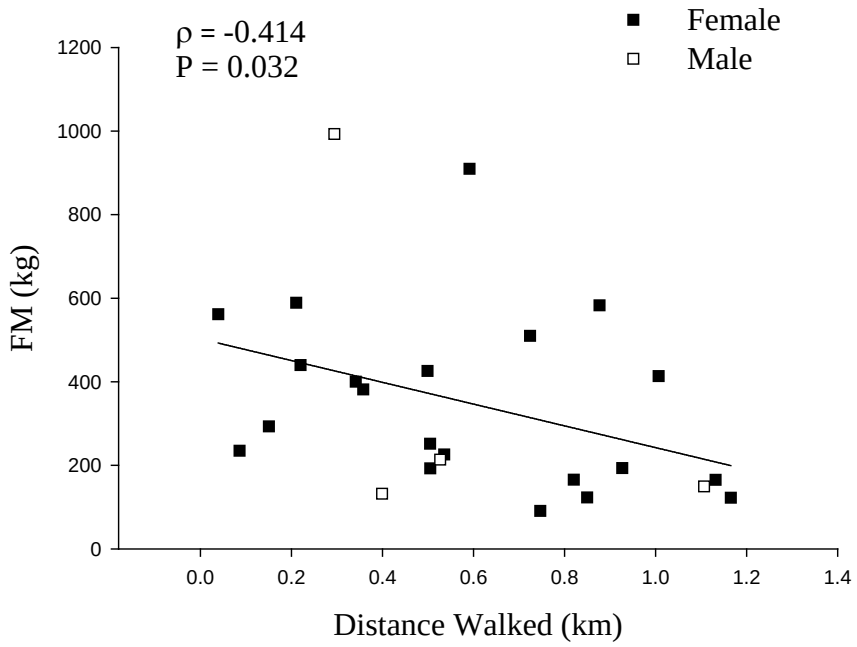

Figure 4. The relationship between distance walked and fat mass. Distance walked is depicted on 60-minute intervals for a minimum of six hours. Analyses were done with females and males together. Different symbols are for representative purposes only.

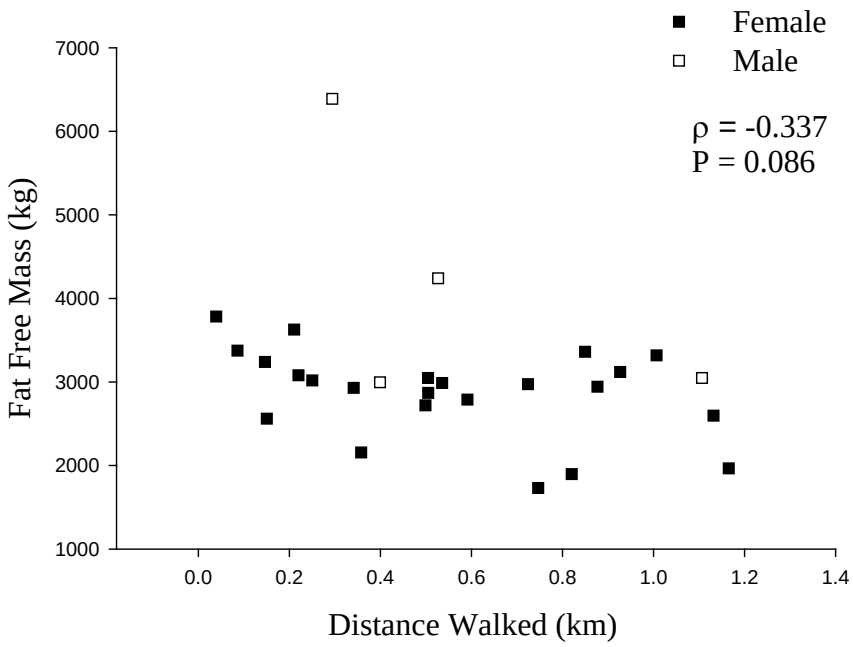

Figure 5. The relationship between distance walked and fat free mass. Distance walked is depicted on 60-minute intervals for a minimum of six hours. Analyses were done with females and males together. Different symbols are for representative purposes only.

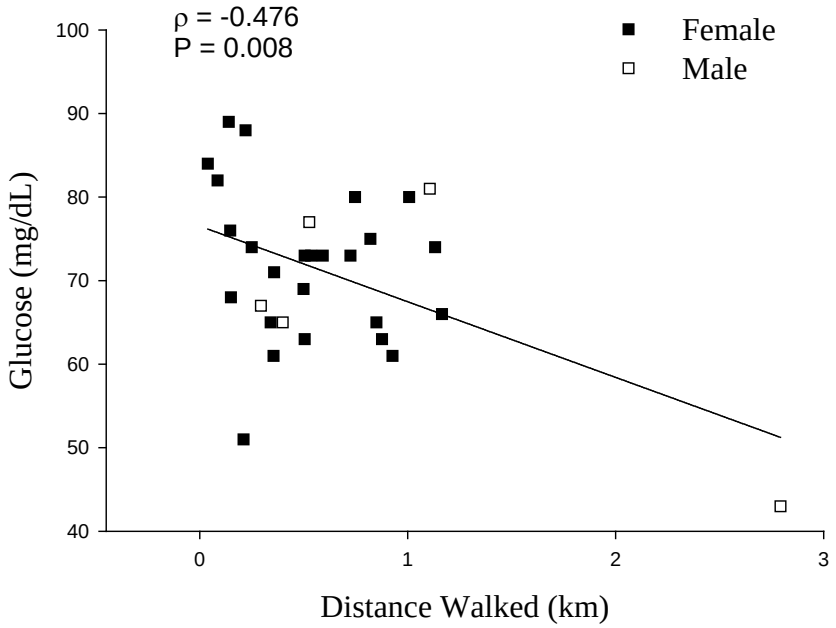

Figure 6. The relationship between distance walked and glucose. Distance walked is depicted on 60-minute intervals for a minimum of six hours. Analyses were done with females and males together. Different symbols are for representative purposes only.

