THE UNIVERSITY OF WESTERN AUSTRALIA

THE PYGMY HIPPOPOTAMUS

(Choeropsis liberiensis)

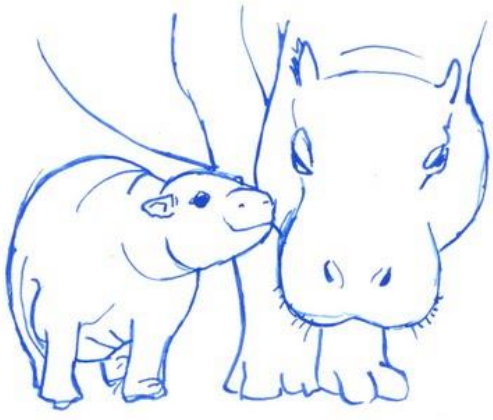
An Enigmatic Oxymoron

How a not-so-small species presents a sizeable conservation challenge

Gabriella L Flacke

DVM, MVSc

This thesis is submitted to fulfill
the requirements for the degree of
Doctor of Philosophy
School of Animal Biology
2016



It is not the critic who counts;
not the man who points out how the strong man stumbles,
or where the doer of deeds could have done better.
The credit belongs to the man who is actually in the arena,
whose face is marred by dust and sweat and blood;
who strives valiantly; who errs,
who comes short again and again,
because there is no effort without error and shortcoming;
but who does actually strive to do the deeds;
who knows great enthusiasms, the great devotions;
who spends himself in a worthy cause;
who at the best knows in the end
the triumph of high achievement,
and who at the worst, if he fails,
at least he fails while daring greatly.

–Theodore Roosevelt

DEDICATION

Dedicated to the memory of loved ones who accompanied me on this journey in spirit –
Phyllis and Ben Johnson; Stephen Johnson; Hildegard and Gerhard Flacke

Caminante, no hay camino, se hace camino al andar.

Traveler, there is no path; the path must be forged as you walk.

–Antonio Machado

SUMMARY

The pygmy hippopotamus (*Choeropsis liberiensis*) is endangered in the wild and has been exhibited in zoological collections since the early 1900s; however, it remains one of the most little known and poorly understood large mammal species in the world. Studies of its biology, physiology and ecology have been limited, both by the often inhospitable nature of its native West African rainforest habitat and by the common misconception that the husbandry and management of the *ex situ* population is already optimized. Most of what we know about this species stems from two radio-telemetry studies conducted in Côte d'Ivoire in the 1980s, a handful of camera-trap and footprint tracking surveys, and observations in zoological facilities; very little analytical, prospective research has been conducted.

The first *Conservation Strategy for the Pygmy Hippopotamus* (Mallon et al. 2011) identified several key areas for further research on the *ex situ* population, including reproductive physiology, polycystic kidney disease (PKD), the female-biased sex ratio, and the potential influence of captivity-associated stress on health and welfare. The overall aim of my PhD research was to address some of these objectives and thereby also provide baseline data essential for further studies. Specifically, I investigated mortality (causes and trends), the demographics of PKD and potential impacts on long-term population viability, reproductive biology of both sexes, and the application of a non-invasive technique for monitoring stress.

The first chapter provides background context and outlines the overall scope of the thesis. Chapter 2 reviews historical and recent literature, creating a framework for the thesis. Several specific issues are discussed in detail, including PKD, the female-biased sex ratio, obesity, the high neonatal mortality rate, and failure of many breeding pairs to reproduce. Chapter 3 identifies primary causes of disease and mortality for newborn, juvenile, and adult pygmy hippos and presents recommendations for veterinary care, husbandry and management. The information in this chapter constitutes the first comprehensive review of mortality data (greater than 400 cases) from the managed population since 1982 and significantly expands on previous

analyses. Chapter 4 describes the demographics of PKD, investigates potential inheritance patterns for this condition based on pedigree, and examines effects of PKD on longevity. This chapter also includes an age-based survival analysis to assess long-term viability based on historical data and current population demographics. The data in the third and fourth chapters were generated in collaboration with the International Studbook keeper at Zoo Basel.

Chapter 5 is the first investigation of female reproductive parameters for this species and represents a collaborative research effort between the Southeast Zoo Alliance for Reproduction and Conservation (SEZARC), Yulee, Florida; the Department of Biomedical Sciences, Unit of Physiology, Pathophysiology and Experimental Endocrinology, University of Veterinary Medicine (Vetmeduni), Vienna, Austria; the Chester Zoo, UK; and the Institute for Breeding and Reproduction of Endangered African Mammals (IBREAM). Together, we characterize the reproductive biology of the female pygmy hippo during gestation, lactation, and the estrus cycle using enzyme immunoassays (EIAs) to measure fecal hormone metabolites. Chapter 6 was also a collaborative venture between SEZARC, Vetmeduni and IBREAM, and examines androgen metabolite patterns throughout the year for male pygmy hippos. We explore one of the potential pitfalls of EIA analysis and describe a substantial cross-reaction between two assays developed to measure the native hormones corticosterone and testosterone. We also describe an EIA that presents biologically relevant patterns in fecal metabolites of cortisol and thus can be used for quantifying individual stress response in pygmy hippos.

The final chapter offers a brief review of overall conclusions and provides recommendations based on research outcomes. Collectively, the information presented in this thesis significantly expands our knowledge base about the pygmy hippo and provides a valuable framework for future studies to further optimize health, reproduction and welfare in the managed population.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

If one advances confidently in the direction of his dreams
and endeavors to live the life which he has imagined,
he will meet with a success unexpected in common hours.
If you have built castles in the air,
your work need not be lost;
that is where they should be.
Now put the foundations under them.

–Henry David Thoreau

The research described in this thesis would not have been possible without the guidance, assistance, support, input, and collaboration of an untold number of kind and dedicated people around the globe. Thank you for collectively providing a strong foundation upon which I could build my castles, and thereby complete this important project.

I foremost extend a heartfelt thank you to everyone who provided funding for my research; without your financial support this project would not have been possible. The University of Western Australia (UWA); UWA Convocation Postgraduate Research Travel Award; UWA Graduate Research School PhD Completion Scholarship; UWA Postgraduate Student's Association Fieldwork and Data Collection Award; American Association of Zoo Veterinarians Wild Animal Health Fund; Center for Conservation of Tropical Ungulates; Omaha's Henry Doorly Zoo and Aquarium; Phoenix Zoo Conservation and Science Grant; Auckland Zoo Small Grants Programme; and the Institute for Breeding Rare and Endangered African Mammals (IBREAM). Additional funding was provided via a crowdfunding campaign by Maria Aguilar, Kerry Allred, Barbara and Henning Clüver, Damon Bell and Katja Geschke, Cherry and Les Bordelon, Jeri and David Brown, Brenda Carlson and Tom Rice, April Conway, Joyce Dixon, Kevin and Jessica Ford, Horst Goede, Gail and Michael Grinder, Franklin Hynes, Peter and Veronika Jirasek, Bruce Mackintosh, Kathy Macleod, Rosemary Martin, Richard Moser, Robyn Owens, Reggie Piencikowski (†), Jeffrey Pollack, Jon and Mary Potter, Chrissi Riedel,

Katherine and Phil Robinson, Keith and Vlasta Ross-Jones, Haley Smith, Miriam Sullivan, Maudelle and Lynn Terry, and Kathy Welter.

To my multiple PhD advisors, I cannot thank you enough for your enduring support and encouragement throughout this challenging journey. You have guided me professionally, taught me well, encouraged perseverance, provided friendship, and remained by my side when the walls came tumbling down; I could not have done it without you. Graeme, Brian, Kris, Linda, Bob and Monique – thank you.

To several anonymous reviewers whose valuable feedback helped to considerably improve my chapters and publications; thank you for your time and thoughtful comments. A big thank you to Phil Robinson and Tanya ten Broeke for proof-reading the thesis, when my eyes were so tired they could no longer see the mistakes.

To my co-authors, professional collaborators, and the veterinarians from the numerous zoological facilities that participated in my studies; thank you for allowing me to conduct research with your animals and for supporting this important project. Your professionalism and dedication to cooperative conservation efforts and animal health is admirable and much appreciated by myself and the pygmy hippos. Cayman Adams, Doug Armstrong, Bob Black, Tim Bouts, James Brown, Roy Burns, Jennifer D'Augostino, Tom deMaar, Walter Dupree, Christie Eddie, Michael Frushour, Kathryn Gamble, Katie Gillespie Jill Gossett, Zoli Gyimesi, Robert Hermes, Jeff Holland, Jamie Huber, Jill Katka, Larry Killmar, Chris Massaro, Lara Metrione, Julie Napier, Christina Ploog, Frank Ridgley, Amy Roberts, Lee Ann Rottman, Lex Salisbury, Franz Schwarzenberger, Beatrice Steck, Laszlo Szilagyi, Suzana Tkalcic, Donna Todd, Joe Tomkins, Sue Walker, Steven Wing, and Sam Winslow.

To all of the zoological facilities that participated in my prospective research (Baton Rouge Zoo; Chicago Zoological Society, Brookfield Zoo; Giraffe Ranch; Gladys Porter Zoo, Brownsville; Jackson Zoo; Lincoln Park Zoo, Chicago; Louisville Zoo; Lowry Park Zoo, Tampa; Oklahoma City Zoo; Omaha's Henry Doorly Zoo and Aquarium; Rum Creek Center for

Conservation of Tropical Ungulates; Zoo Miami), and to those who responded to my enquiries for data concerning mortality in pygmy hippos; your collaboration was essential to the success of this project and has significantly expanded our understanding of this unique and extraordinary species.

To the keepers who introduced me to their pygmy hippos and who diligently collected dung samples for an entire year; it is thankless work and I literally could not have conducted this research without your help and dedication. Your time and efforts are much appreciated – Chris Allen, Jeremy Allen, Daniel Custar, Juan Dominquez, Virginia Edmonds, Jill Gossett, Rolando Grazia, Carl Gyarmaty, Nicole Hill, Tori Hopkins, Susan Hoss, Robyn Jackson, Richard Laird, Shea Leffler, Tabitha Miller, George Morgan, Katie Reimers, Deron Roney, Omar Ruiz, Teresa Shepard, Sarah Smolinski, Ashley Todd, Dana Vinci, Paige Wiggins, Alexis Williamson, and Michelle Wise.

To my University of North Florida and SEZARC lab interns, how can I ever thank you for the countless hours you spent crushing, weighing, and extracting hormones from frozen hippo dung? I had so much fun working with all of you and witnessing your enthusiasm and dedication. I know you will succeed in your future endeavors and enjoy rewarding careers in medicine, science, research and conservation. Your hard work, kindness and support will never be forgotten – Sarah Allred, Kim Daly-Crews, Saleha Khan, Paige Pickering and Kayla Weller.

To those who have also been lucky enough to work with pygmy hippos in West Africa, thank you for guiding me on my journey and sharing your own experiences, knowledge, trials and tribulations with me first-hand. I admire all of your efforts studying this remarkable species in the wild and your struggles to preserve its vanishing rainforest habitat; you are true conservation heroes. Elie Bogui Bandama, Donatien Belé, Waldemar Bülow, April Conway, Knut Hentschel, Inza Koné, Karim Ouattara, Phil Robinson, Leon Sio Toh, and Wei Yeen Yap.

To the three veterinarians who have served as my role-models and who have significantly influenced my life and my career; you have inspired me to do my best, to never give up, to

always be true to myself, and you have shown me that it's OK to be imperfect; in those imperfections lie some of our greatest strengths. To me, you symbolize what our wonderful profession is all about, and you do it with kindness, grace and humility. Thank you for being my mentors and friends. Dave Cooper, David Gold and Mark Davis.

To the veterinary team at the Perth Zoo: Sam Comito, Cathy Cooper, Lisa Hills, Franklin Hynes, Anna Le Souef, Peta Moore, Karen Payne, Michelle Rouffignac, Kate Simon-Menasse, Bec Vaughn-Higgins, Simone Vitali, and Lyn Weir – thank you for the opportunity to work with all of you and for making me feel like part of the family. I will never forget all of the amazing and fascinating local creatures I have become acquainted with through my time working in the vet department.

To my friends in Australia, who provided a listening ear, a shoulder to cry on, a smile or a hug, who stood by my side during good times and bad, and whose love and support made my time in Perth incredibly memorable and special; I will miss you all very much. Damon Bell, Katja Geschke, Pete and Rae Hartley, Pat Intuprapa, Veronica Phillips, Emanuel and Tanja Reiterer, Keith and Vlasta Ross-Jones, Troy and Mai Spencer, Miriam Sullivan, Elf Thavornkanlapachai, Laura Travers, Alisa Wallace, and Erina Young.

To my friends and family from afar, some of whom have known me for decades before this PhD adventure began, who did not give up on me as I faced one seemingly impossible hurdle after another, who provided support across the miles, and who hosted me on my travels in pursuit of the elusive pygmy hippo; thank you for always being there for me. Christina and Brandon Anderson, Penny and Warren Becker, Candace and Shane Cavilee, Diana Dodge, Elizabeth and JD Ferries-Rowe, Heather and Steve Fodor, Kevin and Jessica Ford, Mari Good, Lisa Harrenstien, Karen and Robert Hirschman, Audrey and Jeff Hawken, Peter Jirasek, Betty Lane, Martha Lane, Diana Lilienthal, Susan Monger, Tracy Mott, Tomas Pecha, Jon and Mary Potter, Mike and Kristen Potter, Chrissi Riedel, Kirk Schwartzah, Jon Seltz, Brenda Smith, Jerry Switzer, Hans and Dagmar Strickler, Tanya ten Broeke, Suzana Tkalcic, Steve Urberg, Violet Vanatta, Bill Young, Michele Zawadzki, and Fee Zimmermann.

And last but never least, to my parents. Your unending love and support are the most treasured things in my life. I am so incredibly fortunate. Thank you.

AUTHOR'S DECLARATION

I declare that this thesis is my own account of my research and its content has not previously been submitted for a degree at any tertiary education institution.

Additionally, I declare that I have the explicit permission of all of my co-authors to include the material in this thesis that has been written and/or published with these co-authors.

STATEMENT OF CONTRIBUTIONS

LIST OF PUBLICATIONS

1. Flacke, G.L., Chambers, B.K., Martin, G.B. & Paris, M.C.J., 2015. The pygmy hippopotamus *Choeropsis liberiensis* (Morton, 1849): Bringing to light research priorities for the largely forgotten, smaller hippo species. Der Zoologische Garten NF, 84, pp.234–265.

2. Flacke, G.L., Tkalčić, S., Steck, B., Warren, K. & Martin, G.B. A retrospective analysis of mortality in captive pygmy hippopotamus (*Choeropsis liberiensis*) from 1912 – 2014.
   - This chapter represents a revised manuscript that is currently undergoing a third round of peer-review with Zoo Biology.

3. Flacke, G.L., Tomkins, J.L., Black, R. & Steck, B. Demographics of polycystic kidney disease and implications for captive population viability in pygmy hippopotamus (*Choeropsis liberiensis*).
   - This chapter represents a revised manuscript that is currently undergoing a second round of peer-review with Zoo Biology.

4. Flacke, G.L., Schwarzenberger, F., Penfold, L.M., Walker, S., Martin, G.B., Millar, R. and Paris, M.C.J. The reproductive biology of the female pygmy hippopotamus (*Choeropsis liberiensis*) as characterized by non-invasive endocrine monitoring.
   - A condensed version of this chapter was submitted for peer review to Theriogenology, Oct 2016.

5. Flacke, G.L., Penfold, L.M., Schwarzenberger, F., Martin, G.B., and Paris, M.C.J. Non-invasive monitoring of fecal glucocorticoid and androgen metabolites in pygmy hippopotamus (*Choeropsis liberiensis*).
   - A condensed version of this chapter is planned for submission to the Journal of Zoo and Wildlife Medicine, Nov 2016.

AUTHOR CONTRIBUTIONS

1. GLF conducted the literature review and wrote the manuscript. BKC, GBM and MCJP provided editorial feedback.

2. GLF designed the study, collected the pathology reports, analyzed the data, and wrote the manuscript. ST assisted with interpretation of pathology reports. BS assisted with collection of pathology reports and provided data from the Studbook. KW assisted with data analysis. All co-authors provided editorial feedback.

3. GLF designed the study, collected the data, performed 60% of the data analysis, and wrote the manuscript. JLT performed 20% of the data analysis; RB performed 20% of the data analysis and wrote the code for R. BS provided the data from the Studbook. All co-authors provided editorial feedback.

4. GLF and MCJP designed the study and organized data collection with the zoological facilities (GLF – North America, MCJP – Europe). GLF performed 40% of the laboratory analyses in collaboration with LMP; FS performed 50% of the laboratory analyses; SW performed 10% of the laboratory analyses. GLF analyzed the data, with assistance from FS, LMP, GBM and MCJP. GLF wrote the manuscript, and all co-authors provided editorial feedback.

5. GLF designed the study and organized the data collection with the zoological facilities. GLF performed 85% of the laboratory analyses in collaboration with LMP; FS performed 15% of the laboratory analyses. GLF analyzed the data with assistance from LMP and GBM. GLF wrote the manuscript, and all co-authors provided editorial feedback.

LIST OF ABBREVIATIONS

11,17-DOA	11,17-dioxoandrostane
ABTS	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt
ACTH	Adrenal corticotropic hormone
ADPKD	Autosomal dominant polycystic kidney disease
ANOVA	Analysis of variance
ARPKD	Autosomal recessive polycystic kidney disease
AZA	American Association of Zoos and Aquariums
BSA	Bovine serum albumin
CC	Corticosterone CJM006
CCST	Corticosterone
CEPF	Critical Ecosystem Partnership Fund
CITES	Convention on International Trade in Endangered Species
CL	Corpus luteum
CNS	Central nervous system
CORT	Cortisol
df	Degrees of freedom
EAZA	European Association of Zoos and Aquaria
EEP	European Endangered Species Program
EIA	Enzyme immunoassay
EMCV	Encephalomyocarditis virus
E2a	Total estrogens
E2b	Estradiol 17β R4972
E2c:	Estradiol 17β R0008
F	Inbreeding coefficient
GLM	Generalized linear model
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IBREAM	Institute for Breeding Rare and Endangered African Mammals
ISIS	International Species Information System, now rebranded Species 360
IUCN	International Union for Conservation of Nature and Natural Resources
λ (lambda)	Intrinsic rate of population growth
LTF	Lost to follow-up
mono-P4	Monoclonal progesterone antibody Quidel clone 425
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate

$NaPO_4$,	Sodium phosphate
PdG	Pregnanediol-3-glucuronide
Pg-diol	Pregnanediol
PKD	Polycystic kidney disease
PQL	Penalized likelihood method
poly-P4	Polyclonal progesterone antibody R4859
SSP	Species Survival Plan
Testo	Testosterone C196
UNEP	United Nations Environment Programme

© Dave Parkinson – Pygmy Hippos ‘Zsa Zsa’ and ‘Zola’
Lowry Park Zoo, Tampa

LIST OF FIGURES

LIST OF TABLES

Chapter 1 GENERAL INTRODUCTION

Hans Schomburgk and a Pygmy Hippo – 1913

Das Zwergflußpferd, eine Zoologische Neuheit, *Kosmos Handweiser für Naturfreunde*, 2, p. 62

The ecosystem will not give priority to humans over every other living kind;
neither religion nor conventional thinking can trump ecological limits.

–Richard Lamm

When we try to pick out anything by itself,
we find it hitched to everything else in the universe.

–John Muir

1.1 A Brief Overview of the Pygmy Hippopotamus

The pygmy hippo (*Choeropsis liberiensis*) is one of two extant members of the family Hippopotamidae, the other being the common hippo (*Hippopotamus amphibius*). The first report of this smaller species was made in 1844 by the naturalist Samuel Morton, based on his discovery of several skull fragments during an expedition to Liberia, the country from which the species' name originated (Morton 1844). Endemic to the Upper Guinean Rainforest ecosystem in West Africa, its current distribution is limited to isolated populations within fragmented habitats in Côte d'Ivoire, Guinea, Liberia, and Sierra Leone. The current wild population size remains unknown, but it is estimated to be less than 2500 and is widely considered to be declining (Ransom et al. 2015).

The pygmy hippo is listed as Endangered on the IUCN Red List and is a CITES Appendix II species (Ransom et al. 2015). The primarily threat to survival is habitat loss secondary to numerous anthropogenic factors, specifically logging, mining, agriculture, and an ever-expanding human population. Further pressures include lack of adequate legal protection for the few remaining intact areas of habitat, poaching for bush meat, inadequate infrastructure, and an often unstable political climate within the four range states (Collen et al. 2011; Hoppe-Dominik et al. 2011; Lindsell et al. 2011; Roth et al. 2004).

There have been very few studies of the basic biology, ecology and life history of the pygmy hippo in the wild because the species is elusive in nature and its habitat is often inhospitable (Mallon et al. 2011). The first field research was conducted in 1968 in Liberia (Robinson 1970), and for many decades this initial survey, together with two subsequent radio-telemetry studies conducted in Côte d'Ivoire (Bülow 1987; Hentschel 1990), comprised the only available information. Recently, observational field-studies using camera trapping, transect dung counts, and footprint analysis have provided further data about occurrence and distribution (Collen et al. 2011; Conway 2013; Eshuis 2011; Hillers & Muana 2010; van Heukelum 2011). However,

none of these studies collected data on demography, morphometry, physiology or epidemiology, and therefore relatively little is known about this species compared to its larger relative.

The first pygmy hippo to be exported from West Africa was brought to Dublin, Ireland in 1873; unfortunately the young animal was in such poor health that it died several hours after arrival (Macalister 1873). It was not until almost forty years later in 1912 that the infamous hunter and animal trapper, Hans Schomburgk, successfully exported the first live animals to Carl Hagenbeck's zoo in Hamburg, Germany (Schomburgk 1913). These animals lived into their 40s and reproduced readily, demonstrating that the species could survive and thrive in captivity despite limited understanding of its basic biology, ecology and husbandry. Importation of pygmy hippos from the wild for display and breeding in zoological collections across the globe continued until the early 1980s; the last two wild animals were imported to the Kuala Lumpur Zoo in 1982 (Steck 2016). The wild founder population was sourced as follows: Liberia 89/162; Côte d'Ivoire 25/162; Sierra Leone 8/162; "Africa Unknown" 40/162 (Steck 2016).

1.2 CONTEXT AND SCOPE

In recent decades, several prospective studies conducted in zoological facilities have provided preliminary information concerning reproductive biology, digestive physiology, and population genetics for pygmy hippos. Researchers from the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany, have piloted electro-ejaculation and semen preservation techniques for males (Saragusty et al. 2010). Several studies have investigated digestive physiology, metabolism, and potential impact of diet on obesity (Clauss et al. 2004; Clauss et al. 2007; Schwarm et al. 2006). Researchers from the University of Chester and the Royal Zoological Society of Scotland (RZSS) have developed molecular genetic markers from mitochondrial DNA (Senn et al. 2014). Additionally, veterinary case reports have documented a number of pathological conditions in individual pygmy hippos, including dystocia (Flach et al. 1998), leptospirosis (Cracknell et al. 2011), mycobacteriosis (Bouts et al. 2009), encephalomyocarditis virus (Reddacliff et al. 1997) benign osteoma (Weston et al. 1996),

anaplastic sarcoma (Masters et al. 2014), and lymphoblastic leukemia (McCurdy et al. 2014). Newer anesthetic protocols have also allowed for safer immobilization procedures for diagnostic, medical, and surgical treatments in pygmy hippos, a species notorious for being 'difficult' to anesthetize in the past (Bouts et al. 2012; Miller et al. 2014; Walzer & Stalder 2014).

The zoological community generally considers the pygmy hippo as a species that is relatively easy to manage and will reproduce readily if husbandry conditions are 'appropriate' (Lang 1975; Schanberger & Weinhardt 1997; von Houwald et al. 2007). However, three specific concerns for the health, well-being and sustainability of the *ex situ* population have been proposed: polycystic kidney disease (Nees et al. 2009; Raymond et al. 2000), a female-biased sex ratio of offspring (Saragusty et al. 2012; Zschokke 2002), and a high rate of stillbirth and neonatal mortality, especially for males (Leutenegger 1978; Steck 2016). It is unknown whether these problems are also a concern for the wild population. Additionally, numerous breeding pairs at several zoological facilities worldwide have failed to reproduce, whereas others are genetically over-represented. These issues have the potential to continually reduce genetic diversity and negatively impact the long-term viability of the managed population.

The first Conservation Strategy Action Plan for the pygmy hippo was developed by the IUCN Pygmy Hippo Specialist Group after a 2010 workshop in Liberia that involved collaboration of stakeholders from all four range states including scholars, government agencies, and local and international non-governmental organizations (Mallon et al. 2011). The plan identified actions necessary to achieve defined, long-term conservation objectives, primarily relating to the sparsely studied and poorly understood wild population. However, specific priorities involving the *ex situ* population were also documented, including "to continue research into reproductive biology of pygmy hippos, the biased sex ratio in the captive population and polycystic kidney disease; and establish a pygmy hippo gene bank for wild and captive populations" (Mallon et al. 2011). The first three of these four items are the main subject of this thesis.

Zoological population management for the pygmy hippo is directed through the European Endangered Species Program (EEP) and the North America Species Survival Plan (SSP); the International Studbook is maintained at Zoo Basel in Switzerland. As of 31 December 2015, the Studbook listed 144 male and 255 female pygmy hippos, plus 6 animals of undetermined sex, housed in 140 institutions worldwide (Steck 2016). Maintaining genetic diversity for this closed population requires vigilant breeding management and hence a thorough understanding of the species' reproductive biology. Non-invasive endocrine monitoring is one of the most widely-used tools for evaluating reproductive parameters in non-domestic species under managed care. Specifically, the use of enzyme immunoassays (EIAs) to assess hormone metabolites in saliva, urine and feces has provided valuable data to guide breeding management for many endangered species (Kersey & Dehnhard 2014; Schwarzenberger 2007; Schwarzenberger & Brown 2013). Endocrine monitoring is also necessary for planning and implementing assisted reproductive techniques such as artificial insemination, embryo transfer, and in vitro fertilization (Paris et al. 2007; Pukazhenthi & Wildt 2004). Another topic that has recently garnered considerable attention within the zoological community is the potential impact of chronic stress on reproductive function and overall well-being for the animals under its care, principally because it is challenging to mimic natural environmental, dietary, and social conditions (Morgan & Tromborg 2007). Mason (2010) identified a substantial number of non-domestic species that experience behavioral abnormalities and low reproductive success in comparison to their wild counterparts, at least partially due to captivity-associated stress.

It is therefore essential to be able to monitor objectively both stress and reproductive parameters in wildlife species under managed care. Although several aspects of female reproductive biology have been elucidated for the common hippo using non-invasive endocrine monitoring (Graham et al. 2002; Smith et al. 2000; Wheaton et al. 2006), this information cannot be directly extrapolated to the pygmy hippo because the reproductive physiology and steroid hormone metabolites of even closely related species can differ markedly. For example, different species of rhinoceros, felids, and ursids have different estrous cycle characteristics and produce varied types and amounts of fecal estrogen and progesterone metabolites (Schwarzenberger & Brown

2013). Similarly, metabolites of glucocorticoid stress hormones, primarily cortisol, can also differ markedly among species (Möstl et al. 2002; Möstl et al. 1999; Schatz & Palme 2001; Touma & Palme 2005). Non-invasive methods for evaluating baseline glucocorticoids and individual stress responses have not been developed for either hippo species.

1.3 OBJECTIVES AND STRUCTURE

The overall aim of my PhD was to provide additional data concerning the biology and physiology of the pygmy hippo to help further optimize health, well-being and reproductive management. I began by building upon previous research from the *ex situ* population and designed a study involving both retrospective and prospective aspects to address several of the objectives outlined by the Pygmy Hippo Specialist Group. Specific aims were as follows: *i*) comprehensively review the literature, targeting several myths about this species that have been perpetuated from misinterpretations of historical data and inaccurate translations from previous peer-reviewed publications and non-published reports; *ii*) conduct a survey of the global zoo population to investigate trends in morbidity and mortality; *iii*) determine prevalence and demographics of polycystic kidney disease and assess potential implications of this condition for long-term population viability; *iv*) characterize reproductive biology via longitudinal monitoring of fecal metabolites of estrogens, progestogens, and androgens; and *v*) establish a non-invasive methodology for measuring metabolites of cortisol to monitor individual stress response in pygmy hippos.

The thesis is presented as a series of five scientific manuscripts, some of which have been published and some undergoing peer review. In each chapter, I address one of the specific aims listed above. The chapters are independent, each containing an Abstract, Introduction, Methods, Results, Discussion, Conclusions and Reference section, so there will be some content overlap and reference style will differ between chapters. A comprehensive bibliography is presented at the end of the thesis. A brief final chapter provides overall conclusions and presents several recommendations for future research with this endangered and endearing species.

Chapter 2 THE PYGMY HIPPOPOTAMUS *CHOEROPSIS LIBERIENSIS* (MORTON, 1849): BRINGING TO LIGHT RESEARCH PRIORITIES FOR THE LARGELY FORGOTTEN, SMALLER HIPPO SPECIES

Paul Steinemann of Zoo Basel
Weighs a Newborn Pygmy Hippopotamus

Ernst M. Lang, 1975. *Das Zwergflußpferd*, Wittenberg Lutherstadt: A. Ziemsen Verlag, DDR.

The world is a book,
and those who do not travel
read only one page.

–Saint Augustine of Hippo

The Pygmy Hippopotamus Choeropsis liberiensis (Morton, 1849): Bringing to Light Research Priorities for the Largely Forgotten, Smaller Hippo Species

Das Zwergflußpferd Choeropsis liberiensis (Morton, 1849): Überblick der Literatur und wichtige Forschungsthemen für das oft vergessene, kleinere Flusspferd

Gabriella L. Flacke[a,*], Brian K. Chambers[a], Graeme B. Martin[a] and Monique C. J. Paris[a,b,c,d]

[a] *School of Animal Biology M092, University of Western Australia, 35 Stirling Highway, Crawley 6009 WA, Australia*

[b] *College of Public Health, Medical and Veterinary Services, James Cook University, Solander Drive, Douglas 4811 QLD, Australia*

[c] *Institute for Breeding of Rare and Endangered African Mammals (IBREAM), Edinburgh EH3 6AT, United Kingdom*

[d] *Mammal Research Institute, University of Pretoria, Department of Zoology and Entomology, Private Bag X20, Hatfield 0028, South Africa*

*Corresponding Author E-mail: gflacke@grs.uwa.edu.au

2.1 ABSTRACT

An endangered species, the pygmy hippo (*Choeropsis liberiensis* Morton, 1849) has been housed in captivity since the early 1900s, but systematic, prospective research and peer-reviewed literature remain limited in comparison to other IUCN-listed, charismatic mega fauna. There are just over 350 animals in the *ex situ* population worldwide, so it is an uncommon resident in zoological collections compared to the larger, 'common' or Nile hippo (*Hippopotamus amphibius*). Most published information for the pygmy hippo constitutes descriptive accounts of first-hand experiences in various zoological institutions. Here we review, analyze and provide a synthesis of the pertinent literature, aiming to identify and prioritize focal research topics for optimizing *ex situ* management. The pygmy hippo is continually reported to breed well, so long-term survival of the species, at least in captivity, is assumed, although we identify several reasons to exercise caution. Further, we demonstrate that the common perception amongst zoological institutions that the pygmy hippo is easy to manage and experiences limited health and husbandry issues is erroneous. Specific issues affecting the captive population with potential negative implications for long-term sustainability include polycystic kidney disease (PKD), a female-biased sex ratio, obesity, a high neonatal mortality rate, and failure of many breeding pairs to reproduce. We identify several research priorities to help address these concerns, and how the resulting information can be applied to improve management, health and welfare of pygmy hippos in captivity.

Key words: Husbandry; Polycystic kidney disease (PKD); Pygmy hippo; Reproduction; Sex ratio

2.2 INTRODUCTION

The pygmy hippopotamus (*Choeropsis liberiensis* Morton, 1849), hereafter 'pygmy hippo,' is one of two extant members of the family Hippopotamidae, the other being the common hippopotamus (*Hippopotamus amphibius*). The species is listed as "endangered" on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List and distribution is limited to fragmented populations in Côte d'Ivoire, Guinea, Liberia, and Sierra Leone in West Africa (Eltringham, 1999; Lewison & Oliver, 2008). Primary threats to survival include habitat loss, lack of adequate legal protection, and poaching for bush meat (Lewison & Oliver, 2008; Robinson, 2013). An often unstable political climate in the region also leads to insecurity of protected areas, unregulated logging and hunting, and inhibits scientific research efforts.

The Zoological Society of London, with support from the IUCN Hippo Specialist Group, recently developed the first Conservation Strategy Action Plan for the species (Mallon et al., 2011). They identified a range of detailed actions for achieving integrated conservation goals, including specific priorities for the captive population: "to continue research into reproductive biology of pygmy hippos, the biased sex ratio in the captive population and polycystic kidney disease; and establish a pygmy hippo gene bank" (Mallon et al., 2011). For this management plan to be effective, it must be based on thorough, evidence-based research. Furthermore, to optimize husbandry practices and contribute effectively to integrated conservation strategies, we first need to clearly understand factors influencing pygmy hippo health, physiology, welfare, and successful reproduction in captivity.

A comprehensive examination of pygmy hippo literature unveils a multitude of older first-hand descriptive accounts, often published in symposium proceedings and various other 'grey literature.' Prospective studies are limited. Furthermore, a large portion of the older but valuable and relevant material is written in German and has never been formally translated, thus limiting its accessibility. Moreover, inaccuracies have inadvertently been perpetuated through recurring

citation of outdated sources and erroneous information. To paint a clear, comprehensive picture of our present understanding of this species, we have summarized relevant literature and extracted pertinent material from more archaic and foreign language publications. We need an accurate, easily accessible, and comprehensive baseline reference framework if we are to move forward and appropriately address specific research questions. The information presented here should therefore facilitate future goal-directed pygmy hippo research essential for developing integrated conservation strategies for this endangered species.

2.3 Ex Situ History

The first live pygmy hippo exported from West Africa was brought to Liverpool (en route to Dublin, Ireland) from Sierra Leone in 1873 by John M. Price of the British Colonial Office (Sclater, 1873). The mother had been shot and it was only a few weeks old when captured; unfortunately it was in such poor health upon arrival that it did not survive. The cause of death was "violent inflammation of both lungs," a severe case of pneumonia (Macalister, 1873). It was not until almost forty years later, in 1912, that Hans Schomburgk successfully brought five pygmy hippos from Liberia to Hamburg, Germany, on behalf of the legendary wild animal dealer Carl Hagenbeck (Schomburgk, 1912). Three of these animals, two males and a female, were subsequently sold and moved to the New York Zoological Society, presently the Bronx Zoo (Hornaday, 1912); the other two remained in Germany.

The original New York pair (one male was eventually moved to London) lived into their late 30s and reproduced readily, demonstrating that the species could survive and reproduce in captivity despite limited understanding of its basic biology and husbandry. In fact, when the animals had resided at the zoo for eight years, Hornaday (1920) sang their praises in the New York Zoological Society Bulletin: "Never since we began to worry over the idiosyncrasies and troubles of wild animals have we had any more satisfactory animals than those pygmy hippos. In appetite, health, and general deportment they must be marked 100 percent. No one of the trio

has ever been sick for so much as one day, or missed a meal. They eat their rations cheerfully, gratefully, and copiously."

After the initial importation by Schomburgk, pygmy hippos were actively and continually sourced from the wild for display and breeding in zoos across the globe until the late 1970s when the captive population was believed large enough to be self-sustaining. The last wild-caught animals were imported on the 22nd of September, 1982 to Kuala Lumpur, Malaysia, and only two wild-caught pygmy hippos remain within the captive population at the time of writing – a male in the USA (Oklahoma) and a female in Germany (Duisburg). The wild founder population originally consisted of 162 pygmy hippos sourced primarily from Liberia; however, only 60 of those founders are genetically represented in the extant population (Steck, 2014). The captive population has consistently remained at 300 to 350 for the last 40 years, and the International Studbook is managed by Zoo Basel, Switzerland (Lang, 1975; Steck, 2015).

2.4 PHYLOGENY AND TAXONOMY

Historically, both common and pygmy hippos have been classed with suids (pigs). However, recent molecular phylogenetic studies of suids, ruminants, hippopotamids and cetaceans, all of the order Artiodactyla, have repeatedly identified a sister-group relationship between Hippopotamidae and Cetacea (Shimamura, Yasue, Ohshima, Abe, Kato, Kishiro, Mutsuo, Isao, & Okada, 1997; Ursing & Arnason, 1998; Nomura & Yasue, 1999; Boisserie, Lihoreau, & Brunet, 2005). Comparative morphological analysis of hippos and whales lends further support (Geisler & Uhen, 2003; Fisher, Scott, & Naples, 2007). The previous assignment of hippopotamids to the suborder Suiformes was recently revised and hippos were reclassified under Whippomorpha, a clade containing whales and hippos, within the order Cetartiodactyla (Boisserie et al., 2005; Fisher et al., 2007).

Genus for the pygmy hippo has also been debated at length based on phylogeny, ontogeny, and morphological characteristics. The species was first described by Morton (1844), who labelled the animal *Hippopotamus minor* based on differences between common and pygmy hippo

cranial morphology and dental formula; a few years later, he renamed it *Hippopotamus liberiensis* (Morton, 1849). Leidy (1853), focusing on "striking differences in cranial anatomy" between the two hippo species, subsequently proposed *Choeropsis liberienses*; *Choeropsis* from the Greek for "pig-like" and *liberiensis* to describe geographic origin. Early anatomists and zoologists repeatedly disputed whether the pygmy hippo deserved a separate genus to *Hippopotamus* (Flower, 1887; Chapman, 1894; Renshaw, 1904), but majority opinion eventually supported the distinction. *Choeropsis* was used until Coryndon (1977) placed the pygmy hippo in the genus *Hexaprotodon*, or "six front teeth." Both names appear interchangeably in the scientific literature. Most recently, Boisserie (2005) concluded that the mix of primitive and derived features of the extant pygmy hippo gave it a distinct lineage, validating genus *Choeropsis*, and proposed that genus *Hexaprotodon* be restricted to the fossil lineage mostly found in Asia.

2.5 ANATOMY AND PHYSIOLOGY

We forewarn the reader that most references discussed in this section provide a level of detail that can be both daunting and overwhelming. Here we attempt to dissect these meticulous works and extract the most relevant and useful points for understanding pygmy hippo anatomy in a clinically relevant context while maintaining historical accuracy. Differences between pygmy and common hippos are emphasized, and aspects of morphology that provide insight into ecology and life history traits are highlighted. Understanding how and why the pygmy hippo differs from its larger relative and how it compares to other species such as ruminants and marine mammals, provides direction for improving husbandry and medical management of this species in captivity.

The first general anatomical report was by Morton (1844) who 'discovered' the existence of the smaller hippo species based on his observations of several skull fragments given to him during an expedition to Liberia. Both Morton and Leidy (1853) described six maxillary, or upper, front teeth (one pair of canines and two pairs of incisors) undoubtedly part of the reason for the

pygmy hippo's potential classification as a Hexaprotodont. However, these early scientists also identified only four mandibular, or lower, front teeth. The dental formula (maxillary/mandibular) is as follows: Incisors: 2/1, Canines: 1/1, Premolars: 4/4, Molars: 3/3, in contrast to the common hippo which has two pairs of both maxillary and mandibular incisors (Incisors: 2/2).