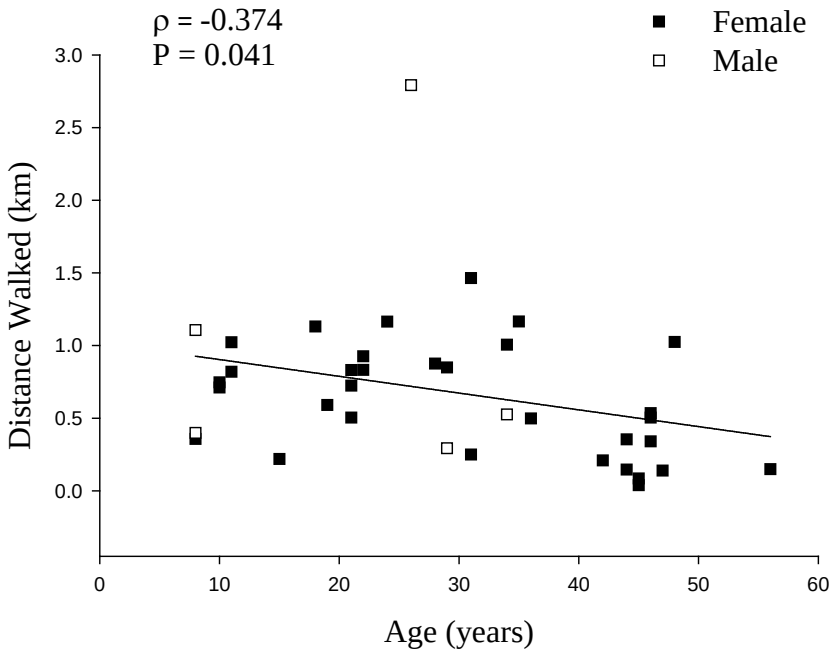

Figure 7. The relationship between age and distance walked. Distance walked is depicted on 60-minute intervals for a minimum of six hours. Analyses were done with females and males together. Different symbols are for representative purposes only.

FAT MASS COMPARED TO FOUR BODY CONDITION SCORING SYSTEMS IN THE ASIAN ELEPHANT (*Elephas maximus)*

by

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In preparation for *Zoo Biology*

Format adapted for dissertation

Abstract

Captive breeding is increasingly viewed as a means of insurance against elephant extinction. However, captive elephant populations are not self-sustaining due to reproductive and health concerns, which may be related to obesity. Categorizing overweight or obesity in elephants relies upon a qualitative index, the body condition score (BCS), which has not previously been validated against a measure of fat mass. The objective of this study was to compare BCS systems against quantified fat mass. Body composition was determined by deuterium dilution in 30 zoo Asian elephants. Each elephant was weighed and received deuterated water orally (0.05 mL/kg). Blood was collected from either the ear or leg prior to and then five times (~ 24, 120, 240, 360, and 480 h) after deuterated water administration. On the same day of deuterated water administration, photographs of the elephant were taken from every 45° angle. The photographs were used to score the elephant based on four different BCS systems (BCS_{Wemmer}, $BCS_{Morfeld}$, $BCS_{Fernando}$, $BCS_{Wijeyamohan}$). Body fat percentages, from deuterium dilution, ranged from 3.54% to 26.60%. The BCS_{Wemmer} ($P = 0.014$), $BCS_{Fernando}$ ($P = 0.002$), and $BCS_{Wijeyamohan}$ ($P = 0.009$) systems were associated with absolute fat mass, while $BCS_{Fernando}$ was associated with relative fat mass ($P = 0.019$). When adjusted for sex, BCS_{Wemmer} ($P = 0.015$; 0.012), $BCS_{Fernando}$ ($P=0.002$; 0.001), and $BCS_{Wijeyamohan}$ ($P = 0.007$; 0.005) were significantly associated with absolute fat mass and relative fat mass, respectively, and $BCS_{Morfeld}$ almost reached significance with relative fat mass ($P = 0.051$). Based on this study sample, the majority of the BCS systems reflect absolute fat mass, while only $BCS_{Fernando}$ predicted relative FM. Results also indicated sexual dimorphism impacts the scoring of elephants.

Introduction

With free-ranging elephant populations rapidly declining, captive breeding is increasingly viewed as a means of insurance against elephant extinction (1, 2). However, most captive elephant populations are not self-sustaining. Over the past decade mortality rates have been greater than birth rates (3). This is in large part due to a high prevalence of reproductive and health issues, including arthritis, dystocia and abnormal ovarian cycles (4-6).

Many of these reproductive and health concerns are believed to be associated with obesity. In fact, in a recent survey of elephants housed in American Zoo and Aquarium (AZA) accredited zoos, 75% of female and 65% of male Asian elephants were determined to be either overweight or obese (7). However, the assessment of overweight and obesity in elephants is based upon a subjective body condition score (BCS), rather than a quantified measure of body fat. Although higher BCSs have been shown to be associated with increased acyclicity, as well as leptin and insulin concentrations (8), it is not clear whether BCS accurately reflect the amount of fat (7, 9-11).

There are several different Asian elephant BCS systems that are commonly used, each involve a qualitative comparison of a photo of the elephant with a range of key skeletal descriptors (e.g., ribs, pelvic bone, backbone) (7, 12). Based on the appearance of these anatomical regions, the elephant is given a numerical score, with lower numbers representing less and higher numbers representing greater amounts of fat (7).

Although BCSs continue to be used and accepted as a way to measure body "fatness" (13, 14), this method was originally developed to assess the soft tissue of livestock to evaluate their nutrition and economic efficiency (15). Therefore, the

tendency to extrapolate BCSs for the measurement of fat, rather than for soft tissue, deviates from its original purpose without any quantitative data supporting such use. Further, the reliability of BCSs is contingent on the variation between and within the individual scoring the elephant (16, 17). Therefore, prior to accepting BCSs as a reliable means of estimating fat in elephants, it is critical to compare BCSs to a more direct measure of total body fat (18).

Due to the elephant's large body size, the best means to estimate total body fat mass (FM) is by deuterium dilution. Deuterium dilution is a non-destructive technique that measures the animal's total body water, which can then be used to estimate fat free mass (FFM) (19). Fat mass is calculated as the difference between body weight and FFM. Deuterium dilution has been validated in a range of animals, from bumblebees to the Atlantic Walrus (20, 21), and we have previously shown the feasibility of using this method to measure body fat in African elephants (22). The primary objective of this study is to compare four different BCS systems with FM estimated by deuterium dilution to determine which scoring system most accurately reflects FM in both female and male zoo Asian elephants.