The aspects of anatomy and physiology that are unique to the pygmy hippo are, not surprisingly, better described than other body systems exhibiting more conserved morphology. Recently, Weston (2000, 2003) extensively described hippo cranial anatomy with comparisons of the two extant species and various extinct species of hippopotamids. She labelled the pygmy hippo as a "living fossil" because it shares more traits with extinct ancestral clades than with the common hippo (Weston, 2000). An important conclusion derived from these morphological comparisons is that the two extant species are not solely the result of ontogenetic scaling, thus the pygmy hippo is not a dwarf species as previously hypothesized (Weston, 2003).

Hegner (1967) identified potential ecological implications of differing ocular morphology: a) the periscope-like location on top of the skull of the eyes of the common hippos serves as an adaptation to an aquatic lifestyle, in contrast to the lateral placement in the pygmy hippo; b) asymmetry of the ciliary body offers the pygmy hippo a more extensive rear field of vision; c) a more extensive tapetum lucidum in the pygmy hippo, a structure associated with improved vision under low-light conditions. Van den Bergh (1971) made the intriguing observation that ocular placement and head shape affords the pygmy hippo a binocular field of vision even when the mouth is gaping open, allowing the animal to advance toward a threat while maintaining visual contact. The pygmy hippo's toes are less webbed than the common hippo's and spread out more widely during weight-bearing, an adaptation to walking through muddy rainforest terrain (Pocock, 1923). The hippopotamids are unique among the Artiodactyla in that all four toes are weight-bearing (Crandall, 1964; Walker, 1964).

The first examination of the pygmy hippo gastrointestinal system was by Macalister (1873), who described four stomachs, similar to the ruminant, and "an elongated triangular gall

bladder." Both hippo species are repeatedly reported to lack a gall bladder entirely (Lang, 1968; Boever, 1978; Taylor & Greenwood, 1986; Jarofke, 1993; Miller, 2003; von Houwald, Macdonald, Pagan, & Steck, 2007; Walzer & Stalder, 2014). However, gall bladder-associated pathology has been documented in several pygmy hippo necropsy reports (Jarofke & Klös, 1982; Flacke et al., Chapter 3). Thus, the previous accounts are either erroneous or reflect an anatomical exception to the rule. The stomach has four compartments, including the non-glandular visceral and parietal 'blindsacs' (also termed left and right diverticula) with their connecting chambers, all of which are lined with papillae of various lengths and separated by mucosal folds (Langer, 1975). These compartments are collectively referred to as the 'fore stomachs.' The final compartment is the customary glandular stomach. The relative chamber size and orientation of the stomach differs between the adult and neonate (Macdonald & Hartman, 1983), and mucosal papillae are more prominent in the adult (Langer, 1975; Endo, Sasaki, Kogiku, Hayashi, Komiya, Narushima, Arishima, & Yamamoto, 2001), reflecting dietary differences between life stages.

Hippos are thus referred to as 'pseudo-ruminants' or 'ruminant-like,' with digestive anatomy and physiology most similar to that of the macropodid marsupials endemic to Australia (Langer, 1975; Clauss, Schwarm, Ortmann, Alber, Flach, Kühne, Hummel, Streich, & Hofer, 2004; Schwarm, Ortmann, Hofer, Streich, Flach, Kühne, Hummel, Castell, & Clauss, 2006; Schwarm, Ortmann, Wolf, Jürgen Streich, & Clauss, 2008). However, hippos exhibit lower dry matter intake, lower energy requirement and basal metabolic rate, longer retention time, and less efficient digestion of forage than ruminants (Clauss et al., 2004; Schwarm et al., 2006). Therefore, the feeding ecology and activity budget of the pygmy hippo is thought to involve relatively less time spent foraging and more time resting (and digesting) compared to a similarly-sized ruminant. These characteristics may explain why pygmy (and common) hippos in captivity easily become overweight when they are offered energy-dense foods or ad-libitum access to forage (Schwarm et al., 2006).

The skin is approximately 1 cm thick, very vascular with extensive subcutaneous fat, and has a rubbery texture (Flach, Furrokh, Thornton, Smith, Parkyn, & Campbell, 1998). Dermal sebaceous glands and sweat glands are absent; instead, subdermal glands secrete an oily clear fluid that protects against sunburn, dehydration and bacterial infection (Eulenberger, 1995; Hashimoto et al., 2007). The skin secretions are reddish brown in common hippos, lending support to the myth that these animals 'sweat blood' (Crandall, 1964; Walker, 1964). There are multiple anecdotal reports describing a white foamy skin secretion produced by wild pygmy hippos, and captive animals often exhibit the same phenomenon during periods of physical exertion, including during mating (Steinmetz, 1937; Lang, 1975; Schubert, 2004; von Houwald et al., 2007).

Both hippo species exhibit a unique behavior of backing against a vertical surface and spreading a mixture of urine and fecal material everywhere with a series of rapid, propeller-like tail movements. Kranz (1982) hypothesized that splitting of the tail hairs functions to increase the hair's surface area and thus improve the efficiency with which dung is spread in the environment. Lochte (1951) described the structure of pygmy hippo hair as stiff bristles comprised of multiple keratin strands. Amongst his extensive collection of mammalian hair samples, he only found a similar conglomerate bristle-like hair structure among the phocids (seals and sea lions), lending further empirical support to a link between aquatic mammals and the Hippopotamidae.

Lobulated kidneys are similar in architecture to those of the Bovidae. A large number of short nephrons are hypothesized to allow the evolution of a larger animal albeit with a limited ability to concentrate the urine (Maluf, 1978; Maluf, 1994). There are no published values for the specific gravity of 'normal' hippo urine. Both hippo species have diffuse epitheliochorial placentation with an umbilical cord containing two arteries and two veins (Macdonald & Bosma, 1985). Benirschke (2007) provides extensive detail concerning gross and histological aspects of the pygmy hippo placenta, complete with multiple full-color photographs to delight anyone interested in histology.

In conclusion, most pygmy hippo anatomical studies provide a level of detail beyond what is necessary for practical clinical application. However, a thorough understanding of morphology offers important clues to ecology and life history traits and allows us to draw conclusions about functionality. Such information is particularly useful in a zoological setting for husbandry staff and veterinary clinicians to optimize management and health care of this species.

2.6 HUSBANDRY

In contrast to many other African ungulates and pachyderms, including the common hippo, that have been kept by humans since ancient times, the pygmy hippo has only been held in captivity for a little over a century. While Roman gladiators were fighting rhinos and lions in the Coliseum, the pygmy hippo was quietly minding its own business, hidden in the rainforests of West Africa. Although it was certainly used locally as a source of meat, it is unlikely that it was kept as a pet or display animal by the indigenous people of the Upper Guinean rain forests. Thus, whereas we have had centuries, even millennia, to improve captive management and husbandry of the common hippo, everything we know about the pygmy hippo stems from after 1912.

Early husbandry summaries for this species include remarkable historical accounts, including the untimely death of several pygmy hippos during the allied bombing of Berlin during WWII (Blaszkiewitz, 1983; see Appendix I). The Association of British Wild Animal Keepers provided a literature review describing the history of pygmy hippos in captivity and general recommendations for husbandry and breeding (Greed, 1983). Most recently, Husbandry Guidelines for the Pygmy Hippopotamus was published as a joint effort of the European Association of Zoos and Aquaria (EAZA) and the American Association of Zoos and Aquariums (AZA) (von Houwald et al., 2007). These guidelines include dietary recommendations, exhibit design options, behavioral training information, and a compilation of personal experiences from zoological institutions worldwide with pygmy hippos in their

collections. There is only one published account of managing the pygmy hippo in captivity on its home continent, at Jos Wildlife Park in Nigeria (Osakwe et al., 1988).

The most detailed history and description of pygmy hippo husbandry, "Das Zwergflußpferd" by Ernst Lang (1975), is based on many years of the author's personal experiences at Zoo Basel and includes multiple excerpts from Büttikofer's (1890) early explorations in Liberia in search of the elusive animal. Later, in partnership with Waldemar Bülow and Knut Hentschel, Lang published a comprehensive encyclopedia chapter, a compilation of excerpts from his 1975 text and the work of his collaborators in Côte d'Ivoire (Lang, Hentschel, & Bülow, 1990). This chapter is the only formal publication to result from the ground-breaking *in situ* research by Bülow (1987) and Hentschel (1990) in the 1980s. It is also the only published English-language translation of their research theses, albeit greatly abbreviated and condensed.

Several zoological facilities house adult pygmy hippos separately except during breeding. Direct and indirect (i.e., camera trap photos, footprint tracking) observation of wild pygmy hippos has repeatedly demonstrated that these animals are predominately solitary, thus it is hypothesized that separate housing more closely mimics their natural biology (Lang, 1975; von Houwald et al., 2007). Many older publications support this viewpoint – "At the Bronx Zoo, we have never had two adults that, regardless of sex, would live peaceably together, except for a pair during the brief estrus periods of the female" (Crandall, 1964). Accounts from the Bristol Zoo describe multiple attempts to house animals together that were unsuccessful because of aggression and fighting, resulting in a strict policy of separation except during breeding (Greed, 1983; Partridge, 1983). We caution zoo management staff who are housing their pygmy hippos communally that this practice has resulted in severe injuries and even death (Jarofke & Klös, 1982; Greed, 1983; Osakwe et al., 1988; Gippoliti & Leoni, 1999; Flacke et al., Chapter 3).

Nevertheless, a separate housing strategy is not uniform throughout the zoo community, and a primary constraint is the availability of sufficient space. Both Rahn (1978) from Zoo Berlin and Thompson & Ryan (2001) from Chicago's Lincoln Park Zoo reported that the majority of zoos they surveyed (24 in Europe; 23 in North America) housed animals communally. Rahn (1978)

maintained that the animals might be aggressive on first encounter but, once accustomed to each other, they could be housed together without difficulty, and advocated that housing in pairs or family groups is easier from a logistical standpoint. Gippoliti & Leoni (1999) from the Rome Zoological Garden acknowledged that the species is solitary, but not necessarily asocial – all three of their animals would often rest together "peacefully" in mud wallows on exhibit. However, they hypothesized that housing adults together or in adjacent enclosures might result in more stereotypies and possibly compromise normal maternal behavior (Gippoliti & Leoni, 1999), presumably secondary to associated stress.

Although it is clearly possible to house certain individuals together without overt aggression or outward signs of stress, there are no controlled studies investigating the potentially detrimental effects of this 'unnatural' social situation. Lang (1975) firmly believed that young animals raised and housed together do not develop natural reproductive physiology and behavior. It is important to note that, even when housed individually, pygmy hippos often share an exhibit area and thus have visual, auditory, and/or olfactory contact. In such situations, the animal may not perceive itself as being solitary or 'isolated' despite the physical separation. Being physically separate but in close proximity to conspecifics may also result in low-level stress with long-term detrimental consequences (von Houwald et al., 2007). Currently there are no data available to determine the relative merits of either approach or associated welfare implications.

There is unlikely to be one 'correct' way to house and manage pygmy hippos because many factors inherently vary between zoological institutions, affecting options and outcomes. On-going first-hand experience coupled with directed research efforts has substantially improved husbandry since pygmy hippos were first exported from West Africa. However, if we wish to optimize captive pygmy hippo welfare, further *ex situ* research is necessary to determine the potential effects of various husbandry practices on stress, reproduction and health, coupled with *in situ* studies of natural ecology and behavior.

2.7 REPRODUCTION

Age ranges for puberty in pygmy hippos are presented in Table 2.1. The youngest captive female to produce a viable offspring was 23 months old at the time of conception, but the average age is eight years (Steck, 2015). Age at first conception in captivity is primarily determined by access to breeding males and availability of appropriate space for placing offspring, thus is more heavily influenced by zoo management practices than by the natural onset of sexual maturity.

Table 2.1 - Average age at puberty or first conception for the pygmy hippo.

Reference	Age (years)
Crandall, 1964	8
Lang, 1968	4 – 5
Lang, 1975	2½ – 5½
Boever, 1978	2
Laws, 1984	4 – 5
Osakwe et al., 1988	4 ♀
Eulenberger, 1995	2 – 4
Schubert, 2004	2½ – 3

Wild common hippo females in sub-Saharan Africa reach sexual maturity, as measured by the presence of ovarian follicles and corpora lutea, between six and 20 years of age, with an average of nine to eleven years (Laws & Clough, 1966; Sayer & Rakha, 1974; Marshall & Sayer, 1976; Smuts & Whyte, 1981). In contrast, puberty in captive females, as measured by regular ovarian cyclicity, occurs between two and a half and four years of age (Dittrich, 1976; Graham, Reid, Webster, Richards, & Joseph, 2002; Wheaton, Joseph, Reid, Webster, Richards, & Savage, 2006). Hypotheses for this disparity include: *a*) a flawed aging technique for wild specimens; *b*) density-dependent factors in wild populations; *c*) differences in nutritional status and energy balance between captive and wild hippos; *d*) unnaturally close proximity of the male in captivity; *e*) accelerated growth rates in captivity; *f*) absence of parasites and infectious diseases

in captivity; *g*) lack of social competition and stress in captivity (Dittrich, 1976; Graham et al., 2002; Wheaton et al., 2006; Macdonald, 2007). Population reduction measures (culling) of wild common hippos also leads to earlier onset of puberty in the remaining females, most likely due to a lack of competition and greater access to resources (Marshall & Sayer, 1976). This finding lends support to the hypothesis that conditions in captivity favor accelerated sexual maturation in common hippos, and it is likely that a similar effect occurs in pygmy hippos.

Average estrous cycle length in pygmy hippos (Table 2.2) is primarily derived from behavioral observations. However, preliminary studies of estrous cycle physiology utilizing hormone metabolites have initially supported behavioral data. Dathe & Kuckelkorn (1989) used skin and salivary secretions to measure progesterone metabolites for two adult females (Table 2.2). Interestingly, regular cyclicity was evident from hormone changes in both skin and salivary secretions, but behavioral signs of estrus were only observed on one occasion during the entire six-month study period. Paris, Millar, Colenbrander, & Schwarzenberger (2008) also found sporadic correlation between behavioral observations and physiological estrus based on analysis of reproductive hormone metabolites from dung samples. We thus caution that detection of estrus may be inconsistent if based on behavioral observations alone.

Table 2.2 - Estrous cycle duration for the pygmy hippo.

Reference	Zoological Facility	Duration (days)
Stroman & Slaughter, 1972	National Zoo Washington DC, USA	28 – 30
Lang, 1975	Zoo Basel, Switzerland	30 – 40 (mean 35)
Boever, 1978	–	38
Partridge, 1983	Bristol Zoo, UK	28
Dathe & Kuckelkorn, 1989	Tierpark Berlin, Germany	27 – 32 (mean 28)
Eulenberger, 1995	–	28 – 30
Paris et al., 1998	–	30 – 36
Thompson, 2002	–	28
Schubert, 2004	–	28 – 40

Common hippos exhibit year-round estrous cycling, but there is a peak of calving events during the rainy season in areas with distinct wet and dry periods (Laws & Clough, 1966; Pienaar, van Wyk, & Fairall, 1966; Marshall & Sayer, 1976; Smuts & Whyte, 1981). Whether wild pygmy hippos exhibit year-round or seasonal estrous cycling and calving remains unknown. Pygmy hippo calves have been born during every month of the year in zoological institutions across the globe, but this does not equate to continuous year-round cycling for this species in the wild. Hentschel (1990) stated that calving may be concentrated at the end of the rainy season in Côte d'Ivoire (August/September). Schomburgk (1913) reported that in Liberia births occur primarily in the dry season (November/December), but he provided no supporting evidence and this inference could only have been made by his local informants. Seasonal births would presumptively be adaptive in habitats such as the Upper Guinean forests with distinct wet and dry seasons affecting resources for offspring, including food availability and marked habitat changes prompted by cyclical flooding during the rains.

Gestation period is approximately 200 days (Table 2.3), and is reported to be a few days shorter for primiparous females (Zschokke & Steck, 2001). It should be emphasized that pregnancy detection in this species can be challenging because the 'classic' behavioral and physical changes are often not obvious (Hornaday, 1920; Hediger, 1946; Lang, 1975). Weaning age varies dramatically (Table 2.3) and is dependent on logistical issues unique to each zoo. Rahn (1978) reported an age range of three to twelve months at weaning in a survey of 24 European zoological facilities. The AZA Pygmy Hippo Husbandry Manual suggested weaning at six to eight months (Thompson & Ryan, 2001). Weaning age in the wild is unknown, but may occur as a yearling based on footprint analysis of mother/offspring pairs in the Taï National Park, Côte d'Ivoire (Hentschel, 1990; van Heukelum, 2011). The question of natural weaning age in wild populations can only be answered through direct observational *in situ* research.

Table 2.3 - Gestation, birth weights, and age at weaning for the pygmy hippo.

Reference	Gestation (days)	Birth weight (kg)	Weaning age (months)
Steinmetz, 1937	201 – 210	6.5 – 7.0	9 – 15
Hediger, 1946	approx. 210	–	–
Roth, 1962	204 – 210	5.0 – 7.0	–
Walker, 1964	201 – 210	3.0 – 4.5	–
Stroman & Slaughter, 1972	192 – 196	3.4 – 6.4	3 – 4
Lang, 1968	190 – 210	4.5 – 6.2	–
Lang, 1975	188 – 200	mean 5.7	10 – 12
Boever, 1978*	206 – 210	–	3 – 4
Leutenegger, 1978	187 – 204	3.6 – 7.0	–
Greed, 1983	193 – 208	5.1 – 5.3	–
Partridge, 1983	193 – 201	–	6 – 13
Laws, 1984	190 – 210	–	–
Taylor & Greenwood, 1986*	186 – 270	–	–
Jarofke, 1993*	approx. 200	–	–
Zschokke & Steck, 2001	187 – 214 (mean 199)	2.2 – 7.1 (mean 5.2)	–
Miller, 2003*	188 – 210[1]	4.0 – 8.0	6 – 12
Schubert, 2004	187 – 214	5.5 – 6.5	6

[1]Referencing Eltringham (1999), who was in turn referencing Lang (1975) and others
*Serial editions of the textbook "Zoo and Wild Animal Medicine"

Leutenegger (1978) reviewed all pygmy hippo births from 1919 to 1975, totaling 219 calves. The overall calf survival rate was 62%; calf mortality most commonly occurred within the first month after parturition (Leutenegger, 1978). Higher birth weights were correlated with a greater likelihood of survival beyond three months of age. A steady increase in neonate survival over time was attributed to greater experience in maintaining the species in captivity and increasing awareness that parturition takes place on land (Leutenegger, 1978). Pygmy hippo calves have drowned soon after birth, even in shallow pools, but other calves have been born in shallow water without detrimental consequences. Lang (1975) reported that all primiparous females at Zoo Basel and several other European zoos produced offspring that were either stillborn or didn't survive beyond the first year of life; he predicted the same for the captive population at

large. However, Leutenegger's (1978) comprehensive evaluation of studbook data revealed 55% calf survival for primiparous mothers. This striking contradiction evidences the potential problems with personal observations and communications – they may be correct within context but should not be extrapolated to broad population-wide conclusions without supportive evidence-based research.

Eventually, we hope that assisted reproductive technologies, such as artificial insemination, will become available for enhancing genetic diversity and for facilitating the breeding of pygmy hippos that are physically distant from each other. Much less is known about reproductive physiology in males, but Saragusty, Hildebrandt, Bouts, Göritz, & Hermes (2010) have started by investigating semen collection and preservation techniques. Refined, standardized methods for non-invasive monitoring of estrous cycles, identifying timing of ovulation, and early detection of pregnancy are also needed.

2.8 Female-biased Sex Ratio

A larger percentage of female calves coupled with higher mortality rates for male calves have resulted in population-wide skewed sex ratio (Table 2.4). This trend was first reported by Zoo Basel, where one prolific breeding pair produced 38 offspring, ten male and 28 female, a sex ratio of 0.263 (26.3% males) (Lang 1968). Stroman & Slaughter (1972) similarly noted a 0.333 (33.3% males) sex ratio for 24 calves born at the National Zoo in Washington DC between 1927 and 1955. Saragusty, Hermes, Hofer, Bouts, Göritz, & Hildebrandt (2012) analyzed data from the 2008 International Studbook for pygmy hippos surviving to five years of age and reported a ratio of 0.383 (38.3% males), significantly different to the expected ratio of 0.5 ($p < 0.0001$).

Table 2.4 - Sex ratios in the *ex situ* pygmy hippo population. The actual ratios may vary slightly as animals of unknown sex were not included in the calculations.

Reference	Source	% males	Total animals of known/unknown sex
Stroman & Slaughter, 1972	National Zoological Park, Washington DC, USA	33.3	24/0
Leutenegger, 1978	International Studbook	45.6	271/19
Wirz-Hlavacek, Zschokke, & Studer, 2001	International Studbook	40.9	879/39
Thompson, 2002	North American Regional Studbook, Chicago, USA	37.5	48/1
Steck, 2008	International Studbook	42.5	1,089/32
Steck, 2014	International Studbook	42.0	1,361/69
Steck, 2015	International Studbook	39.0*	364/3*

*Extant population at the end of 2014

Why is it important to unravel the mystery of the female-biased sex ratio? First, an excess of females in the captive population can cause significant management issues for zoos and affect husbandry practices. Second, if a biased sex ratio also occurs in wild populations, its adaptive significance might provide clues to the ecology and reproductive physiology of the species. Last and perhaps most importantly, if the phenomenon is specific to captivity or is linked to certain husbandry factors, it may indicate a response to underlying physiological and physical stressors with implications for long-term health and population viability.

Thus, the critical question becomes: is the female-biased sex ratio an artefact of captivity or a reflection of normal pygmy hippo biology? Fisher's (1930) sex-allocation theory predicts an equal number of male and female offspring unless differential investment is required or there is a form of competition. The Trivers-Willard Hypothesis focuses on differential investment and holds that mothers in 'good condition' at the time of conception tend to produce more sons (Trivers & Willard, 1973). However, 'good condition' is an ill-defined descriptor that likely encompasses not only nutritional state but also implies normal physiological processes, a lack of chronic stress, and the absence of underlying disease. Alternatively, the Local Resource Competition Hypothesis maintains that competition for access to locally limited resources

within small groups of related individuals selectively favors the evolution of a sex ratio biased toward the dispersing sex (Clark, 1978). It is unknown whether the assumptions governing this hypothesis – e.g., one sex disperses and other competes with their same-sex parent for local resources essential for reproduction – apply to wild pygmy hippo populations. The only study of home range size in wild pygmy hippos reported smaller territories for females than males, and overlap of one male's home range with that of several females (Bülow, 1987). The smaller territory size for females suggests that they may experience more resource competition than males, but the sample size in this study was very limited (n = 1 male, n = 5 females) and confounding factors were numerous.

Zschokke (2002) examined relationships between sex ratio and several environmental and husbandry factors and concluded that some of these factors do play a role because the sex ratio varied dramatically among zoological institutions. Specifically, with increased quantity and quality of food, described as "favorable feeding conditions", less male calves are produced; on the other hand, female pygmy hippos housed in groups versus singly had more male calves (Zschokke, 2002). Boonyarittichaikij (2010) investigated a more extensive list of individual and zoo-related factors and also found natal sex ratio to vary significantly between the 33 zoos in her study. Diet, degree of human contact, and husbandry-related stress were all potentially correlated with offspring sex ratio, but none were statistically significant (Boonyarittichaikij, 2010).

In other mammalian species, including humans, both diet and stress, via their effects on parental hormone levels, can affect sex ratio (James, 1996; Rosenfeld & Roberts, 2004). In rats and hamsters, higher maternal stress levels led to a female-biased offspring ratio (Lane & Hyde, 1973; Pratt & Lisk, 1989). The physiological mechanisms through which stress at conception and/or during pregnancy affects sex ratio are not entirely understood, but one possibility is sex-selective embryonic processes mediated by changes in the levels of glucocorticoid and reproductive hormones (Krackow, 1995). Other potential mechanisms include a skewed X:Y ratio of semen (the 'primary' sex ratio), selective inactivation of one type of sperm by the

female (a variation of cryptic female choice), and selective resorption of embryos of the more costly sex (Krackow, 1995).

Saragusty et al. (2012) used fluorescence *in situ* hybridization to determine the primary sex ratio in semen collected by electro-ejaculation of male pygmy hippos under general anesthesia. They found an average of 43% Y-chromosome spermatozoa, significantly different to the expected 50%, and proposed that males can adjust the ratio of X- and Y-chromosome spermatozoa and thus avoid father–son competition for territories and breeding rights (Saragusty et al., 2012), a variant of the Local Resource Competition Hypothesis. Although an intriguing contribution to the sex ratio question, only ten males were sampled (approximately 7% of the captive male population), all originating from European zoos. A broader, population-wide study, including evaluation of wild animals, is necessary to determine if the findings are generally applicable on a population-wide basis.

It is not known if wild pygmy hippo populations exhibit a female-biased sex-ratio. Hentschel (1990) reported a male to female ratio of 1:2 for 15 adults that he captured in Taï National Park for telemetry and translocation research. This observation could be explained by sex differences in ability to avoid trapping, but we are left with the intriguing possibility that a female-biased sex ratio serves some sort of adaptive ecological purpose for this species. Rigorous *in situ* investigations are logistically challenging, but essential if we are to go beyond the observations of the captive population. Indeed, in their IUCN Species Survival Commission Report – "Conservation Strategy for the Pygmy Hippopotamus" – (Mallon et al., 2011) highlighted research on the sex ratio as a conservation priority.

2.9 General Health and Medicine

As early as the 1940s, the pygmy hippo was described as extremely hardy, rarely suffering from illness (Hediger, 1946). In the Handbook of Zoo Medicine, originally published in 1976 as "Zootierkrankheiten," Lindau (1982) astutely states that "hippopotamuses are extremely difficult patients for the veterinarian since clinical examinations are of a very limited nature."

He goes on to say that fortunately "hippopotamuses rarely experience problems." Detailed literature describing medical and surgical conditions for pygmy hippos is indeed limited. Lindau's (1982) chapter provided the first synopsis of illnesses in captive common and pygmy hippos, based mostly from personal communications between the author and other zoo veterinarians. Specific infectious diseases included: one case of Salmonellosis with recovery after systemic antibiotic therapy (R. Göltenboth, pers. comm.); one case of Pasteurellosis resulting in death (P. Weilenmann, pers. comm.); and two cases of enterotoxaemia and ensuing death the day after arrival from West Africa (P. Weilenmann, pers. comm.). Non-infectious causes of mortality included aspiration of amniotic fluid followed by suffocation in a neonate (D. M. Jones, pers. comm. 1974) and generalized neoplasia of unknown type (R. Göltenboth, pers. comm.). Other medical conditions described were dystocia and wounds inflicted by conspecifics. Parasites were listed as inconsequential in captive hippos, as per reports from zoos in Berlin and Köln.

Jarofke & Klös (1982) conducted a world-wide survey regarding disease and immobilization protocols for pygmy hippos. The survey was sent to all 138 zoological institutions holding the species at the time, and they received data from 45 institutions for a total of 147 animals. The most commonly reported problems were various dermatological issues including dermatitis and abscesses, occurring primarily in colder weather. Wounds and injuries inflicted by conspecifics as the result of aggressive behavior were also common, especially neonates and juveniles attacked by adults. Gastrointestinal problems included diarrhea and obstipation and dental issues consisting of canine 'tusk' overgrowth, often necessitating immobilization for corrective measures. Other less common medical issues included glomerulonephritis (n = 3), grass awn ocular foreign body (n = 3), keratitis (n = 1), umbilical hernia (n = 2), ovarian neoplasia (n = 1) and several reports of dystocia. As reported by Lindau (1982), parasites were considered unimportant for pygmy hippos. Common causes of mortality included enteritis, abscess, trauma (primarily in young animals), and gastrointestinal foreign body obstruction (Jarofke & Klös, 1982).

More recently published zoological medicine textbooks include a limited drug formulary for hippopotamids; controlled pharmacological studies have not been conducted in either species (Eulenberger, 1995; Miller, 2003). Hematology and serum biochemistry reference ranges for pygmy hippos are available from Walzer & Stalder (2014), but are derived from a limited number of animals. We strongly recommend that veterinary clinicians continually report hematology and serum chemistry results from their pygmy hippo patients to the International Species Information Systems (ISIS) database, and indicate whether the data are from healthy or ill animals, in order to improve reference range validity.

A comprehensive review of all primary literature reporting disease and mortality for pygmy hippos is presented chronologically in Table 2.5. Malocclusion resulting in canine tusk overgrowth occurs fairly frequently, similar to common hippos. Lagomorphs (rabbits) and rodents are also frequently affected by malocclusion and dental pathology (Legendre, 2002; Harcourt-Brown, 2007). The incisors are most frequently affected because they grow continually through the animal's lifetime, as do hippo canine teeth. Contributing etiological factors in rabbits and rodents include poor nutrition (processed, soft foods and inadequate amounts of coarse plant matter), mandibular and maxillary malformations, genetic influences, inappropriate husbandry, and inadequate Vitamin D levels from insufficient exposure to natural sunlight (Legendre, 2002; Harcourt-Brown, 2007). A combination of similar factors likely plays a role in the development of this condition in hippopotamids as well.

Table 2.5 - Disease, morbidity and mortality in pygmy hippos. Each report concerns a single adult animal unless otherwise noted.

Reference	Condition
Cohrs, 1952	Abortion secondary to (unidentified) protozoal infection (two events for the same female)
Schulze, 1955	Severe interstitial nephritis and cystitis, renal failure
Bush, Lemken, & Moore, 1972	Uterine prolapse
Tijskens, 1973	Malocclusion and canine tooth overgrowth
Gray & Bush, 1974	Malocclusion and canine tooth overgrowth
Fábián, 1976	Meconium-associated obstipation (neonate)

Reference	Condition
Franz, Heymann, & Zscheile, 1978	Umbilical hernia (one year old female)
Graf, 1981	Seizures and convulsions followed by acute death due to undetermined toxin exposure
Heuschele, Doyle, Hooker, Gottling, & Kawanabe, 1982	Demonstration of antibodies to infectious bovine rhinotracheitis virus (aclinical)
Miller & Boever, 1983	Recurrent rectal prolapse, subsequent rectal stricture (adult female)
Partridge, 1983	Dermatitis and dermal abscesses
Pearce, Gustavo, Gulland, & Knight, 1985	Arthritis, chronic dermatitis
Osakwe et al., 1988	Gastro-intestinal parasites (hookworm)
Kumar, Singh, & Husni, 1990	Ventral abdominal puncture (trauma) followed by surgical repair
Schüppel, Kinne, & Reinacher, 1994	Demonstration of antibodies to Bornavirus (aclinical)
Weston, Fagella, Burt, Crowley, & Moore, 1996	Benign osteoma of the oral cavity Canine tooth overgrowth
Reddacliff, Kirkland, Hartley, & Reece, 1997	Encephalomyocarditis virus infection and acute death
Flach et al., 1998	Dystocia secondary to fetal oversize/fetal death
Gippoliti & Leoni, 1999	Dermal abscesses, associated with colder weather
Hildebrandt & Göritz, 1999	Uterine leiomyoma
Johnston, 2002	Malocclusion and canine tooth overgrowth (2 adult animals)
Mbaya, Aliyu, Nwosu, & Ibrahim, 2008	Anaplasmosis – *Anaplasma marginale* (aclinical)
Wings, Hatt, Schwarm, & Clauss, 2008	Gastroliths (gravel) – incidental finding on necropsy
Bouts, Vordermeier, Flach, & Routh, 2009	Mycobacteriosis (aclinical)
Cracknell, Stidworthy, & Holliman, 2011	Leptospirosis – *Leptospira icterohaemorrhagica*
Masters, Franklinos, Feltrer, Pocknell, Bolt, Smith, & Molenaar, 2014	Anaplastic sarcoma – oral cavity

The mycobacteriosis case report by Bouts, Vordermeier, Flach, & Routh (2009) warrants further discussion. Many zoological institutions include routine screening for organisms in the *Mycobacterium tuberculosis* complex as part of quarantine and zoonotic disease management programs. The pygmy hippo in question had positive results on several ante mortem tests for mycobacterial infection, but was subsequently negative at post mortem based on gross,

histological, and culture evidence. Jarofke & Klös (1982) reported a similar scenario for one female pygmy hippo that was euthanized after a positive intradermal skin test. A detailed analysis of testing options available for mycobacteriosis is beyond the scope of this review, but a consistent, reliable ante mortem test that is both highly sensitivity and specific is not currently available and domestic animal tests have not been validated for pygmy hippos. Bouts et al. (2009) hypothesized that cross-reaction with non-tuberculous environmental mycobacterial proteins may have produced false-positive results. To date, only one case of *Mycobacterium tuberculosis* complex has been histologically confirmed in the pygmy hippo (Flacke et al., Chapter 3) and one in the common hippo (Lindau, 1982). Although hippopotamids are susceptible to tuberculosis, it is not a common disease in either species in captivity. Since the implications of positive diagnostic tests can be severe, including euthanasia of non-infected (potentially valuable breeding) animals of an endangered species, we advise judicious interpretation of test results in conjunction with the clinical picture and potential transmission risks. Furthermore, hippo aquarium exhibits incorporating fish should closely monitor the quality of the fish to preclude potential contact with aquatic mycobacterial species that can subsequently complicate interpretation of screening tests.

None of the disease conditions in Table 2.5 is unique to the Hippopotamidae but some, such as chronic dermatitis, are probably unique to captivity and often linked to husbandry shortfalls. For the common hippo, skin problems in captivity are often associated with poor water quality and dermal dehydration secondary to being housed indoors in the winter months (Reifinger, Kübber-Heiss, & Linhart, 1997; Clyde, Wallace, & Pocknell, 1998; Helmick, Rush, Ogburn, Trupkiewicz, & Garner, 2007; Spriggs & Reeder, 2012), and the etiology in pygmy hippos is undoubtedly similar. Leiomyoma of the reproductive and gastrointestinal tracts is not infrequent in older pygmy hippos (Flacke et al., Chapter 3), but only one published account is available (Hildebrandt & Göritz, 1999). It is unknown if any of the conditions listed in Table 2.5 also occur in wild populations. Continued vigilance in investigating and reporting various causes of morbidity and mortality, both rare and common, will contribute to an evolving framework to help veterinarians and animal keepers improve pygmy hippo health and welfare.

2.10 POLYCYSTIC KIDNEY DISEASE (PKD)

Polycystic kidney disease (PKD) is a degenerative condition where cystic dilation replaces functional renal tissue. It is the most common genetic cause of renal failure in humans, with the great majority of cases being inherited rather than acquired (Igarashi & Somlo, 2007). PKD in pygmy hippos was first identified by Jarofke and Klös (1982) as an incidental finding in the animal euthanized due to a positive intradermal tuberculin test (described above). PKD has since been reported both as an incidental finding at necropsy and as a cause of kidney failure and associated clinical signs including lethargy, anorexia, weight loss, and polyuria (Raymond, Eaton, & Montali, 2000; Nees, Schade, Clauss, Steinmetz, Ehrensperger, Steck, & Hatt, 2009). The condition has been identified from originally wild-caught pygmy hippos and from animals born in captivity. It appears to be more common in females, but this may simply mirror the higher proportion of females in the captive population.

Both Raymond et al. (2000) and Nees et al. (2009) hypothesized that PKD in pygmy hippos exhibits a familial pattern of inheritance, similar to humans, because several affected animals were genetically related. Furthermore, Nees et al. (2009) proposed PKD as a mechanism to account for the female-biased sex ratio. Autosomal recessive PKD in humans often manifests as stillbirth or death in the first few weeks of life (Zerres, Rudnik-Schöneborn, Steinkamm, Becker, & Mücher, 1998; Wilson, 2004), and a similar phenomenon could preferentially affect male pygmy hippo neonates. We advocate a simple method to test this hypothesis – conducting necropsies on all non-viable calves to evaluate both gross and histological renal structure.

Significant intra-familial variability in clinical severity of inherited PKD in humans points to genetic and environmental modifying factors that influence disease progression (Torres et al., 2007). It is unknown whether husbandry conditions such as diet, housing, management practices, stress, or other environmental factors may contribute to the development of PKD in pygmy hippos. If the disease is linked to captivity-associated factors, then we would expect a low prevalence in wild populations. The only definitive way to determine if PKD is present in

the wild population is to perform ultrasonography or necropsy on adult animals in the wild, logistically a very challenging prospect. The majority of the founder population (89 of 162 animals) originated from Liberia and procurement expeditions often sourced multiple animals from the same limited geographic areas, so a particular genetic make-up may be over-represented in the captive population. If continued research supports a familial inheritance pattern, developing genetic markers for ante-mortem testing should be explored. A comprehensive understanding of the prevalence, demographics, and etiology of PKD in pygmy hippos is necessary to guide management and breeding recommendations.

2.11 OBESITY

For almost a century the pygmy hippo has, perhaps unwisely, been designated a naturally 'fat' creature. Hornaday (1920) opined that "the fat and always rotund condition of the female is so pronounced that the usual signs of maternity are negligible… her fat round body is shaped like a barrel." Hediger (1946) described pygmy hippos as "plump, round, and fatty animals," but went on to sing their praises nonetheless. Similarly, both Stroman & Slaughter (1972) and Thompson & Ryan (2001) described the pygmy hippo as "naturally round and fat." However, these descriptions are not supported by camera trap photos of wild pygmy hippos throughout their range in West Africa that repeatedly portray the animals as (subjectively) thinner and sleeker than their captive counterparts (Collen, Howard, Konie, Daniel, & Rist, 2011; Eshuis, 2011; Conway, 2013).

An adult male pygmy hippo shot by Heslop (1944) in Nigeria weighed 450 lbs (204 kg). Hentschel (1990) weighed two (live) adult female pygmy hippos in Taï National Park; they tipped the scales at 165 and 170 kg. These three weights are the only substantiated data available from wild animals. Body weight reported for captive pygmy hippos varies widely (Table 2.6), but other than 180 – 200 kg reported by Eulenberger (1995) the range extends notably above the highest weight for a wild animal (the 204 kg adult male). The sample size of objective data for wild pygmy hippos is indeed very small, and obtaining morphometric

measurements, including accurate body weights, from wild animals is a research priority for this species.

Table 2.6 - Body weight of the pygmy hippo in captivity.

Reference	Weight Range (kg)
Pilleri, 1962	200 – 275
Crandall, 1964	Up to 231
Walker, 1964	160 – 180 or 160 – 240
Lang, 1968	180 – 260
Lang, 1975	192 – 273 ♂ 179 – 267 ♀
Boever, 1978*	200 – 250
Laws, 1984	180 – 275
Taylor & Greenwood, 1986*	200 – 250
Jarofke, 1993*	200 – 250
Eulenberger, 1995	180 – 200
Thompson, 2002	160 – 270
Miller, 2003*	160 – 350
Steck, 2015	180 – 260

*Serial editions of the textbook "Zoo and Wild Animal Medicine"

Zoo literature reports higher body weights in captive animals than for their wild conspecifics in many species, suggesting lack of physical activity, overfeeding, inappropriate diet and, in some cases, contraception of females as contributing factors. Although many historical dietary recommendations are now understood to be inappropriate, for example feeding four liters of commercial horse pellets daily (Stroman & Slaughter, 1972) or a diet consisting of 75% corn, maize, cabbage, and other root crops (Osakwe et al., 1988), obesity in pygmy hippos is likely still exacerbated by a relative oversupply of highly fermentable sugars including commercial fruits and cereal-based pellets (Schwarm et al., 2006). Additionally, wild pygmy hippos spend a large percentage of their time foraging, thus consuming energy while ingesting multiple small quantities of food over time (Bülow, 1987; Hentschel, 1990). In contrast, zoos generally provide the entire ration as one or two daily feedings.