Methods

Animals

This study was approved by the Institution Animal Care and Use Committee of the University of Alabama, Birmingham (UAB), the Smithsonian Conservation Biology Institute (SCBI), and participating zoos. A total of 21 zoos were contacted, of which eight zoos participated and were visited between June 2016 and October 2017. Twenty-five

female (≥8 years of age) and five male (≥8 years of age) Asian elephants housed in seven accredited North American institutions were studied. Female elephants were not pregnant, but four females had calves (calf age: 1.5 to 4.5 years). Male elephants were not in musth at the time of the study.

Body Composition

Body composition was assessed as previously described (22). In brief, using the institutions' scales, elephants were weighed to the nearest pound or five pounds. Zoo personnel collected venous blood from an ear or leg vein prior to deuterated water administration to determine background isotope enrichment. An oral dose of (99.9% APE) deuterium oxide (0.05 mL D_2O/kg of weight; DLM-4-1000, Cambridge Isotopes, Tewksbury, MA) was administered using bread (Publix®, Birmingham, AL) as the vehicle. Bread was weighed (to the closest 0.01 g, Pioneer, Ohaus, Pine Brook, NJ), deuterated water was carefully added, and the bread was reweighed. The difference in weight represented the dose of deuterated water. On average, each elephant received four pieces of bread with approximately 40-50 g of deuterated water per piece. Post deuterium administration, blood (~9 mL) was collected at regular intervals (~24, 120, 240, 360, and 480 h). Whole blood samples sat up to 30 minutes in an airtight container to allow for coagulation, and were then centrifuged to separate serum. Serum samples were aliquoted, and frozen at a minimum of -20 °C until it was shipped on dry ice overnight to UAB. Samples were then kept in a frost-free -80 °C freezer until analysis.

As we previously described (22), isotope ratio mass spectroscopy (Finningan Delta V Advantage, Thermo Fisher Scientific, USA) analysis was carried out by UAB's

Nutrition Obesity Research Center's Metabolism Core with guidance and support from the Energetics Research Group at the University of Aberdeen, Aberdeen, Scotland. In brief, $^{2}H/^{1}H$ delta value was converted to parts per million, and used to calculate FFM based on the mammalian hydration constant (23). Fat free mass was then subtracted from body weight to infer FM.

Body Condition Score (BCS)

Four different BCS systems were identified in the literature, and each employ different scoring criteria. In elephants, the use of BCS systems have been coopted with lower scores to imply less fat and higher scores to imply greater amounts of fat. The BCS system developed by Morfeld and colleagues (2016), $BCS_{Morfeld}$, is based on three anatomical regions (ribs, pelvic bone, and backbone) using a 1 to 5 scoring system. The BCS system developed by Wemmer and colleagues (2006), BCS_{Wemmer}, is based on six anatomical regions (head, scapula, thoracic region, the area in front of the pelvic bone, backbone, pelvic bone) using a 0 to 11 scoring system. The BCS system developed by Fernando and colleagues (2011), $BCS_{Fernando}$, compares the test elephant to five reference photographs preassigned a score of 1, 3, 5, 7, and 9. The test elephant could be given one of the preassigned scores if it looks like the elephant in the reference photograph, or if it falls between the reference photographs, an even score is given, resulting in a scoring system from 0 to 10. The BCS system developed by Wijeyamohan and colleagues (2014), $BCS_{Wijeyamohan}$, is based on reference photographs coupled with a dichotomous key, scoring elephants from 1 to 10.

For each elephant, a set of photographs was taken by an observer around the elephant from approximately every 45° angle along the horizontal plane (≥8 photographs per elephant) on the same day deuterated water was administered. The photographs were used to score the test elephant based on the aforementioned BCS systems. To assess intra- and inter-assessor variability, three assessors scored each elephant three times, with a minimum of one week between scoring. Photographs were randomized prior to each scoring session. Scores were generated by DEC (the initials of the assessor) and two assessors who were trained by DEC. Intraclass correlations (ICC) were done to evaluate the correlation within and between assessors' scores. Intra-rater reliability for $BCS_{Morfeld}$, BCS_{Wemmer}, $BCS_{Fernando}$, and $BCS_{Wijeyamohan}$, ICC (2,1) = 0.760-0.908, 0.832-0.970, 0.756-0.971, and 0.776-0.971, respectively. Inter-reliability reliability for $BCS_{Morfeld}$, BCS_{Wemmer}, $BCS_{Fernando}$, and $BCS_{Wijeyamohan}$, ICC (2,1) = 0.584-0.744, 0.765-0.831, 0.597-0.824, and 0.591-0.655, respectively. Within each assessor, ICC (2,1) = 0.756 – 0.970. The first round of scoring showed the strongest inter-assessor reliability, ICC (2,1) = 0.655 – 0.831. Therefore, BCSs from the first round of scoring were averaged across assessors to determine the final BCSs for each elephant. There were no significant effects on the primary model outcomes when BCSs were used exclusively from one assessor's scoring or from other time points.

Statistical analyses

Statistical analyses for the primary models were performed using SAS v9.4 statistical software (SAS Institute, Cary, NC, USA), while secondary sensitivity analyses were performed using R (R Development Core Team, 2008). All statistical analyses were

determined prior to examining the data unless otherwise stated. Although body composition was conducted on 30 elephants, two elephants were excluded because they did not ingest their total deuterated water amount, and we were unable to determine the exact amount ingested. Therefore, calculating body composition accurately was not possible. Thus, all models included 28 elephants.

The primary models to address our main hypothesis were simple linear models regressing FM, FFM, body weight, or relative fat on each BCS system. Sex and age were then included in the primary model as covariates. Secondary sensitivity analyses were then conducted. Generalized linear mixed models (GLMM) regressed FM, FFM, body weight, or relative fat on each BCS system, with familial relationships treated as a random effect. To address familial relationship, a pedigree file was created (24). Then, zoo as a random effect and sex and age as covariates were included in the GLMM. Relative FM was determined by the residual for each elephant when fat was regressed on weight. Relative FM is the amount of fat the elephant has after taking into account body size, as overall FM and FFM typically increase with body size.

Descriptive statistics were assessed for the total sample, and then by sex. Significance was set at $P < 0.05$ (2-tailed).

Results

Descriptive statistics for the entire population and by sex (females: n=23 from seven zoos, mean age 31 ± 3.0 years, age range 8 – 56 years; males: n=5 from five zoos; mean age 21 ± 5.4 years; age range 8 – 34 years) are presented in Table 1. Body

composition was estimated by deuterium dilution based on the individual's washout curve. Corresponding BCSs for each elephant by BCS system are presented in Table 2.