Hippopotamids have comparatively lower energy requirements and basal metabolic rates than ruminants (Clauss et al., 2004), so even a small excess in energy intake can lead to weight gain. Schwarm et al. (2006) recommend roughage-only diets and regular weighing to monitor body condition. Housing and feeding pygmy hippos separately also facilitates control of dietary intake. Specific recommendations concerning nutritional composition, dietary requirements, and feeding for the pygmy hippo are available from several well-researched sources, such as the EAZA and AZA Husbandry Manual (von Houwald et al., 2007). This document extensively references a report from the Nutritional Advisory Group wherein Lintzenich & Ward (1997) detailed dietary recommendations for a multitude of ungulates. They classify the pygmy hippo as a non-ruminant generalist browser similar to the black rhinoceros (*Diceros bicornis*). However, a more restricted strategy is suggested by information on the foraging habits of wild pygmy hippos (Bülow, 1987; Hentschel, 1990), and dentition supports an intermediate feeding strategy that combines browsing and grazing in varying proportions, depending on nutritional requirements and availability (Lang, 1975).

Maintaining pygmy hippos at ideal body weight is essential from a health and welfare perspective as obesity worsens many pathological conditions of the musculoskeletal system, most notably arthritis and foot problems. Standardized criteria for subjectively assessing body condition are an essential component for developing appropriate nutritional management strategies. Direct observational studies combined with fecal collection for dietary composition analysis from wild pygmy hippos throughout their range are necessary to establish diet and feeding strategies definitively. Additional data concerning weight and body condition from wild pygmy hippos are also highly desirable to help establish normal morphometric measures. Finally, further research is needed to elucidate the potential influence of obesity on reproductive capacity.

2.12 POPULATION GENETICS

The last known pygmy hippo export from West Africa occurred in the early 1980s. Maintaining genetic diversity in captive populations involves vigilant breeding management with the overall goals of reducing long-term loss of founder genomes and maintenance of genotypic variation. Such systematic breeding requires knowledge of parental pedigrees and population genetic structure. However, the traditional method of calculating inbreeding coefficients and mean kinship values is via analysis of Studbook data, but such data inaccurately assumes equal relatedness among founders, often contains errors in recorded pedigrees, and cannot clearly identify genetically under represented animals. Furthermore, certain founder pygmy hippos are dramatically over-represented, a phenomenon first identified by Greed (1983) that continues through to the present (Steck, 2014).

Saragusty et al. (2012) used 2008 studbook data to calculate a mean kinship coefficient of 0.031, an average inbreeding coefficient of 0.0666, and a gene diversity of 0.9699. Although these values present a fairly positive genetic scenario, they are not based on molecular genetic analysis and they assume that founder animals are unrelated, and may therefore inaccurately reflect the true genetic state of the captive population. Even a brief analysis of capture and import history reveals that most animals were sourced from limited geographic areas, often several at the same time, increasing the likelihood that the founding population included many fathers, mothers, sons, daughters, brothers, sisters and first cousins. A molecular genetic database for the pygmy hippo would be an invaluable tool for holistic conservation efforts targeting both captive and wild populations. The recent identification of multiple DNA markers for the species is an essential first step toward developing such a database (Senn et al., 2014). A comparison of the genetic structure of wild and captive populations via molecular markers will help delineate relatedness and help determine whether inbreeding depression is a concern for the captive population.

2.13 ANESTHESIA

An important limiting factor for tackling the above list of unresolved issues with captive management is handling a large, dangerous and sometimes disagreeable pachyderm. Although some procedures can be performed non-invasively or with behavioral training (e.g. ultrasound scans; collection of saliva, feces, urine, or even blood), many other techniques (e.g. tusk trimming, semen sampling in males) are not feasible without sedation or anesthesia for both safety and welfare reasons. However, a review of anesthesia protocols for either common or pygmy hippos quickly uncovers a persistent theme – hippopotamids are 'difficult' to anesthetize. Difficulties include: accurate weight estimation for dosage calculations; anesthetic drug protocol selection; a thick dermal layer that is challenging to penetrate with a needle or dart; a thick layer of subcutaneous fat leading to variable systemic drug absorption; limited peripheral vascular access; redundant pharyngeal tissue complicating endotracheal intubation; bradycardia; hypoventilation, hypoxemia and apnea; thermoregulatory issues due to a high volume to surface area ratio; and a high fatality rate (Miller, 2003; Miller, 2007; Miller, Fleming, Citino, & Hofmeyr, 2014; Walzer & Stalder, 2014).

There are a number of case reports describing anesthesia for the pygmy hippo (Table 2.7), but many of the older pharmacological agents have fallen out of favor because of frequent undesirable side effects (including death) and the recent availability of safer, more effective combinations. For example, etorphine has resulted in numerous adverse effects and inconsistent depth of anesthesia and phencyclidine is no longer commercially available. The diversity of anesthetic drug combinations and protocols reported in Table 2.7 point to the difficulty in achieving consistent, safe, reliable immobilizations in this species. Stress levels immediately prior to immobilization, highly dependent on individual animal temperament and the degree of handling to which the animal is accustomed, also play an important role in the variability of anesthetic efficacy.

Learning from the trials and tribulations of their predecessors, veterinary anesthetists have recently developed protocols employing a combination of ketamine, medetomidine (or detomidine), and butorphanol (Table 2.7) that have proven more effective and reliable than potent opioids like etorphine or carfentanil. Bouts, Hermes, Gasthuys, Saragusty, Taylor, Routh, & Hildebrandt (2012) safely anesthetized 14 adult pygmy hippos with a combination of 1.2 mg/kg ketamine and 0.08 mg/kg medetomidine followed by endotracheal intubation and maintenance on oxygen and isoflurane gas, and experienced minimal complications and no mortalities. Several similar combination protocols using butorphanol in addition to, or in place of, ketamine are also reported to be safe and effective (Miller, 2007; Miller et al., 2014; Walzer & Stalder, 2014).

The only known successful immobilizations of wild pygmy hippos were conducted in the Taï National Park, in the 1980s by Dr. Knut Hentschel. He first immobilized a pygmy hippo in the Abidjan zoo, where a total dose of 300 mg ketamine was sufficient to induce anesthesia. The dose needed to achieve the same result for wild animals and to allow the safe placement of telemetry collars proved to be substantially higher, ranging from 1100 to 3300 mg ketamine per animal, with an average dose of 1500 mg (Hentschel, 1990). Three pygmy hippos were successfully immobilized in 1982 and eight more in 1985; there were no losses under anesthesia with this ketamine-only protocol (Hentschel, 1990).[1]

[1] Throughout Hentschel's multi-year field study, there were two mortalities in Taï: one in 1982, several days after capture while being held in a field enclosure; the second in 1985, from injuries sustained in a pitfall capture trap. During Waldemar Bülow's field study of the pygmy hippos translocated from Taï to Azagny National Park in early 1986, three animals were lost: (i) a telemetry-collared female died after becoming entangled in tree roots and drowning; (ii) two males died shortly after arrival in Azagny (several months after their only anesthetic episode), probably succumbing to a combination of dehydration and exertional myopathy syndrome as a result of the transport. Anesthetic deaths did not occur during either study.

Table 2.7 - Anesthesia protocols used for captive pygmy hippos, in chronological order, illustrating the diversity of anesthetic agents and variable efficacy. Blank sections indicate the data was not provided.

Author	Reason for Immobilization	Patient Weight	Protocol	Reversal	Effect
Manton & Jones, 1971		120 kg ♀	Etorphine 2 mg and Acepromazine 25 mg	Nalorphine 100 mg	-Initial excitation, marked salivation, adequate anesthetic depth
Bush, Lemken, & Moore, 1972	Uterine prolapse	Adult ♀	Phencyclidine 200 mg and Combelen* 400 mg	Not administered	-Adequate for surgery, but minimal muscle relaxation and intermittent arousal
Alford, Burkhart, & Johnson, 1974		Adult	Etorphine 6mg	Diprenorphine 12 mg	
Gray & Bush, 1974	Malocclusion	Adult ♂	Phencyclidine 250 mg and Acepromazine 250 mg		-Recumbent within 20 min, sufficient for tusk trim and skull radiographs
Boever, 1978		Juvenile Adult	Etorphine 2 – 5 mg Etorphine 4 – 7 mg		
Franz, Heymann, & Zscheile, 1978	Umbilical hernia repair	102 kg ♀	Phencyclidine 100 mg and Combelen* 50 mg		-Adequate for surgery but minimal muscle relaxation -Same doses inadequate for suture removal 10 days later
Jarofke & Klös, 1982	Laceration repair	Adult	Ketamine 1650 mg and Xylazine 200 mg		-Insufficient plane of anesthesia

Author	Reason for Immobilization	Patient Weight	Protocol	Reversal	Effect
Lindau, 1982 ***via R. Göltenboth, pers. comm.**	Wound treatment	Adult ♀	Ketamine 10 mg/kg, with Xylazine 2 mg/kg and Combelen* 2 mL		-Heavily sedated but still stumbling and aggressive
Miller & Boever, 1983	Rectal prolapse	Adult ♀	Etorphine 5 mg and Xylazine 10 mg	Diprenorphine 10 mg	-Adequate for surgery; minimal muscle relaxation
Pearce, Gustavo, Gulland, & Knight, 1985	Examination and treatment of dermatitis and polyarthritis	216 kg ♀	a. Xylazine alone b. Ketamine and Xylazine c. Immobilon** alone d. Immobilon** and Xylazine e. Etorphine and Xylazine	Diprenorphine at 1.3x the Etorphine dose	-21 immobilizations in total -Xylazine alone not effective -Effects of other combinations variable and are summarized in the publication
Kumar, Singh, & Husni, 1990	Ventral puncture wound repair		Immobilon** 2.25 mg	Diprenorphine 1 mL	-Adequate sedation and analgesia for surgical repair
Jarofke, 1993		Adult	a. Xylazine 100 – 150 mg and Immobilon** 2 – 3 mg b. Xylazine 1.5 mg/kg, with Polamivet% 10 mL and Combelen* 5 mL		

Author	Reason for Immobilization	Patient Weight	Protocol	Reversal	Effect
Weston, Fagella, Burt, Crowley, & Moore, 1996	Surgery for oral mass removal and tusk trim	270 kg ♂	-*Oral premedication:* Detomidine 44 µg/kg and Diazepam 0.5 mg/kg -*Induction:* Atropine 20 µg/kg and Ketamine 0.2 mg/kg Multiple top-up doses with Butorphanol, Ketamine, Detomidine, and Hyaluronidase	Yohimbine 0.11 mg/kg	-Mild sedation after premedication and initial injections; multiple top-up injections given before endotracheal intubation and maintenance on O_2 and isoflurane was possible
Kawamura, Hibino, Nakamura, Hashikawa, & Tamamura, 1996	Malocclusion, tusk trims	300 kg ♂ 250 kg ♀	a. Etorphine 4 mg and Xylazine 200 mg b. Etorphine 4 mg and Azaperone 160 – 180 mg c. Ketamine 15 mg/kg and Xylazine 2 mg/kg	a. Diprenorphine 8 mg b. Diprenorphine 8 mg c. No reversal given	-Tremors seen with etorphine, prolonged renarcotization after diprenorphine injection -Acute respiratory arrest and death of the female 21 min after injection with protocol b.
Flach et al., 1998	Caesarean section	200 kg ♀	Etorphine 2 – 3 mg, with Xylazine 100 – 150 mg and Ketamine 150 mg	Diprenorphine 2x the Etorphine dose and Yohimbine 0.125 mg/kg	-Sufficient for endotracheal intubation and maintenance on O_2 and halothane
Morris, Bicknese, Janssen, Loudis, Shima, Sutherland-Smith, & Young, 2001	Tusk trims	Adult	Ketamine 1.0 mg/kg		-Adequate sedation, good recovery

Author	Reason for Immobilization	Patient Weight	Protocol	Reversal	Effect
Johnston, 2002	Dental procedures	Adult ♂ Adult ♀	Ketamine 1.0 mg/kg and Medetomidine 0.08 mg/kg		-Sufficient for endotracheal intubation and maintenance on O_2 and isoflurane
Kreeger & Arnemo, 2002		Adult	Etorphine 2.5 mg and Xylazine 125 mg	Diprenorphine 5.0 mg and Yohimbine 0.125 mg/kg	
Miller, 2003, 2007; **Walzer & Stalder, 2014**			a. Carfentanil 7.5 mg/kg and Xylazine 0.08 mg/kg b. Telazol[@] 2.2 – 3.5 mg/kg and Midazolam 0.1 mg/kg c. Medetomidine 0.036 mg/kg and Butorphanol 0.2 mg/kg d. Ketamine 1.25 mg/kg and Butorphanol 0.018 mg/kg e. Detomidine 0.05 mg/kg and Butorphanol 0.15 mg/kg	a. Naltrexone 100x Carfentanil dose and Yohimbine 0.1 – 0.3 mg/kg c. Atipamizole 5x the Medetomidine dose and Naltrexone 3x the Butorphanol dose e. Yohimbine 0.1 – 0.3 mg/kg and Naltrexone 0.4 – 0.6 mg/kg	

Author	Reason for Immobilization	Patient Weight	Protocol	Reversal	Effect
Bouts, Hermes, Gasthuys, Saragusty, Taylor, Routh, & Hildebrandt, 2012	General health assessment, trans-rectal ultrasound, electro-ejaculation	250kg est. ♂ and ♀	Ketamine 1.2 mg/kg and Medetomidine 0.08 mg/kg	Atipamezole 0.4 mg/kg	-Sufficient for endotracheal intubation and maintenance on O_2 and isoflurane
Miller et al., 2014			a. Ketamine 0.8 – 2.0 mg/kg, with Detomidine 0.07 – 0.08 mg/kg and Butorphanol 0.15 – 0.2 mg/kg b. Medetomidine 0.035 mg/kg and Butorphanol 0.2 mg/kg	a. Atipamezole 3–5x the Detomidine dose and Naltrexone 5x the Butorphanol dose b. Atipamezole 3–5x the Medetomidine dose and Naltrexone 3–5x the Butorphanol dose	-Overall ratings for procedures were good to excellent -Sometimes used in combination with oral premedication: Midazolam 0.1 mg/kg or Diazepam 0.5 mg/kg or Detomidine 0.045 mg/kg

*Combelen: 10mg propionylpromazine per mL
**Immobilon: 2.45mg etorphine with 10mg acepromazine per mL
%Polamivet: 2.5mg levomethadone with 0.125mg fenpipramide per mL
@Telazol: 50mg tiletamine and 50mg zolazepam per mL

Ketamine-only protocols for general anesthesia have become uncommon for multiple reasons, including hyperthermia, muscle rigidity, 'rough' recoveries, and CNS alterations akin to hallucinations. Nevertheless, the drug was safe and effective for the only immobilizations ever conducted with wild pygmy hippos (n = 11). However, any future immobilizations of wild pygmy hippos should rather employ multi-drug combination protocols similar to those that have worked well for captive animals (Miller et al., 2014). Hentschel's experience predicts that substantially higher dose rates will be necessary for wild pygmy hippos, a fair warning for those planning *in situ* immobilization. Advances in the realm of field anesthetic equipment have made monitoring devices such as pulse oximetery, capnography, and blood gas analysis, as well as portable inhalant anesthetic machines, feasible for remote field conditions. Therefore, this infamously 'difficult' to anesthetize species can now be handled with more experience, care and precaution, both in captivity and in the wild, than was possible in the past.

2.14 CONCLUSIONS

As a result of this comprehensive review, we conclude that further research in the following key areas is essential to guide collaborative, integrated conservation efforts for the pygmy hippo and to help optimize health, husbandry, welfare, and reproduction in captivity:

- Elucidating *in situ* social structure, behavioral ecology, feeding strategies, and nutritional requirements such that husbandry practices in captivity can be adjusted accordingly;

- Establishing and comparing baseline stress levels for both wild and captive populations;

- Assessing the potential for captivity-induced stress to alter normal physiology, behavior, or contribute to disease processes;

- Determining the prevalence and demographics of PKD to guide future population-wide breeding recommendations;

- Examining the potential influence of external factors, including diet, hormones, and microbial pathogens, in the development of PKD;

- Developing standard, consistent, non-invasive methods for monitoring estrous cycles and diagnosing pregnancy;

- Refining and streamlining the processes for collecting and preserving semen;

- Clarifying underlying reasons for the lack of breeding success experienced by many zoos;

- Elucidating the influence of obesity on reproductive physiology and general welfare;

- Determining underlying mechanisms and potential ecological significance of the female-biased sex ratio;

- Developing a 'family tree,' using molecular genetic tools, to guide captive breeding efforts and maximize representation of unique genomes.

Most importantly, prospective *in situ* research is needed to deduce the 'normal' parameters for a wide variety of ecological and physiological phenomena, ranging from morphometrics and reproductive physiology to habitat utilization and population demographics. Knowing whether wild pygmy hippos also have polycystic kidney disease or a biased sex ratio will influence hypotheses about these phenomena in captive animals. Meanwhile, continued investigations with the captive population through carefully considered, step wise, hypothesis-driven analysis is crucial, especially for elucidating the degree to which captive husbandry conditions affect various physiological and pathological processes. Humans have kept wildlife species in captivity for millennia – we can never perfectly replicate a natural ecological environment for these animals, but our desire to optimize their welfare while under our care should never fade.

2.15 ACKNOWLEDGEMENTS

Funding to support the primary author's efforts in compiling this document was provided by the University of Western Australia and the Institute for Breeding of Rare and Endangered African Mammals (IBREAM). The authors are grateful to Beatrice Steck of Zoo Basel, Christie Eddie of Omaha's Henry Doorly Zoo, Dr. Phillip Robinson of the University of Toledo and Dr. Robert Hermes of the Leibniz Institute for Zoo and Wildlife Research (IZW) for providing supporting information and various elusive documents we have cited in this review.

2.16 LITERATURE CITED

Alford, B. T., Burkhart, R. L., & Johnson, W. P. (1974). Etorphine and diprenorphine as immobilizing and reversing agents in captive and free-ranging mammals. *J. Am. Vet. Med. Assoc.*, *164*(7), 702–705.

Blaszkiewitz, B. (1983). Haltung und Zucht des Zwergflußpferdes (*Choeropsis liberiensis* Morton 1849) im Zoologischen Garten Berlin. *Bongo*, *7*, 71–78.

Benirschke, K. (2007). East African River Hippopotamus & Pygmy Hippopotamus. In: Comparative Placentation. Retrieved from <http://placentation.ucsd.edu/hippofs.htm>

Boever, W. J. (1978). Artiodactyla. In M. E. Fowler (Ed.), *Zoo and Wild Animal Medicine* (pp. 771–815). Philadelphia, Pennsylvania: W.B. Saunders Company.

Boisserie, J.-R. (2005). The phylogeny and taxonomy of Hippopotamidae (Mammalia: Artiodactyla): a review based on morphology and cladistic analysis. *Zool. J. Linn. Soc.*, *143*(1), 1–26.

Boisserie, J.-R., Lihoreau, F., & Brunet, M. (2005). Origins of Hippopotamidae (Mammalia, Cetartiodactyla): towards resolution. *Zool. Scr.*, *34*(2), 119–143.

Boonyarittichaikij, R. (2010). *Studying the effect of factors that potentially influence the sex ratio of captive pygmy hippopotamus (Choeropsis liberiensis)*. MSc Thesis, Utrecht University.

Bouts, T., Hermes, R., Gasthuys, F., Saragusty, J., Taylor, P., Routh, A., & Hildebrandt, T. B. (2012). Medetomidine-ketamine-isoflurane anaesthesia in pygmy hippopotami (*Choeropsis liberiensis*) - a case series. *Vet. Anaesth. Analg.*, *39*(1), 111–118.

Bouts, T., Vordermeier, M., Flach, E., & Routh, A. (2009). Positive skin and serologic test results of diagnostic assays for bovine tuberculosis and subsequent isolation of Mycobacterium interjectum in a pygmy hippopotamus (*Hexaprotodon liberiensis*). *J. Zoo Wildl. Med.*, *40*(3), 536–542.

Bülow, W. (1987). *Untersuchungen am Zwergflußpferd, Choeropsis liberiensis im Azagny - Nationalpark, Elfenbeinküste*. Diplomarbeit, Zoologischen Institut Braunschweig.

Bush, M., Lemken, R., & Moore, J. A. (1972). Prolapsed uterus in a pygmy hippopotamus. *J. Am. Vet. Med. Assoc.*, *140*, 651.

Büttikofer, J. (1890). *Reisebilder aus Liberia, Bd. 2*. Leiden: E. J. Brill.

Cameron, E. Z. (2004). Facultative adjustment of mammalian sex ratios in support of the Trivers–Willard hypothesis: evidence for a mechanism. *Proc. R. Soc. London B*, *271*, 1723–1728.

Chapman, H. C. (1894). Notes on *Choeropsis liberiensis* (Morton). *Proc. Acad. Nat. Sci. Philadelphia*, 185–187.

Clark, A. B. (1978). Sex ratio and local resource competition in a prosimian primate. *Science (80-.).*, *201*, 163–165.

Clauss, M., Schwarm, A., Ortmann, S., Alber, D., Flach, E. J., Kühne, R., … Hofer, H. (2004). Intake, ingesta retention, particle size distribution and digestibility in the Hippopotamidae. *Comp. Biochem. Physiol. Part A*, *139*(4), 449–459.

Clubb, R., Rowcliffe, M., Lee, P., Mar, K. U., Moss, C., & Mason, G. J. (2008). Compromised survivorship in zoo elephants. *Science (80-.).*, *322*, 1649.

Clyde, V. L., Wallace, R. S., & Pocknell, A. M. (1998). Dermatitis caused by group G beta-hemolytic Streptococcus in Nile hippos (*Hippopotamus amphibius*). In *Proceedings of the American Association of Zoo Veterinarians and American Association of Wildlife Veterinarians Joint Conference* (pp. 221–225). Omaha, Nebraska.

Cohrs, P. (1952). Protozoen als Ursache wiederholter Fehlgeburten beim Zwergflußpferd (*Choeropsis liberiensis*). *Der Zool. Garten NF*, *19*, 192–195.

Collen, B., Howard, R., Konie, J., Daniel, O., & Rist, J. (2011). Field surveys for the endangered pygmy hippopotamus *Choeropsis liberiensis* in Sapo National Park, Liberia. *Oryx*, *45*(01), 35–37.

Conway, A. L. (2013). *Conservation of the Pygmy Hippopotamus (Choeropsis liberiensis) in Sierra Leone, West Africa*. PhD Thesis, University of Georgia, Athens.

Coryndon, S. C. (1977). The taxonomy and nomenclature of the Hippopotamidae (Mammalia, Artiodactyla) and a description of a two new fossil species. *Proc. R. Netherlands Acad. Arts Sci. B*, *80*(2), 61–88.

Cracknell, J. M., Stidworthy, M., & Holliman, A. (2011). Leptospirosis in a pygmy hippopotamus (*Choeropsis liberiensis*). In *Proceedings of the American Association of Zoo Veterinarians Annual Conference* (pp. 35–37). Kansas City, Missouri.

Crandall, L. S. (1964). Hippopotamuses. In *The Management of Wild Mammals in Captivity* (pp. 530–543). Chicago, Illinois: The University of Chicago Press.

Dathe, H. H., & Kuckelkorn, B. (1989). Progesteronnachweis in Sekreten des Zwergflußpferdes (*Choeropsis liberiensis* Morton, 1844). *Der Zool. Garten NF*, *59*(3), 201–208.

Davies, M. J. (2006). Evidence for effects of weight on reproduction in women. *Reprod. Biomed. Online*, *12*(5), 552–561.

Dittrich, L. (1976). Age of sexual maturity in the hippopotamus. *Int. Zoo Yearb.*, *16*(1), 171–173.

Endo, H., Sasaki, M., Kogiku, H., Hayashi, Y., Komiya, T., Narushima, E., … Yamamoto, M. (2001). Anatomy and histology of the stomach in a newborn pygmy hippopotamus (*Choeropsis liberiensis*). *Mammal Study*, *26*, 53–60.

Eshuis, H. (2011). *Habitat preference and activity pattern of the pygmy hippopotamus analyzed by camera trapping and GIS*. MSc Thesis, Wageningen University.

Eulenberger, K. (1995). Flußpferde. In R. Göltenboth & H. G. Klös (Eds.), *Krankheiten der Zoo- und Wildtiere* (pp. 246–255). Berlin: Blackwell Wissenschafts-Verlag.

Fábián, L. (1976). Darmpech-Obstipation („Fohlenkolik“) bei neugeborenem Zwergflußpferd, *Choeropsis liberiensis*. *Der Zool. Garten NF*, *46*(6), 452–454.

Fisher, R. E., Scott, K. M., & Naples, V. L. (2007). Forelimb myology of the pygmy hippopotamus (*Choeropsis liberiensis*). *Anat. Rec.*, *290*, 673–693.

Flach, E. J., Furrokh, I. K., Thornton, S. M., Smith, J., Parkyn, J. P., & Campbell, E. J. (1998). Caesarean section in a pygmy hippopotamus (*Choeropsis liberiensis*) and the management of the wound. *Vet. Rec.*, *143*, 611–613.

Flower, W. H. (1887). On the pygmy hippopotamus of Liberia, *Hippopotamus liberiensis* (Morton), and its claims to distinct generic rank. *Proc. Zool. Soc. London*, 612–614.

Franz, W., Heymann, H., & Zscheile, D. (1978). Immobilisierung und Nabelbruchoperation beim Zwergflusspferd (*Choeropsis liberiensis*). *Erkrankungen Der Zootiere Verhandlungsbericht*, *20*, 197–200.

Geisler, J. H., & Uhen, M. D. (2003). Morphological support for a close relationship between hippos and whales. *J. Vertebr. Paleontol.*, *23*(4), 991–996.

Gippoliti, S., & Leoni, A. (1999). The pygmy hippopotamus at Rome Zoological Garden. *Int. Zoo News*, *46*(6), 335–339.

Graf, Z. (1981). Über den Verlust eines Zwergflußpferdes im Budapester Zoo. *Erkrankungen Der Zootiere Verhandlungsbericht*, *23*, 389–390.

Graham, L. H., Reid, K., Webster, T., Richards, M., & Joseph, S. (2002). Endocrine patterns associated with reproduction in the Nile hippopotamus (*Hippopotamus amphibius*) as assessed by fecal progestagen analysis. *Gen. Comp. Endocrinol.*, *128*, 74–81.

Gray, C. W., & Bush, R. M. (1974). Malocclusion in a pygmy hippopotamus. In *National Zoological Park 18-Month Report* (Vol. 1, p. 41). Washington, D.C.: Smithsonian Institution Press.

Greed, G. R. (1983). Husbandry and breeding of the pigmy hippopotamus (*Choeropsis liberiensis*). In *Proceedings Symposium 7, Association of British Wild Animal Keepers* (pp. 10–23).

Harcourt-Brown, F. M. (2007). The progressive syndrome of acquired dental disease in rabbits. *J. Exot. Pet Med.*, *16*(3), 146–157. http://doi.org/10.1053/j.jepm.2007.06.003

Hashimoto, K., Saikawa, Y., & Nakata, M. (2007). Studies on the red sweat of the Hippopotamus amphibius. *Pure Appl. Chem.*, *79*, 507–517.

Hediger, H. (1946). Die Baseler Zwergflußpferd-Zucht. *Zool. Garten Basel*, *74*, 23–29.

Hegner, B. (1967). Zur Morphologie des Auges von *Choeropsis liberiensis* und *Hippopotamus amphibius* (Mammalia, Artiodactyla, Hippopotamidae). *Acta Zool.*, *48*(1-2), 59–85.

Helmick, K. E., Rush, E. M., Ogburn, A. L., Trupkiewicz, J. G., & Garner, M. (2007). Dermatopathy in captive hippopotamus (*Hippopotamus amphibius*). In *Proceedings of the American Association of Zoo Veterinarians, American Association of Wildlife Veterinarians and Association of Zoos and Aquariums Joint Conference* (p. 92). Knoxville, Tennessee.

Hentschel, K. M. (1990). *Untersuchung zu Status, Ökologie und Erhaltung des Zwergflusspferdes (Choeropsis liberiensis) in der Elfenbeinküste*. Doktorandarbeit, Technischen Universität Carolo-Wilhelmina, Braunschweig.

Heslop, I. R. P. (1944). The pigmy hippopotamus. *Field*, *183*, 588.

Heuschele, W. P., Doyle, L. G., Hooker, P. A., Gottling, K. L., & Kawanabe, P. S. (1982). Current status of some important viruses of domestic ruminants in captive wild ruminants in the USA. In *Proceedings of the American Association of Zoo Veterinarians Annual Conference* (pp. 94–121). New Orleans, Louisiana.

Hildebrandt, T. B., & Göritz, F. (1999). Use of Ultrasonography in Zoo Animals. In M. E. Fowler & R. E. Miller (Eds.), *Zoo and Wild Animal Medicine* (4th ed., pp. 41–54). Philadelphia, Pennsylvania: W.B. Saunders Company.

Hornaday, W. T. (1912). Our pygmy hippopotami. *New York Zool. Soc. Bull.*, 16(52), 877–879.

Hornaday, W. T. (1920). Birth of a Pygmy Hippopotamus. *New York Zool. Soc. Bull.*, *23*(1), 11–13.

Igarashi, P., & Somlo, S. (2007). Polycystic kidney disease. *J. Am. Soc. Nephrol.*, *18*, 1371–1373.

James, W. H. (1996). Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *J. Theor. Biol.*, *180*, 271–286.

Jarofke, D. (1993). Hippopotamidae (Hippopotamus). In M. E. Fowler (Ed.), *Zoo and Wild Animal Medicine, Current Therapy* (3rd ed., pp. 522–525). Philadelphia, Pennsylvania: W.B. Saunders Company.

Jarofke, D., & Klös, H. G. (1982). Immobilisierung und Krankheiten von Zwergflusspferden: Auswertung einer Umfrage bei mehr als 100 Zoologischen Gärten. *Erkrankungen Der Zootiere Verhandlungsbericht*, *24*, 361–374.

Johnston, N. W. (2002). Atraumatic malocclusion in two pygmy hippos (*Choeropsis liberiensis*). *J. Vet. Dent.*, *19*(3), 144–147.

Kawamura, H., Hibino, S., Nakamura, A., Hashikawa, H., & Tamamura, F. (1996). A comparison between etorphine hydrochloride and a combination of xylazine hydrochloride and ketamine hydrochloride for the immobilization of pygmy hippopotamus, Choeropsis liberiensis. *J. Japanese Assoc. Zool. Gard. Aquariums*, *37*, 113–116.

Krackow, S. (1995). Potential mechanisms for sex ratio adjustment in mammals and birds. *Biol. Rev.*, *70*, 225–241.

Kranz, K. R. (1982). A note on the structure of tail hairs from a pygmy hippopotamus (*Choeropsis liberiensis*). *Zoo Biol.*, *1*, 237–241.

Kreeger, T. J., & Arnemo, J. M. (2002). *Handbook of Wildlife Chemical Immobilization, International Edition*. Fort Collins, Colorado: Wildlife Pharmaceuticals, Inc.

Kumar, R., Singh, J., & Husni, M. M. (1990). Anaesthesia and repair of a ventral hernia in a pygmy hippopotamus (*Choeropsis liberiensis*). *Indian Vet. J.*, *67*(2), 166–167.

Lane, E. A., & Hyde, T. S. (1973). Effect of maternal stress on fertility and sex ratio: A pilot study with rats. *J. Abnorm. Psychol.*, *82*(1), 78–80.

Lang, E. M. (1968). Das Zwergflußpferd. In B. Grzimek (Ed.), *Grzimek's Tierleben - Enzyklopädie des Tierreiches, Bd. 13* (pp. 118–120). Zürich: Kindler Verlag AG.

Lang, E. M. (1975). *Das Zwergflußpferd*. Wittenberg Lutherstadt: A. Ziemsen Verlag, DDR.

Langer, P. (1975). Macroscopic anatomy of the stomach of Hippopotamidae Gray, 1821. *Zentralblatt Für Veterinärmedizin C*, *4*, 334–359.

Laws, R. M. (1984). Hippopotamuses. In D. Macdonald (Ed.), *The Encyclopaedia of Mammals* (pp. 506–511). New York, New York: Facts on File, Inc.

Laws, R. M., & Clough, G. (1966). Observations on reproduction in the hippopotamus (*Hippopotamus amphibius* LINN). *Symp. Zool. Soc. London*, *15*, 117–140.

Legendre, L. F. J. (2002). Malocclusions in guinea pigs, chinchillas and rabbits. *Can. Vet. J.*, *43*, 385–390.

Leidy, J. (1853). On the osteology of the head of hippopotamus, and a description of the osteological characters of a new Genus of Hippopotamidæ. *J. Acad. Nat. Sci. Philadelphia*, *2*, 207–224.

Leutenegger, M. (1978). Pygmy hippopotamus *Choeropsis liberiensis* births in captivity. *Int. Zoo Yearb.*, *18*(1), 234.

Lewison, R., & Oliver, W., IUCN SSC Hippo Specialist Subgroup. (2008). *Choeropsis liberiensis*. IUCN 2012; IUCN Red List of Threatened Species. Version 2012.1. Available from www.iucnredlist.org. Accessed 19.09.2012.

Lim, C. E. D., & Cheng, N. C. L. (2011). Obesity and reproduction. *J. Aust. Tradit. Med. Soc.*, *17*(3), 143–145.

Lindau, K.-H. (1982). Hippopotamuses. In R. Göltenboth & D. Jarofke (Eds.), *Handbook of Zoo Medicine* (pp. 216–223). New York: Van Nostrand Reinhold Company.

Lintzenich, B. A., & Ward, A. M. (1997). Hay and Pellet Ratios: Considerations in Feeding Ungulates. In *Nutrition Advisory Group Handbook* (p. Fact Sheet 006). Retrieved from <http://www.nagonline.net/Home/Site Map.htm>

Lochte, T. (1951). Untersuchungen an Haaren eines neugeborenen Nilpferdes und eines Zwergflußpferdes. *Der Zool. Garten NF*, *18*, 119–124.

Macalister, A. (1873). The anatomy of *Choeropsis liberiensis*. *Proc. R. Irish Acad.*, *2*, 494–500.

Macdonald, A. A. (2007). The Reproductive Biology of the Pigmy Hippopotamus (*Choeropsis liberiensis*) with comparative observation on the Common Hippopotamus (*Hippopotamus amphibious*). In F. von Houwald, A. A. Macdonald, O. Pagan, & B. Steck (Eds.), *Husbandry Guidelines for the Pygmy Hippopotamus (Hexaprotodon liberiensis)* (pp. 86–100). Basel: Zoo Basel, Switzerland.

Macdonald, A. A., & Bosma, A. A. (1985). Notes on placentation in the Suina. *Placenta*, *6*(1), 83–91.

Macdonald, A. A., & Hartman, W. (1983). Comparative and functional morphology of the stomach in the adult and newborn pigmy hippopotamus (*Choeropsis liberiensis*). *J. Morphol.*, *177*, 269–276.

Mah, P. M., & Wittert, G. A. (2010). Obesity and testicular function. *Mol. Cell. Endocrinol.*, *316*, 180–186.

Mallon, D., Wightman, C., De Ornellas, P., & Ransom, C. (2011). *Conservation Strategy for the Pygmy Hippopotamus*. Gland, Switzerland & Cambridge, UK: IUCN Species Survival Commission.

Maluf, N. S. R. (1978). Anatomy of the kidneys of a newly born pigmy hippopotamus (*Choeropsis liberiensis* Morton). *Zentralblatt Für Veterinärmedizin C*, *7*, 28–48.

Maluf, N. S. R. (1994). Renal anatomy of the pigmy hippopotamus (*Choeropsis liberiensis*): An overview. *Zentralblatt Für Veterinärmedizin C*, *23*, 189–204.

Manton, V. J. A., & Jones, P. M. (1971). Whipsnade Park Report 1970. *The Zoological Society of London, Scientific Report*, 1969–1971, 553.

Marshall, P. J., & Sayer, J. A. (1976). Population ecology and response to cropping of a hippopotamus population in eastern Zambia. *J. Appl. Ecol.*, *13*(2), 391–403.

Masters, N., Franklinos, L., Feltrer, Y., Pocknell, A., Bolt, D., Smith, S., & Molenaar, F. M. (2014). Successful chemotherapy of an oral anaplastic sarcoma in a pygmy hippopotamus (*Hexaprotodon liberiensis*). In *Proceedings of the International Conference on Diseases of Zoo and Wild Animals* (p. 78). Warsaw, Poland.

Mbaya, A. W., Aliyu, M. M., Nwosu, O., & Ibrahim, U. I. (2008). Captive wild animals as potential reservoirs of haemo and ectoparasitic infections of man and domestic animals in the arid-region of Northeastern Nigeria. *Vet. Arh.*, *78*(5), 429–440.

Miller, M. (2007). Hippopotami. In G. West, D. Heard, & N. Caulkett (Eds.), *Zoo Animal and Wildlife Immobilization and Anesthesia* (pp. 579–584). Ames, Iowa: Blackwell Publishing.

Miller, M. A. (2003). Hippopotamidae (Hippopotamus). In M. E. Fowler & R. E. Miller (Eds.), *Zoo and Wild Animal Medicine* (5th ed., pp. 602–612). St. Louis, Missouri: Saunders Elsevier.

Miller, M., Fleming, G. J., Citino, S. B., & Hofmeyr, M. (2014). Hippopotamidae. In G. West, D. Heard & N. Caulkett (Eds.), *Zoo Animal and Wildlife Immobilization and Anesthesia* (2nd ed., pp. 787–795). Ames, Iowa: John Wiley & Sons, Inc.

Miller, R. E., & Boever, W. J. (1983). Repair of a rectal stricture and prolapse in a pygmy hippopotamus (*Choeropsis liberiensis*). *J. Zoo Wildl. Med.*, *14*(2), 63–66.

Morris, P. J., Bicknese, B., Janssen, D., Loudis, B., Shima, A., Sutherland-Smith, M., & Young, L. (2001). Chemical restraint of juvenile east African river hippopotamus (*Hippopotamus amphibius kiboko*) at the San Diego Zoo. In D. Heard (Ed.), *Zoological Restraint and Anesthesia* (pp. 10–14). Ithica, New York: International Veterinary Information Systems <www.ivis.org>.

Morton, S. G. (1844). On a supposed new species of hippopotamus. *Proc. Acad. Nat. Sci. Philadelphia*, *2*, 14–17.

Nees, S., Schade, B., Clauss, M., Steinmetz, H. W., Ehrensperger, F., Steck, B., & Hatt, J.-M. (2009). Polycystic kidney disease in the pygmy hippopotamus (*Hexaprotodon liberiensis*). *J. Zoo Wildl. Med.*, *40*(3), 529–535.

Nomura, O., & Yasue, H. (1999). Genetic relationships among hippopotamus, whales, and bovine based on SINE insertion analysis. *Mamm. Genome*, *10*, 526–527.

Osakwe, M. E., Meduna, A. J., Kigbu, E. E., & Ishaya, P. D. (1988). Management of pigmy hippopotamus and West African manatee in Jos Wildlife Park. *Niger. F.*, *53*, 175–178.

Paris, M., Millar, R., Colenbrander, B., & Schwarzenberger, F. (2008). Non-invasive assessment of female reproductive physiology in the pygmy hippopotamus (*Choeropsis liberiensis*). In *Proceedings of the 16th International Congress on Animal Reproduction* (p. 17). Budapest, Hungary.