The primary model investigated the relationship between each BCS system and body composition measures and body weight. BCS_{Wemmer} (R^2=0.210, P=0.037), $BCS_{Fernando}$ (R^2=0.305, P=0.014), and $BCS_{Wijeyamohan}$ (R^2=0.234, P=0.023) significantly predicted FM (Figure 1). Only $BCS_{Fernando}$ was associated with relative FM (R^2=0.185, P=0.019).

When the primary model was adjusted for sex and age, all BCS systems were significantly associated with FM (P<0.026), FFM (P<0.018), and body weight (P<0.032). BCS_{Wemmer} (R^2=0.395, P=0.030), $BCS_{Fernando}$ (R^2=0.506, P=0.002), and $BCS_{Wijeyamohan}$ (R^2=0.432, P=0.013) were associated with relative FM.

When the primary model was adjusted for sex, BCS_{Wemmer} (R^2=0.214, P=0.015; R^2=0.394, P=0.014, respectively), $BCS_{Fernando}$ (R^2=0.313, P=0.002; R^2=0.506, P=0.001, respectively), and $BCS_{Wijeyamohan}$ (R^2=0.255, P=0.007; R^2=0.431, P=0.006, respectively) were significantly associated with FM and relative FM. $BCS_{Morfeld}$ (P=0.051) was marginally significant with relative FM.

When the primary model was adjusted for age, all BCS systems were significantly associated with FM (P<0.022), FFM (P<0.040), and body weight (P<0.014). $BCS_{Fernando}$ (R^2=0.200, P=0.020) was associated with relative FM.

To account for possible effects related to genetic similarities, familial relatedness was included as a random effect. BCS_{Wemmer} (P=0.020), $BCS_{Fernando}$ (P=0.0002), $BCS_{Wijeyamohan}$ (P=0.020) were significantly associated with FM. None of the BCS systems were significantly associated with FFM (P>0.083), and only $BCS_{Wijeyamohan}$ was

significantly associated with body weight (P=0.038). $BCS_{Fernando}$ was significantly associated with relative FM (P=0.020).

To account for possible effects related to residing in the same zoo, zoo was included as a random, with age and sex included as covariates in the familial relatedness GLMM model. All BCS systems associated with FM (P<0.005) and residual FM (P<0.024). None of the BCS systems were significantly associated with FFM (P>0.163), although BCS_{Wemmer} almost reached significant (P=0.055). BCS_{Wemmer} and $BCS_{Fernando}$ were significantly associated with body weight (P<0.028), while $BCS_{Wijeyamohan}$ almost reached significance (P=0.058).

Discussion

This study investigated how four different BCS systems associated with body composition measured by deuterium dilution and body weight in zoo Asian elephants. To our knowledge, this is the first study to examine whether BCSs correspond to a rigorous measure of adiposity in elephants. Specifically, we found three out of four BCS systems significantly associated with absolute FM, unadjusted, while only $BCS_{Fernando}$ associated with relative FM, unadjusted.

The primary linear regression model tested if each BCS system could predict FM of the elephant. The Wemmer, Fernando, and Wijeyamohan BCS systems were able to significantly predict absolute FM, while the Fernando BCS also predicted relative FM. These results likely reflect the flexible scoring range for the systems. For example, the Wemmer, Fernando, and Wijeyamohan BCS systems score the elephants on a minimum of a 1-10 scale, while the Morfeld system only scores the elephants on a 1- 5 scale. By

having a smaller range of scores, the Morfeld system provides little differentiation and flexibility in assigning elephants to each score. Indeed, when examining the distribution of scores, there is substantial overlap between those elephants scored as a 4 and those scored as a 5.

Family relatedness was then included in the primary model as a random effect to account for potentially correlated residuals attributed to genetic relatedness. For example, the BCS of related elephants may be more similar because of a genetic predisposition to a certain body shape. However, these results were nearly identical to the results from the primary linear regression model.

When age and sex were accounted for in the primary model, all the BCS systems were significantly associated with absolute FM, FFM, and body weight. The Fernando and Wijeyamohan systems were also associated with relative FM. Following exploratory analyses, it was determined that the relationship between the Morfeld system and absolute FM, FFM, and body weight was mediated through age. This was also true for the relationship between the Wemmer, Fernando, and Wijeyamohan systems with FFM and body weight. Interestingly, the relationship between all the BCS systems and relative fat was mediated through sex, except for the Fernando system which independently predicts absolute and relative FM. This suggests factors related to being a male or female elephant influences the ability of the Wemmer and Wijeyamohan systems to predict the elephant's relative fat mass. Therefore, for the majority of the BCS systems tested, there appears to a be a sex bias in relation to relative FM. Relative fat mass is the amount of fat the elephant has after considering the animal's body size; therefore, relative fat is likely more important than absolute fat mass in terms of the impact of fat on overall health.

Sexual dimorphism is apparent in the elephant, with males being much larger and heavier compared to their female counterparts. The four included BCS systems assumed the criteria used to score the elephant is the same for males and females; however, this is probably not appropriate and our results support this. Similar to other species (25), based on our deuterium dilution results, males overall have significantly greater FFM and relatively less FM compared to females. This suggests a male could be scored a 4 due to their greater FFM deposition obscuring bone structures, while a female could be scored a 4 due to their greater FM obscuring bone structures. Both elephants receive the same score, but have entirely different body compositions. This may explain why the authors of the BCS systems did not see a significant difference in BCS by sex.

The Wemmer system consistently had the highest ICC results, both in terms of intra- and inter-rater reliability, while the Wijeyamohan system typically had the lowest. The stronger correlation between the Wemmer scores is likely attributed to the detailed and clear description of scoring for each anatomical region of the elephant. In comparison, the Wijeyamohan method directly compares the focal elephant photograph to a series of reference photographs, with an accompanying description. However, the reference photographs for the Wijeyamohan system were placed on multiple pages throughout the publication and left more room for individual interpretation. Descriptors must be clearly defined to allow assessors certainty of their interpretations (26), and this ambiguity may have led to the lower ICC results.

Scoring accuracy may also be contingent upon the pictures each BCS system uses as their examples. The difference in lighting and background, black and white versus color photographs, and size of the photograph may all impact how the elephant is scored.

There does not seem to be a standardized protocol for which photographs are used. Ultimately, the uniformity of the photographs may prove easier for the assessor to consistently score the elephant and future systems should consider the standardization of photographs.