Partridge, J. (1983). The management of the pygmy hippopotamus (*Choeropsis liberiensis*) at Bristol Zoo. *Int. Zoo News*, *30*(3), 28–41.

Pasquali, R. (2006). Obesity, fat distribution and infertility. *Maturitas*, *54*, 363–371.

Pasquali, R., & Gambineri, A. (2006). Metabolic effects of obesity on reproduction. *Reprod. Biomed. Online*, *12*(5), 542–551.

Pearce, P. C., Gustavo, C., Gulland, F., & Knight, J. (1985). Immobilization of a pygmy hippopotamus (*Choeropsis liberiensis*). *J. Zoo Anim. Med.*, *16*(3), 104–106.

Pienaar, U. de V., van Wyk, P., & Fairall, N. (1966). An experimental cropping scheme of hippopotami in the Letaba River of the Kruger National Park. *Koedoe*, *9*, 1–33.

Pilleri, G. (1962). Zur Anatomie des Gehirnes von *Choeropsis liberiensis* Morton (Mammalia, Artriodactyla). *Acta Zool.*, *43*(2-3), 229–245.

Pocock, R. I. (1923). The external characteristics of the pigmy hippopotamus (*Choeropsis liberiensis*) and of the Suidæ and Camelidæ. *Proc. Zool. Soc. London*, *35*, 531–549.

Pratt, N. C., & Lisk, R. D. (1989). Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (*Mesocricetus auratus*). *J. Reprod. Fertil.*, *87*, 763–769.

Rachoń, D., & Teede, H. (2010). Ovarian function and obesity - interrelationship, impact on women's reproductive lifespan and treatment options. *Mol. Cell. Endocrinol.*, *316*, 172–179.

Rahn, P. (1978). On housing the pygmy hippopotamus in pairs: a survey of zoo practice. *Int. Zoo Yearb.*, *18*(1), 187–190.

Raymond, J. T., Eaton, K. A., & Montali, R. J. (2000). A disease in captive pygmy hippopotamuses (*Choeropsis liberiensis liberiensis*) anatomically resembling polycystic kidney disease. In *Proceedings of the American Association of Zoo Veterinarians and International Association for Aquatic Animal Medicine Joint Conference* (p. 302). New Orleans, Louisiana.

Reddacliff, L. A., Kirkland, P. D., Hartley, W. J., & Reece, R. L. (1997). Encephalomyocarditis virus infections in an Australian zoo. *J. Zoo Wildl. Med.*, *28*(2), 153–157.

Reifinger, V. M., Kübber-Heiss, A., & Linhart, P. (1997). Streptokokkenseptikämie und Candidiasis bei einem sieben Tage alten Flusspferd (*Hippopotamus amphibius*) mit Missbildung der grossen herznahen Gefässe. *Erkrankungen Der Zootiere Verhandlungsbericht*, *38*, 395.

Renshaw, G. (1904). The Pigmy Hippopotamus. In *Natural History Essays* (pp. 113–125). London, UK: Sherratt & Hughes.

Robker, R. L. (2008). Evidence that obesity alters the quality of oocytes and embryos. *Pathophysiology*, *15*, 115–121.

Rosenfeld, C. S., & Roberts, R. M. (2004). Maternal diet and other factors affecting offspring sex ratio: A review. *Biol. Reprod.*, *71*, 1063–1070.

Roth, H. H. (1962). Mitteilung über die Zwergflußpferdzucht in Gelsenkirchen. *Der Zool. Garten NF*, *26*, 327–331.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., & Hildebrandt, T. B. (2012). Male pygmy hippopotamus influence offspring sex ratio. *Nat. Commun.*, *3*, 1–5.

Saragusty, J., Hildebrandt, T. B., Bouts, T., Göritz, F., & Hermes, R. (2010). Collection and preservation of pygmy hippopotamus (*Choeropsis liberiensis*) semen. *Theriogenology*, *74*, 652–657.

Sayer, J. A., & Rakha, A. M. (1974). The age of puberty of the hippopotamus (*Hippopotamus amphibius* Linn.) in the Luangwa River in eastern Zambia. *East African Wildl. J.*, *12*, 227–232.

Schomburgk, H. (1912). On the trail of the pygmy hippo – an account of the Hagenbeck expedition to Liberia. *New York Zool. Soc. Bull.*, *16*(52), 880–884.

Schomburgk, H. (1913). Das Zwergflußpferd, eine zoologische Neuheit. *Kosm. Handweiser Für Naturfreunde*, *2*, 62–65.

Schubert, T. (2004). Haltung von Zwergflusspferden. In W. Puschmann (Ed.), *Zootierhaltung* (pp. 636–639). Frankfurt, Germany: Verlag Harri Deutsch.

Schulze, W. (1955). Nephritis beim Zwergflußpferd. *Der Zool. Garten NF*, *21*, 188.

Schwarm, A., Ortmann, S., Hofer, H., Streich, W. J., Flach, E. J., Kühne, R., … Clauss, M. (2006). Digestion studies in captive Hippopotamidae: a group of large ungulates with an unusually low metabolic rate. *J. Anim. Physiol. Anim. Nutr. (Berl).*, *90*, 300–308.

Schwarm, A., Ortmann, S., Wolf, C., Jürgen Streich, W., & Clauss, M. (2008). Excretion patterns of fluid and different sized particle passage markers in banteng (*Bos javanicus*) and pygmy hippopotamus (*Hexaprotodon liberiensis*): two functionally different foregut fermenters. *Comp. Biochem. Physiol. Part A*, *150*, 32–39.

Sclater, P. L. (1873). Remarks on the Liberian hippopotamus. *Proc. Zool. Soc. London*, 434.

Senn, H., O'Donoghue, P., McEwing, R., & Ogden, R. (2014). Hundreds of SNPs for the Endangered pygmy hippopotamus. *Conserv. Genet. Resour.*, *6*(1), 535–538.

Shimamura, M., Yasue, H., Ohshima, K., Abe, H., Kato, H., Kishiro, T., … Okada, N. (1997). Molecular evidence from retroposons that whales form a clade within even-toed ungulates. *Nature*, *388*, 666–670.

Smuts, G. L., & Whyte, I. J. (1981). Relationships between reproduction and environment in the hippopotamus *Hippopotamus amphibius* in the Kruger National Park. *Koedoe*, *24*, 169–185.

Spriggs, M., & Reeder, C. (2012). Treatment of vasculitis and dermatitis in a 59-yr-old Nile hippopotamus (*Hippopotamus amphibius*). *J. Zoo Wildl. Med.*, *43*(3), 652–656.

Steck, B. (Ed.). (2014). *Pygmy Hippopotamus Choeropsis liberiensis (Morton, 1844) International Studbook 2013* (20th ed.). Basel: Zoo Basel, Switzerland.

Steck, B. (Ed.). (2015). *Pygmy Hippopotamus Choeropsis liberiensis (Morton, 1844) International Studbook 2014* (21st ed.). Basel: Zoo Basel, Switzerland.

Steinmetz, H. (1937). Beobachtungen über die Entwicklung junger Zwergflußpferde im Zoologischen Garten Berlin. *Der Zool. Garten NF*, *9*(6), 255–263.

Stroman, H. R., & Slaughter, L. M. (1972). The care and breeding of the pygmy hippopotamus. *Int. Zoo Yearb.*, *12*(1), 126–131.

Taylor, D., & Greenwood, A. (1986). Hippopotamidae (Hippopotamus). In M. E. Fowler (Ed.), *Zoo and Wild Animal Medicine* (2nd ed., pp. 967–969). Philadelphia, Pennsylvania: W.B. Saunders Company.

Taylor, V. J., & Poole, T. B. (1998). Captive breeding and infant mortality in Asian elephants: A comparison between twenty western zoos and three eastern elephant centers. *Zoo Biol.*, *17*, 311–332.

Thompson, S. D. (2002). *North American Regional Studbook for the Pygmy Hippopotamus (Hexaprotodon liberiensis)*. (E. Brown, Ed.). Chicago, Illinois: Lincoln Park Zoo.

Thompson, S. D., & Ryan, S. (2001). *AZA Pygmy Hippopotamus Husbandry Manual*. Chicago, Illinois: Lincoln Park Zoo.

Torres, V. E., Harris, P. C., & Pirson, Y. (2007). Autosomal dominant polycystic kidney disease. *Lancet*, *369*, 1287–1301.

Trivers, R. L., & Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science (80-.).*, *179*, 90–92.

Ursing, B. M., & Arnason, U. (1998). Analyses of mitochondrial genomes strongly support a hippopotamus-whale clade. *Proc. R. Soc. London B*, *265*, 2251–2255.

Van den Bergh, H. K. (1971). Can the pygmy hippopotamus, *Choeropsis liberiensis* (Morton), look through its open mouth? *Der Zool. Garten NF*, *40*, 167–171.

Van Heukelum, M. (2011). *In search of the elusive Pygmy Hippo; Establishment of methods to determine population structure of Pygmy Hippos in Taï National Park, and assessment of their role in seed dispersal*. MSc Thesis, Wageningen University.

Von Houwald, F., Macdonald, A. A., Pagan, O., & Steck, B. (Eds.). (2007). *Husbandry Guidelines for the Pygmy Hippopotamus (Hexaprotodon liberiensis)*. Basel: Zoo Basel, Switzerland.

Walker, E. P. (1964). Hippopotamuses. In *Mammals of the World, Vol. 2* (pp. 1367–1370). Baltimore, Maryland: The Johns Hopkins Press.

Walzer, C., & Stalder, G. (2014). Hippopotamidae (Hippopotamus). In R. E. Miller & M. E. Fowler (Eds.), *Fowler's Zoo and Wild Animal Medicine* (8th ed., pp. 584–592). Philadelphia, Pennsylvania: Saunders Elsevier.

Weston, E. M. (2000). A new species of hippopotamus *Hexaprotodon lothagamensis* (Mammalia: Hippopotamidae) from the late Miocene of Kenya. *J. Vertebr. Paleontol.*, *20*(1), 177–185.

Weston, E. M. (2003). Evolution of ontogeny in the hippopotamus skull: using allometry to dissect developmental change. *Biol. J. Linn. Soc.*, *80*, 625–638.

Weston, H. S., Fagella, A. M., Burt, L., Crowley, K., & Moore, T. (1996). Immobilization of a pygmy hippopotamus (*Choeropsis liberiensis*) for the removal of an oral mass. In *Proceedings of the American Association of Zoo Veterinarians Annual Conference* (pp. 576–581). Puerto Vallarta, Mexico.

Wheaton, C. J., Joseph, S., Reid, K., Webster, T., Richards, M., & Savage, A. (2006). Body weight as an effective tool for determination of onset of puberty in captive female Nile hippopotami (*Hippopotamus amphibious*). *Zoo Biol.*, *25*, 59–71.

Wilson, P. D. (2004). Polycystic kidney disease. *N. Engl. J. Med.*, *350*, 151–164.

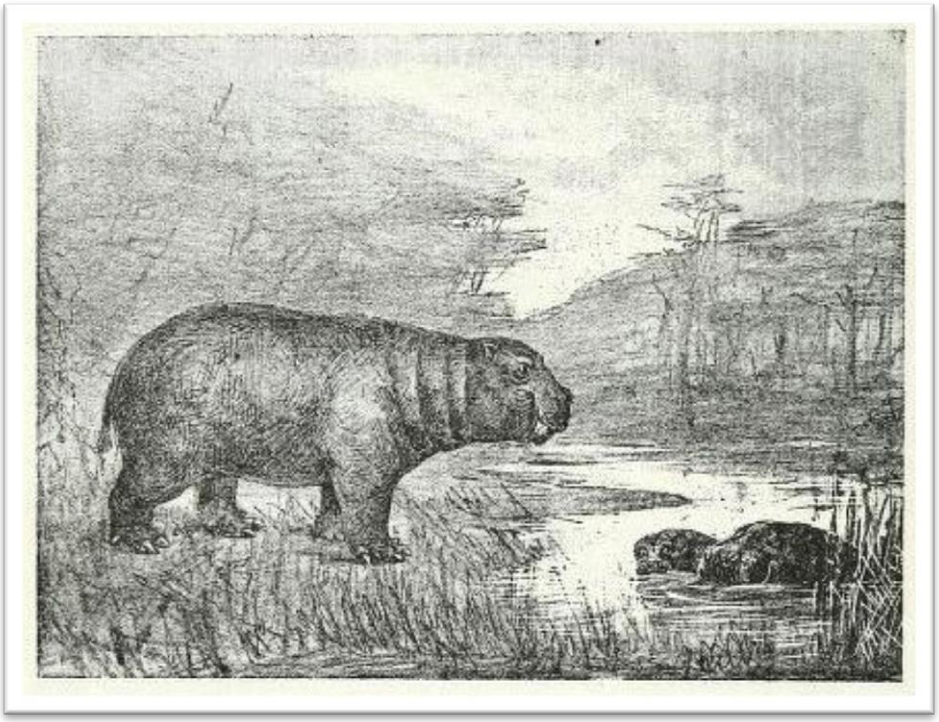
Wings, O., Hatt, J.-M., Schwarm, A., & Clauss, M. (2008). Gastroliths in a pygmy hippopotamus (*Hexaprotodon liberiensis* Morton 1844). *Senckenb. Biol.*, *88*(2), 345–348.

Zerres, K., Rudnik-Schöneborn, S., Steinkamm, C., Becker, J., & Mücher, G. (1998). Autosomal recessive polycystic kidney disease. *J. Mol. Med.*, *76*, 303–309.

Zschokke, S. (2002). Distorted sex ratio at birth in the captive pygmy hippopotamus, *Hexaprotodon liberiensis*. *J. Mammal.*, *83*(3), 674–681.

Zschokke, S., & Steck, B. (2001). Tragzeit und Geburtsgewicht beim Zwergflusspferd, *Hexaprotodon liberiensis*. *Der Zool. Garten NF*, 71, 57–61.

Chapter 3 A RETROSPECTIVE ANALYSIS OF MORTALITY IN CAPTIVE PYGMY HIPPOPOTAMUS (*CHOEROPSIS LIBERIENSIS*) FROM 1912 – 2014



The Liberian Hippo – Johann Büttikofer, 1890

Reisebilder aus Liberia, Band 2, Leiden: E. J. Brill

Behold the hippopotamus!
We laugh at how he looks to us,
And yet in moments dark and grim,
I wonder how we look to him.

–Ogden Nash

A Retrospective Analysis of Mortality in Captive Pygmy Hippopotamus (*Choeropsis liberiensis*) from 1912 – 2014

Gabriella L. Flacke,[1*] Suzana Tkalčić,[2] Beatrice Steck,[3] Kristin Warren,[4] and Graeme B. Martin[1]

[1]*School of Animal Biology, University of Western Australia, Crawley, Australia*

[2]*Western University of Health Sciences, College of Veterinary Medicine, Pomona, CA, USA*

[3]*Zoo Basel, Basel, Switzerland*

[4]*College of Veterinary Medicine, School of Veterinary and Life Sciences, Murdoch University, Murdoch, Australia*

***Address for correspondence**: Gabriella Flacke, School of Animal Biology M092, University of Western Australia, 35 Stirling Highway, Crawley 6009 WA, Australia; +61 470 137 217; gflacke@grs.uwa.edu.au

3.1 ABSTRACT

The pygmy hippopotamus (*Choeropsis liberiensis*) is an IUCN Red List Endangered species (CITES Appendix II) that has been housed in zoological collections since 1912. As wild populations continue to decline throughout the species' range, successful *ex situ* breeding and management, including an understanding of morbidity and mortality, are of upmost importance. This study is the first comprehensive review of mortality data from the captive population since 1982 and significantly expands on previous analyses. We solicited necropsy reports from 129/187 zoological institutions worldwide that currently or previously held pygmy hippos and received data for 404 animals (177 ♂, 220 ♀, 7 undermined sex), representing 43% of pygmy hippos that have died in captivity. Mortality in neonates was primarily due to perinatal causes (51.8% – stillbirth, failure to thrive, weakness, poor suckling reflex, maternal neglect) or parent-inflicted trauma (28%). Common causes of mortality in adult and geriatric animals included cardiovascular disease (16%), degenerative musculoskeletal conditions (10%), obstructive gastrointestinal disease (9%), and renal insufficiency (13%), sometimes associated with advanced polycystic kidney disease (PKD). Although not the direct cause of mortality, a number of adult and geriatric pygmy hippos were also overweight to obese. Infectious causes of mortality in included leptospirosis and encephalomyocarditis virus, the latter usually presenting as acute death and cardiovascular demise. This comprehensive overview presents a useful guide for recommendations in preventative veterinary care and for improved husbandry and management of pygmy hippos in captivity.

Key words: encephalomyocarditis virus (EMCV), leptospirosis, obesity, pathology, polycystic kidney disease (PKD), stillbirth

3.2 INTRODUCTION

The pygmy hippopotamus (*Choeropsis liberiensis*), hereafter 'pygmy hippo,' is listed as an endangered species on the International Union for the Conservation of Nature and Natural Resources (IUCN) Red-List. It is endemic to the Upper Guinean ecosystem of West Africa where its range is limited to fragmented rainforest habitats in Côte d'Ivoire, Liberia, Sierra Leone and Guinea. The size of the wild population is estimated to be less than 2,500 (Ransom et al. 2015). Threats to survival include: habitat loss and fragmentation secondary to logging, mining and agriculture; lack of adequate legal protection for the few remaining intact areas of habitat; poaching for bush meat; and an often unstable political climate in all four range states (Hoppe-Dominik et al. 2011; Mallon et al. 2011; Roth et al. 2004).

The first pygmy hippos were exported from the wild in 1912 and went to Carl Hagenbeck's Zoo in Hamburg, Germany. The *ex situ* population is managed through a Species Survival Plan (SSP) Program in North America, a European Endangered Species Program (EEP) in European facilities, and the International Studbook is maintained at Zoo Basel (Switzerland). The average lifespan for pygmy hippos in captivity is 35 to 40 years; longevity in the wild is unknown. At the end of 2015, the Studbook listed 377 animals (146.225.6) in 140 zoos and private collections worldwide. Most facilities hold two to three animals, usually a breeding pair plus a calf (Steck 2015). Average gestation is 200 days and pygmy hippos are is primarily monotocous. The first captive birth in this species occurred in 1919 at the Bronx Zoo in New York; unfortunately the calf had a congenital deformity and died within 24 hours (Hornaday 1920). Successful reproduction is an important part of conservation efforts for any endangered species held in captivity. However, for pygmy hippos historically high mortality rates of up to 59% in the first year of life (Leutenegger 1978) may threaten the long-term viability of the *ex situ* population. Additionally, pygmy hippos exhibit a female-skewed sex ratio in captivity due to a higher percentage of female calves coupled with higher mortality rates for male calves (Flacke et al. 2015; Zschokke 2002). The sex ratio for the extant population at the end of 2015

was 39.3% male for animals of known sex (Steck 2015), and the associated shortage of breeding-age males presents significant population management issues.

Even though the pygmy hippo has been held in zoological facilities for just over a century, information concerning preventative health care and principal causes of morbidity and mortality is limited. The only previous review was conducted more than 30 years ago by Jarofke and Klös (1982) who reported the most common causes of mortality to be enteritis, abscess, trauma (primarily in young animals), and gastrointestinal foreign body obstruction. Two earlier case reports from European zoos described severe, chronic interstitial nephritis in a 14-year old female (Schulze 1955) and acute neurological disease, seizures and death in an adult male due to suspected toxin exposure (Graf 1981). More recently, a handful of case reports identified single mortality events due to a mycobacterial infection, leptospirosis, lymphoblastic leukemia, and encephalomyocarditis virus (EMCV) (Bouts et al. 2009; Cracknell et al. 2011; McCurdy et al. 2014; Reddacliff et al. 1997). Two additional case series accounts identified 10 animals, all female, affected by polycystic kidney disease (PKD) and categorized the condition as a possible health concern for the *ex situ* population overall (Nees et al. 2009; Raymond et al. 2000). There is no information about morbidity or mortality in wild pygmy hippos other than one incident of leopard predation of a juvenile (Hentschel 1990).

Routine gross post-mortem examination and histopathology are essential tools for detecting diseases and pathological conditions that can potentially reduce long-term population health and viability in zoological collections. The identification of mortality patterns in rare species requires careful examination and monitoring of trends over time. Species-specific husbandry and veterinary protocols, such as those summarized in the Pygmy Hippo Husbandry Manual (von Houwald et al. 2007), can also be optimized through a comprehensive analysis of common causes of morbidity and mortality.

Therefore, the objective of our global retrospective review of necropsy data from zoological collections was to provide a cross-institutional, longitudinal analysis of mortality in pygmy hippos. We aimed to identify trends, common disease conditions and potential risk factors for

mortality that could improve captive management of this species. Additionally, we anticipated that a population-wide overview would indicate whether mortality patterns have changed over time as management and husbandry practices have been modified. We also expected the data to provide evidence to direct veterinarians in preventative care, diagnostic and treatment decisions for optimizing both individual animal and *ex situ* population health.

3.3 MATERIALS AND METHODS

3.3.1 DATA COLLECTION

We solicited necropsy reports from 129 of the 187 zoological institutions and private facilities worldwide that have held pygmy hippos since 1912 and received responses from 121 institutions: Africa – 8; Asia – 9; Australia – 4; Europe – 59; North America – 37; South America – 4. A total of 985 (400.530.55) known mortality events were recorded between 1912 and 31st December 2014 (Steck 2015). We were able to request necropsy reports for a subset of these animals (n = 517) from the 121 institutions that responded to our enquiry. We received information for 404, or 41% of known mortalities through to the end of 2014 (Table 3.1). For 166 (41%) of these animals the cause of mortality was reported without supporting documentation (no gross pathology or histopathological report provided). Reports on gross pathology only (no histopathology) were provided for 80 hippos (20%) and complete gross necropsy and histopathological reports were provided for 158 hippos (39%).

Table 3.1 - Demographic distribution of the mortality cases for pygmy hippos represented in this study.

Age class	Number of pygmy hippos		Sex Ratio (% ♂)
Neonate (0 to 30 days)	193	85.101.7	45.7
Juvenile (31 days up to 3 years)	27	13.14.0	48.1
Adult (3 to 30 years)	121	54.67.0	44.6
Geriatric (30+ years)	63	25.38.0	39.7
Total	404	177.220.7	44.6

3.3.2 CAUSES OF MORTALITY

All necropsy reports were reviewed by a veterinary pathologist. It was not possible to determine the cause of death in all cases as the quality and completeness of the reports varied dramatically. We assumed that the information concerning cause of mortality reported by the submitting institutions was correct and made no further attempts to confirm reported pathology results. For the purposes of data analysis, we assumed that a lack of information concerning a specific organ system indicated an absence of pathology. When we were unable to determine etiology and/or organ system from the information provided, we placed the animal into an "undetermined" category. For hippos that were euthanized for welfare reasons, the cause of death was categorized based on the primary reason for euthanasia and/or the primary pathologic process identified on necropsy. In the absence of sufficient information, we listed the organ system as 'euthanasia' and the cause of death as 'undetermined.' We assigned each pygmy hippo to only one category that most accurately represented the ultimate cause of death, even though many animals experienced morbidity within more than one etiology and/or organ system category.

3.3.3 AGE CATEGORIES

We tabulated the data based on gender and age as follows: neonate 0–30 days; juvenile 31 days up to 3 years; adult 3–30 years; geriatric 30+ years (Table 3.1). Age categories have not previously been defined for this species, so we based 'adult' status on average age at puberty and/or first successful reproduction reported in captivity (Flacke et al. 2015). The 'geriatric' category was based on the maximum age reported for this species in captivity, 35–47 years (Steck 2015). Longevity in captivity is unknown as the oldest individual is still alive (49 years) at the time of writing.

3.3.4 DATA ANALYSIS

The primary cause of mortality for each hippo was classified according to the principal etiology: anesthesia-related; anomaly/congenital; degenerative; infectious/inflammatory; neonatal/perinatal; neoplasia; nutritional; reproductive; toxin; trauma. The 'anesthesia-related'

category refers to a mortality event while anesthetized for a diagnostic or medical/surgical procedure without significant findings to explain the cause of death on post-mortem examination or histopathology. The 'neonatal/perinatal' category includes premature birth, stillbirth, failure to thrive and/or nurse, weakness, maternal neglect, and dystocia. We further categorized cause of mortality as primarily related to one of the following organ systems: cardiovascular; dental; endocrine; gastrointestinal/digestive; hematologic; hepatic; integumentary; multi-systemic; musculoskeletal/neuromuscular; neurologic; ocular; renal/urinary; reproductive; and respiratory. The 'multi-systemic' category included cachexia/starvation, dehydration, drowning, electric shock, exertional myopathy, heat-stroke, hypothermia, trauma, enterotoxemia and septicemia.

Table 3.2 - Causes of mortality by etiology and within age class for captive pygmy hippos between 1912 and 2014.

	Overall ***n* = 404 (%)**	**Neonate** ***n* = 193 (%)**	**Juvenile** ***n* = 27 (%)**	**Adult** ***n* = 121 (%)**	**Geriatric** ***n* = 63 (%)**
Anesthesia-related	15 (3.7)	0 (0)	2 (7.4)	11 (9.1)	2 (3.2)
Anomaly / congenital	5 (1.2)	4 (2.1)	1 (3.7)	0 (0)	0 (0)
Degenerative	58 (14.4)	0 (0)	0 (0)	23 (19)	35 (55.6)
Infectious / inflammatory	103 (25.5)	27 (14)	11 (40.7)	54 (44.6)	11 (17.5)
Neonatal / perinatal	99 (24.5)	99 (51.3)	0 (0)	0 (0)	0 (0)
Neoplasia	7 (1.7)	0 (0)	1 (3.7)	2 (1.7)	4 (6.3)
Nutritional	13 (3.2)	4 (2.1)	2 (7.4)	7 (5.8)	0 (0)
Reproductive	1 (0.2)	0 (0)	0 (0)	1 (0.8)	0 (0)
Toxin	1 (0.2)	0 (0)	0 (0)	1 (0.8)	0 (0)
Trauma	77 (19.1)	55 (28.5)	6 (22.2)	10 (8.3)	6 (9.5)
Undetermined	25 (6.2)	4 (2.1)	4 (14.8)	12 (9.9)	5 (7.9)

We calculated prevalence of mortality within the 11 etiology (Table 3.2) and 16 organ system (Table 3.3) categories, both overall and by age class. We determined an overall neonatal

mortality rate for the captive population using International Studbook data: total number neonatal deaths divided by the total number of births from 1919–2014 and for a subset of time periods similar to Leutenegger's (1978) review of neonatal and juvenile mortality. Additionally, we determined mortality rate for juveniles between 31 days and one year of age for the same time periods to determine overall mortality rate in the first year of life. We also grouped the data for the overall time period 1919 to 2014 by sex, excluding the 55 calves of undetermined sex. We used chi-square analysis to evaluate potentially significant differences in mortality rates between different time periods; statistical significance was set at $P < 0.05$. We were unable to discern clear longitudinal trends in mortality parameters (organ system or etiology) over time within any age class, and thus did not perform statistical analyses for these parameters.

Table 3.3 - Causes of mortality by organ system and within age class for captive pygmy hippos between 1912 and 2014.

	Overall ***n* = 404 (%)**	**Neonate** ***n* = 193 (%)**	**Juvenile** ***n* = 27 (%)**	**Adult** ***n* = 121 (%)**	**Geriatric** ***n* = 63 (%)**
Cardiovascular	38 (9.4)	4 (2.1)	4 (14.8)	21 (17.4)	9 (14.3)
Dental	5 (1.2)	0 (0)	0 (0)	4 (3.3)	1 (1.6)
Endocrine	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Euthanasia	7 (1.7)	0 (0)	0 (0)	3 (2.5)	4 (6.3)
GI/digestive	21 (5.2)	4 (2.1)	1 (3.7)	16 (13.2)	0 (0)
Hematologic	9 (2.2)	1 (0.5)	4 (14.8)	3 (2.5)	1 (1.6)
Hepatic	4 (1)	0 (0)	1 (3.7)	2 (1.7)	1 (1.6)
Integumentary	2 (0.5)	0 (0)	0 (0)	1 (0.8)	1 (1.6)
Multisystemic	118 (29.2)	75 (38.9)	11 (40.7)	21 (17.4)	11 (17.5)
Musculoskeletal / neuromuscular	20 (5)	2 (1)	0 (0)	8 (6.6)	10 (15.9)
Neurologic	17 (4.2)	4 (2.1)	1 (3.7)	8 (6.6)	4 (6.3)
Ocular	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Renal/urinary	26 (6.4)	2 (1)	1 (3.7)	9 (7.4)	14 (22.2)
Reproductive	4 (1)	0 (0)	0 (0)	4 (3.3)	0 (0)
Respiratory	21 (5.2)	5 (2.6)	2 (7.4)	10 (8.3)	4 (6.3)
Undetermined	112 (27.7)	96 (49.7)	2 (7.4)	11 (9.1)	3 (4.8)

3.4 RESULTS

3.4.1 STUDY POPULATION

We received mortality data for 404 (177.200.7) pygmy hippos. The demographic distribution of these 404 hippos is presented in Table 3.1 and was comprised of 47.8% neonates, 6.7% juveniles, 29.9% adults and 15.6% geriatric animals. The age distribution reflects a mortality pattern heavily skewed toward neonates with limited juvenile mortality. The majority of animals were found dead (324; 80.2%) and 54 (13.4%) were euthanized; for the remaining 26, the necropsy report did not state if the animal died or was euthanized. In 6.2% of cases the cause of mortality or reason for euthanasia could not be determined from the information we received; these are listed as 'undetermined' in Table 3.2.

3.4.2 NEONATES (0–30 DAYS)

Nearly half of all mortalities in our study occurred in this age category (193/404; 48%). The overall neonatal mortality rate from 1919 to 2014 was 30.5%. The most prevalent etiology for the neonatal age class was 'perinatal' causes (*n* = 99; 51%) primarily due to stillbirth (*n* = 67/99). Of the 99 calves that experienced perinatal mortality or were stillborn, 45 were male, 50 were female and 4 were of unknown sex. Other common causes included premature/failure to thrive (*n* = 8), maternal neglect (*n* = 7) and hypothermia/collapse (*n* = 3). The large number of overall mortalities listed in the 'undetermined' organ system category in Table 3.3 (*n* = 112) primarily represent these perinatal cases (*n* = 97/112; 87%), including the 67 stillbirths. Mortality due to trauma was also frequent in neonates (*n* = 55; 29%); just over half of these mortalities were caused by one or both parents (*n* = 28/55). Three cases of trauma were secondary to dystocia. Mortality due to infectious/inflammatory conditions in neonates (*n* = 27; 14%) included enterotoxemia/septicemia (*n* = 13) and omphalophlebitis with septicemia (*n* = 3). Six neonates drowned and two died of aspiration pneumonia. Twinning in captivity has been recorded on thirteen occasions (*n* = 26 calves); 18 calves were stillborn or died within the first

two weeks of life, seven survived to adulthood, and one was 10 months old at the time of writing.

3.4.3 JUVENILES (31 DAYS TO 3 YEARS)

Mortality in juveniles was uncommon compared to other age categories (27/404; 7%) and was primarily a result of infectious/inflammatory conditions (*n* = 11) and trauma (*n* = 6). Infectious causes of mortality included encephalomyocarditis virus (EMCV; *n* = 3), pneumonia (*n* = 2), enterotoxemia/septicemia (*n* = 2), meningitis (*n* = 1) and pyelonephritis (*n* = 1). Two juveniles drowned. One death was secondary to colonic impaction and ensuing necrotic enteritis. There were two anesthetic deaths, one case of acute hemolytic anemia of unknown etiology, and one mortality secondary to exertional myopathy following transfer to another facility.

3.4.4 ADULTS (3 TO 30 YEARS)

Thirty percent (121/404) of mortalities in our study were adults. The principal causes of mortality in this age class were infectious/inflammatory and degenerative conditions, mainly cardiovascular disease (*n* = 21; 17%), multisystemic disorders (*n* = 21; 17%) and gastrointestinal disease (*n* = 16; 13.2%). Mortality due to cardiovascular disease included EMCV (*n* = 7), cardiac arrest under anesthesia (*n* = 5), dilated cardiomyopathy (*n* = 3), cardiac failure secondary to myocardial fibrosis and degeneration (*n* = 3), hypovolemic shock after severe trauma and blood loss (*n* = 2), and cardiovascular collapse (n = 1). Multisystemic disorders causing mortality encompassed enterotoxemia/septicemia (*n* = 8), trauma from conspecifics (*n* = 6), drowning (*n* = 2), exertional myopathy after transfer between zoos (*n* = 2), cachexia (*n* = 1), carcinomatosis (*n* = 1) and electric shock (*n* = 1). Two animals died as a result of the fire-bombing raids in Germany during World War II. Gastrointestinal disease secondary to ingestion of foreign material was diagnosed as the cause of mortality in seven adults. Sources included sand/gravel (*n* = 3), trichobezoar – not hippo hair (*n* = 1), rubber ball (*n* = 1), children's shoe (*n* = 1) and a plastic bag (*n* =1). Gastrointestinal perforation and peritonitis as a cause of death were also caused by gastric ulceration (*n* = 1), intussusception (*n* =1), and intestinal volvulus (*n* = 1). Mortality due to respiratory disease in adults (*n* = 10) included

interstitial and (lobar) bronchopneumonia (*n* = 7), aspiration pneumonia (*n* = 1), pneumothorax (*n* = 1) and pulmonary edema (*n* = 1). Two females died secondary to dystocia; in one case a Caesarian section was performed in time to save the calf but the dam died under anesthesia. Several adult and geriatric pygmy hippos were also noted to be overweight, based on body weight recorded at necropsy.

3.4.5 GERIATRIC (30+ YEARS)

Not surprisingly, mortality in geriatric pygmy hippos was primarily due to degenerative conditions (*n* = 35/63; 56%). Twenty-five of these animals were euthanized and seven were found dead; for the remaining three the manner of death was not reported. Necropsy reports for geriatric animals often described multiple comorbid degenerative conditions affecting more than one organ system. Degenerative causes of mortality primarily affected the renal/urinary (*n* = 14), musculoskeletal/neuromuscular (*n* = 10) and cardiovascular systems (*n* = 9). Arthritis, degenerative joint disease, ankylosing spondylitis, lameness, impaired mobility, posterior paresis, and inability to rise/stand were the most common reasons for euthanasia in this age class. Mortality due to trauma in geriatric hippos was principally the result of injuries from conspecifics (*n* = 4/6); one animal drowned and one fell into a construction pit in its enclosure. Although malignant neoplasia was an uncommon cause of death overall for the pygmy hippos (< 2%), the majority of cases (*n* = 4/7) occurred in geriatric animals. A list of malignant and benign neoplasms reported in this study is presented in Table 3.4.

Table 3.4 - Additional necropsy findings in pygmy hippos.

Number of affected animals indicated in parentheses. Malignant neoplasms were the cause of death unless otherwise indicated (*). One pygmy hippo (†) was affected by two neoplastic conditions; neither was the cause of death

Category	Necropsy finding
Neoplasia, malignant	Adrenal cortical carcinoma (1)*†
	Bronchogenic carcinoma (1)*
	Carcinomatosis (1)
	Fibrosarcoma, oral cavity (1)
	Epithelial carcinoma, pulmonary (1)
	Hemangiosarcoma (1)
	Hemangiosarcoma or melanoma (1)
	Hepatocelluar carcinoma (1)
	Leiomyosarcoma, gastric (1)*
	Lymphoblastic leukemia (1)
Neoplasia, benign	Leiomyoma, gastrointestinal (8)
	Leiomyoma, uterine (1)
	Leiomyoma, urinary bladder (1)
	Adrenal neuroblastoma (1)
	Bronchioalveolar adenoma (1)†
	Mandibular osteoma (1)
	Thyroid cystadenoma (1)
Trauma	Neonate, from parent (28)
	Juvenile, from parent (1)
	Adult, from conspecific (7)
	Adult, from another species (1)
Anomaly/Congenital	Urethral & vaginal atresia (1)
	Hind limb deformity (1)
	Hydrocephalus (1)
	Cardiac abnormality (2)
	Unilateral renal agenesis, incidental (3) ♂ = 2, ♀ = 1
	Umbilical hernia (2)
Incidental	Buckshot in various tissues (2)

3.4.6 EUTHANASIA

Degenerative conditions were limited to adult and geriatric age groups and were the primary reason for euthanasia for animal welfare reasons (*n* = 34/54; 63%). These conditions were predominately musculoskeletal/neuromuscular disorders (*n* = 12) and renal disease (*n* = 12). Other conditions prompting euthanasia included central nervous system disease (*n* = 4), chronic subcutaneous and dermal abscessation (*n* = 3) and severe trauma from conspecifics (*n* = 2).

3.4.7 INFECTIOUS DISEASE

Mortality due to infectious diseases was diagnosed in 60 pygmy hippos. Those confirmed by diagnostic testing or histopathologic findings included EMCV (*n* = 10), leptospirosis (*n* = 2), anthrax (*n* = 1), and *Mycobacterium tuberculosis* complex (*n* = 1). The latter case was euthanized due to suspected mycobacteriosis, but necropsy and histopathology findings did not support the diagnosis (see Bouts et al. 2009). An additional five cases were suspected to have died of EMCV based on gross post-mortem findings (no histopathology report available), but additional diagnostic testing either was not performed or was inconclusive. EMCV presented as acute death due to cardiovascular collapse with moderate to marked myocarditis, myocardial necrosis, degeneration, fibrosis, and ensuing cardiac failure. Bacterial pneumonia was diagnosed within all age classes for nine pygmy hippos overall; only two included microbial culture results. Enterotoxemia and/or septicemia was the cause of death in 24 hippos, primarily neonates (*n* = 13) and adults (*n* = 8). Twelve of these cases included microbial culture results. Overall, microbial pathogens were cultured and identified from a total of 18 animals (Table 3.5). Gastrointestinal parasites were not reported for any of the pygmy hippos in our study.

Table 3.5 - Microbial pathogens cultured from 18 pygmy hippos, including 12 with enterotoxemia and/or septicaemia.

Each microbe was cultured from a single animal unless otherwise indicated. Several animals were culture positive for more than one microbe.

Organ system	Bacteria or fungi
Bronchopneumonia	*Pasteurella multocida*
	Escherichia coli
Dermal/subcutaneous abscesses	*Proteus morganii*
	Streptococcus iniae
	Streptococcus spp.
Enteritis, hemorrhagic	*Clostridium spp.*
Enterotoxemia/septicemia	*Edwardsiella tarda*
	Escherichia coli (n = 7)
	Pasteurella multocida
	Proteus morganii
	Proteus spp.
	Salmonella spp.
	Streptococcus dysgalactiae
	Streptococcus iniae
	Streptococcus spp. (n = 2)
Gastritis	*Candida spp.*
Meningitis	*Escherichia coli*
	Streptococcus spp.
Meningoencephalitis	*Escherichia coli*
Nephritis & hepatitis	*Leptospira interrogans* serovar *icterohaemorrhagiae*
Omphalophlebitis	*Escherichia coli*

3.4.8 RENAL DISEASE

Overall, 20 pygmy hippos (9 adult, 11 geriatric) died or were euthanized due to renal failure. Common causes of renal insufficiency included glomerulonephritis, glomerulosclerosis, glomerulonephropathy, interstitial nephritis, and interstitial fibrosis. There was one case of a suppurative pyelonephritis. Polycystic kidney disease, defined as 3 or more cysts distributed between both kidneys (Nees et al. 2009), was identified in 56 (37.6%) adult and geriatric pygmy

hippos. However, it was only diagnosed as a clinically significant cause of renal failure and mortality in 15 of these cases; in the remaining 41 it was considered an incidental finding at necropsy.

3.4.9 NEUROLOGIC DISEASE

Seventeen pygmy hippos died or were euthanized due to neurologic disease; four neonates (hydrocephalus, meningitis), one juvenile (meningitis), eight adults and four geriatric animals. In the adult and geriatric hippos, clinical signs included seizures, head-pressing, altered mentation, ataxia, and paresis/paralysis. Specific causes of mortality included cerebral hemorrhage/infarct (n = 6), meningoencephalitis (n = 3), encephalo/leukomalacia (n = 3), cerebral edema (n = 1), and multifocal brainstem neoplasia (n = 1).

3.4.10 DENTAL DISEASE

Seven pygmy hippos (1.7%) were diagnosed with advanced dental wear or dental abscessation; five of these hippos died of resulting starvation/cachexia from inability to properly masticate. Four of these animals were wild-caught as adults, thus their actual age was not known and they may have been much older than the number of years they lived in captivity.

3.4.11 ANESTHETIC DEATHS

Anesthetic-related deaths occurred in 15 pygmy hippos in this study: 2 juvenile, 11 adult, and 2 geriatric. In most cases the animal's health was already compromised and anesthesia was performed for diagnostic and treatment purposes, but in at least three cases an elective procedure was being performed (e.g. tusk trims, umbilical hernia repair).

3.4.12 LONGITUDINAL TRENDS

Trends in population-wide mortality rates over time for both neonates and within the first year of life are presented in Table 3.6. Although mortality for both groups decreased between the initial time frame (1919–1940) and all subsequent time frames, this decrease was not statistically significant for either group (neonates: $\chi^2 = 2.21$, $df = 3$, $P = 0.530$; ≤ 1 year: $\chi^2 =$

5.96, $df = 3$, $P = 0.114$). The mortality rate for both age classes across the study period overall (1919–2014) was nearly identical to the mortality rate for the most recent time period from 1976 to 2014 (neonates: $\chi^2 = 0.023$, $df = 1$, $P = 0.879$; ≤ 1 year: $\chi^2 = 0.121$, $df = 1$, $P = 0.728$). There were no discernable trends in primary cause of mortality (etiology) or organ system over time within any age class. Mortality rate was higher for male calves during the neonatal period, but was the same for the two sexes after 30 days of age (Table 3.6).

Table 3.6 - Mortality rate (%) for neonates and within the first year of life for pygmy hippos. The data are presented overall from 1919 to 2014 and during a subset of four time frames. The overall data are also presented by sex, with calves of unknown sex excluded, illustrating that males experience a higher neonatal mortality rate than females.

Time Period	Neonatal (0-30 d) mortality rate (%)	Mortality rate 31 d to 1 yr (%)	Overall mortality rate 1st year of life (%)
1919–2014	30.5	4.8	35.3
	33.0 ♂ 25.8 ♀	4.7 ♂ 4.7 ♀	37.7 ♂ 30.5 ♀
1919–1940	40.0	4.4	44.4
1941–1960	32.8	14.1	46.9
1961–1975	29.6	3.9	33.5
1976–2014	30.2	4.4	34.5

3.4.13 UNDETERMINED CAUSES

Overall, older reports tended to contain less comprehensive information than newer reports and were thus more difficult to interpret. We were unable to determine the etiology associated with the cause of death or reason for euthanasia in 6% of hippos ($n = 25$), predominately due to lack of detail in the associated necropsy reports. For over a quarter of the mortalities included in our study ($n = 112$) the primary organ system was undetermined. The majority of these mortalities were due to neonatal/perinatal causes ($n = 97/112$), including the 67 stillbirths.

3.4.14 LIMITATIONS

As with any retrospective study using data from a multitude of institutions within a longitudinal time frame, there were several logistical limitations. Since we categorized the data by primary

cause of mortality or primary morbidity prompting euthanasia for welfare reasons, we were unable to quantitatively evaluate multi-factorial problems and comorbidities. Additional limitations included marked variation in record-keeping styles, level of detail provided, and terminology within necropsy reports. Hand-written reports were sometimes difficult to decipher, and some meaning may have been lost in translation for reports written in a language other than English. We also experienced a geographic reporting bias: zoological facilities from North America, Europe, and Australia had a greater than 90% response rate to our enquiries, whereas facilities in Southeast Asia, Africa, and Central/South America had a 60–80% response rate. All of these factors have the potential to influence our results. Additionally, the cause of mortality (etiology) remains undetermined for 6% of our study animals and for the additional 59% of the captive population for which we have no data. However, the age structure and gender ratio in our study population (43.8% ♂, 54.5% ♀; 1.7% undetermined gender) was very similar to that of the overall Studbook population (Steck 2015), indicating that the morbidity and mortality trends we identified in our subset of pygmy hippos are likely to apply on a population-wide basis.

3.5 DISCUSSION

Our comprehensive overview of mortality by age class in captive pygmy hippos reveals a number of pathological conditions and comorbidities previously unreported for this species. Overall, the two primary categories of mortality were infectious/inflammatory conditions (all age groups) and neonatal/perinatal causes.

3.5.1 NEONATAL MORTALITY

The high proportion of perinatal deaths in this study and the greater than 30% overall neonatal mortality rate are cause for concern. Population-wide, neonatal deaths are more common in male (33%) than female (26%) calves; however, in our sampled sub-population the frequency of neonatal mortality was similar for males (21%) and females (25%). The underlying reasons for the higher neonatal mortality rate in males are poorly understood, but this phenomenon is a

cause for concern from both an individual animal health and a population management perspective. Although it is encouraging that the overall mortality rate for neonates and within the first year of life dropped by 10–11% between the first two decades of captive breeding (1919–1940) and the most recent time frame (1976–2014), these differences were not statistically significant and the current mortality rates are too high, especially for an endangered species with limited numbers in captivity.

The Studbook was first implemented as a management tool in the mid-1970s (Lang 1975) and substantially enhanced communication and information-sharing between zoos with pygmy hippos. Shortly thereafter important alterations in husbandry and management practices were more widely implemented, including improved hygiene standards, recognition that this species gives birth on land, not in the water, and removal of the male from the enclosure prior to birth. However, despite these developments, both neonatal mortality and mortality within the first year of life have remained essentially unchanged since the 1960s, indicating a need for further optimization of husbandry and suggesting that some additional, as yet undetermined, factors play an important role in calf survival.

Stillbirths contributed substantially to the high mortality rate for this age class and are also of major concern. Unfortunately, the underlying reason for the stillbirth was not determined in any of these cases, so the implementation of preventative measures or proactive veterinary care presents a challenge. Additionally, many neonates were reported as succumbing to ill-defined causes such as “failure to thrive and/or nurse,” “premature,” “maternal neglect” or “weakness.” The ultimate cause of mortality in these cases was elusive, also making it difficult to suggest preventative strategies. One potential criterion for predicting survivability is the birth weight – calves that weigh between 5 and 6.5 kg at birth are more likely to survive, in contrast to those below that range (Lang 1975; N. Robert 2011, unpublished data). Continual measurement of birth weights for both live and dead calves across the captive population will help provide further evidence for a correlation of birth weight and survival success. Furthermore, it is important to maintain the calf’s environment above 20 °C with adequate humidity for at least

the first few weeks of life to mimic the pygmy hippo's natural tropical habitat. Although it may be tempting to hand-rear calves that are not thriving, this practice is not uniformly recommended and should only be considered after consultation with the regional program coordinator and international studbook keeper.

A high prevalence of neonatal mortality due to stillbirths and other perinatal causes has been documented for a number of other mammalian species in zoological collections. Giant anteaters (*Myrmecophaga tridactyla*) experience a near 50% neonatal mortality rate, primarily due to stillbirth, mis-mothering, and trauma from the male (Patzl et al. 1998). For Arabian gazelles (*Gazella arabica*), Soares et al. (2015) reported 46.1% of neonatal (< 15 days) mortality in 256 cases at a wildlife research center in Saudi Arabia as due to stillbirth and maternal neglect. In cotton-top tamarins (*Saguinus oedipus*), neonatal (< 1 week) mortality in the North American SSP population was 22.5% and was primarily attributed to stillbirth, maternal neglect and trauma (Leong et al. 2004). For the jaguar (*Panthera onca*), Hope and Deem (2006) attributed 20% of overall mortality in the captive population in North American zoos to stillbirths and perinatal causes. For the cheetah (*Acinonyx jubatus*), Wielebnowski (1996) described a juvenile (< 6 months) mortality rate of 32% among those born over a 24-year period in five North American breeding facilities, 31% of which were due to stillbirth and other perinatal causes. In Asian elephants (*Elephus maximus*), Taylor and Poole (1998) reported a 25% stillbirth rate across 20 zoos in North America and Europe, far exceeding the maximum of 3% for three elephant orphanages and forestry centers in Southeast Asia. The latter example clearly demonstrates a marked difference between captive and wild populations. Longevity and mortality data are not available for wild pygmy hippos so we cannot make comparisons with the *in situ* population. However, similar to the Asian elephant, the pygmy hippo is a large-bodied, long-lived species adapted to a tropical rainforest environment with limited fluctuation in environmental conditions and resource availability, life-history traits that predict limited neonatal and juvenile mortality.

The high rates of stillbirth and neonatal mortality in pygmy hippos, and in myriad other mammalian species in captivity, point to an urgent need for more research to investigate the underlying mechanisms. Obesity in the mother has been proposed as a primary cause for the higher incidence of stillbirth for Asian elephants in captivity (Taylor & Poole 1998). Although there is no data for wildlife, numerous studies support a strong link between maternal obesity, stillbirth, and neonatal mortality in humans across the globe (Cresswell et al. 2012; Kristensen et al. 2005; Nohr et al. 2007) as well as in non-human primates, rodents, and domestic animal models (Frias et al. 2011; Li et al. 2011). Many pygmy hippos in captivity, including several adult and geriatric animals in our study, are overweight or obese compared to their wild conspecifics (Flacke et al. 2015; G. Flacke 2016, unpublished data). We hypothesize that maternal body condition may be influencing the rate of stillbirth and other perinatal causes of mortality in pygmy hippos as has been demonstrated in other species.

Trauma was the second-leading cause of mortality for neonates; just over half were parent-inflicted. In the past, it was not understood that the male poses a danger to the newborn calf so many neonates were killed by their fathers. It is now a standard practice to separate the male when the birth is pending but, in some cases, it was not known that the female was pregnant and the newborn calf was killed by the male before he could be separated. In several cases the female crushed the calf, we assume inadvertently, though obesity may also contribute to this problem.

Enterotoxemia and/or septicemia were the most common multisystemic causes of mortality in neonates, but the source of the bacterial infection was definitively diagnosed in only three of 24 cases (omphalophlebitis). It is essential to perform a neonatal examination within 24 hours of birth to ensure that the umbilicus is clean and to keep the mother and calf's environment as clean as possible, free of fecal contamination, and at an appropriate temperature. Mortality from aspiration of milk, formula or meconium was reported for several calves, thus we advise zoos to carefully inspect the oral cavity during neonatal examination and to exercise caution when bottle feeding if hand-rearing is indicated.

3.5.2 CARDIOVASCULAR DISEASE AND EMCV

With the exception of EMCV, mortality due to cardiovascular disease was primarily diagnosed in adult and geriatric animals. Degenerative disease conditions leading to cardiac failure included cardiomyopathy, myocardial fibrosis/sclerosis and valvular endocardiosis. Acute death due to EMCV (n = 10) was the primary infectious cause of mortality affecting the cardiovascular system. The diagnosis was confirmed by IFA or viral isolation in eight cases and based on histopathological findings combined with clinical history for the other two. Nine of the ten cases were reported from the southeastern United States along the Gulf Coast; the tenth from Australia.

EMCV is a Picornavirus with ubiquitous, world-wide distribution that is transmitted by rodent carriers (Thomson et al. 2001). Most mammalian species are susceptible to infection and animals are often found dead without any prior clinical signs of illness (Backues 2008). Sporadic outbreaks have been reported in free-ranging wildlife populations and among zoological collections in North America (Gaskin et al. 1980; Wells et al. 1989), Europe (Canelli et al. 2010; Lamglait et al. 2015) and Australia (Reddacliff et al. 1997; Seaman & Finnie 1987). Seroconversion and survival after infection has not been documented in pygmy hippos, but serological data is lacking. We only identified one case where a clinically healthy pygmy hippo was serologically screened for the virus as part of a multi-species survey to evaluate EMCV antibody titers as an indication of exposure and immune response; results were negative (Atkins & Backues 2005).

The situation with EMCV leads us to strongly recommend that all cases of acute death in pygmy hippos be submitted to a reference laboratory for serology testing and virus isolation whenever post-mortem findings indicate a cardiovascular etiology. Commercial vaccines for EMCV are not currently available, but experimental vaccines (inactivated and modified live) have been used in clinical trials and outbreak situations (Gaskin et al. 1987; Hunter et al. 1998; Kilburn et al. 2011; McLelland et al. 2005; Osorio et al. 1996; Wells et al. 1989). Although seroconversion and efficacy are variable, side-effects were not reported after experimental

vaccination in numerous other ungulate species including llamas (*Llama glama*), guanacos (*Lama glama guanicoe*), Bactrian camels (*Camelus bactrianus*), dromedary camels (*Camelus dromedarius*), Barbary sheep (*Ammotragus lervia*), gerenuk (*Litocranius walleri*), blackbuck (*Antilope cervicapra*), black duikers (*Cephalophus niger*), lowland tapirs (*Tapirus terrestris*), Baird's tapirs (*Tapirus bairdii*), Malayan tapirs (*Tapirus indicus*), babirusas (*Babyrousa babyrussa*), and collared peccaries (*Tayassu tajacu*) (Backues 2008; Kilburn et al. 2011; McLelland et al. 2005). We therefore advise that all resident pygmy hippos are vaccinated if a zoological facility experiences an outbreak of EMCV. Additionally, as outbreaks have repeatedly been linked to rodent population eruptions, rodent control and facility hygiene are of utmost importance in controlling transmission. Finally, we recommend that zoos planning an inter-facility transfer to or from an area with high EMCV prevalence (e.g., southeastern USA) perform serological screening as part of the pre-shipment examination. This approach will help us develop a greater understanding of EMCV epidemiology in this species.

3.5.3 RESPIRATORY DISEASE

The primary respiratory disease across all age classes was pneumonia, including aspiration, interstitial and bronchopneumonia. Although pulmonary radiography is challenging in adults, we recommend standing thoracic radiographs for pygmy hippos that show increased respiratory effort, tachypnea, dyspnea, or elevated white blood cell counts indicative of systemic inflammation or infection. Empirical broad-spectrum antimicrobial therapy may also be indicated.

3.5.4 GASTROINTESTINAL DISEASE

Gastroenteritis and foreign body obstruction were often linked to husbandry and management including feeding on a sandy substrate (sand colic) and exhibit designs where visitors are able to drop materials into areas accessed by hippos. We therefore recommend the use of feeding troughs to help prevent inadvertent substrate ingestion when outdoor exhibits contain sandy or soil substrate, and that enclosures are designed or modified where possible to prevent zoo visitors from inadvertently dropping items in areas that can be accessed by the hippos.

3.5.5 RENAL DISEASE AND PKD

Mortality due to renal disease in adult and geriatric pygmy hippos was primarily degenerative in nature, with conditions such as glomerulosclerosis, glomerulonephritis, interstitial fibrosis and tubulointerstitial nephritis leading to chronic renal failure. There was one mortality secondary to renal failure from a confirmed case of leptospirosis (Cracknell et al. 2011). A second mortality associated with leptospirosis was strongly suspected based on clinical signs of renal failure, elevated serum urea and creatinine, and positive titers for *Leptospira interrogans, serovars bratislava* and *autumnalis* combined with severe tubulointerstitial nephritis at necropsy; however, immunohistopathology results were negative. There are several commercial vaccine products for leptospirosis and their use in pygmy hippos should be considered in geographic areas where the disease is common or endemic. We caution that vaccine safety and efficacy trials have not been conducted in this species. Like EMCV, leptospirosis is transmitted by a rodent reservoir (Adler & de la Peña Moctezuma 2010) emphasizing the importance of ongoing pest control measures combined with appropriate hygiene standards in zoological facilities.

PKD was diagnosed in 38% (n = 17 ♂, 39 ♀) of adult and geriatric pygmy hippos but was considered the ultimate cause of death in only 8%. Nonetheless, this figure is much higher than the reported incidence in humans (0.125%), where PKD is a heritable disorder (Wilson 2004). The clinical significance of this condition in pygmy hippos remains enigmatic, although some animals with advanced disease had accompanying evidence of chronic renal insufficiency based on elevated blood urea nitrogen and creatinine values and clinical signs associated with uremia. Advanced PKD was also implicated in one adult female with neurological disease and multifocal hemorrhagic cerebral infarcts, deemed likely to be secondary to systemic hypertension associated with chronic renal disease (Nielsen et al. 2015). Six pygmy hippos died of hemorrhagic cerebral infarcts; four of these animals also had renal lesions and two were diagnosed as uremic. It is possible that some of the hippos diagnosed with cardiovascular disease at necropsy, particularly arteriosclerosis, were affected by occult hypertension secondary to chronic renal disease.

Management and treatment options for pygmy hippos affected by PKD are limited. However, given the high prevalence and potential for severe secondary complications, we urge clinicians to perform ultrasound of the kidneys whenever possible and to monitor renal function via serial serum chemistry analysis if PKD is diagnosed. A database for renal function tests for both affected and non-affected individuals would be a valuable tool for guiding clinical decisions in pygmy hippos known to be affected by PKD. Additionally, we recommend further investigation into the heritability of the condition in this species and into whether PKD has the potential to significantly compromise the long-term viability of the captive population.

3.5.6 DEGENERATIVE MUSCULOSKELETAL DISEASE

Adult and geriatric hippos were also commonly affected by osteoarthritis and neuromuscular disorders. Mobility issues, including inability to rise or stand, were the reason for euthanasia in fourteen animals. The fact that many captive pygmy hippos are overweight likely contributes to the severity of musculoskeletal problems as these animals age. Maintaining an optimal body condition (see Appendix II) is expected to reduce the incidence of obesity-related health problems and help improve mobility, as has been documented in domestic animals and humans (Berenbaum et al. 2013; Laflamme 2012; Marshall et al. 2009). Additionally, older pygmy hippos are likely to be affected by some degree of mild to moderate (and in some cases marked) degenerative joint disease since advanced age has been directly linked with certain pathological conditions, including osteoarthritis, in numerous other mammalian species commonly kept in zoological collections (Föllmi et al. 2007). Any dysfunction of the musculoskeletal system has the potential to negatively impact animal welfare and zoo veterinarians should therefore consider managing these animals with analgesics and inflammatory mediators as indicated by clinical signs of pain/lameness.

3.5.7 TRAUMA

Mortality due to trauma in adult and geriatric pygmy hippos was caused by a conspecific in more than 50% of cases and, in one case, by a common hippo (*Hippopotamus amphibius*). One pygmy hippo in a multi-species exhibit died of hemorrhage and hypovolemic shock after it

sustained a jugular laceration from an oryx (*Oryx beisa callotis*). These events were rare but facilities that choose to house pygmy hippos in mixed-species exhibits need to be aware of the associated risks. Furthermore, keeping the male separate from the female except during breeding helps reduce conspecific aggression and mortality, although this management option may be limited by zoo-specific logistics. In these situations, zoos must endeavor to implement methods for timely pregnancy diagnosis via ultrasound or fecal hormone metabolite analysis so that the male can be removed before parturition to reduce the risk of trauma to the neonate.

3.5.8 ANESTHESIA-RELATED DEATH

Anesthetic deaths primarily occurred in adults (n = 13/15), likely because anesthetic procedures are uncommon in neonates and juveniles. The majority of these mortalities were prior to the implementation of newer, multi-modal anesthetic protocols with improved safety and efficacy compared to ultra-potent opioids (Bouts et al. 2012; Walzer & Stalder 2014). Additionally, the animals were often already debilitated at the time and required anesthesia for diagnostics and treatments, making an anesthetic procedure inherently more risky.

3.5.9 FUTURE RECOMMENDATIONS

Continued vigilance in monitoring trends in morbidity and mortality is essential for ongoing optimization of health and welfare for this species. We therefore encourage zoos to perform systematic, comprehensive necropsies using the EEP & SSP standard protocol for all pygmy hippo mortality events, especially as the predominant limitation in this study was incomplete or unclear necropsy reports. Overall, we were unable to conclude the cause of mortality in 6% of cases and the organ system could not be determined in 28% of cases. For 12 hippos, the etiology remained elusive, even for the pathologist, despite a thorough gross necropsy and histopathology report, but for the remaining hippos assigned to this category the information provided lacked sufficient detail to make an assessment.

We were unable to discern any clear trends in mortality parameters (organ system or etiology) over time or within any age class. However, we caution the veterinary clinician that

comorbidities often contribute to or exacerbate the clinical presentation of disease conditions in adult and geriatric pygmy hippos. Although there have been some notable improvements in management of this species over time (von Houwald et al. 2007), we have identified a number of areas where morbidity and mortality can potentially be reduced through husbandry modifications and veterinary procedures. Moreover, mortality in the neonatal period and during the first year of life have not significantly decreased over the last century, suggesting a need for further optimization of husbandry and management during these time frames. The high neonatal mortality rate, coupled with the skewed sex ratio (Flacke et al. 2015; Zschokke 2002), hinders breeding efforts and may threaten the long-term viability of the captive population, especially since importation of new genetic material from wild populations is unlikely in the foreseeable future.

3.6 CONCLUSIONS

- The population-wide mortality trends identified in this study can be used to guide preventative and proactive health care for pygmy hippos in captivity and provide a solid framework for future studies.

- Improved record-keeping, comprehensive necropsies using standardized protocols, and serology, virology and microbial culture for definite diagnosis when indicated, together with timely submission of mortality data to the international studbook keeper are of utmost importance to further our understanding of morbidity and mortality in this species.

- The high mortality rate within the first year of life is a concern, especially within a captive breeding program for an endangered species. The underlying causes of most perinatal mortalities, including stillbirths, are poorly understood, as are the reasons for the higher neonatal mortality rate in males. It is therefore difficult to make specific recommendations for reducing mortality in this age class. Future research to

systematically analyze risk factors associated with stillbirth and early mortality is recommended.

- Although EMCV was not a common cause of mortality overall, the acute onset of death, often in the absence of clinical signs, makes this viral disease a primary concern, especially in an outbreak situation. Serological screening is recommended in endemic areas to help further characterize the epidemiology of EMCV in pygmy hippos.

- PKD was diagnosed in over 37% of adult and geriatric pygmy hippos but was only considered the cause of death in a quarter of these animals. Further investigation is warranted into the potential heritability mechanisms and clinical significance of PKD for long-term population viability.

- Many adult and geriatric pygmy hippos in this study were overweight. Maintaining ideal body condition is strongly recommended for individual animal health and welfare reasons; obesity may also be linked to stillbirths and poor calf survival rates.

- Euthanasia for animal welfare reasons was primarily due to degenerative conditions in older adult and geriatric pygmy hippos, most notably musculoskeletal and neuromuscular disorders. Post-mortem examination in this cohort usually identified multiple comorbidities.

3.7 ACKNOWLEDGEMENTS

The authors have no conflicts of interest to declare. Funding for this research was provided by the University of Western Australia and the Institute for Breeding and Reproduction of Endangered African Mammals (IBREAM). We thank Dr Bethany Jackson of Murdoch University, College of Veterinary Medicine, for assistance with data analysis and Mrs Rita Runnels for the body condition chart drawings. We are also indebted to those individuals who facilitated communication with several zoos and who assisted with translation of foreign

language reports: Patmasuda Intuprapa, Dr Monique Paris, Tomas Pecha, Dr Pablo Rodriguez, Dr Shiho Sumigama, Mai Tanimoto and Rujiporn Thavornkanlapachai. Additionally, we are grateful to the two anonymous reviewers whose valuable feedback helped to considerably improve our manuscript.

We wholeheartedly extend our gratitude to all of the zoological institutions and private facilities that responded to our requests for data and shared their necropsy reports; without their participation we could not have conducted this study: Aalborg Zoo, Adelaide Zoo, Attica Zoological Park, Bali Zoo, BioParc de Dou, BREC's Baton Rouge Zoo, Bristol Zoological Gardens, Brookfield Zoo, Bronx Zoo, Budapest Zoo and Botanical Garden, Cango Wildlife Ranch, Center for Conservation of Tropical Ungulates, Chester Zoo, Chiangmai Night Safari, Cincinnati Zoo and Botanical Garden, Cleveland Metroparks Zoo, Colchester Zoo, Copenhagen Zoo, Dallas Zoo, Dierenpark Wissel, Dusit (Bangkok) Zoological Park, Edinburgh Zoo, Emerald Animal World, Fondazione Bioparco di Roma, Fort Worth Zoo, GaiaZOO, Givskud Zoo, Gladys Porter Zoo, Greater Vancouver Zoo, Honolulu Zoo, Houston Zoo, Jackson Zoological Park, Jardin Zoologico de Cuidad Buenos Aires, Johannesburg Zoological Gardens, Kedar Country Lodge, Krakow Zoo, Kristiansand Zoo, Las Aguilas Jungle Park, Lichtenburg Game and Breeding Centre, Lincoln Park Zoo, Lisbon Zoo, Lithuanian Zoo, London Zoo, Louisville Zoological Garden, Nakhon Ratchasima Zoological Park, Marwell Wildlife, Maryland Zoo in Baltimore, Melbourne Zoo, Memphis Zoo, Mokopane Biodiversity Conservation Centre, Nanki Shirahama Adventure World, National Zoological Gardens of South Africa, Pairi Daiza, Ol Jogi Ltd, Omaha's Henry Doorly Zoo & Aquarium, Parc Zoologique de Paris, Parque de la Naturaleza de Cabarceno, Parque Zoologico Buin, Parc Zoologic de Barcelona, Perth Zoo, Philadelphia Zoo, Reid Park Zoo, Riyadh Zoological Gardens, Rostov-on-Don Zoo, Rotterdam Zoo, Safari Wild, Saint Louis Zoological Park, San Antonio Zoo & Aquarium, San Diego Safari Park, San Diego Zoo, San Francisco Zoo, Seoul Zoo, Singapore Zoological Gardens, Smithsonian National Zoological Park, South Lakes Wild Animal Park, Szeged Zoo, Taman Safari Indonesia I, Tampa's Lowry Park Zoo, Taronga Zoo, Tiergarten Hoyerswerda, Tierpark Berlin, Tierpark Chemnitz, Toronto Zoo, Utah's Hogle Zoo,

Whipsnade Wild Animal Park, Wilhelma Zoo, Zoo Antwerpen, Zoo Aquarium de Madrid, Zoo Carmona, Zoo de Cerza, Zoo Duisburg, Zoo Dvur Kralove, Zoo Gdansk, Zoo in der Wingst, Wroclaw Zoo LLC, Zoo Jaderberg, Zoo Kosice, Zoo Leipzig, Zoologischer Garten Basel, Zoologischer Garten Berlin, Zoologischer Garten Halle, Zoologick a Botanick Zahrada Plzen, Zoologicka Zahrada Bratislava, Zoologicka Zahrada Jihlava, Zoologicka Zahrada Olomouc, Zooloski vrt Zagreb, ZOOM Erlebniswelt Gelsenkirchen, Zoo Miami, Zoo New England, Zoo Parc Overloon, and Zoo Zürich.

3.8 LITERATURE CITED

Adler, B., and A. de la Peña Moctezuma. 2010. Leptospira and leptospirosis. *Vet. Microbiol.* 140:287–296.

Atkins, A., and K. Backues. 2005. Serologic survey for encephalomyocarditis virus in zoological institutions throughout the United States and Canada. *In* Proceedings of the American Association of Zoo Veterinarians (AAZV), American Association of Wildlife Veterinarians (AAWV), Association of Zoos and Aquariums (AZA) / Nutrition Advisory Group (NAG) Joint Conference. Omaha, Nebraska. 284.

Backues, K.A. 2008. Encephalomyocarditis virus infection in zoo animals. *In* Zoo and Wild Animal Medicine. M.E. Fowler and R.E. Miller, editors. Saunders Elsevier, St. Louis, Missouri. 75–78.

Berenbaum, F., F. Eymard, and X. Houard. 2013. Osteoarthritis, inflammation and obesity. *Curr. Opin. Rheumatol.* 25:114–118.

Bouts, T., R. Hermes, F. Gasthuys, J. Saragusty, P. Taylor, A. Routh, and T.B. Hildebrandt. 2012. Medetomidine-ketamine-isoflurane anaesthesia in pygmy hippopotami (*Choeropsis liberiensis*) - a case series. *Vet. Anaesth. Analg.* 39:111–118.

Bouts, T., M. Vordermeier, E. Flach, and A. Routh. 2009. Positive skin and serologic test results of diagnostic assays for bovine tuberculosis and subsequent isolation of Mycobacterium interjectum in a pygmy hippopotamus (*Hexaprotodon liberiensis*). *J. Zoo Wildl. Med.* 40:536–542.

Canelli, E., A. Luppi, A. Lavazza, D. Lelli, E. Sozzi, A.M.M. Martin, D. Gelmetti, E. Pascotto, C. Sandri, W. Magnone, and P. Cordioli. 2010. Encephalomyocarditis virus infection in an Italian zoo. *Virol. J.* 7:1–7. doi:10.1186/1743-422X-7-64.

Cracknell, J.M., M. Stidworthy, and A. Holliman. 2011. Leptospirosis in a pygmy hippopotamus (*Choeropsis liberiensis*). *In* Proceedings of the American Association of Zoo Veterinarians Annual Conference. Kansas City, Missouri. 35–37.

Cresswell, J.A., O.M.R. Campbell, M.J. De Silva, and V. Filippi. 2012. Effect of maternal obesity on neonatal death in sub-Saharan Africa: Multivariable analysis of 27 national datasets. *Lancet*. 380:1325–1330.

Flacke, G.L., B.K. Chambers, G.B. Martin, and M.C.J. Paris. 2015. The pygmy hippopotamus *Choeropsis liberiensis* (Morton, 1849): Bringing to light research priorities for the largely forgotten, smaller hippo species. *Der Zool. Garten NF*. 84:234–265.

Föllmi, J., A. Steiger, C. Walzer, N. Robert, U. Geissbühler, M.G. Doherr, and C. Wenker. 2007. A scoring system to evaluate physical condition and quality of life in geriatric zoo mammals. *Anim. Welf.* 16:309–318.

Frias, A.E., T.K. Morgan, A.E. Evans, J. Rasanen, K.Y. Oh, K.L. Thornburg, and K.L. Grove. 2011. Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. *Endocrinology*. 152:2456–2464.

Gaskin, J.M., T.L. Andresen, J.H. Olsen, E.E. Schobert, D. Buesse, J.D. Lynch, M. Walsh, S. Citino, and D. Murphy. 1987. Encephalomyocarditis in zoo animals: Recent experiences with the disease and vaccination. *In* Proceedings of the International Conference on Zoological and Avian Medicine. Oahu, Hawaii. 491.

Gaskin, J.M., M.A. Jorge, C.F. Simpson, A.L. Lewis, J.H. Olson, E.E. Schobert, E.P. Wollenman, C. Marlowe, and M.M. Curtis. 1980. The tragedy of encephalomyocarditis virus infection in zoological parks of Florida. *In* Proceedings of the American Association of Zoo Veterinarians Annual Conference. Washington, D.C. 1–7.

Graf, Z. 1981. Über den Verlust eines Zwergflußpferdes im Budapester Zoo. *Erkrankungen der Zootiere Verhandlungsbericht*. 23:389–390.

Hentschel, K.M. 1990. Untersuchung zu Status, Ökologie und Erhaltung des Zwergflusspferdes (*Choeropsis liberiensis*) in der Elfenbeinküste. Technischen Universität Carolo-Wilhelmina, Braunschweig.

Hope, K., and S.L. Deem. 2006. Retrospective study of morbidity and mortality of captive jaguars (*Panthera onca*) in North America: 1982 – 2002. *Zoo Biol.* 25:501–512.

Hoppe-Dominik, B., H.S. Kühl, G. Radl, and F. Fischer. 2011. Long-term monitoring of large rainforest mammals in the Biosphere Reserve of Taï National Park, Cote d'Ivoire. *Afr. J. Ecol.* 49:450–458.

Hornaday, W.T. 1920. Birth of a Pygmy Hippopotamus. *New York Zool. Soc. Bull.* 23:11–13.

Hunter, P., S.P. Swanepoel, J.J. Esterhuysen, J.P. Raath, R.G. Bengis, and J.J. van der Lugt. 1998. The efficacy of an experimental oil-adjuvanted encephalomyocarditis vaccine in elephants, mice and pigs. *Vaccine*. 16:55–61.

Jarofke, D., and H.G. Klös. 1982. Immobilisierung und Krankheiten von Zwergflusspferden: Auswertung einer Umfrage bei mehr als 100 Zoologischen Gärten. *Erkrankungen der Zootiere Verhandlungsbericht*. 24:361–374.

Kilburn, J.J., D.P. Murphy, M. Titus, M.E. Payton, and K.A. Backues. 2011. Vaccination of llamas, Llama glama, with an experimental killed encephalomyocarditis virus vaccine. *J. Zoo Wildl. Med.* 42:65–68.

Kristensen, J., M. Vestergaard, K. Wisborg, U. Kesmodel, and N.J. Secher. 2005. Pre-pregnancy weight and the risk of stillbirth and neonatal death. *BJOG An Int. J. Obstet. Gynaecol.* 112:403–408.

Laflamme, D.P. 2012. Obesity in dogs and cats: What is wrong with being fat? *J. Anim. Sci.* 1653–1662.

Lamglait, B., A. Joris, A. Romey, L. Bakkali-Kassimi, and K. Lemberger. 2015. Fatal encephalomyocarditis virus infection in an African savanna elephant (*Loxodonta africana*) in a French zoo. *J. Zoo Wildl. Med.* 46:393–396.

Lang, E.M. 1975. Das Zwergflußpferd. A. Ziemsen Verlag, DDR, Wittenberg Lutherstadt.

Leong, K.M., S.P. Terrell, and A. Savage. 2004. Causes of mortality in captive cotton-top tamarins (*Saguinus oedipus*). *Zoo Biol.* 23:127–137.

Leutenegger, M. 1978. Pygmy hippopotamus *Choeropsis liberiensis* births in captivity. *Int. Zoo Yearb.* 18:234.

Li, M., D.M. Sloboda, and M.H. Vickers. 2011. Maternal obesity and developmental programming of metabolic disorders in offspring: Evidence from animal models. *Exp. Diabetes Res.* Article ID:9 pages.

Mallon, D., C. Wightman, P. De Ornellas, and C. Ransom. 2011. Conservation Strategy for the Pygmy Hippopotamus. IUCN Species Survival Commission, Gland, Switzerland & Cambridge, UK.

Marshall, W.G., B.A. Bockstahler, D.A. Hulse, and S. Carmichael. 2009. A review of osteoarthritis and obesity: Current understanding of the relationship and benefit of obesity treatment and prevention in the dog. *Vet. Comp. Orthop. Traumatol.* 22:339–345.

Mccurdy, P., C. Sangster, S. Lindsay, and L. Vogelnest. 2014. Acute lymphoblastic leukemia in a pygmy hippopotamus (*Hexaprotodon liberiensis*). *J. Zoo Wildl. Med.* 45:906–910.

McLelland, D.J., P.D. Kirkland, K.A. Rose, R.J. Dixon, and N. Smith. 2005. Serologic responses of Barbary sheep (*Ammotragus lervia*), Indian antelope (*Antilope cervicapra*), wallaroos (*Macropus robustus*), and chimpanzees (*Pan troglodytes*) to an inactivated encephalomyocarditis virus vaccine. *J. Zoo Wildl. Med.* 36:69–73.

Nees, S., B. Schade, M. Clauss, H.W. Steinmetz, F. Ehrensperger, B. Steck, and J.-M. Hatt. 2009. Polycystic kidney disease in the pygmy hippopotamus (*Hexaprotodon liberiensis*). *J. Zoo Wildl. Med.* 40:529–535.

Nielsen, A.W.N., T. van Dreumel, G. Crawshaw, C.J. Dutton, S.R. Hollamby, A.R. Pastor, G.L. Flacke, and D.A. Smith. 2015. Haemorrhagic stroke in a pygmy hippopotamus. *In* Proceedings of the International Conference on Diseases of Zoo and Wild Animals. Leibniz Institute for Zoo and Wildlife Research (IZW) & European Association of Zoo and Wildlife Veterinarians (EAZWV), Barcelona, Spain. 190.

Nohr, E.A., M. Vaeth, B.H. Bech, T.B. Henriksen, S. Cnattingius, and J. Olsen. 2007. Maternal obesity and neonatal mortality according to subtypes of preterm birth. *Obstet. Gynecol.* 110:1083–1090.

Osorio, J.E., G.B. Hubbard, K.F. Soike, M. Girard, S. van der Werf, J.-C. Moulin, and A.C. Palmenberg. 1996. Protection of non-murine mammals against encephalomyocarditis virus using a genetically engineered Mengo virus. *Vaccine*. 14:155–161.

Patzl, M., F. Schwarzenberger, C. Osmann, E. Bamberg, and W. Bartmann. 1998. Monitoring ovarian cycle and pregnancy in the giant anteater (*Myrmecophaga tridactyla*) by faecal progestagen and oestrogen analysis. *Anim. Reprod. Sci.* 53:209–219.

Ransom, C., P.T. Robinson and B. Collen. 2015. *Choeropsis liberiensis*. The IUCN Red List of Threatened Species 2015: e.T10032A18567171 http://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T10032A18567171.en.

Raymond, J.T., K.A. Eaton, and R.J. Montali. 2000. A disease in captive pygmy hippopotamuses (*Choeropsis liberiensis liberiensis*) anatomically resembling polycystic kidney disease. *In* Proceedings of the American Association of Zoo Veterinarians and International Association for Aquatic Animal Medicine Joint Conference. New Orleans, Louisiana. 302.

Reddacliff, L.A., P.D. Kirkland, W.J. Hartley, and R.L. Reece. 1997. Encephalomyocarditis virus infections in an Australian zoo. *J. Zoo Wildl. Med.* 28:153–157.

Roth, H.H., B. Hoppe-Dominik, M. Mühlenberg, B. Steinhauer-Burkart, and F. Fischer. 2004. Distribution and status of the hippopotamids in the Ivory Coast. *Afr. J. Ecol.* 39:211–224.

Schulze, W. 1955. Nephritis beim Zwergflußpferd. *Der Zool. Garten NF*. 21:188.

Seaman, J.T., and E.P. Finnie. 1987. Acute myocarditis in a captive African elephant (Loxodonta africana). *J. Wildl. Dis.* 23:170–171.

Soares, J.F., H. Pereira, F.S. Desta, M. Sandouka, and W. Macasero. 2015. Causes of mortality of captive Arabian gazelles (*Gazella arabica*) at King Khalid Wildlife Research Centre, Kingdom of Saudi Arabia, from 1988 to 2011. *J. Zoo Wildl. Med.* 46:1–8.

Steck, B. ed. . 2015. Pygmy Hippopotamus Choeropsis liberiensis (Morton, 1844) International Studbook 2014. 21st ed. Zoo Basel, Switzerland, Basel.

Taylor, V.J., and T.B. Poole. 1998. Captive breeding and infant mortality in Asian elephants: A comparison between twenty western zoos and three eastern elephant centers. *Zoo Biol.* 17:311–332.