To further improve future BCS systems, the six anatomical regions provided by the Wemmer system were included in a stepwise regression analysis, in addition to the surface area of the thoracic region, to predict FM. The six anatomical regions used in the Wemmer system reflect those used by the Morfeld system. Of the six anatomical regions used (temporal depression in the head, pronouncement of the scapula, visibility of the ribs, depression in front of the pelvic bone, visibility of the lumbar vertebrae viewed from behind the elephant, and visibility of the pelvic bone) and the surface area, the stepwise selection resulted in a model with only two explanatory variables, the ribs and the surface area of the thoracic region. The lumbar vertebrae although originally significant, was not significant after adjusting for the ribs. The other anatomical regions relied upon may reflect anatomical changes associated with age rather than nutritional status. For example, Albl (1976) took a series of direct body measurements to investigate their relationship with subcutaneous fat and muscle mass in wild African elephants. Albl found that most of the direct measurements were indicative of age and not nutritional status. Of relevant measurements, the temporal dent and the scapular depression were found to be associated with age. Further, in other species, older age is associated with increased muscle loss (28), particularly in females (29). The clear pronouncement of anatomical regions, like the pelvic bone, may be related to age-related muscle loss rather than fat stores. These results should also be considered in future BCS system development and refinement.

Elephants exhibit sexual dimorphism, yet BCS systems have been generalized to the entire species. The inclusion of both males and females in this study provided the opportunity to demonstrate that there are inherent sex differences within most BCS systems, albeit with a smaller sample size of males. In addition, by using multiple assessors to score each elephant multiple times, it was possible to examine which BCS system proved most consistent. This is valuable information as BCS is a tool widely used by individuals of various backgrounds. Because only elephants under human care were used in this study, it is not known if the results carry over to free-ranging populations. Nevertheless, this was the first step required in determining the validity of BCS systems for elephants.

The use of deuterium dilution to quantify total FM was the major strength of this study. Deuterium, a non-radioactive isotope of hydrogen, replaces hydrogen in water molecules, allowing the measurement of total body water (30). There is a relationship between total body water and FFM in a mammal, termed the hydration constant, ultimately allowing for body composition quantification (31). Although assumptions were made (e.g., appropriate hydration constant used) and deuterium dilution has not been validated by total carcass analysis in Asian elephants, the method appears to be robust over time and species (16, 21, 32-35).

In conclusion, this study suggested that while subjective BCS can be used to reflect absolute body fat in Asian elephants, a wider scoring range improves the overall predictability of absolute body fat. In the future, the development or the refinement of current BCS systems for each sex that include only those measures that regressed onto actual FM measured by deuterium dilution could minimize assessors' errors leading to

clearer fat classifications. It is critical to have a valid BCS system as BCSs are consistently used in Asian elephant husbandry, welfare and research. This ultimately will allow for the identification of individuals of which require intervention to improve overall wellness.

Acknowledgments

The authors thank Dr. Kenda Rigdon and Ms. Lindsay Pappas for scoring the elephants. Dr. Barbara Gower and Mrs. Cindy Zeng at the UAB Nutrition Obesity Research Center's Metabolism Core for their assistance with mass spectroscopy. Dr. Paul Lin for assistance with statistical analyses. The authors thank Columbus Zoo and Aquarium, Fort Worth Zoo, Little Rock Zoo, Oklahoma City Zoo, Oregon Zoo, Saint Louis Zoological Park, and Santa Barbara Zoo for their agreement to participate in this study. Specifically, thank you to the zoos' elephant keepers and elephants. They made this study possible. A special thank you to the Birmingham Zoo and Pat Flora and his elephant team for their continuous support, help, and input with method improvement. The opinions expressed herein are those of the authors and not necessarily those of any other organization with which the authors are affiliated.

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Table 1. Sample characteristics of the study sample.

	Total Sample N=28	**Females N=23**	**Males N=5**
Age (years)	29.1 ± 2.7	31.0 ± 3.01[a]	20.6 ± 5.4[b]
Weight (kg)	3506 ± 200	3273 ± 149[a]	4580 ± 772[b]
Fat Free Mass (kg)	3143 ± 170	2911 ± 121[a]	4208 ± 616[b]
Fat Mass (kg)	363 ± 46	361 ± 46	372 ± 161
Body Fat (%)	9.91 ± 0.95	10.54 ± 1.06	7.01 ± 1.74
$BCS_{Morfeld}$ (1-5)	4 ± 0.14	4 ± 0.16[a]	5 ± 0.24[b]
BCS_{Wemmer} (0-11)	7.5 ± 0.31	7.5 ± 0.35[a]	8.5 ± 0.60[b]
$BCS_{Fernando}$ (0-10)	7 ± 0.28	7 ± 0.32[a]	8 ± 0.51[b]
$BCS_{Wijeyamohan}$ (1 -10)	7 ± 0.24	6 ± 0.25[a]	7 ± 0.60[b]

Different letters represent significant differences within the row. $P < 0.05$.

Table 2. Body composition by deuterium dilution and BCSs for each elephant.

ID	Weight (kg)	FFM (kg)	FM (kg)	BF%	BCS_M (1-5)	BCS_W (0-11)	BCS_F (1-10)	BCS_{Wi} (1-10)
201	3343	3121	222	6.65	3	5.5	6	5
202	3699	2789	910	24.59	4	9.0	9	7
203	3483	2974	510	14.64	4	7.0	6	6
204	4345	3783	562	12.93	5	9.0	8	7
205	3611	3376	235	6.51	3	7.0	6	6
206	4819	4004	815	16.91	4	8.5	8	7
207	2854	2561	293	10.27	3	5.0	5	5
208	3313	3120	193	5.84	4	8.5	7	6
209	3733	3319	414	11.08	5	9.5	8	7
210	2089	1966	123	5.87	4	8.5	7	6
211	3198	3028	150	4.68	5	9.0	8	8
212	3520	3080	440	12.51	5	10.0	10	9
213	2538	2156	382	15.05	5	10.0	9	9
214	4216	3527	589	13.97	4	9.5	8	8
215	3128	2996	132	4.23	5	8.5	8	8
216	7382	6389	993	13.45	5	10.0	9	9
217	3484	3361	123	3.54	4	7.5	6	6
218	2762	2597	165	5.99	4	6.5	6	6
219	3526	2943	583	16.53	5	8.5	8	7
220	4740	4367	373	7.88	4	7.5	7	6
221	2064	1898	166	8.04	3	5.5	5	5
222	1823	1732	91	4.99	4	8.5	7	6
223	3062	2869	193	6.30	4	6.0	6	5
224	3329	2929	400	12.02	4	7.5	7	7
225	3300	3048	252	7.63	4	6.5	6	6
226	3146	2720	426	13.54	4	6.5	7	6
227	3214	2988	226	7.04	2	4.0	3	4
228	4454	4240	214	4.80	4	6.5	6	6

FFM: Fat free mass; FM: Fat mass; BF%: Body fat percent; BCS_M: $BCS_{Morfeld}$; BCS_W: BCS_{Wemmer}; BCS_F: $BCS_{Fernando}$; BCS_{Wi}: $BCS_{Wijeyamohan}$.