Thomson, G.R., R.G. Bengis, and C.C. Brown. 2001. Picornavirus infections. *In* Infectious Diseases of Wild Mammals. E.S. Williams and I.K. Barker, editors. Iowa State University Press, Ames, Iowa. 119–130.

von Houwald, F., A.A. Macdonald, O. Pagan, and B. Steck eds. . 2007. Husbandry Guidelines for the Pygmy Hippopotamus (*Hexaprotodon liberiensis*). Zoo Basel, Switzerland, Basel.

Walzer, C., and G. Stalder. 2014. Hippopotamidae (Hippopotamus). *In* Fowler's Zoo and Wild Animal Medicine. R.E. Miller and M.E. Fowler, editors. Saunders Elsevier, Philadelphia, Pennsylvania. 584–592.

Wells, S.K., A.E. Gutter, K.F. Soike, and G.B. Baskin. 1989. Encephalomyocarditis virus : Epizootic in a zoological collection. *J. Zoo Wildl. Med.* 20:291–296.

Wielebnowski, N. 1996. Reassessing the relationship between juvenile mortality and genetic monomorphorism in captive cheetahs. *Zoo Biol.* 15:353–369.

Wilson, P.D. 2004. Polycystic kidney disease. *N. Engl. J. Med.* 350:151–164.

Zschokke, S. 2002. Distorted sex ratio at birth in the captive pygmy hippopotamus, Hexaprotodon liberiensis. *J. Mammal.* 83:674–681.

Chapter 4 Demographics of Polycystic Kidney Disease and Implications for Captive Population Viability in Pygmy Hippopotamus (*Choeropsis liberiensis*).

Pygmy Hippo – Center for Conservation of Tropical Ungulates

There is wisdom in turning as often as possible
from the familiar to the unfamiliar;
it keeps the mind nimble,
it kills prejudice,
and it fosters humor.

–George Santayana

Demographics of Polycystic Kidney Disease and Implications for Captive Population Viability in Pygmy Hippopotamus (*Choeropsis liberiensis*)

Gabriella L. Flacke,[1*] Joseph L. Tomkins,[1,2] Robert Black,[1] and Beatrice Steck[3]

[1]*School of Animal Biology, University of Western Australia, Crawley, Australia*

[2]*Center for Evolutionary Biology, School of Animal Biology, University of Western Australia, Crawley, Australia*

[3]*Zoo Basel, Basel, Switzerland*

Running head: Polycystic kidney disease in pygmy hippos

***Address for correspondence:** Gabriella Flacke, School of Animal Biology M092, University of Western Australia, 35 Stirling Highway, Crawley 6009 WA, Australia; +61 470 137 217; E-mail: gflacke@grs.uwa.edu.au

4.1 ABSTRACT

Polycystic kidney disease (PKD) was previously diagnosed at necropsy in several pygmy hippopotami (*Choeropsis liberiensis*) from the Smithsonian National Zoo and Zoo Basel, suggesting a threat to the long-term viability of the captive population. We determined the incidence and demographics of PKD in captive pygmy hippos; we tested whether the condition is linked to pedigree or affects long-term population viability; we investigated the mode of inheritance; and we examined the effects of PKD on longevity. Thirty-seven percent of 149 adult pygmy hippos were affected by PKD, and it was more common in females, controlling for the sex-ratio. There was no significant correlation between inbreeding coefficient (F) and PKD, nor was there a significant difference in prevalence between wild-caught and captive-born animals. PKD did not affect longevity, overall or within any age class. Prevalence increased significantly with age, but most hippos were beyond their reproductive prime before they developed clinical signs associated with PKD and thus fecundity was not affected. For the overall studbook population, survival for both adults and juveniles has increased in zoos over time. However, longevity for both captive-born and inbred hippos ($F > 0$) is significantly shorter than longevity for their wild-caught and outbred counterparts. Demographic projections indicated the extant population will likely experience a slow increase over time. We conclude that PKD can cause clinically significant morbidity and mortality in individual pygmy hippos, but the condition is not a primary concern for long-term viability of the captive population despite the limited number of remaining founder genomes.

Key words: inbreeding coefficient, life table, longevity, pedigree, population viability, survival analysis

4.2 INTRODUCTION

The pygmy hippopotamus (*Choeropsis liberiensis*), hereafter 'pygmy hippo,' is listed as an endangered species on the Red List of the International Union for the Conservation of Nature and Natural Resources (IUCN; Ransom et al. 2015). It is endemic to the Upper Guinean ecosystem of West Africa where its range is limited to fragmented rainforest habitats in Côte d'Ivoire, Liberia, Sierra Leone and Guinea. The size of the wild population is estimated to be less than 2,500 animals (Ransom et al. 2015). Threats to the survival of this species include: loss and fragmentation of habitat due to logging, mining and agriculture; lack of adequate legal protection for the few remaining intact areas of habitat; poaching for bush meat; and instability of the political climate in all four range states (Hoppe-Dominik et al. 2011; Mallon et al. 2011; Roth et al. 2004).

The captive population is managed through a Species Survival Plan (SSP) Program in North America and a European Endangered Species Program (EEP) in European facilities, and the International Studbook is maintained at Zoo Basel in Switzerland (Steck 2015). Over the last century, captive breeding has been ongoing with intermittent addition of animals from the wild population until the last documented import in 1982. Longevity in the wild is unknown and the oldest captive animal was 49 years old at the time of writing. However, maximum lifespan for pygmy hippos in captivity is generally between 35 to 47 years (Flacke et al. 2015).

Due to the limited number of pygmy hippos in captivity, circumstances that negatively influence health and welfare can have a significant impact on the success of captive breeding programs for this endangered species. Polycystic kidney disease (PKD) is a degenerative condition where fluid-filled cystic structures progressively replace functional renal tissue (Wilson 2004). PKD has been reported in a number of domestic species including rodents (Cowley et al. 1993), Persian cats (Biller et al. 1996), and bull terrier dogs (O'Leary et al. 1999). The first two cases of PKD in pygmy hippos (1 ♂, 1 ♀) were identified in a mortality survey by Jarofke and Klös (1982), both as incidental findings at necropsy. More recently, PKD

was identified at necropsy in 10 additional pygmy hippos, all female, four wild-caught and six captive-born (Nees et al. 2009; Raymond et al. 2000). Some of these animals also exhibited clinical signs consistent with renal failure. Nees et al. (2009) classified PKD as a possible health concern for the *ex situ* population overall and, because several affected animals in their retrospective study were genetically related, they hypothesized that PKD in pygmy hippos may exhibit a dominant inheritance pattern.

In humans, PKD is one of the most common hereditary disorders and is the most common genetic-based cause of renal failure (Igarashi & Somlo 2007). The condition exhibits both autosomal dominant (ADPKD) and recessive (ARPKD) inheritance patterns. ADPKD is the predominant form and is characterized by a slow progression of cystic dilation over time with a delay of clinical signs until later in adulthood (Grantham 2008; Igarashi & Somlo 2007; Wilson 2004). Although the clinical course is extremely variable, 50% of people with ADPKD exhibit chronic renal failure by the age of 60 (Peters & Breuning 2001). Overall, life-expectancy is reduced compared to the general population due to both renal failure and secondary complications (Grantham 2008; Torres et al. 2007).

It is unknown if PKD in pygmy hippos is predominately a heritable trait as in humans, rodents, bull terrier dogs and domestic cats (Biller et al. 1996; Cowley et al. 1993; O'Leary et al. 1999; Wilson 2004), or whether husbandry conditions such as diet, management practices, stress or other environmental factors may play a contributing role. Significant intra-familial variability in the clinical severity of ADPKD in humans points to a combination of genetic and environmental modifying factors influencing disease progression (Torres et al. 2007). It also remains unknown if PKD also occurs in wild pygmy hippos; however, the majority of original founders to the captive population (89 of 162 animals) originated from Liberia and procurement expeditions often sourced multiple animals from the same limited geographic areas (Flacke et al. 2015). Additionally, inbreeding (full and half sibling or parent-offspring mating) is documented in the Studbook on multiple occasions in the early history of the captive population (Steck 2015). Thus, a particular genetic make-up may be over-represented.

The long-term effects of PKD on overall longevity and fecundity in pygmy hippos are of primary relevance to *ex situ* population viability and conservation efforts, especially as a limited number of founder genomes remain (Steck 2015). Thus, our retrospective study aimed to develop a greater understanding of PKD in this species by testing several hypotheses. We anticipated PKD would exhibit equal prevalence in wild-caught and captive-born animals, but expected a higher prevalence in females as previously noted (1 ♂, 11 ♀). Based on Nees et al.'s (2009) preliminary data, we predicted PKD would be linked to pedigree and would exhibit an autosomal dominant inheritance pattern, akin to the primary form in humans. Furthermore, we predicted a lack of correlation between PKD and inbreeding coefficient (*F*), as dominant conditions are inherited whether or not there is inbreeding. We also expected PKD to reduce longevity in affected animals as it does in humans. Finally, we conducted an age-based survival analysis to project long-term captive population trends and assess viability based on historical data and current population demographics.

4.3 Materials and Methods

4.3.1 Polycystic Kidney Disease (PKD)

In humans, the number and distribution of cysts necessary to diagnose PKD increases with age (Grantham 2008), but this methodology was difficult to extrapolate to pygmy hippos because the number and distribution of cysts is often not reported on necropsy reports. We therefore defined the condition as three or more cysts distributed between both kidneys (Gabow 1993) in accordance with the earlier description of PKD in pygmy hippos by Nees et al. (2009). As part of a population-wide mortality survey we received necropsy data for 404 pygmy hippos from 1919 through the end of 2014, including information concerning PKD status for 367 of these hippos (163.197.7). Additionally, one female hippo (age 22 years) was diagnosed ante mortem, via ultrasound, as unaffected by PKD. Of these 368 animals, we only included adults (≥3 years) in our demographic analyses (n = 149; 66.83.0) because the condition was not reported in neonatal or juvenile animals.

Many necropsy reports were incomplete and only described gross pathologic changes without reporting a lack of pathology in (assumingly) normal organ systems. However, PKD is typically an obvious condition on post-mortem, so if renal pathology was not mentioned we classified the hippo as unaffected by PKD. We also assumed the information provided by the submitting institutions concerning PKD status was correct and made no further attempts to confirm reported pathology results.

4.3.2 Demographic, Pedigree, and Survival Analysis

Determining the exact age of wild-caught animals is challenging if not impossible for most species. Thus, Studbook data concerning birth dates for these animals are often arbitrarily assigned to fulfill software requirement and are not a true reflection of age at the time of import. In the pygmy hippo Studbook, the majority of wild-caught animals with estimated birth dates were assigned an age between 1 and 5 years at the time of transfer to a zoo. Given that the majority of pygmy hippos were imported as adults, we chose to assign all wild-caught animals used in our analyses an age of 3 years at the time of Studbook acquisition rather than equating the import date with the birth date. We recognize that this method of age estimation has the potential to skew our data for wild-caught animals toward a younger age at death because many hippos were likely older than 3 years at the time of capture.

We calculated prevalence of PKD for the 149 adults overall and by age class (≥3–9; 10–19; 20–29; 30–39; 40+ years old), sex, and origin (wild versus captive-born). Additionally, we used pedigree data to investigate potential inheritance patterns for the subset of these 149 animals where the PKD status of the sire (n = 14), dam (n = 6), or both (n = 48) was also known. The PKD status of one or both parents was unknown for 81 hippos.

We used data from the 2015 edition of the Studbook (Steck 2015) and ASreml3 (Gilmour et al., 2009) to construct a pedigree for the 149 adults to analyze factors potentially influencing the likelihood of developing PKD and to determine if there is a genetic component to the condition.

PKD as the dependent variable was modelled as a binomial trait with a logit link function using the penalized likelihood method (PQL; Gilmour et al. 2009). PQL does not allow for specific hypothesis testing of the random effects (such as the additive genetic variance) using log-likelihoods, but does provide an estimate of the heritability and its standard error on the logistic scale (Gilmour et al. 2009). The full PQL model included the random effects of 'genetic ID' and 'zoo of death;' genetic ID is equivalent to the Studbook number. 'Date of death' was not included in the model because PKD is a grossly evident condition with an equal probability of being detected irrespective of when the necropsy was performed. Fixed effects included sex, origin, age at time of death, and inbreeding coefficient (*F*). Both sex and origin were categorical variables whereas age at death and *F* were continuous variables.

We used SPSS for Windows version 24 (Statistical Package for the Social Sciences, Chicago IL, USA) to calculate predicted probabilities of a pygmy hippo developing PKD from a generalized linear model (GLM) with the same data set (n = 149) and variables as above except for the exclusion of genetic ID. We used the modified Wald method to calculate standard errors (SE) and confidence intervals (CI).

We also used SPSS for Windows version 24 and a GLM to test whether PKD reduces overall longevity with age in days as the continuous dependent variable. We then grouped the data according to age class as previously described. PKD tends to develop later in life, so this categorization allowed us to determine whether PKD reduces longevity within any specific age class. We only included pygmy hippos ≥10 years of age (n = 125) in this second analysis because the youngest animal affected by PKD was 11 years old.

We also used 2015 Studbook data and SPSS version 24 to conduct Kaplan Meier survival analysis and to test whether longevity is affected by origin, sex, or inbreeding coefficient (*F*). For these analyses we evaluated survival in days for all live-born pygmy hippos listed in the Studbook from the first successful import (1912) through a census date of 31 December 2014. Wild-caught animals were assumed to be 3 years old at the time of entry to the Studbook as described above. We included animals from both zoological and private institutions because a

substantial subset of the Studbook population has been kept in private facilities over the years, including 23% of the extant population at the end of 2014 (Steck 2015). Hippos listed as 'lost to follow up' (LTF) were also included in our analyses with the LTF date as a live census date. However, we excluded animals with unknown birth or death dates that were not listed at LTF. Animals of undetermined sex (n = 59) were also excluded, although we recognize this exclusion has the potential to skew results toward higher estimates of longevity because the majority of these hippos were neonates or juveniles at the time of death. Additionally, we excluded captive-born hippos with an unknown pedigree due to lack of information about parentage because F could not be determined. We assumed, per the Studbook, that F for all wild-caught animals is 0.

Wild-caught animals were only included in the analysis for survival by origin because their actual birth date is unknown, although these hippos were at least as old as the number of years they lived in captivity. Additionally, for survival by origin we only analyzed data for hippos 5 years and older to remove the effects of juvenile mortality in captivity and habituation in wild-caught animals. However, for the analyses to test the effects of sex and inbreeding coefficient on longevity in captive-born, known-age animals, we initially included hippos of all ages. Thus, our data set for the survival analysis by origin comprised 59.73 wild-caught and 215.362 captive-born animals greater than five years of age. Our data set to compare survival between sexes and to determine the effect of inbreeding comprised 485.684 captive-born hippos. For inbreeding analysis, we grouped animals as follows: $F = 0$; $F > 0$ and ≤ 0.25 (full-sibling mating); $F > 0.25$. The maximum inbreeding coefficient in our cohort was 0.3887. We considered $P \leq 0.05$ as statistically significant for all analyses.

4.3.3 Age-Based Population Models and Demographic Projections

We used 2015 Studbook data, R v. i386 3.2.3 (R Foundation for Statistical Computing 2016, available at https://www.r-project.org/) and popbio package v. 2.4.2 (Stubben and Milligan 2007) within R to generate life tables and age-based population models.

Pygmy hippos in captivity exhibit a birth flow rather than a birth pulse pattern, with a similar number of calves being born during all months of the year (Steck 2015). Birth of offspring can occur as early as 3 years but also depends on individual zoo factors, especially the availability of a mate. Weaning age in captivity is also influenced by zoo factors including space limitations and the timing of animal transfers between institutions. Logistical issues therefore strongly affect generation time in captive populations. Lifespan in captivity is 35 to 47 years and maximum reproductive age is considered to be 35 years (Steck 2015), although the oldest female to reproduce was at least 38 years old (wild-caught) and the oldest living pygmy hippo of known age (a male) will be 50 years old in October 2016.

As of 31st December 2014, the International Studbook listed 1454 pygmy hippos (585.810.59), representing both the historical and extant population, of which 58.1% of animals of known sex were female (Steck 2015). Using this population and other information from the 2015 Studbook concerning birth and death dates, we extrapolated a subset of data to generate life tables and a Leslie transition matrix model for deceased females, excluding stillbirths. We analyzed data for females only because they are the reproductively limiting sex. Further, we excluded females whose age could not be determined due to undetermined birth or death dates. Thus, we removed all wild-caught animals, those listed in the Studbook as LTF, and all other females with uncertain birth or death dates from the dataset. The final cohort used in our analyses represented all live-born, deceased female pygmy hippos of known age from the first live import in 1912 through the end of 2014 (n = 407). Of these 407 females, 101 reproduced and gave birth to a total of 561 (218.311.32) offspring. The sex of each offspring was determined from the Studbook. For the 32 calves of undetermined sex we calculated the number of females by multiplying by 0.581, the proportion of females in the overall Studbook population at the end of 2014.

We first constructed a life table using birth and death dates to determine the number of female hippos (n_x) alive in each age class (x in years), and the age-specific survival (p_x), the probability that an individual will survive from time x to time $x+1$, for all age classes. Similarly, we

calculated cumulative survival (l_x), the proportion of the starting number of individuals that remained alive at the end of each time interval ($l_x = n_x / n_0$). We then used n_x and l_x to calculate life expectancy (E_x), the average lifespan remaining for an animal in age class *x*, for all age classes (Krebs 1999). The maximum age class *x* for our dataset was 41 years. We then determined each female's age at each birth event to generate values for fecundity (m_x), or the number of female offspring per female during each time interval $x = 1$ year. For each age class, m_x is equal to the total number of female offspring (*tf*) born to all female hippos of age *x* divided by the total number of females hippos (n_x) of age *x*.

We also created a model for living female pygmy hippos using a censor date of 31 December 2014 and vertical cohort construction in order to compare annual population growth rate, lambda (λ), between the historical data and the extant population. The maximum age class *x* for living female hippos was 42 years. For this model, fecundity (m_x) was based on number of offspring through the end of 2014.

The first set of life tables for both the living and deceased female datasets was generated according to a birth-pulse model and subsequently converted to a birth-flow model using the methods described by Ebert (1999; pp. 89–91). The birth pulse model we used assumes that all births occur simultaneously for all females at the beginning of each time step, whereas the birth flow model concentrates births in the middle of each time step. In both models births occur prior to deaths. Raw data for both models are presented in Appendix III.

We then constructed Leslie transition matrices with one-year time steps up to the maximum age class using the life table data from the birth flow models. The initial transition matrix was generated using $f_x = p_0 \bullet m_x$, where f_x is the age-specific fecundity function per year and p_0 is the age-specific survival, or the probability that an individual will survive from birth to one year of age (Ebert 1999). We used this first matrix to obtain an estimated population growth rate, asymptotic lambda (λ_{asympt}) and elasticity values (e_i) for each age class. Elasticities are a measure of how responsive the population growth rate (λ) would be to changes in any element

in the matrix; larger values indicate a greater change in λ per change in the associated matrix element. We then constructed a second matrix using $f_x = \lambda \bullet m_x$ to calculate stable age (c_x) and reproductive value (v_x) distributions. λ_{asympt} year^{-1} is a demographic projection of the annual asymptotic growth rate that a given population will exhibit once it reaches the stable age distribution (c_x). The stable age distribution for extant females was generated according to Caughley (1977).

We estimated the variance of λ_{asympt} year^{-1} using a demographic stochasticity projection to calculate the variance of f_x using the $f_x = \lambda \bullet m_x$ transition matrix and the "multiresultm" function in the R popbio package. Because λ is constant, $var(f_x) = var(\lambda) \bullet m_x$ and therefore $var(f_x) = \lambda^2 \bullet var(m_x)$, where $var(m_x)$ was determined from the number of females alive at each age and the number of female offspring they produced. We then used the "multiresultm" function to project a population with the age distribution for the 222 living female hippos according to the appropriate transition matrix and the $var(m_x)$ matrix as reconstructed by "mutliresultm" for each of 50 time steps (years). Keeping track of the total number of hippos in the population at each time step, we calculated λ year^{-1} as exp[(ln (final total numbers) – ln (starting total numbers)) / number of time steps that the population exceeded two individuals]. We performed 1000 iterative simulations and used 2.5 and 97.5% quantiles to estimate demographic stochasticity of λ_{asympt} year^{-1}. We considered values of $P \leq 0.05$ as statistically significant for the analyses described in this section.

4.4 RESULTS

4.4.1 PKD PREVALENCE

Of the 149 (66.83.0) adult pygmy hippos in our demographic analysis, 56 (17.39.0) animals were affected by PKD, but it was only considered a clinically significant cause of renal failure and mortality in 15 of these hippos. In the remaining 41 animals, the disease was described as an incidental finding at necropsy. Many of these hippos exhibited extensive cyst formation,

often designated as “severe” bilateral PKD, with cysts ranging from a few mm to several cm in size. However, the approximate number and distribution of cysts was not mentioned in the majority of necropsy report.

The prevalence of PKD is presented in Table 4.1, overall and by age class, sex, and origin. Only two hippos less than 20 years old were affected, an 11 year-old male and an at least 19 year-old female, but the second animal was wild-caught so its exact age is unknown. The condition was more prevalent in females than males ($\chi^2 = 6.19$, $df = 1$, $P = 0.013$). There was similar prevalence in wild-caught and captive-born animals ($\chi^2 = 2.38$, $df = 1$, $P = 0.123$). Prevalence increased significantly with age class ($\chi^2 = 26.80$, $df = 4$, $P < 0.001$). The Studbook indicates that 60 (28.32.0) of the 162 pygmy hippos imported from the wild are considered founders (Steck 2015). Of these 60 founders, we were able to determine PKD status for 28 animals – 14 (4.10) were affected and 14 (11.3) were unaffected. Thirteen pygmy hippos were reported to have extrarenal cysts; 11 of these animals were also affected by PKD (Table 4.2).

Table 4.1 - Prevalence of PKD in 149 pygmy hippos diagnosed at necropsy.

The data are presented for adult (≥3 years) hippos overall, for males versus females, by age class, and for wild-caught versus captive-born animals.

	Overall			**Age class**					**Origin**	
	≥3	**≥3 ♂**	**≥3 ♀**	**3–9**	**10–19**	**20–29**	**30–39**	**40+**	**Wild**	**Captive**
***n* (PKD)**	56	17	39	0	2	16	29	9	22	34
***n* (total)**	149	66	83	24	30	40	44	11	46	103
Prevalence (%)	37.6	25.8	47.0	0.0	6.7	40.0	65.9	81.8	47.8	33.0

Table 4.2 - Occurrence and distribution of extrarenal cysts in 13 of 149 pygmy hippos, including 11 animals with PKD.

Wild-caught animals were assumed to be 3 years old at the time of import.

Studbook Number	Sex	Sire	Dam	Year of death	Age	PKD	Extrarenal cysts
12	F	Wild	Wild	1944	26.5	Yes	Thyroid
96	F	26	77	1988	41.5	Yes	Thyroid Ovaries
144	F	Wild	Wild	1985	30	Yes	Thyroid Duodenum Bladder
153	M	Wild	Wild	1987	31	No	Thyroid Duodenum
175	F	122	87	1992	30.5	Yes	Liver
184	M	Wild	Wild	1978	18	No	Thyroid
242	F	Wild	Wild	2009	46.5	Yes	Thyroid
245	M	Wild	Wild	1991	27	Yes	Thyroid
247	M	Wild	Wild	2001	37	Yes	Thyroid
250	M	Wild	Wild	2007	43.5	Yes	Liver
251	F	Wild	Wild	2007	43	Yes	Pancreas
257	F	Wild	Wild	1983	19	Yes	Ovaries
387	F	156	127	2007	33	Yes	Thyroid

4.4.2 *Demographic, Pedigree and Survival Analysis*

The demographics and pedigree for the 56 pygmy hippos affected by PKD are presented in Table 4.3. For several PKD (+) hippos, one (primarily the dam) or both parents were also affected, indicating a potential genetic basis for the disease. However, pedigree analysis for the 48 of 149 pygmy hippos in our study where the PKD status of both parents was known did not support autosomal dominant inheritance (Table 4.4). Additionally, for the 54 hippos where the PKD status of the dam was known, PKD (+) and PKD (–) dams produced both PKD (+) and PKD (–) offspring with similar frequency (Table 4.5). Similarly, there was no statistically significant correlation between PKD status of the sire and the offspring for the 62 hippos where

the PKD status of the sire was known (Table 4.5). Thus, PKD in pygmy hippos did not exhibit an autosomal dominant inheritance pattern.

However, PKD may exhibit X-linked dominant inheritance in this species given the significantly higher prevalence in females. With this inheritance pattern, PKD (+) by PKD (+) crosses will produce 100% PKD (+) female offspring and 50% PKD (+) male offspring. Of the 149 pygmy hippos of known PKD status where the PKD status of both parents was also known, there were six hippos (2 ♂, 4 ♀) with two PKD (+) parents. Both males were PKD (+) and two of the four females were PKD (+). X-linked dominant inheritance dictates that all four females should be positive; however, the two PKD (–) females were 8 and 10 years old at the time of death. Thus, their negative status at the time of death cannot be considered reflective of their PKD status later in life because the youngest PKD (+) hippo in our cohort was 11 years old and only one other PKD (+) hippo was younger than twenty.

X-linked dominant inheritance also dictates that PKD (–) dams will produce 0% PKD (+) female offspring if the sire is also PKD (–), but will produce 100% PKD (+) female offspring if the sire is PKD (+). All male offspring born to PKD (–) dams will also be PKD (–) regardless of the sire's PKD status. Our data set had nine PKD (–) by PKD (–) crosses, producing 4 males and 5 females. Of these nine offspring, two were less than 10 years old at the time of death and thus their PKD status cannot be considered reflective of their status later in life (see above). For the remaining seven hippos, the PKD status of four animals supported X-linked dominant inheritance while the PKD status of three animals did not. Additionally, we only had two PKD (–) dam by PKD (+) sire crosses in our dataset, and these were from the same breeding pair. Both offspring were female. One was PKD (+), as predicted by X-linked dominant inheritance, but the other was PKD (–). However, again the negative female was only 11 years old at the time of death and thus almost certainly too young to have developed PKD. We cannot rule out that this female wouldn't also have been positive if she had lived as long as her full sibling, who was 22 at the time of death and PKD diagnosis. Thus, although some of our pedigree data support X-linked dominant inheritance, our sample size for older adult pygmy hippos of known

PKD status with two parents of known PKD status was too small to support or refute this inheritance pattern.

Table 4.3 - Demographics of PKD in 56 of 149 pygmy hippos diagnosed at necropsy.

Wild-caught animals were assumed to be 3 years old at the time of import. If the PKD status is of the sire and/or dam is not indicated, it was unknown. ID = studbook number.

ID	Sex	Age	Year of death	Sire ID	Sire PKD status	Dam ID	Dam PKD status	Notes
3	M	42.5	1952	Wild		Wild		
12	F	26.5	1944	Wild		Wild		
42	F	38	1967	Wild		Wild		
77	F	43	1980	Wild		Wild		
80	F	37.5	1978	26		24		Sire of 24 had PKD; status for dam of 24 unknown
81	F	32	1970	Wild		Wild		
87	F	33.5	1976	29	(–)	42	(+)	
96	F	41.5	1988	26		77	(+)	
112	F	27	1979	29	(–)	87	(+)	
120	F	37.5	1985	Wild		Wild		
133	F	29	1985	29	(–)	42	(+)	
144	F	30.5	1985	Wild		Wild		
175	F	30.5	1992	122	(–)	87	(+)	
178	F	37	1999	153	(–)	77	(+)	
192	M	34	1994	Wild		Wild		
204	F	31	1991	Wild		Wild		
205	F	23.5	1984	Wild		Wild		
207	F	31.5	1995	153	(–)	99		Sire had extra-renal cysts in thyroid and duodenum; dam of 99 had PKD
213	F	36.5	1997	Wild		Wild		
226	F	22	1984	Wild		Wild		
242	F	46.5	2009	Wild		Wild		
245	M	27	1991	Wild		Wild		
247	M	37	2001	Wild		Wild		
250	M	43.5	2007	Wild		Wild		

ID	Sex	Age	Year of death	Sire ID	Sire PKD status	Dam ID	Dam PKD status	Notes
251	F	43	2007	Wild		Wild		
253	F	43	2007	Wild		Wild		
257	F	19	1983	Wild		Wild		
267	F	37	2005	101	(–)	103		Dam of 103 had PKD
282	M	27	1996	220		200		Dams of both 220 and 200 had PKD
283	M	41	2010	209		197		Dam of the dam of 209 had PKD
286	M	32.5	1999	Wild		Wild		
287	F	35.5	2002	Wild		Wild		
289	F	26.5	1996	203		204	(+)	
291	F	40	2010	256	(–)	257	(+)	
292	M	43.5	2013	122	(–)	145	(–)	Neither parent affected but dam of 145 had PKD
330	M	28.5	2001	153	(–)	96	(+)	Sire had extra-renal cysts in thyroid and duodenum
337	F	39	2012	101	(–)	267	(+)	
345	M	37	2005	Wild		Wild		
387	F	33	2007	156		127		Parents of 156 were full siblings; no info on PKD status of either
392	M	37.5	2012	274		253	(+)	
403	F	34	2007	255	(–)	257	(+)	
418	M	39	2014	211	(–)	212		
424	F	35	2010	290		300		
447	F	35	2011	285		265		Parents (285 and 265) were full siblings; their dam had PKD
449	F	28.5	2005	273	(–)	133	(+)	
452	F	34	2010	153	(–)	144	(+)	Sire had extra-renal cysts in thyroid and duodenum

ID	Sex	Age	Year of death	Sire ID	Sire PKD status	Dam ID	Dam PKD status	Notes
464	M	27	2004	295		299	(–)	Dam of 299 had PKD
473	F	35	2012	281	(–)	314	(–)	Neither parent affected but dam of 281 had PKD; PKD status of 314's parents unknown
474	M	35	2012	157		234		Parents (157 and 234) were full siblings; their dam had PKD
530	F	32.5	2012	273	(–)	198	(–)	Neither parent affected but dam of 198 had PKD
552	M	31	2011	379		380		Parents were half-sibling (same sire); dam of 379 had PKD
568	F	33	2013	286	(+)	287	(+)	
626	F	15	2008	595		596		
687	F	26.5	2011	445		478		Parents (445 and 478) were full siblings; no info on PKD status of either
757	M	11.5	1999	421	(–)	444		Dam of 421 had PKD
870	F	22	2014	330	(+)	326	(–)	Dams of 330 and 326 also both had PKD

Table 4.4 - Analysis of inheritance pattern for PKD in 48 pygmy hippos with two parents of known PKD status and for an additional 20 hippos where the PKD status of one parent was known.

The observed proportions are compared to the expected pattern for autosomal dominant inheritance, Code N = affected; n = not affected; ? = PKD status unknown.

Parent phenotype	Code	Offspring PKD (+) proportion	Offspring PKD (+) percent	Predicted % of PKD (+) offspring with autosomal dominant inheritance
2 parents PKD (–)	n x n	3 of 9	33	0
1 parent PKD (+) **1 parent PKD (–)**	N x n	12 of 33	36	50
2 parents PKD (+)	N x N	1 of 6	17	75
1 parent PKD (+) **1 parent unknown**	N x ?	5 of 6	83	n/a
1 parent PKD (–) **1 parent unknown**	n x ?	3 of 14	21	n/a

Table 4.5 - The prevalence of PKD for pygmy hippos where the PKD status of the dam (*n* = 54) or sire (*n* = 62) was also known.

Status of parent	Number of offspring PKD (+)	Number of offspring PKD (–)	Total	χ^2	*P*
Dam (+)	15	25	40	0.041	0.839
Dam (–)	5	9	14		
Sire (+)	2	7	9	0.097	0.756
Sire (–)	18	35	53		

For the 149 hippos of known PKD status, our analysis of the additive genetic variance initially included the random effects of 'zoo of death' and 'genetic ID.' However, 'zoo of death' had a nearly zero variance component (0.6×10^{-6}) that was 'fixed at the boundary' by the program, or too small to measure accurately. Therefore 'zoo of death' was excluded from the analysis and the final model was based on genetic ID as the single random effect. The heritability of PKD was estimated as 0.16 ± 0.46 on the underlying logistic scale. The standard error is larger than

the heritability suggesting that there is no significant narrow sense heritability of PKD. For the fixed effects in the model, neither origin (wild or captive-born) nor inbreeding coefficient (*F*) significantly affected the probability of a developing PKD, whereas both sex and age were significant factors (Table 4.6).

Table 4.6 - Fixed effects included in the ASreml3 penalized likelihood (PQL) model to determine if each variable influences the probability of a pygmy hippo developing polycystic kidney disease.

The data set used in this model included 149 hippos; 56 were PKD (+) and 93 were PKD (–). Origin refers to birth status; *df* = degrees of freedom.

Variable	*df* Numerator	*df* Denominator	F	*P*
μ	1	19.1	26.86	< 0.001
Sex (♂ vs ♀)	1	140.0	6.04	0.016
Origin (wild vs captive)	1	99.7	0.01	0.916
Age in years	1	140.0	32.03	< 0.001
Inbreeding coefficient (*F*)	1	104.3	0.01	0.916

Table 4.7 - General linear model of between-subject effects for PKD in 149 pygmy hippos within five age classes, with a dependent categorical variable of age in days.

Origin was included in the model to control for a potential difference in longevity between the two groups. Age classes were 10–19; 20–29; 30–39; and 40+ years. *F* = inbreeding coefficient; *df* = degrees of freedom. R squared = 0.947; adjusted R squared = 0.943.

Source	*df*	Mean square (survival in days)	F	*P*
Corrected model	11	1631.105	223.008	< 0.001
Intercept	1	40915.423	5594.048	< 0.001
Sex	1	.003	0.000	0.985
Origin	1	8.526	1.166	0.282
Age class	4	3053.503	417.482	0.000
PKD	4	9.167	1.253	0.291
F	1	0.764	0.104	0.747
Error	137	7.314		
Total	149			
Corrected total	148			

Summation of results across age classes from the GLM to analyze the effect of PKD on survival showed that longevity was similar in affected and unaffected animals regardless of age class (Fig. 4-1; Table 4.7; $P = 0.291$). Median survival did not differ between wild and captive pygmy hippos with PKD (Table 4.7; $P = 0.282$) and there was no significant effect of sex, indicating that longevity is similar in males and females affected by PKD (Table 4.7; $P = 0.985$). Predicted probability analysis showed an earlier age of onset for PKD in females, but over time both sexes develop the condition at the same rate (Fig. 4-2). Median survival was also similar between inbred ($F > 0$) and non-inbred ($F = 0$) animals affected by PKD (Table 4.7; $P = 0.747$).

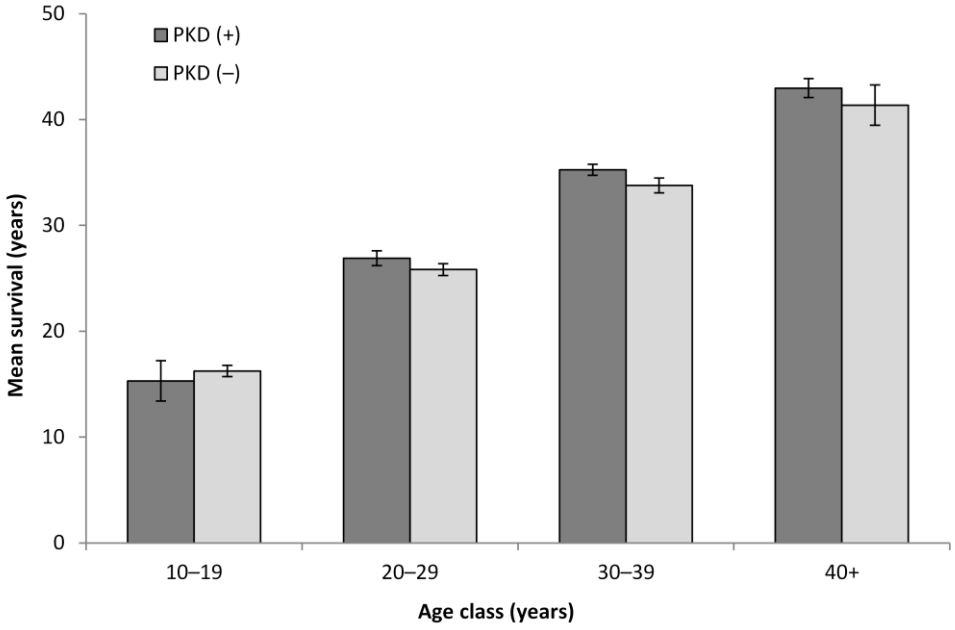


Fig. 4-1 - Mean survival in years for pygmy hippos affected (+) and not affected (–) by PKD for each age class in the sampled population ($n = 149$).

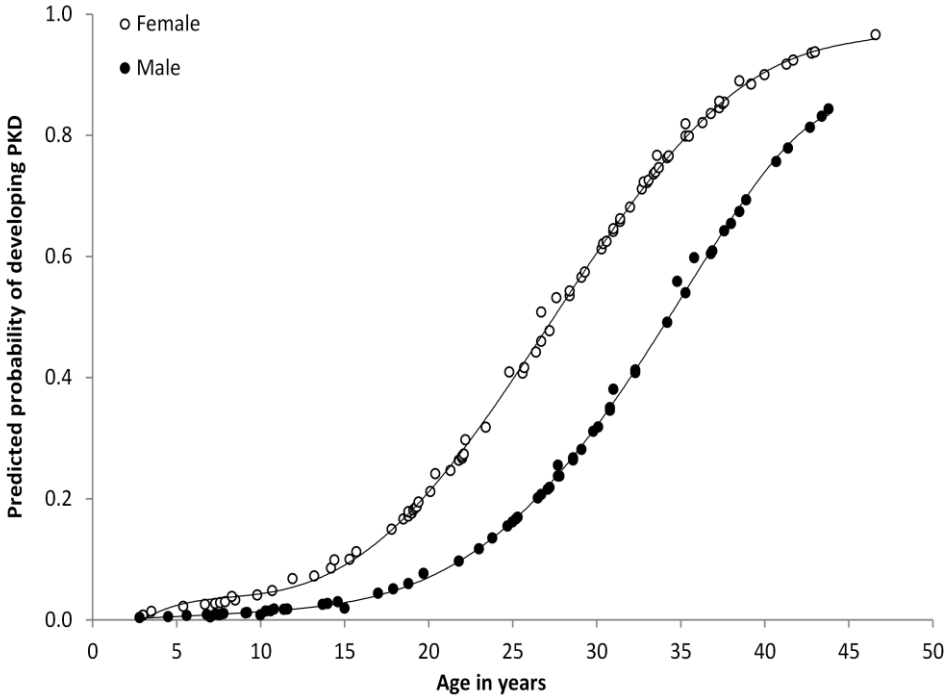


Fig. 4-2 - Predicted probability of developing PKD with increasing age in male (*n* = 66) and female (*n* = 83) pygmy hippos**.**

There is an earlier onset of the condition in females but both sexes develop PKD at the same rate as evidenced by the similar slope of the polynomial trend lines.

Since PKD status was known for only 149 animals, it was omitted from the Kaplan Meier survival analysis we used to evaluate factors affecting longevity of pygmy hippos in the overall population. We first pooled the data for wild-caught (*n* = 70.82) and captive born (*n* = 485.684) hippos of all ages and both sexes and performed Kaplan Meier survival analyses for the population overall, stratified by five time frames (1912–1939; 1940–1959; 1960–1979; 1980–1999; and 2000–2015), to test if longevity changed over the study period. Median survival increased over time (Fig. 4-3), with a significant difference in survival between the most recent time frame (2000 to 2015) compared to all previous ones (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 142.5$, $df = 4$, $P < 0.001$). Additionally, a generalized linear model with binomial distribution and a logit link function, with time frame as a fixed factor and no covariates or other effects showed that the probability of a newborn pygmy hippo surviving to adulthood (≥ 3 years) has significantly increased over time ($\chi^2 = 192.2$, $df = 4$, $P < 0.001$). Thus, for subsequent survival analyses we also stratified the factor of interest by time frame to account for differences in survival over the century-long study period.

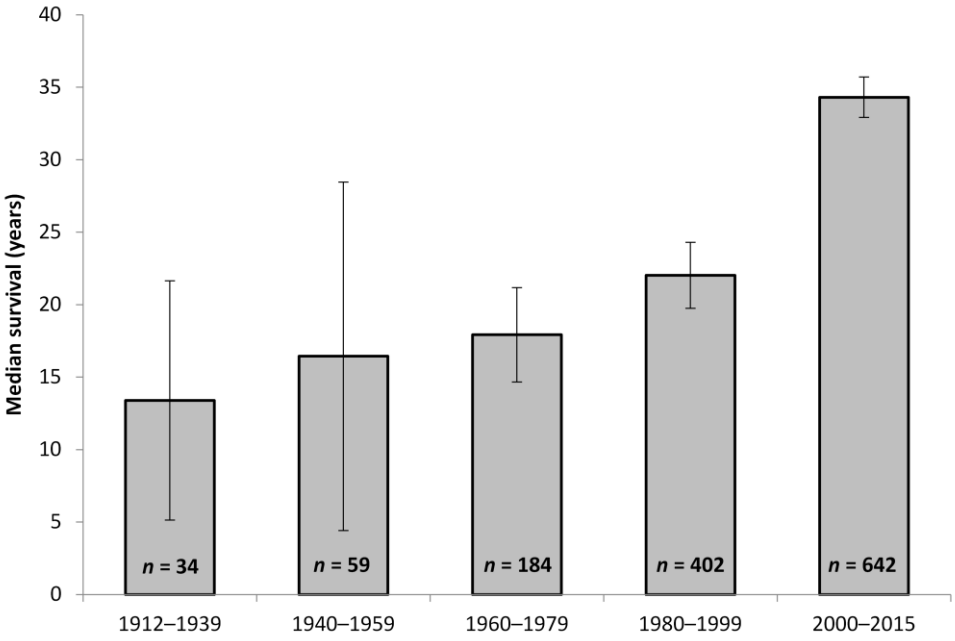


Fig. 4-3 - Median survival in years for pygmy hippos in the *ex situ* population within five subset time periods between 1912 and 2015.

The data represent a pooled sample of all ages and both sexes for wild-caught (*n* = 70.82) and captive-born (*n* = 485.684) animals; error bars signify 95% confidence intervals.

There was no significant difference in median survival between wild-caught and captive-born hippos aged ≥ 5 years overall (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 0.11$, $df = 1$, $P = 0.740$). However, when the data were stratified by time frame, wild-caught hippos survived significantly longer than captive-born hippos (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 28.803$, $df = 1$, $P < 0.001$). For all subsequent survival analyses we only examined captive-born hippos. Median survival overall, controlling for time frame, was significantly longer for females (15.5 years) than for males (12.7 years) (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 6.89$, $df = 1$, $P = 0.009$). To eliminate the influence of first-year mortality, we performed an additional analysis of survival by sex for hippos aged ≥ 1 year, also controlling for time frame. For this model, there was no significant difference in median survival between the two sexes (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 0.004$, $df = 1$, $P = 0.950$). Thus, first-year mortality significantly affects median survival for males but not for females.

Controlling for sex, non-inbred ($F = 0$) hippos survived significantly longer (median = 14.1 years) than hippos with $F > 0$ and ≤ 0.25 (median 12.1 years) or hippos with $F > 0.25$ (median = 7.8 years) overall (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 7.33$, $df = 2$, $P = 0.026$). When the effects of first-year mortality were removed by only including hippos aged ≥ 1 year in the model, there was no significant difference in median survival for the three levels of inbreeding: 26.5 years for $F = 0$; 27.7 years for $F > 0$ and ≤ 0.25; 24.8 years for $F > 0.25$ (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 10.28$, $df = 2$, $P = 0.006$). However, when also controlling for time frame to account for both changes in survival over time and the lack of hippos with $F > 0$ prior to 1960, non-inbred hippos aged ≥ 1 year experienced significantly greater longevity (Kaplan Meier Generalized Wilcoxon test $\chi^2 = 10.23$, $df = 2$, $P = 0.006$), especially in the most recent time frame between 2000 and 2015 (33.5 years for $F = 0$; 32.8 years for $F > 0$ and ≤ 0.25; 28.9 years for $F > 0.25$).

4.4.3 AGE-BASED POPULATION MODELS AND DEMOGRAPHIC PROJECTIONS

The life table and Leslie matrix results for λ_{asympt} year^{-1}, generation times, maximum reproductive values (v_x), and highest elasticities (e_i) are provided in Table 4.8. For both models, population growth rate was marginally lower with birth flow calculations than with birth pulse calculations; λ_{asympt} year^{-1} for birth pulse calculations is presented in Appendix III. For the deceased female model, λ_{asympt} year^{-1} was < 1.0, signifying a slow decrease in population size over time, whereas λ_{asympt} year^{-1} was > 1.0 for the extant population, indicating a slow increase.

In addition to performing demographic projections from the Leslie transition matrix, we also calculated λ_{asympt} year^{-1} for the extant captive population of female pygmy hippos using $N_t = N_0 \bullet \lambda^t$ (Ebert, 1999). Because the last pygmy hippo was imported from the wild in 1982 and the population has been genetically closed since that time, we included only females that were alive from 1983 ($N_0 = 184$) through the end of 2014 ($N_t = 222$), for a λ_{asympt} of 1.006 year^{-1}. Both this calculation and the model results for extant females are reflective of recent population trends. Pygmy hippo numbers have slowly increased since 1982 with the exception of the period

between 2000 and 2002, when 45 animals that had been lost to follow-up for several decades were removed from the managed population because no updates had been provided to the Studbook for at least a decade (Fig. 4-4).

Table 4.8 - Life table and Leslie transition matrix birth flow projections for deceased female pygmy hippos of known age (*n* = 407) and for the extant population (*n* = 222) through the end of 2014.

Generation times were calculated from life table data according to Ebert (1999) as Σ *xlxmx* / Σ *lxmx*. CI = confidence interval.

		Deceased ♀s (*n* = 407)	**Living ♀s (*n* = 222)**
λ year^{-1}		0.9640	1.0082
95% CI for λ year^{-1}	Lower	0.9466	1.0023
	Upper	0.9716	1.0134
Generation time, *T* (years)		15.0	15.1
Maximum reproductive (v_x) values		p_2–p_7 v_x > 2.50	p_2–p_7 v_x > 1.40
Highest elasticities (e_i)		p_0–p_6 Σ 0.4178	p_0–p_6 Σ 0.4715

Generation time for female pygmy hippos was similar for both models at approximately 15 years (Table 4.8). The highest values for elasticity (e_i) were for the youngest age classes in both models; $p_0 – p_6$ or 0 to 7 years of age (Table 4.8). These data provides insight into what part(s) of the life cycle should be emphasized in conservation strategies for this endangered species. Emphasis should be placed on preserving or improving transitions with the largest elasticity values. The maximum reproductive values (v_x) were from 2 to 8 years of age for both models, meaning that pygmy hippos in this age range have the highest overall reproductive potential (Table 4.8).

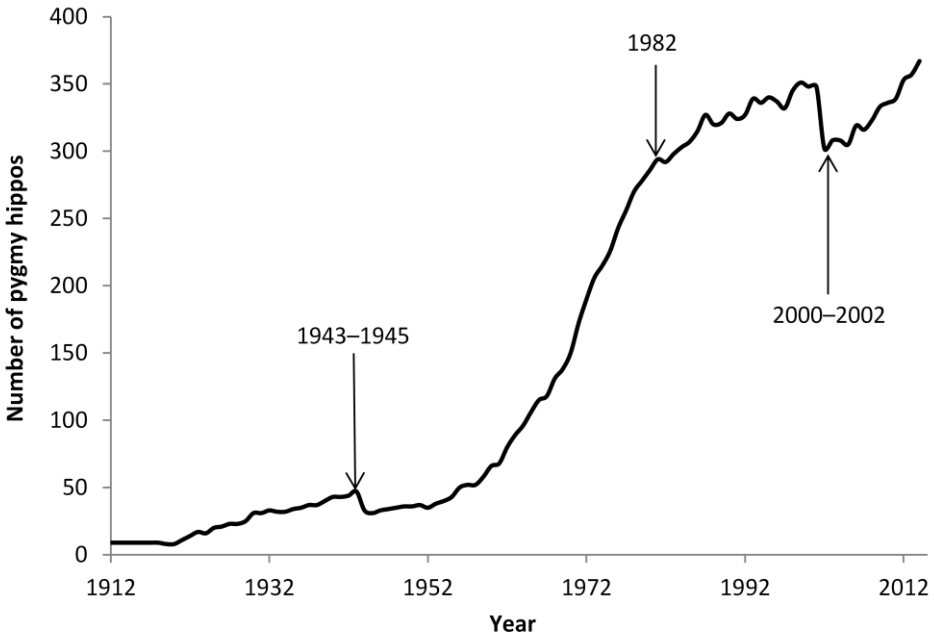


Fig. 4-4 - Annual census of pygmy hippos in the Studbook population from 1912 through the end of 2014.

Numbers increased dramatically from the 1950s until the early 1980s when imports from the wild ceased. A noticeable decrease occurred from 1943–1945 when many animals in Europe did not survive World War II. Another significant decrease from 2000–2002 is attributable to 45 animals from both zoos and private institutions being designated as lost to follow up (LTF) and thus removed from the managed population.

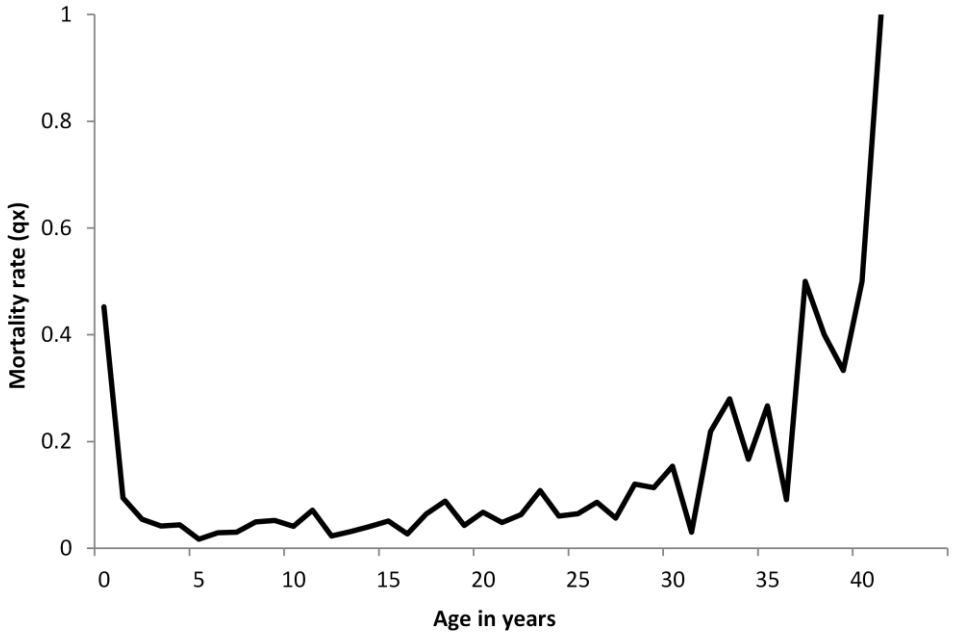


Fig. 4-5 - Mortality rate (q_x) for captive-born female pygmy hippos of known age (n = 407).

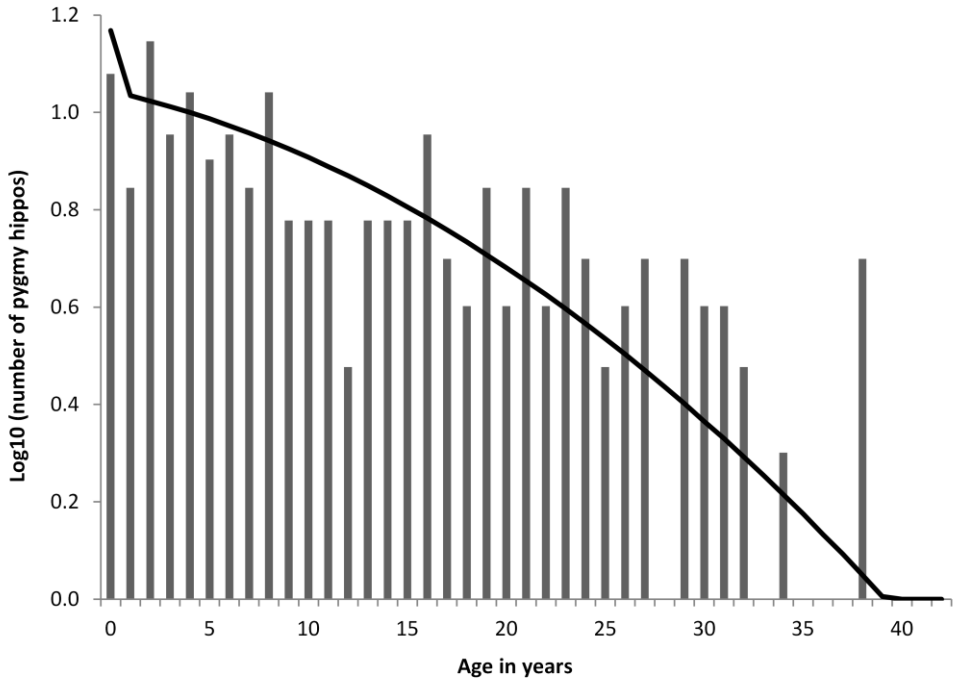


Fig. 4-6 - Stable age (c_x) distribution (solid line) compared with actual age distribution (bars) for the extant Studbook population of female pygmy hippos at the end of 2014 (n = 222).

As indicated by the age-specific survival (p_x) values in Appendix III, mortality in the first year of life (excluding stillbirths) is high, between 26 and 45%. Mortality rate then drops to below 10% until about 30 years of age, when it increases again due to senescence (Fig. 4-5). Mortality rate (q_x) was calculated as $q_x = 1 - p_x$ for each age class.

The stable age distribution (c_x) curve for the extant population also shows a marked decrease in survivorship from age 0 to 1 year followed by a smooth, constant decrease at a slower rate from 2 years of age and onward (Fig. 4-6). This initial rapid decline is attributable to the high mortality rate in the first year of life. Fig. 4-6 also shows that age distribution for the 222 living females does not mirror the c_x curve, indicating that the current population is not very close to the stable state. Depending on both external and internal factors influencing cumulative survival (l_x) and fecundity (m_x), a population can either exhibit a smooth convergence toward the stable age distribution (c_x) and λ_{asympt}, or it may be subject to significant oscillations. Thus, because c_x for each age class is an abstract value, it is usually interpreted in the context of the damping ratio (ρ) (Ebert 1999). If $\rho \leq 1.0$, convergence will not occur; the larger the value of $\rho > 1.0$, the

more rapidly the population will converge on the stable age distribution. For our data set ρ = 1.111 for the 222 living females, indicating a very slow convergence to stable age distribution if age-specific survival (p_x) and m_x remain constant.

4.5 DISCUSSION

4.5.1 PREVALENCE, DEMOGRAPHICS AND PEDIGREE

The prevalence of PKD in captive pygmy hippos (37%) is striking compared to ADPKD humans (0.125%; Wilson 2004). However, it is similar to the prevalence reported for purebred Persian Siamese, American curl and Scottish fold cats (16–49%; Rey 2013) and bull terrier dogs in Australia (26%; O'Leary et al. 1999). Purebred domestic animals are more homozygous than cross breeds, explaining the higher prevalence of genetic disorders such as PKD. Although the pygmy hippo Studbook indicates a low mean kinship (0.03) and average inbreeding coefficient (0.06) for the extant captive population of pygmy hippos, the analyses that generate these data assume that all founding animals are unrelated. Early inbreeding due to a limited pool of animals, the over-representation of certain genotypes, and a potential X-linked dominant inheritance pattern may all play a role in the high prevalence of PKD in this species.

Progressive growths of cysts over time and the delay of clinical signs until later in adulthood are the characteristic course of disease with ADPKD in humans; this is consistent with the patterns we observed in pygmy hippos. Two observations from the ASreml3 pedigree model are suggestive concerning inheritance patterns in the pygmy hippo. First, the lack of significant narrow sense heritability does not support an additive genetic basis to the condition such as would be predicted by Falconer's threshold model (Falconer and Mackay 1996) where numerous genes with minor effects act synergistically to influence the expression of a dichotomous character. This finding therefore implicates an environmental cause, recessive inheritance, or dominant gene action. Second, the nonsignificant relationship with inbreeding coefficient supports either a non-genetic basis for PKD in pygmy hippos, or dominant gene action, since inbreeding would only affect the prevalence of a recessive genetic condition.

However, analysis of the pedigree was not consistent with an autosomal dominant mechanism, particularly because this inheritance pattern dictates that two PKD (−) parents cannot produce a PKD (+) offspring. Our data also did not support an autosomal recessive inheritance pattern as several PKD (+) by PDK (+) crosses produced PKD (−) offspring. X-linked recessive inheritance is also unlikely as we would expect the prevalence to be higher in males than females and our results show the opposite. On the other hand, X-linked dominant inheritance is a possibility given the higher prevalence in females and our limited pedigree data that support this pattern. However, the number of hippos with known PKD status for both parents was too small to definitively support or refute X-linked dominance. Interpreting the mode of inheritance was complicated by the fact that some of the hippos diagnosed as PKD (−) were almost certainly too young at the time of death (<10 years) to have developed the condition, even if they were going to later in life. Additionally, some cases of PKD may not have been reported if they were considered incidental because many older necropsy reports were incomplete and only described pathology directly related to the ultimate cause of death.

PKD in pygmy hippos may also be characterized by incomplete penetrance, more than one gene mutation, or variable gene expression influenced by interaction of genotype and environment. In humans, different genetic loci for ADPKD can result in variations in disease onset and severity and non-genetic factors are believed to influence the development and progression of ADPKD (Gabow 1993; Grantham 2008); the same is true in Persian cats (Biller et al. 1996; Rey 2013). Similar to human and rodent models (Gabow 1993; Peters & Breuning 2001; Torres et al. 2007), non-genetic factors such as urinary infections, multiple pregnancies, and exogenous estrogen compounds (e.g. dietary phytoestrogens in certain food plants) may also play a role in the progression of cyst formation in pygmy hippos with PKD. The higher prevalence of PKD in females, controlling for sex-ratio, may further point toward an influence of estrogens in the disease process.

The prevalence of PKD increased with age, similar to ADPKD in humans. Because PKD is a late-onset condition and appears to be slowly progressive from a clinical standpoint, affected

hippos generally won't experience PKD-associated clinical signs or mortality until later in life. Similarly, as reproduction is concentrated in the first two decades of life, PKD doesn't appear to negatively influence fitness because affected individuals have already lived long enough to reproduce. If there is a genetic component, the later onset of disease would promote the propagation and maintenance of PKD in the population.

Even though PKD does not impair reproductive capacity, affected animals still experience significant morbidity and secondary complications, especially in the later stages of PKD. The clinical significance of this condition in pygmy hippos remains enigmatic, but those with advanced disease eventually develop chronic renal insufficiency based on elevated blood urea nitrogen and creatinine values and clinical signs associated with uremia. It is likely that many of the comorbidities encountered in humans and domestic animals occur in pygmy hippos as well, negatively affecting health and welfare. These include chronic hypertension; arteriolar sclerosis; intracranial aneurisms; chronic back, abdominal and flank pain; cyst infections, pyelonephritis, hematuria, and urinary tract infections (Gabow 1993; Grantham 2008; O'Leary et al. 1999; Rey 2013). For example, advanced PKD was associated with multifocal hemorrhagic cerebral infarcts (stroke) secondary to chronic renal disease and systemic hypertension in a 22-year old female pygmy hippo (Nielsen et al. 2015).

Management and treatment options for pygmy hippos affected by PKD are unfortunately limited. Most of the modalities available in human medicine would be difficult or impossible to implement in pygmy hippos, or indeed in most zoo animals, due to logistical and financial constraints. Pharmacological agents that block biochemical cellular pathways of cyst formation and hence slow cyst progression are still undergoing pre-clinical trials and are not readily available (Torres & Harris 2007; Wilson 2004); implementation of such therapies in pygmy hippos may become a viable option in the future.

PKD-affected pygmy hippos were reported to have extrarenal cysts in the ovaries, duodenum, bladder, pancreas, liver, and most commonly the thyroid. In humans, extrarenal cysts are common with ADPKD, especially in the liver and pancreas (Grantham 2008), but also in the

spleen, thyroid and central nervous system (Gabow 1993). It is unknown if extrarenal cysts in hippos are associated with PKD or are simply incidental. It is also important to distinguish PKD from simple renal cysts; these are common in older animals and are usually limited in number, incidental and benign (K. Terio 2015, pers. comm.). Older necropsy reports, especially those lacking a histopathology component, may not have clarified this distinction, further complicating interpretation of pedigree data.

4.5.2 SURVIVAL ANALYSIS

We note that survival analyses only take into account the random effects incorporated in the model (PKD, origin, sex, *F*) and that many other random effects can impact survival and longevity, both in captivity and in wild populations. Nevertheless, we have identified general trends and potential factors that can be targeted to improve survival in captive pygmy hippos. Overall, median survival for PKD (+) and PKD (−) animals was similar regardless of origin, sex, age class, or inbreeding coefficient, so PKD does not significantly affect longevity in the captive population. From the perspective of *ex situ* conservation for this endangered species, these observations are encouraging given the high prevalence of the condition.

Screening the wild population for PKD would provide insight concerning prevalence in comparison with captive animals, but would be logistically challenging. We can therefore only speculate if the development of PKD is influenced or precipitated by factors associated with captivity. It is also possible that longevity in the wild is limited in comparison with a protective zoo environment, making an age-associated disease like PKD irrelevant from a fitness perspective and removing it from selective pressure.

There was no significant difference in median survival for wild-caught versus captive-born hippos aged ≥ 5 years overall; however, when the data were stratified by time frame, median survival for wild-caught animals was significantly longer. These contrasting results emphasize the importance of accounting for changes in survival and life expectancy over time and demonstrate that husbandry for this species has improved significantly over the last century.

The initial analysis would lead to the comforting conclusion that longevity is not affected by captivity-associated environmental factors. On the other hand, greater median survival for wild-caught-animals maintained under the same husbandry conditions as captive-born animals suggests that maternal effects may influence longevity in this species. Maternal effects have been shown to influence viability, phenotype and longevity for numerous invertebrate species (Fox et al. 2003; Jann & Ward 1999; Lynch & Ennis 1983; Mousseau & Dingle 1991), but examples in mammals are limited to mice and humans (Chen et al. 2009; Korpelainen 1999). If the age, nutritional status, environment, and/or stress level of the mother during pregnancy can influence these same parameters in pygmy hippos, then our results raise concern that captivity-associated factors may negatively impact longevity. Overall, the results of our survival by origin analyses must be interpreted with caution, since estimating all wild-caught animals to be 3 years old at the time of import can bias longevity calculations.

Results of the survival by sex analyses indicate that first-year mortality is a significant factor affecting median survival for male pygmy hippos. A higher neonatal mortality rate for male calves has been previously noted as contributing to the female-skewed sex ratio in the captive population (Zschokke 2002). Potential underlying mechanisms for the higher male neonatal mortality rate have not yet been explored. Increasing male calf survival would help address the management issue of 'excess' females that are currently excluded from the *ex situ* breeding population due to limited availability of breeding-age males.

The inverse relationship between *F* and survival for captive-born pygmy hippos provides additional evidence that management efforts aimed at reducing inbreeding are essential. A similar effect of *F* on survival has been demonstrated for the cheetah (*Acinonyx jubatus*) at the five most successful captive breeding facilities in North America, where juvenile mortality for inbred cubs was significantly higher than for non-inbred cubs (Wielebnowski 1996). Inbreeding coefficients are calculated from Studbook pedigree data and assume that $F = 0$ for all wild-caught animals, undoubtedly leading to an underestimate of relatedness. Because the captive population of pygmy hippos is relatively small, continued vigilance in maintaining

heterozygosity is essential. In the future, techniques utilizing genetic markers may prove useful to guide captive breeding efforts by identifying relatedness that reaches beyond available pedigree data (Knief et al. 2015).

4.5.3 AGE-BASED POPULATION MODELS AND DEMOGRAPHIC PROJECTIONS

Our results indicate the number of pygmy hippos in the managed population may continue to slowly increase over time. However, there are several real-world barriers that can hinder expected population growth, most notably exhibit space within the zoo community. Additionally, the model we used to generate the demographic projections assumes that age-specific birth, fertility and mortality rates will remain constant into the future. Since these population attributes are dynamic variables that are constantly changing, the data need to be interpreted with caution and frequent reassessment of population viability is imperative.

Nevertheless, the information still provides insight into historic and future population trends, as indicated by the steady increase in the number of pygmy hippos over the last 35 years since imports from the wild have ceased. Additionally, despite no new wild imports the population quickly recovered from the 'decline' associated with Studbook reconciliation between 2000 and 2002. Moreover, with a projected population increase of λ_{asympt} = 1.008 year^{-1} from 2014 onward, pygmy hippos under managed care can be considered demographically viable in the long-term. Indeed, the total number of living females increased from 222 animals in our model at the end of 2014 to 225 animals by the end of 2015 (λ = 1.014 year^{-1}).

The demographic projections and elasticity (e_i) values indicate that improving survival in the first six years of life, especially for age 0 to 1 year, will have the greatest positive influence on overall population growth rate. Thus, focusing on improving survival in the youngest age classes would have the largest positive influence on λ, assuming all other parameters remain unchanged. However, as overall there are > 40 components for both p_x and m_x for both models, and since all of these components must add to 1.0 in the matrix, the resulting individual

elasticity (e_i) values per component are very small. Therefore, the survival and fecundity of every pygmy hippo in the captive population is important for the overall long-term viability of this endangered species.

Fig. 4-5 indicates a high mortality rate for very young animals, especially from birth to one year. It is only from age 5 onward that the mortality rate stabilizes until age 17, when it slowly starts to increase again until senescence, when it increases dramatically. One would expect improved survival of neonates and young animals in comparison with wild populations since conditions in captivity remove many of the factors associated with early mortality. However, no comparative data are available concerning mortality in the wild. Regardless, improving reproductive success in captivity is a fundamental goal of the captive breeding program, and our data support concentrating efforts on improving survival in the youngest age classes as this will have the maximum benefit for intrinsic population growth rate.

Similarly, the transition matrix projections show the highest reproductive values (v_x) for females between 2 to 7 years of age. Therefore, breeding efforts should commence when females first start cycling, usually around 3 years of age, especially for genetically valuable animals. Logistical constraints such as lack of suitable mates or limited space often mandate delayed and/or less frequent breeding, but research has shown this approach to have long-term detrimental effects on fertility in many species (Hermes et al. 2006; Penfold et al. 2014). In our models v_x remains high (≥ 0.5 year^{-1}) until age 23 to 25 years, indicating that female pygmy hippos retain significant reproductive potential until at least their mid-20s. However, due to the limited number of males in the population and other logistical constraints, many females are not provided breeding opportunities until they are in their teens. These hippos often experience limited reproductive success compared to those that reproduce earlier.

The generation time of approximately 15 years for both models is slightly longer than the 13.7 years calculated by Pacifici et al. (2013) using age at first reproduction and reproductive life span for pygmy hippos in captivity, and the mean of 13.1 years for both sexes reported in the 2010 North American SSP Population Analysis and Breeding Transfer Plan (Eddie et al. 2010).

However, because reproductive potential in captivity is strongly limited by access to mates and logistical limitations imposed on social structures, generation time varies markedly between zoological facilities and is not likely to represent the natural biology of this species in the wild.

4.6 CONCLUSIONS AND RECOMMENDATIONS

Sound scientific management and breeding of pygmy hippos in the *ex situ* population is imperative given that this endangered species lives in a diminishing, fragmented habitat in West Africa, there are no accurate estimates for wild population numbers, and so little is known about their wild conspecifics. However, captive management presents many logistical, financial, and sometimes ethical challenges that are complicated by the limited number of remaining founder genomes, the failure of many pairs to reproduce, and the high prevalence of PKD. Moving hippos between facilities for outbreeding is expensive, time-consuming, and stressful for the animals. Additionally, if we are successful in reducing juvenile mortality and improving captive breeding efforts, then space constraints begin to play a larger role. If allowing a female to breed early and often helps maintain and improve lifetime reproductive potential, would euthanasia of 'surplus' pygmy hippos be an acceptable management practice when options to accommodate offspring are exhausted?

Although PKD does not appear to limit fecundity or longevity in pygmy hippos, it does pose a clinical problem for the health and welfare of the affected animal. Captive breeding programs could be restricted to unaffected animals and lineages in an effort to reduce prevalence. However, since the degree of genetic versus environmental contribution to the development of PKD remains unclear and the inheritance mechanism still needs to be definitively elucidated, there is no guarantee that breeding negative animals will reduce the prevalence of this condition. Additionally, complete elimination of all PKD (+) pygmy hippos from the breeding pool will substantially reduce the genetic diversity of the captive population, perhaps resulting in the appearance of other detrimental characteristics, and cannot be recommended at this time.

Given the high prevalence and potential for severe secondary complications, we urge clinicians to perform ultrasound examinations when possible and, if PKD is diagnosed, to monitor renal function and screen for secondary urinary tract infections via serial serum chemistry and urinalysis. A database of baseline normal and abnormal renal function tests for both PKD (+) and (−) pygmy hippos would provide a valuable tool for guiding clinical decisions. Moreover, awareness of a hippo's PKD status will allow veterinarians to adapt their differential diagnosis list accordingly whenever the animal is unwell.

It is essential that full necropsies and histopathology are performed in all cases, preferably using the standardized protocol available from the EEP/SSP, and we cannot overemphasize the importance of reporting negative findings. If PKD is diagnosed, the approximate number and distribution of cysts should always be reported as this will help differentiate PKD from simple renal cysts and can be used to develop a grading system. A standardized grading system, akin to what is used for PKD diagnosis in humans, would provide an objective guideline for assessing severity. It is also advisable to bank renal tissue for potential future identification of genetic markers. Timely reporting of necropsy findings to the international Studbook keeper and EEP/SSP veterinary advisor is essential to maintain a central database for this vital information.

Because controlled genetic trials are logistically prohibitive, additional demographic data are necessary to definitively establish if the inheritance pattern of PKD is X-linked dominant. For the majority of hippos in our study the PKD status of one or both parents was unknown, and the accuracy of some historical data concerning PKD status remains questionable. Thus, we must re-emphasize that high-quality, accurate pathology data and clear, timely communication with the Studbook keeper are of utmost importance. The development of a genetic marker for this condition would offer further clarity concerning heritability and provide a useful diagnostic tool. Additionally, more research is needed to determine if environmental factors influence the development of PKD and to what degree.

Despite a projected increase in population size over time, we must continue to make informed management decisions that help maintain genetic diversity. We must similarly strive to

minimize inbreeding as it has a negative impact on longevity. We should also provide early breeding opportunities for genetically valuable females and aim to improve neonate and juvenile survival. Continued reassessment of population viability over time is also recommended to account for ongoing changes in demographic parameters.

4.7 ACKNOWLEDGEMENTS

The authors have no conflicts of interest to declare. Funding for this research was provided by the University of Western Australia, a UWA Graduate Research School PhD Completion Scholarship, and the Institute for Breeding and Reproduction of Endangered African Mammals (IBREAM). JLT was funded by an Australian Research Council Future Fellowship (FT110100500). We wholeheartedly extend our gratitude to all of the zoological institutions and private facilities that responded to our requests for data and shared their necropsy reports; without their participation we could not have conducted this study. Finally, we are also indebted to those individuals who facilitated communication with several zoos and who assisted with translation of foreign language reports: Patmasuda Intuprapa, Dr Monique Paris, Tomas Pecha, Dr Pablo Rodriguez, Dr Shiho Sumigama, Mai Tanimoto, Dr Suzana Tkalčić, and Rujiporn Thavornkanlapachai.

4.8 LITERATURE CITED

Biller, D.S., S.P. DiBartola, K.A. Eaton, S. Pflueger, M.L. Wellman, and M.J. Radin. 1996. Inheritance of polycystic kidney disease in Persian cats. *J. Hered.* 87:1–5.

Caughley, G. 1977. Analysis of vertebrate populations. John Wiley & Sons, New York, NY.

Chen, J.H., M.S. Martin-Gronert, J. Tarry-Adkins, and S.E. Ozanne. 2009. Maternal protein restriction affects postnatal growth and the expression of key proteins involved in lifespan regulation in mice. *PLoS One*. 4:e4950. doi:10.1371/journal.pone.0004950.

Cowley, B.D., S. Gudapaty, A.L. Kraybill, B.D. Barash, M.A. Harding, J.P. Calvet, and V.H. Gattone. 1993. Autosomal-dominant polycystic kidney disease in the rat. *Kidney Int.* 43:522–534.

Ebert, T.A. 1999. Plant and animal populations: Methods in demography. San Diego State University Academic Press, San Diego, CA.

Eddie, C., M. Maher, and C. Groome. 2010. Population Analysis and Breeding and Transfer Plan - Pygmy Hippopotamus (*Choeropis liberiensis liberiensis*) Species Survival Plan. Chicago, Illinois. 17 pp.

Falconer, D.S., and Mackay, T.F.C. 1996. Introduction to quantitative genetics. Longman Group Ltd., Essex, United Kingdom.

Flacke, G.L., B.K. Chambers, G.B. Martin, and M.C.J. Paris. 2015. The pygmy hippopotamus *Choeropsis liberiensis* (Morton, 1849): Bringing to light research priorities for the largely forgotten, smaller hippo species. *Der Zool. Garten NF*. 84:234–265.

Fox, C.W., M.L. Bush, and W.G. Wallin. 2003. Maternal age affects offspring lifespan of the seed beetle, *Callosobruchus maculatus*. *Funct. Ecol.* 17:811–820.

Gabow, P.A. 1993. Autosomal dominant polycystic kidney disease. *N. Engl. J. Med.* 329:332–342.

Gilmour, A.R., Gogel, B.J., Cullis, B.R., and Thompson, R. 2009. ASReml user guide release 3.0. Hemel Hempstead, UK: VSN International Ltd, www.vsni.co.uk

Grantham, J.J. 2008. Autosomal dominant polycystic kidney disease. *N. Engl. J. Med.* 349:1477–1485.

Hermes, R., T.B. Hildebrandt, C. Walzer, F. Göritz, M.L. Patton, S. Silinski, M.J. Anderson, C.E. Reid, G. Wibbelt, K. Tomasova, and F. Schwarzenberger. 2006. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). *Theriogenology*. 65:1492–1515.

Hoppe-Dominik, B., H.S. Kühl, G. Radl, and F. Fischer. 2011. Long-term monitoring of large rainforest mammals in the Biosphere Reserve of Taï National Park, Cote d'Ivoire. *Afr. J. Ecol.* 49:450–458.

Igarashi, P., and S. Somlo. 2007. Polycystic kidney disease. *J. Am. Soc. Nephrol.* 18:1371–1373.

Jann, P., and P.I. Ward. 1999. Maternal effects and their consequences for offspring fitness in the yellow dung fly. *Funct. Ecol.* 13:51–58.

Jarofke, D., and H.G. Klös. 1982. Immobilisierung und Krankheiten von Zwergflusspferden: Auswertung einer Umfrage bei mehr als 100 Zoologischen Gärten. *Erkrankungen der Zootiere Verhandlungsbericht*. 24:361–374.

Knief, U., G. Hemmrich-Stanisak, M. Wittig, A. Franke, S.C. Griffith, B. Kempenaers, and W. Forstmeier. 2015. Quantifying realized inbreeding in wild and captive animal populations. *Heredity (Edinb).* 114:397–403.

Korpelainen, H. 1999. Genetic maternal effects on human life span through the inheritance of mitochondrial DNA. *Hum. Hered.* 49:183–185.

Krebs, C.J. 1999. Estimation of Survival Rates. *In* Ecological Methodology. C.J. Krebs, editor. Addison Wesley Educational Publishers, Inc, Menlo Park, CA. 499–539.

Lynch, M., and R. Ennis. 1983. Resource availability, maternal effects, and longevity. *Exp. Gerontol.* 18:147–165.

Mallon, D., C. Wightman, P. De Ornellas, and C. Ransom. 2011. Conservation Strategy for the Pygmy Hippopotamus. IUCN Species Survival Commission, Gland, Switzerland & Cambridge, UK.

Mousseau, T.A., and H. Dingle. 1991. Maternal effects in insect life histories. *Annu. Rev. Entomol.* 36:511–534.

Nees, S., B. Schade, M. Clauss, H.W. Steinmetz, F. Ehrensperger, B. Steck, and J.-M. Hatt. 2009. Polycystic kidney disease in the pygmy hippopotamus (*Hexaprotodon liberiensis*). *J. Zoo Wildl. Med.* 40:529–535.

Nielsen, A.W.N., T. van Dreumel, G. Crawshaw, C.J. Dutton, S.R. Hollamby, A.R. Pastor, G.L. Flacke, and D.A. Smith. 2015. Haemorrhagic stroke in a pygmy hippopotamus. *In* Proceedings of the International Conference on Diseases of Zoo and Wild Animals. Leibniz Institute for Zoo and Wildlife Research (IZW) & European Association of Zoo and Wildlife Veterinarians (EAZWV), Barcelona, Spain. 190.

O'Leary, C.A., B.M. Mackay, R. Malik, J.E. Edmondston, W.F. Robinson, and C.R. Huxtable. 1999. Polycystic kidney disease in bull terriers: an autosomal dominant inherited disorder. *Aust. Vet. J.* 77:361–366.

Pacifici, M., L. Santini, M. Di Marco, D. Baisero, L. Francucci, G. Grottolo Marasini, P. Visconti, and C. Rondinini. 2013. Generation length for mammals. *Nat. Conserv.* 5:87–94.

Penfold, L.M., D. Powell, K. Traylor-Holzer, and C.S. Asa. 2014. "Use it or lose it": characterization, implications, and mitigation of female infertility in captive wildlife. *Zoo Biol.* 33:20–28.

Peters, D.J., and M.H. Breuning. 2001. Autosomal dominant polycystic kidney disease: modification of disease progression. *Lancet*. 358:1439–1444.

Ransom, C., Robinson, P.T., and Collen, B. 2015. *Choeropsis liberiensis*. The IUCN Red List of Threatened Species 2015: e.T10032A18567171 http://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T10032A18567171.en.

Raymond, J.T., K.A. Eaton, and R.J. Montali. 2000. A disease in captive pygmy hippopotamuses (*Choeropsis liberiensis liberiensis*) anatomically resembling polycystic kidney disease. *In* Proceedings of the American Association of Zoo Veterinarians and International Association for Aquatic Animal Medicine Joint Conference. New Orleans, Louisiana. 302.