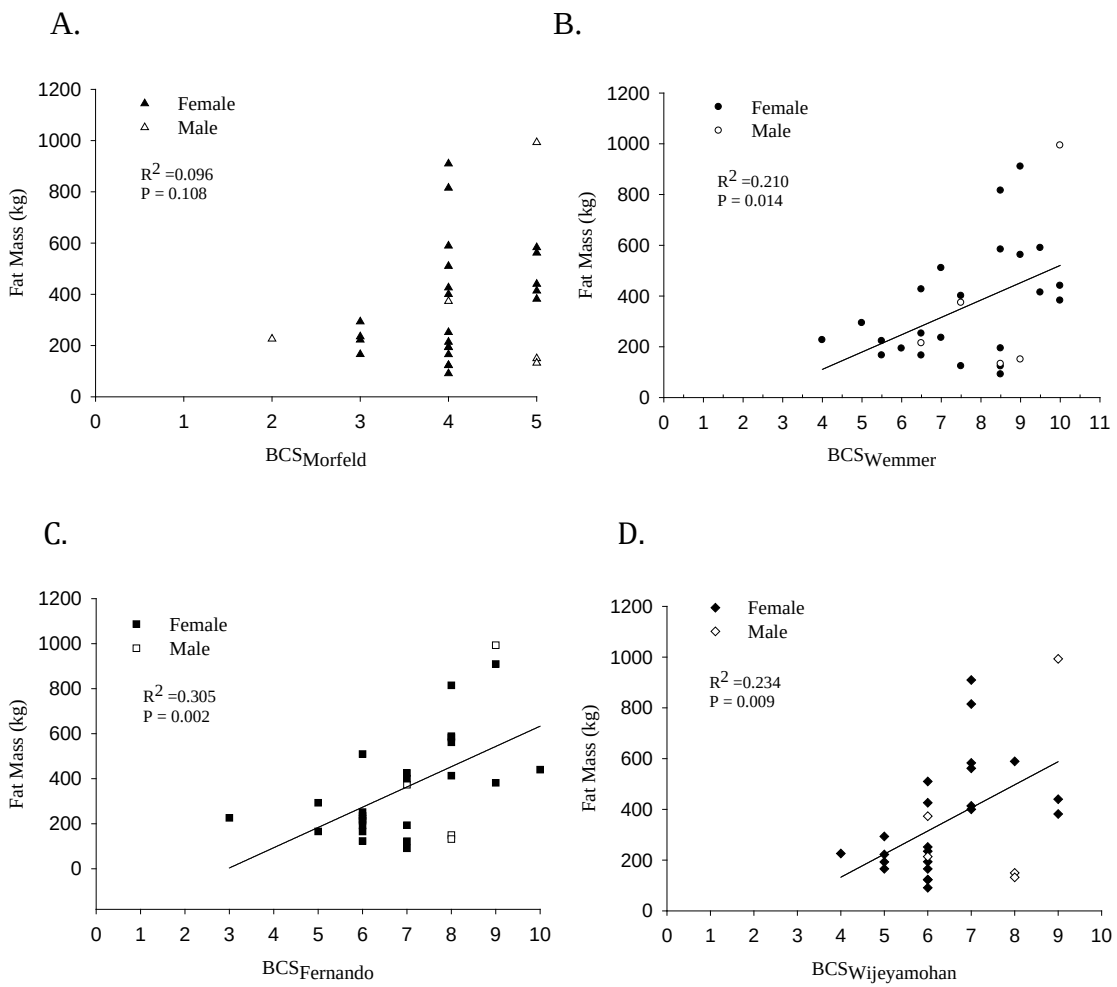

Figure 1. Distribution of FM, unadjusted, by the Morfeld BCS system (A), Wemmer BCS system (B), Fernando BCS system (C), and Wijeyamohan BCS system (D).

GENERAL DISCUSSION

Currently, zoo African and Asian elephant populations are not self-sustaining. For approximately every five deaths annually, there are only three births (6). If this trend does not reverse, the United States' zoo population may be reproductively nonviable within the next few decades (6). For this reason, the AZA and Elephant Species Survival Plan Committee has emphasized understanding the observed morbidities and poor reproduction in these populations to ultimately foster population growth (133). Therefore, in an effort to build upon the literature and further understand the link between excess fat mass and related co-morbidities, the focus of this dissertation was to quantify total fat and investigate its relationship with reproductive cycling status and metabolic health in zoo African and Asian elephants.

The primary aims of these studies were to 1) quantify adiposity and characterize its relationship with metabolic, inflammatory, and reproductive health in zoo African elephants; 2) quantify adiposity and characterize its relationship with metabolic, inflammatory, activity levels, and reproductive health in zoo Asian elephants; and 3) validate multiple BCS systems against adiposity in zoo Asian elephants. We observed a significant species difference regarding the relationship between fat mass and cycling status. Whereas fat mass did not predict cycling status in African elephants, it did in Asian elephants. Asian elephants with greater fat mass were more likely to be cycling. In African elephants there was a positive correlation between fat mass and metabolic biomarkers. Further, in Asian elephants, those elephants who walked more had lower fat mass, in addition to glucose levels compared to elephants who walked less. These data

suggest increased fat mass is not a significant risk factor for impaired reproductive cycling in either African or Asian elephants; however, fat mass may contribute to other metabolic dysfunctions, while physical activity may improve metabolic function. We observed that the current method to classify elephants as obese, the body condition score, reflects absolute FM more so than relative FM, and is age and sex biased depending on which specific system is used.

Discrepancy in obesity definitions

Hippocrates described a person as healthy when the four basic fluids that filled the body were in balance, while excess in fluids caused obesity (134). Over 2000 years later, there is still no clear agreement on what constituents a person with obesity. For example, obesity has been defined and redefined depending on the individual/institution as greater than 32%, 34% and even 38% body fat (135, 136) (137), with the most widely used definition of obesity as a BMI $\geq$30 kg/m^2 (138). Body mass index cut-offs originated from the concept of an "ideal weight," developed by an insurance company.

The Metropolitan Life Insurance Company (MLIC) was concerned with the association between body weight and mortality; therefore, MLIC examined said relationship by categorizing approximately 4 million of their policy holders by sex, height, and weight, with further differentiation based on body frame size (139). While the first tables categorized "ideal" weight (140), the tables were later reclassified to "desirable" weight (141), and ultimately changed to "height to weight" tables (142). These "height to weight" tables became the foundation for the current BMI cut-offs for underweight, normal, overweight, and obese individuals (143, 144).

Since the development of the MLIC "height to weight" tables, there has not been a shortage of proposed weight by height classifications. In 1975, the Fogarty Center Conference on Obesity recommended a BMI cut-off of 20.1 to 25 kg/m^2 for men and 18.7 to 23.8 kg/m^2 for women. In 1985 the National Institutes of Health (NIH) classified overweight as BMI $\geq$ 27.8 kg/m^2 for men and $\geq$ 27.3 kg/m^2 for women (22). In the same year the U.S. Department of Agriculture and U.S. Department of Health, Education and Welfare classified overweight as a BMI of 25 to 26 kg/m^2 for men and 24 to 25 kg/m^2 for women. Five years later the BMI cutoffs were changed yet again, this time to reflect age differences. Ultimately, sex and age were combined to state that overweight and obesity was a BMI $\geq$ 25 kg/m^2. In 1995, WHO Expert Committee on Physical Status assigned levels of BMI at 25, 30 and 40 kg/m^2 by an "arbitrary method of association between BMI and mortality" (15). In 1998, the NIH redefined overweight as a BMI of 25 to 29.9 kg/m^2 and obese as a BMI $\geq$ 30kg/m^2 (2). Not until 2010, did the BMI cut-offs recommended for Americans match those of the international communities (16).

There is no clear consensus on what constitutes a person with obesity even though humans are arguably the most studied species in relation to obesity. Further, the widely accepted definition of obesity does not rely upon a measurement of actual FM, but rather weight. If the concept of obesity in the context of humans is not clearly understood and defined, how, with a dearth of studies, can we confidently classify an elephant as obese.

Nevertheless, leading professionals subjectively categorize elephants under human care as obese (9, 145-147) because they appear to be heavier (148) and have higher BCSs (44) compared to their wild counterparts. This has led to hypotheses that obesity may be related to health and reproductive concerns observed in zoo elephants,

including foot problems, arthritis, and abnormal reproductive cycles (8-10, 12, 34, 42, 149). Although some, or all, of these health concerns may be related to excess fat, until we know what abnormal or excess fat accumulation (i.e., obesity) is for an elephant, or even the actual quantitative amount of fat, we should use the term cautiously.

Prior to our studies, and to our knowledge, there were no documented reports on *in vivo* body composition of the elephant. Clauss and colleagues reported on one African elephant, which had 244 kg of adipose tissue, not including subcutaneous fat, at the time of necropsy. Our studies documented African elephants with approximately 136 to 713 kg and Asian elephants with approximately 91 to 993 kg of total body fat. Although these results provided a snap shot of a small proportion of zoo elephants at one point in time, they served as a requisite starting point to understand adiposity in elephants. Additional research is needed to understand how fat, not simply body size or weight, relates to non-communicable diseases and morbidities to define an elephant with obesity.

Adiposity and BCS

BCSs are quick and inexpensive methods acting as a valuable tool in animal welfare. However, in general, it appears that BCS reflects the elephant's overall size to a greater extent than relative fat. Further, sex and age may greatly influence the BCS the elephant receives. Our results should offer guidance as to when and how to best use the BCS tool.

The BCS may be better suited to identify elephants that are malnourished versus "obese". For instance, large quantities of FFM may be inflating some BCSs, particularly for males, impairing the use of BCS for categorizing obesity. Rather, BCS should be a

tool geared to those animals ranking at the lower end of the scale. A state of undernourishment is characterized by a reduction in both FFM and fat mass, making the anatomical regions used for scoring more pronounced. This could be particularly useful with aging elephants. Fat free mass progressively declines during life (151) and is a contributing factor to a decline in gait speed (152), indicative of functional limitation (153). Further, weight loss is a strong predictor of mortality in advanced age, independent of disease (154). Therefore, tracking BCS and weight changes longitudinally in older elephants can quickly raise a red flag when condition deteriorates.

Reproductive cycling

Acyclicity rates have slowly continued to increase in both zoo African and Asian elephants over time (16, 155); however, acyclicity disproportionately effects zoo African elephants compared to zoo Asian elephants. Whereas approximately 52% of African elephants exhibit abnormal reproductive cycling, approximately 27% of Asian elephants exhibit abnormal reproductive cycling (16). Such a disparity suggests a species difference, which is supported by our findings. While there were no significant differences in fat mass between cycling and non-cycling African elephants, non-cycling Asian elephants trended to have less FM compared to cycling elephants, with higher fat mass significantly predictive of cycling.

Acyclicity has previously been linked to subjective obesity categorization. Freeman and colleagues demonstrated a correlation between high BMI and acyclicity in African elephants (17). More recently, Morfeld and colleagues demonstrated that higher BCSs were associated with an increase prevalence of acyclicity. Comparatively,

acyclicity is not associated with being overweight in Asian elephants (27), which appear to be heavier than their wild counterparts (27) and overall tend to have higher BCSs compared to African elephants (43). Body condition scores capture the total size of the elephant, rather than FM. Therefore, the relationship being detected with acyclicity may actually be body size, reflecting older elephants. It is suggested that African elephants experience indeterminate body length growth (157), leading older elephants to generally be larger. Indeed our results, and recently published accounts show a strong relationship between age and acyclicity (16).

The relationship between age and acyclicity is not likely to be related to menopause as it is generally agreed upon that elephants do not go through a true menopause. It appears that although the follicle reserve can be depleted in older elephants, for the most part, the elephant ovary is able to supply oocytes for ovulation through the entire lifespan of the elephant (29). Further, AMH concentrations, which is used as a marker for the number of healthy oocytes within the follicular reserve (31, 32), are not significantly different between cycling and noncycling elephants (30).

Rather the relationship between age and acyclicity may be linked to the elephant's role within the zoo herd. In the wild, an elephant herd is comprised of related females and their immature offspring. The herd is led by the matriarch, which is often the oldest and largest female. The matriarch is a repository of social information and she is responsible for building and maintaining the close bonds within herd mates. Unlike wild herds, many zoo herds are comprised of unrelated individuals; nevertheless, they still must live in social accord. Maintaining such harmony may fall upon the most dominant female, which, again, is generally the largest individual. It has been postulated that the energy

that goes into peace keeping among the unrelated individuals in a zoo herd, which may include incompatible females, compromises ovarian function (158). Almost at all institutions with both cycling and non-cycling females, the most dominant female was non-cycling (33). Interestingly, in Asian elephants, a relationship between dominance and cycling status does not appear to be present (159), suggesting a major species difference in how social dynamics may impact reproduction. It is acknowledged that African elephants, in regards to cycling status, are more sensitive to changes in their social and overall environment compared to Asian elephants (27). The social undertones may explain why African elephants disproportionately exhibit abnormal ovarian cycling status compared to Asian elephants, rather than the physiological effects of excess fat.

Adiposity, metabolic function and the role of activity

Irrespective of the role of fat on reproductive cycling status, excess fat can have other negative implications on reproductive and overall health in elephants. Elephants experience calving problems, characteristic of humans and other species with obesity, including stillbirths (146), dystocia (146), decreased flexibility in the pelvic region (160), muscle fatigue (160), and producing larger offspring (160). Birthing larger calves is an interesting phenomenon paralleling current trends observed in human babies and may be reflecting maternal effects. Maternal effects are nongenetic factors that contribute to changes in the offspring's phenotype (161), which in part can occur physiologically in utero (162) and may impact the offspring across their lifespan (163, 164).

As in other mammals (165), glucose is the major energy source for the elephant fetus and the placenta (166). Glucose is transported across the placental barrier from the

maternal blood into fetal circulation, and in elephants, is facilitated by the GLUT-1 and GLUT-3 isoforms (166). In humans, a surplus of intrauterine glucose stimulates hypertrophy and hyperplasia of pancreatic β-cells and adipocytes, increases the uptake of free fatty acid and storage of triglycerides in fetal adipocytes (167, 168), and promotes fetal de novo lipogenesis (169). This too may be occurring in zoo elephants, collectively contributing to larger calves being born, in addition to predisposing the calf to lifelong metabolic dysfunction and susceptibility to excess fat accrual. If indeed this is happening, it will only perpetuate an obesogenic cycle.

Maternal effects may also be impacting the sexual development of the offspring. There has been a decrease in menarche age with the rise in maternal and overall human obesity (170). Similarly, zoo-born calves begin cycling earlier compared to their wild counterparts. Cycling has been documented as early as four years of age in zoo Asian elephants (3, 159) and eight years of age in African elephants (8). Comparatively free ranging elephants typically reach sexual maturation between 10-14 years of age (3, 171, 172). Elephant gestation lasts for approximately 20 to 22 months (8), a long exposure window for the fetus. Speculatively, this exposure window may be impacting the observed precocious reproductive development via maternal effects related to the mother's body composition and metabolic profile. Future studies should focus on furthering our understanding of the intrauterine environment and related endocrine profiles, coupled with retrospective studies on offspring phenotypes and metabolic profiles.

In humans, it is acknowledged that metabolic health is improved via physical activity induced skeletal muscle activation (173). Skeletal muscle is the primary site for

insulin mediated glucose disposal (174) and fatty acid oxidation (175). Reduced activity could lead to impaired metabolic (174, 176), glycemic (177), and lipidemic (178) control. Our results suggest increased activity may improve metabolic health in elephants.

In addition to the metabolic benefits, increased activity can benefit the aging elephant population. In the human elderly, walking protects against a loss of physical function (179), and can reduce pain associated with arthritis (180). Our results demonstrate older elephants walk less, and it is acknowledged that there is a high prevalence of foot and joint issues in these populations (10, 145). Therefore, an emphasis should be placed on getting these elephants moving. Indeed, several zoos, particularly those with older elephants, already implement elephant yoga sessions and exercise regiments to improve flexibility and ease of walking. Our results and the literature support such programs and early implementation.

Final conclusions

The studies presented here were conducted to assess body composition and characterize the relationship between fat and reproductive and metabolic health in zoo African and Asian elephants. Excess adiposity does not appear to be related to abnormal reproductive cycles in either African or Asian elephants, but can be contributing to metabolic perturbations. Therefore, it may prove more useful in terms of understanding elephant morbidity and mortality to shift the focus from “ideal weight/adiposity” to “dangerous weights/adiposity”.

These studies were the first to quantify total body fat *in* vivo in African and Asian elephants and provided a necessary starting point for understanding the physiology

between adiposity and health concerns in these populations. However, future research needs to be conducted with more elephants from various backgrounds (i.e., captive versus semi-captive; European zoos versus North American zoos) and should include controlled studies for further elucidation. Specifically, how does aging impact body composition changes in such a long-lived animal, and are there sex differences? To promote fat loss and preservation of lean mass, should calories simply be restricted or high glycemic index (GI) foods be switched for lower GI foods, and what additive role does increased activity play? To improve metabolic health, should zoo elephants be placed on a weight cycling diet to mimic ecological changes elephants evolved to anticipate? By focusing on the body composition of the elephant, and not only their weight or BCS, we can begin to answer these questions. From this information we can put together a more comprehensive picture of what constitutes obesity in elephants. Until then, we should use the term "obesity" in relation to elephants cautiously

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APPENDIX: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVALS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 22-Feb-2016

TO: Nagy, Timothy R

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 22-Feb-2016.

Protocol PI: Nagy, Timothy R

Title: Novel Assessments of Body Composition and Relation to Metabolic Status and Fat Stores in Asian Elephants—Daniella Chusyd

Sponsor: Smithsonian Institution

Animal Project Number (APN): IACUC-20310

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 26-Jun-2015

TO: Nagy, Timothy R

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 26-Jun-2015.

Protocol PI: Nagy, Timothy R

Title: Body composition and inflammation in Overweight and Obese Cycling and Non-cycling Zoo African elephants (Loxodonta africana)

Sponsor: Eppley Foundation for Research, Inc.

Animal Project Number (APN): IACUC-10172

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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