Rey, M.S. 2013. Feline hereditary and congenital kidney diseases. *IVIS Vet. Focus*. 23:10–16.

Roth, H.H., B. Hoppe-Dominik, M. Mühlenberg, B. Steinhauer-Burkart, and F. Fischer. 2004. Distribution and status of the hippopotamids in the Ivory Coast. *Afr. J. Ecol.* 39:211–224.

Steck, B. ed. . 2015. Pygmy Hippopotamus *Choeropsis liberiensis* (Morton, 1844) International Studbook 2014. 21st ed. Zoo Basel, Switzerland, Basel.

Stubben, C.J., and Milligan, B.G. 2007. Estimating and analyzing demographic models using the popbio package in R. *J. Stat. Softw*. 22:11.

Torres, V.E., and P.C. Harris. 2007. Polycystic kidney disease: genes, proteins, animal models, disease mechanisms and therapeutic opportunities. *J. Intern. Med.* 261:17–31.

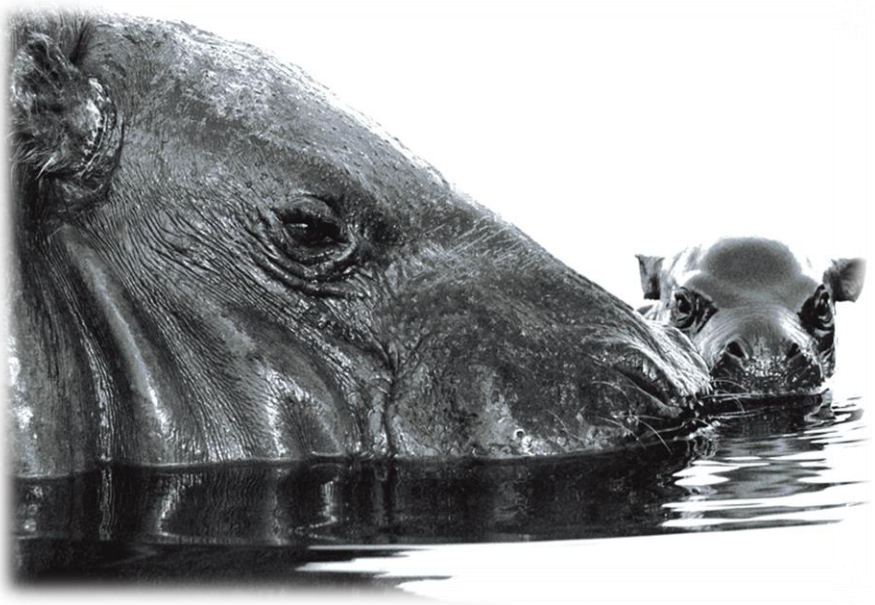
Torres, V.E., P.C. Harris, and Y. Pirson. 2007. Autosomal dominant polycystic kidney disease. *Lancet*. 369:1287–1301.

Wielebnowski, N. 1996. Reassessing the relationship between juvenile mortality and genetic monomorphorism in captive cheetahs. *Zoo Biol.* 15:353–369.

Wilson, P.D. 2004. Polycystic kidney disease. *N. Engl. J. Med.* 350:151–164.

Zschokke, S. 2002. Distorted sex ratio at birth in the captive pygmy hippopotamus, *Hexaprotodon liberiensis*. *J. Mammal.* 83:674–681.

Chapter 5 THE REPRODUCTIVE BIOLOGY OF THE FEMALE PYGMY HIPPOPOTAMUS (*CHOEROPSIS LIBERIENSIS*) AS CHARACTERIZED BY NON-INVASIVE ENDOCRINE MONITORING



A Pygmy Hippo Mother and Calf
Center for Conservation of Tropical Ungulates

Patience is the companion of wisdom.

–Saint Augustine of Hippo

The Reproductive Biology of the Female Pygmy Hippopotamus (*Choeropsis liberiensis)* as Characterized by Non-Invasive Endocrine Monitoring

Gabriella L. Flacke [a,b,*], Franz Schwarzenberger [c], Linda M. Penfold [d], Susan L. Walker [e], Graeme B. Martin [a], Robert Peter Millar [b,f,g], and Monique C. J. Paris [a,b,f,h]

[a] *School of Animal Biology, University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia*

[b] *Institute for Breeding Rare and Endangered African Mammals (IBREAM), Edinburgh EH3 6AT, United Kingdom*

[c] *Department of Biomedical Sciences, Unit of Physiology, Pathophysiology and Experimental Endocrinology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria*

[d] *South East Zoo Alliance for Reproduction & Conservation, 581705 White Oak Road, Yulee, FL 32097, USA*

[e] *Chester Zoo, Upton-by-Chester CH2 1LH, United Kingdom*

[f] *Mammal Research Institute and Centre for Neuroendocrinology, University of Pretoria, Department of Zoology and Entomology, Pretoria 0084, South Africa*

[g] *Institute for Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town 7925, South Africa*

[h] *College of Public Health, Veterinary and Medical Sciences, James Cook University, Townsville 4811, Australia*

[*]**Corresponding author:** Gabriella L. Flacke, DVM, MVSc; School of Animal Biology, M092; The University of Western Australia; Crawley WA 6009, Australia; Email: gflacke@grs.uwa.edu.au

5.1 ABSTRACT

The pygmy hippopotamus (*Choeropsis liberiensis*) is endangered in the wild and very little is known about its reproductive biology. In zoological facilities, this species experiences a number of reproductive issues that complicate breeding management, including a high rate of stillbirths and failure of many pairs to reproduce. We therefore conducted a comprehensive study to evaluate reproductive cycles and pregnancy in the pygmy hippo using enzyme immunoassays (EIAs) for fecal hormone metabolite analysis. Fresh fecal samples were collected twice weekly for a one to three year period from 36 females housed at 24 zoological institutions. Samples were analyzed in three separate laboratories using five progestogen and three estrogen EIAs. Fecal extracts and standards produced parallel displacement curves between 20% and 80% binding for each EIA; however, only three of the five progestogen EIAs (*Pg-diol*: 5β-pregnane-3α,20α-diol 3HS:BSA; *PdG*: pregnanediol-3-glucuronide R13904; *mono-P4*: Quidel clone 425) accurately reflected of reproductive events. In contrast, all three estrogen EIAs (*E2a*: estradiol-17β-OH 17-HS:BSA; *E2b*: estradiol 17β R0008; *E2c*: estradiol 17β R4972) consistently reflected reproductive events. Average estrous cycle length was 31.8 ± 7.4 days based on estrogen metabolite peaks and 30.9 ± 7.3 days based on nadir to nadir progestogen metabolite concentrations. Cyclical patterns in both estrogen and progestogen metabolites were detected throughout the year, indicating a lack of seasonality. Estrogen metabolite peaks were also observed during pregnancy and lactation, suggesting that follicular development occurs during both reproductive states. Pregnancy was most reliably demonstrated by elevation in progestogen metabolites (Pg-diol or PdG) in the second half of gestation. Average gestation length was 203 ± 4 days for 15 pregnancies. This overview of the reproductive biology of the female pygmy hippo provides valuable data for guiding long-term breeding management for this endangered species and a serves as a baseline for future studies addressing the potential influence of social structure, diet, body condition, and other husbandry factors on estrous cycling and reproduction.

Keywords: Enzyme-linked immunoassay (EIA), estrogen, estrous cycle, gestation, pygmy hippo, progestogens

5.2 INTRODUCTION

The pygmy hippopotamus (*Choeropsis liberiensis*) – hereafter referred to as pygmy hippo – is classified as endangered by the International Union for the Conservation of Nature (Ransom et al. 2015) and is ranked 21st worldwide among mammals by Programme EDGE (www.edgeofexistence.org) as a priority for conservation action (Isaac et al. 2007). The pygmy hippo is endemic to the Upper Guinean Rainforest ecosystem in the West African countries of Côte d'Ivoire, Guinea, Liberia and Sierra Leone. Wild population size is uncertain but is estimated at < 2500 and is thought to be declining due to ongoing habitat loss and poaching (Mallon et al. 2011; Ransom et al. 2015). Our understanding of the biology of this species in the wild is limited and data pertinent for developing effective conservation strategies are lacking. The first Conservation Strategy Action Plan was developed by the IUCN Pygmy Hippo Specialist Group in 2010 (Mallon et al. 2011) and one of several research priorities was to describe basic reproductive biology for both sexes.

There is no information concerning reproduction in wild pygmy hippos, but some general aspects of this species' reproductive biology are known from animals under managed care (Flacke et al. 2015). For example, both males and females reach sexual maturity between three and four years of age and can remain reproductively active into their third decade. The gestation period is approximately 200 days after which a single calf is born; twin births are rare. There are no external signs of pregnancy except for enlargement of the mammary glands within a few days of parturition. The female is assumed to be polyestrous as births occur throughout the year in both northern and southern hemispheres, and conception is possible within a few weeks of perinatal mortality (Steck 2016). Historically, certain pairs have reproduced readily and often, and are therefore genetically over-represented in the extant population (Flacke et al. 2015). This trend continues because numerous breeding pairs at several zoological facilities worldwide have failed to reproduce despite regular estrous behavior and/or mating being observed by husbandry staff. Other pairs have repeatedly experienced perinatal calf mortality or stillbirth (Steck 2016). These issues, together with a high neonatal mortality rate (> 30%), have limited the success of

captive breeding and could reduce the long-term genetic diversity of the managed population, especially as imports from the wild ceased after 1982.

Finding solutions to these problems is hampered by a poor basic knowledge of the reproductive processes in this species, particularly the absence of hormone patterns. A clear way forward is to use enzyme immunoassays (EIAs) for the non-invasive assessment of endocrine processes that present insights into reproductive biology and improved breeding management (Schwarzenberger 2007). These assays were originally established to quantify native hormones in serum or urine and exhibit varying degrees of cross-reactivity with the multitude of hormone metabolites excreted in the feces. Although some EIAs have been developed with antibodies to species-specific fecal metabolites, financial and logistical limitations make this approach impractical in a broader conservation context given the sheer number of taxa that need attention. It is therefore more common to test several EIAs and determine which demonstrates the most biologically relevant patterns. For some taxa, such as different species of tapir (J. Brown 2015, pers. comm.), this approach can prove challenging due to limited cross-reactivity between available antibodies and that species' particular repertoire of fecal hormone metabolites. In these scenarios collaborations between institutions are essential, especially where endangered species with limited numbers in the *ex situ* population are concerned, because not all endocrine laboratories will have access to the same selection of EIAs. Additionally, the challenges associated with shipping biological samples internationally can impose further limitations on developing non-invasive endocrine monitoring tools for rarer species.

Endocrine assessment of reproductive events in female pygmy hippos is limited to one study, with only two females, that used a radio-immunoassay to analyze progesterone hormone metabolites in skin secretions and saliva over a six-month period (Dathe & Kuckelkorn 1989). For the common hippo (*Hippopotamus amphibius*), on the other hand, non-invasive methods for confirming pregnancy, monitoring the reproductive cycle, pinpointing estrus, and identifying the timing of puberty have already been established (Graham et al. 2002; Smith et al. 2000; Wheaton et al. 2006). However, this information cannot be directly extrapolated to the pygmy

hippo because the reproductive physiology and steroid hormone metabolites of even closely related species can differ markedly. For example, different species of rhinoceros, felids, and ursids have different estrous cycle characteristics and produce variable types and amounts of fecal estrogen and progesterone metabolites (Schwarzenberger & Brown 2013). Thus, non-invasive protocols for evaluating reproductive events in the pygmy hippo are needed to facilitate estrous cycle monitoring, pregnancy diagnosis and prediction of parturition, especially as blood sample collection is difficult and only representative of a single point in time. Additionally, it is important to determine whether the pygmy hippo exhibits induced versus spontaneous ovulation and whether it is a seasonal breeder because these two characteristics impact both natural and assisted breeding efforts.

Therefore, our overall objective in this study was to characterize the basic reproductive biology of female pygmy hippos under managed care using fecal hormone metabolite analysis to define hormone profiles during the estrous cycle, pregnancy and lactation. Specific aims were to: 1) demonstrate biological relevance for measuring fecal metabolites of estrogen and progestogens via enzyme immunoassay; 2) establish a standardized method for pregnancy detection; 3) determine the length of the estrous cycle via physiologic rather than behavioral assessment; 4) test for seasonality of the estrous cycle.

5.3 MATERIALS AND METHODS

5.3.1 ANIMALS AND SAMPLE COLLECTION

Thirty-six female pygmy hippos from 13 European and 9 North American zoological institutions were included in this study; 33 were sexually mature (≥ 3 years) at the time sampling commenced (Appendix IV). Fecal samples were collected twice weekly and stored frozen at −20°C until extraction and analysis. Occasionally, biweekly sampling was possible and the interval between samples was up to two weeks. Reproductive events, including behavioral estrus, mating and parturition, were recorded in conjunction with the timing of fecal sample collection (Appendix IV).

5.3.2 GASTROINTESTINAL TRANSIT TIME

The lag time between patterns of steroid secretion in the blood and subsequent metabolite excretion in the feces is correlated with gastrointestinal transit time (Palme et al. 1996; Schwarzenberger et al. 1996a). We determined gastrointestinal transit time for one male and one female pygmy hippo housed at the same facility and fed the same diet. We used an easily identifiable fecal marker (glitter; Sulyn Industries, Coral Springs, Florida, USA) mixed with grain. We recorded the time from ingestion until the first and last passage of glitter in the feces for both hippos.

5.3.3 REPRODUCTIVE HORMONE METABOLITE ANALYSIS

Hormone analysis was conducted in three separate laboratories (designated Lab A, Lab B, and Lab C; see Appendix IV) using previously established fecal hormone metabolite extraction and EIA techniques. Modifications to these techniques are described below.

5.3.4 FECAL HORMONE EXTRACTION

Lab A (Vienna, Austria) performed fecal extraction according to Schwarzenberger et al. (2000). Briefly, 0.5 g wet fecal material and 0.5 mL water were mixed with 4 mL reagent grade methanol and vortexed for 30 min. After centrifugation, 1 mL of the methanol solution was transferred to a clean vial and mixed with 0.25 mL of a 5% $NaHCO_3$ solution and 5 mL diethyl ether. The mixture was then vortexed for 30 s, centrifuged for 10 min, and the extract supernatant held at −20°C for 30 min. Finally, the supernatant ether phase was placed in a clean vial, evaporated to dryness, and re-suspended in 0.5 mL assay buffer.

Lab B (Chester, UK) performed fecal extraction with methods adapted from Walker et al. (2002). Following manual homogenization of the sample, 0.5 g of wet fecal material was mixed with 4.5 mL methanol (reagent grade, Sigma-Aldrich, Dorset, UK) and 0.5 mL of distilled water. Samples were vortexed for 30 s, rotated continuously on an orbital shaker at room temperature overnight and centrifuged for 20 min the following day. The supernatant was air-dried in a water bath at 55°C in a fume cupboard and then re-suspended in 1 mL of methanol.

Lab C (SEZARC, Yulee, FL, USA) performed fecal extraction using methods as previously described by Metrione et al. (2008). Briefly, after manual homogenization of the sample, 0.5 g wet fecal material was mixed with 4 mL methanol (reagent grade, Fisher Scientific, Fair Lawn, NJ, USA) and 1 mL reverse osmosis-purified water. Samples were then shaken in a Glas-Col Large Capacity Mixer (Glas-Col LLC, Terre Haute, IN, USA) for 20 min at 90 rpm followed by centrifugation for 10 min. Next 200µL of the supernatant was transferred to 12 mm x 75 mm tubes (Perfector Scientific, Atascadero, CA, USA) containing 800µL assay buffer (0.1 M $NaPO_4$, 150 mM NaCl, 0.1% BSA) to make a 1:5 dilution. Samples were stored at −20°C until analysis.

5.3.5 *ENZYME IMMUNOASSAYS & VALIDATION*

Italicized terms are those we have used to refer to the individual hormone assays throughout the remainder of our study. Cross-reactivities for all of the assays are provided in Appendix V. All samples, controls, and standards were assayed in duplicate.

Lab A analyzed fecal extract aliquots using previously performed group-specific EIAs with rabbit-origin polyclonal antibodies against the following: *i*) 20-oxo-pregnane (5α-pregnane-3β-ol-20-one 3HS:BSA, Schwarzenberger et al. 1996b); *ii*) pregnanediol (*Pg-diol*: 5β-pregnane-3α,20α-diol 3HS:BSA, Schwarzenberger et al. 1993); *iii*) total estrogen (*E2a*: estradiol-17β-OH 17-HS:BSA, Patzl et al. 1998). The intra- and inter-assay coefficients of variation for all assays were < 10% and < 15%, respectively. The minimum assay sensitivities were 2.6 pg/well for 20-oxo-pregnane, 5.5 pg/well for Pg-diol, and 0.5 pg/well for E2a. Serial dilutions of fecal extracts yielded a displacement curve parallel to the standard curve for all three EIAs. There was no evidence of matrix interference for Pg-diol or E2a, demonstrated by significant recovery of pooled fecal extract added to the standards. Recovery was not assessed for 20-oxo-pregnanes because this assay did not demonstrate the anticipated rise in progestogens during pregnancy or during expected luteal phases after estrous behavior. As the data for this assay were not biologically relevant, it was not used for further sample analysis.

Lab B analyzed all fecal extract aliquots using a monoclonal progesterone antibody (*mono-P4*: Quidel clone 425) and an estrogen metabolite antibody (*E2b*: estradiol 17β R4972) developed by Coralie Munro, University of California, Davis, CA, USA (Walker et al. 2008). The intra- and inter-assay coefficients of variation for all assays were < 10% and < 15%, respectively. The minimum assay sensitivities were 0.78 pg/well for mono-P4 and 1.25 pg/well for E2b. Assays were validated by demonstrating parallelism between standard curves and serial dilutions of fecal extracts: CL425 (sample % binding = 24.10 + 0.87 [standard % binding], $r^2 = 0.980$, $F_{1,7} = 293$, $P < 0.001$); E2b (sample % binding = 5.985 + 0.770 [standard % binding], $r^2 = 0.991$, $F_{1,7} = 809$, $P < 0.001$). There was no evidence of matrix interference, demonstrated by significant recovery of pooled fecal extract added to the standards: CL425 (observed = 4.13 + 0.98 [expected], $r^2 = 0.999$, $F_{1,7} = 8694$, $P < 0.001$); E2b (observed = 7.86 + 2.19 [expected], $r^2 = 0.986$, $F_{1,7} = 514$, $P < 0.001$). Both the mono-P4 and the E2b EIAs were biologically validated and used for sample analysis at Lab B.

Lab C analyzed fecal extracts using a monoclonal progesterone antibody (mono-P4: Quidel clone 425), a polyclonal progesterone antibody (poly-P4: R4859), a pregnanediol antibody (PdG: pregnanediol-3-glucuronide, R13904) and an estrogen metabolite antibody (E2c: estradiol 17β R0008), all developed by Coralie Munro, University of California, Davis, CA, USA (Munro et al. 1991). A double-antibody EIA protocol was used as previously described for each assay (Barnes et al. 2016; Metrione et al. 2008; Stoops et al. 2014) with the following specific component dilutions: (1) antibody solution was diluted in assay buffer at 1:20,000 for mono-P4, 1:25,000 for poly-P4, PdG, and E2c; (2) HRP conjugate was diluted in assay buffer at 1:40,000 for mono-P4, 1:400,000 for poly-P4, 1:250,000 for PdG, and 1:300,000 for E2c; (3) standards were 3.9–1000 pg/well for mono-P4, poly-P4 and E2c and 0.019–10 ng/well for PdG.

A pool of randomly selected sample extracts was prepared for both pregnant and non-pregnant females from dates spanning the collection period. Parallel displacement curves were generated by comparing serial dilutions of these pooled extracts (mono-p4 & poly-P4, 1:30–1:1920; PdG & E2c, 1:4–1:1024) with known standards for each hormone antibody. Sample and standard

dilutions were made in assay buffer and the optimal dilution yielding approximately 50% binding was as follows for each assay: mono-P4, 1:250 non-pregnant, 1:500 pregnant (sample % binding = 471.2 + 55.2 [standard % binding], r^2 = 0.801, $F_{1,7}$ = 14.1, P < 0.001); poly-P4, 1:250 non-pregnant, 1:500 pregnant (sample % binding = 1173 + 24.7 [standard % binding], r^2 = 0.844, $F_{1,7}$ = 18.9, P < 0.001); PdG, 1:100 for both pregnant and non-pregnant (sample % binding = 0.774 + 0.006 [standard % binding], r^2 = 0.874, $F_{1,7}$ = 24.3, P = 0.001); E2c, 1:50 for both pregnant and non-pregnant (sample % binding = 140.7 + 13.1 [standard % binding], r^2 = 0.831, $F_{1,7}$ = 17.3, P = 0.002).

Microtiter plates (96-well; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were coated with 150 µL of 0.010 mg/mL goat anti-rabbit IgG (Arbor Assay, Ann Arbor, MI, USA) and incubated overnight at room temperature (approximately 20°C). Plates were then washed with 0.008% Tween 20 (400µL in 5 L RO water; Sigma Aldrich, St. Louis, MO, USA) wash buffer to remove unbound antibody. Next 250µL of blocking buffer (Catalog # X-109; Arbor Assays, Ann Arbor, MI, USA) was added to each well and plates were again incubated at room temperature for an additional 12 h. Finally, the blocking buffer was removed and plates were dried, packed into airtight containers, and stored at 4°C.

For the assays, 50 µL standards, controls and diluted fecal extracts were added to each well followed by 50 µL HRP-conjugate solution and finally 50 µL of antibody. After incubating for 2 h at room temperature, plates were washed four times with 300 µL 0.008% Tween 20 wash buffer and 100 µL color substrate solution (ABTS; 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt, Sigma Aldrich, St. Louis, MO, USA) was added to each well. Plates were read at 405 nm using an optical density plate reader (Dynex Technologies, Chantilly, VA, USA) when the zero standard reached the target optical density of 1.00. The intra- and inter-assay coefficients of variation for all assays were < 10% (average 2.58%) and < 20% (average 8.07%), respectively. The minimum assay sensitivities, determined at 90–95% binding, were 10.1 pg/well for mono-P4, 11.0 pg/well for poly-P4, 18.6 pg/well for PdG, and 4.04 pg/well for E2c.

There was no evidence of matrix interference for PdG, as demonstrated by significant recovery (107% at 1:100) of pooled fecal extract added to hormone standards (0.19–100 ng/well; $F_{1,17}$ = 5397, $y = 1.030x + 0.002$, $r^2 = 0.998$, $P < 0.001$). For E2c, recovery was 119% at 1:50, indicating the assay was slightly overestimating of the amount of hormone in the samples (0.039–10.0 ng/well; $F_{1,15} = 714$, $y = 1.607x + 9.381$, $r^2 = 0.992$, $P < 0.001$). The mono-P4 and poly-P4 EIAs were not biologically validated as neither clearly demonstrated a rise in progestogen metabolites during pregnancy. Thus, recovery was not assessed and neither EIA from Lab C was investigated further. Estrous cyclicity was evaluated using the PdG EIA to demonstrate luteal phases; both the PdG and E2c EIAs were used to examine hormone profiles before, during, and after gestation. Data are reported as µg/g fecal wet weight for mono-P4, poly-P4 and PdG, and as ng/g fecal wet weight for E2c.

5.3.6 DATA ANALYSIS

We plotted hormone concentrations over the sampling period (1 to 3 years) for each pygmy hippo. We first calculated non-pregnant baseline values for each EIA by averaging the lowest 10% of the samples for each female. We subsequently calculated concentrations for each EIA as a percent of each animal's baseline. We characterized progestogen metabolite profiles by examining luteal phases and estrogen metabolite profiles by observing estrogen peaks indicative of follicular phases. We defined a luteal phase as any period when progestogen immunoreactivity was at least 50% above baseline for >14 days. We defined the peak luteal values for each cycle as the highest progestogen metabolite level during the cycle, and the nadir luteal concentration as progestogen metabolite level closest to baseline on either side of the peak. We defined an estrogen metabolite peak as the highest value within a group of samples that was greater than 10 days apart and greater than 2.5-fold (250%) above baseline for E2a and greater than 2-fold (200%) above baseline for E2b and E2c. We then determined the length of the estrous cycle by *i*) the interval between two estrogen metabolite peaks or *ii*) the onset of one luteal phase until the onset of the next. For some profiles we used a 3-point moving averaging to reduce background noise and clarify peak and nadir patterns; however, the values for actual peak and nadir metabolite levels were calculated from the non-averaged data for each female.

Due to the numerous sources of variability associated with measuring immunoreactive metabolites in fecal material, in some cases we made subjective observations to distinguish between true cyclic patterns and random fluctuations in the data. We also assessed estrous cycle length based on direct observations of mating or behavioral estrus by husbandry staff and compared these data (when available) with the hormone metabolite profiles for each hippo.

We calculated the length of gestation from the date of a confirmed mating event until parturition; for some pregnancies, the date of mating was unknown and we were unable to determine the length of gestation. We evaluated both progestogen and estrogen metabolites before, during and after gestation to determine if there is follicular activity during pregnancy, if there is a period of lactational anestrus, and when the first post-partum estrus occurs. For some study females, we could only generate partial pregnancy profiles as the sampling periods did not overlap with the full length of gestation. We calculated the mean and standard deviation (± SD) for estrogen and progestogen metabolite concentrations by combining data for all females during pregnancy, in the non-pregnant state overall, and during peak luteal phase. The data for pregnancy comprised all hormone concentrations from mating until parturition; we calculated the overall mean as well a separate mean for the first and the second half of gestation (E2a & Pg-diol: n = 7 pregnancies, n = 256 samples; E2b & mono-P4: n = 3 pregnancies, n = 96 samples; E2c & PdG: n = 8 pregnancies, n = 341 samples). The data for non-pregnancy included all hormone concentrations throughout the estrous cycle and during periods of acyclicity (E2a & Pg-diol: n = 16 hippos, n = 1683 samples; E2b & mono-P4: n = 9 hippos, n = 719 samples; E2c: n = 8 hippos, n = 321 samples; PdG: n = 16 hippos, n = 1237 samples). The data for peak luteal phase comprised the highest concentration of progestogen metabolites during each luteal phase for each female (Pg-diol: n = 15 hippos, n = 174 samples; mono-P4: n = 7 hippos, n = 58 samples; PdG: n = 15 hippos, n = 120 samples). We first used Shapiro-Wilk tests for normality and then used either paired t-test analysis (2 groups) or ANOVA (>2 groups) to compare estrogen and progestogen immunoreactivity in the different reproductive states (pregnancy, non-pregnant, and peak luteal phase).

5.4 RESULTS

5.4.1 GASTROINTESTINAL TRANSIT TIME

The time between consumption of the fecal marker and the first passage of glitter in the feces was 20 h for the female and 30 h for the male. Peak glitter excretion occurred between 30 and 46 h for the female and between 48 to 70 h for the male. Thus, gastrointestinal transit time for pygmy hippos can vary between individuals fed the same diet, but ranges from one to three days. Hormone metabolite levels measured in the feces are therefore likely to represent endocrine events that occurred in the previous 24 to 48 h. However, for simplification of results, reproductive events are reported as if they occurred on the same day as the feces were voided. Behavioral reports of estrus up to 72 h before endocrine events indicative of estrus were considered temporally associated.

5.4.2 ASSESSMENT OF EIAS FOR BIOLOGICAL RELEVANCE AT LAB C

A comparison of fecal progestogen immunoreactivity for mono-P4, poly-P4 and PdG from a pregnant female at Lab C is shown in Fig. 5-1. The patterns for mono-P4 and poly-P4 were nearly identical in both trends over time and metabolite concentrations. However, neither exhibited a sustained rise at any point during gestation but rather fluctuated significantly with frequent returns to baseline. Additionally, the timing of parturition could not be identified with either of these EIAs. On the other hand, PdG immunoreactivity rose at the time of conception and remained elevated until parturition with a transient decrease at mid-gestation. PdG immunoreactivity did not return to baseline levels until parturition, even during the temporary decrease, and the timing of parturition was evident. Based on these outcomes, we chose to only use the PdG EIA at Lab C for assessing fecal progestogen metabolites.

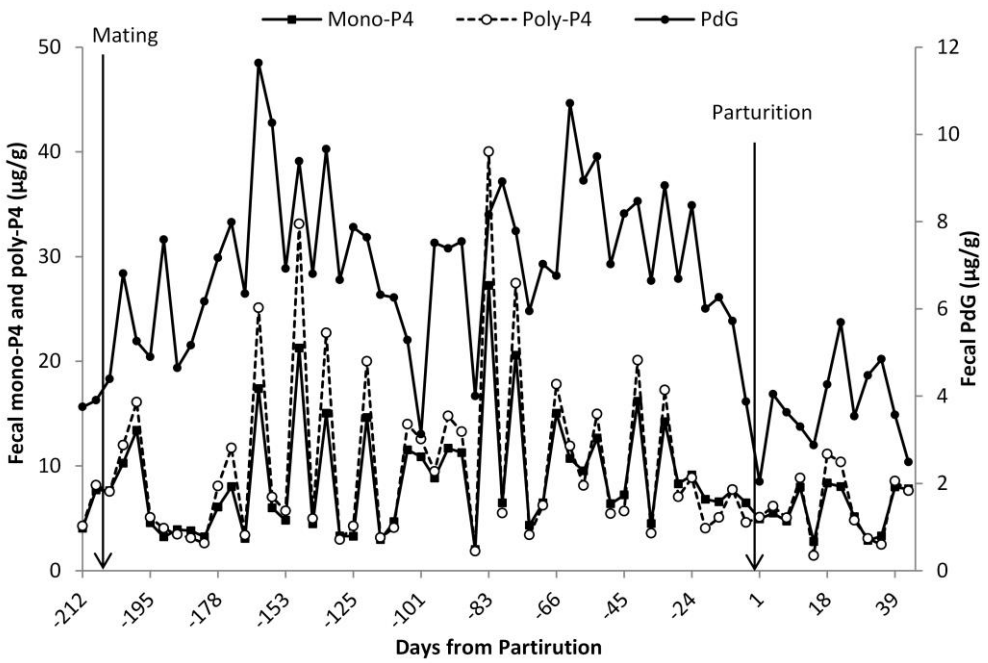


Fig. 5-1 - Fecal progestogen metabolite patterns for the mono-P4, poly-P4 and PdG EIAs during gestation for one female pygmy hippo at Lab C.

The data are oriented around parturition (Day 0). PdG immunoreactivity shows a clear, sustained elevation throughout gestation, except for a short decrease mid-gestation (–100 d), followed by a rapid return to baseline at parturition. Neither mono-P4 nor poly-P4 demonstrates these trends. Baseline is 2.5 µg/g for mono-P4, 1.8 µg/g for poly-P4, and 2.8 µg/g for PdG.

5.4.3 REPRODUCTIVE PATTERNS – GESTATION

Mean ± SD, minimum and maximum hormone metabolite levels for each EIA are presented in Table 5.1 for both pregnant and non-pregnant pygmy hippos. Mean progestogen metabolite concentrations were significantly higher during pregnancy than in the non-pregnant state for all three progestogen EIAs (Pg-diol, n = 16 hippos, $P < 0.001$; PdG, n = 16 hippos, $P < 0.001$; mono-P4, n = 9 hippos, $P < 0.001$). Compared to baseline, concentrations during pregnancy were up to 144-fold higher for Pg-diol, up to 9-fold higher for PdG, and up to 12-fold higher for mono-P4. Progestogen metabolite concentrations were higher during the second half of gestation than during the first half for both Pg-diol and PdG (Fig. 5-2); this trend was not noted for mono-P4. Mean progestogen metabolite concentrations were also significantly higher during pregnancy overall than during peak luteal phase for Pg-diol (n = 7 pregnancies, $P < 0.001$) and PdG (n = 8 pregnancies, $P = 0.010$), up to 9-fold higher for Pg-diol and up to 4-fold higher for

PdG. However, values during the first half of gestation did not differ from peak luteal values (Pg-diol, $P = 0.143$; PdG, $P = 0.131$), whereas during the second half of gestation they were significantly higher (Pg-diol, $P < 0.001$; PdG, $P < 0.001$). For mono-P4, on the other hand, mean progestogen metabolite concentrations throughout pregnancy were lower than peak luteal concentrations ($n = 3$ pregnancies, $P < 0.001$).

The average length of gestation for 15 pregnancies (12 females) was 203 ± 4 days. Progestogen metabolite concentrations (Pg-diol and PdG) rose to more than 50% above baseline values within 17 ± 15 days after mating and conception. Concentrations then fluctuated considerably during the first half of gestation but overall exhibited a slow, steady increase followed by a discernible decrease mid-gestation, around 90 to 100 days post-conception. This decrease was followed by a prominent rise in progestogen metabolites throughout the second half of gestation, reaching peak levels (mean = 31-fold above baseline values) just before parturition followed by a rapid return to baseline values within 13 ± 8 days (Fig. 5-2). These patterns were readily evident in some pregnancies (Fig. 5-3a) whereas in others there was considerable variation, complicating interpretation (Fig. 5-3b). In the latter situation three-point averaging helped to clarify these patterns. The gestational patterns observed with Pg-diol and PdG were not as clearly discernable with mono-P4; however, this EIA was only used for two pregnancies (Fig. 5-5).

Table 5.1 - Mean (± SD) values for minimum, maximum, and baseline concentrations (per g feces) of reproductive hormone metabolites in female pygmy hippos during pregnancy and the estrous cycle.

Results are for six different EIAs assayed in three separate laboratories (Designated A, B and C).

	Lab A Pg-diol µg/g	**Lab B mono-P4 ng/g**	**Lab C PdG µg/g**	**Lab A E2a ng/g**	**Lab B E2b (R4972) ng/g**	**Lab C E2c (R0008) ng/g**
Pregnant	14.3 ± 9.4	1137 ± 319	5.3 ± 1.6	1.3 ± 0.4	121 ± 13.9	611 ± 341
Range	0.19 – 59.8	313 – 4187	1.16 – 19.8	0.01 – 17.5	15.6 – 218	132 – 2922
***n* pregnancies**	7	3	8	7	3	8
Non-pregnant	3.2 ± 1.7	917 ± 271	3.2 ± 1.1	1.3 ± 0.7	74.7 ± 24.2	388 ± 186
Range	0.02 – 26.9	50 – 7084	0.66 – 13.9	0.09 – 43.8	17.1 – 261	43 – 1052
***n* hippos**	16	9	16	16	9	8
Cycle length (days)	30.2 ± 7.4	31.8 ± 5.9	30.7 ± 8.7	31.2 ± 7.1	32.6 ± 7.6	
Range	14 – 46	17 – 42	14 – 49	14 – 48	17 – 46	–
***n* cycles**	157	50	80	147	34	
Peak luteal	6.2 ± 3.5	2150 ± 682	4.7 ± 1.1	–	–	–
Range	0.97 – 26.9	683 – 7084	2.3 – 9.9			
Nadir luteal	1.3 ± 0.8	498 ± 185	1.8 ± 0.5	–	–	–
Range	0.12 – 4.8	171 – 1180	0.66 – 3.18			
Peak follicular	–	–	–	4.5 ± 2.9	142 ± 47.7	–
Range				0.55 – 43.8	57.0 – 262	
Baseline	0.93 ± 0.61	354 ± 162	1.9 ± 0.6	0.30 ± 0.10	44.2 ± 14.6	252 ± 173
Range	0.27 – 2.8	152 – 733	1.1 – 3.2	0.14 – 0.56	25.5 – 62.1	99 – 618
***n* hippos**	16	9	16	16	9	8

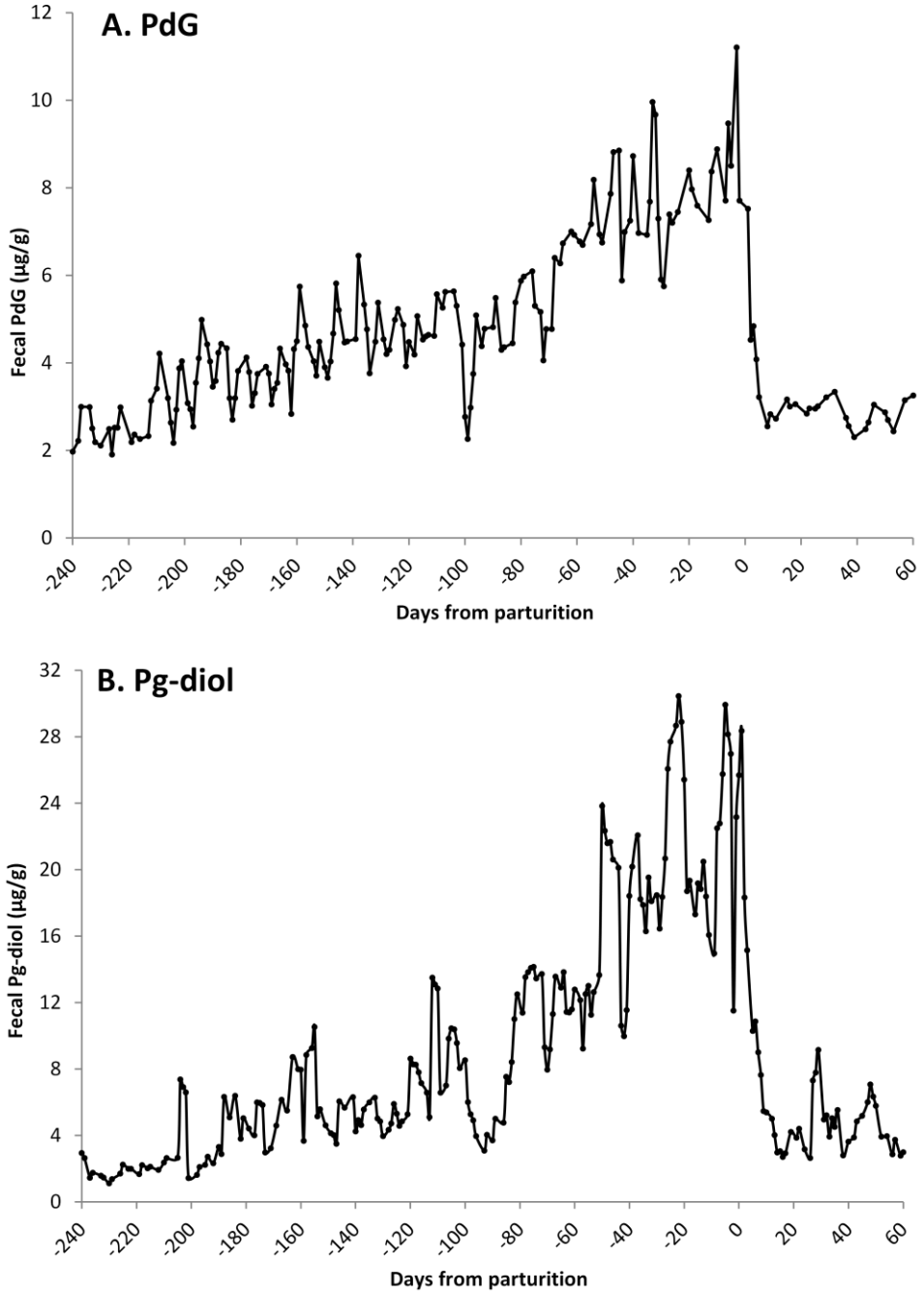


Fig. 5-2 - Mean fecal progestogen metabolite concentrations before, during, and after gestation in the pygmy hippo.

Standard errors are not shown for clarity. Each data point comprised samples for all females spanning a 3 d time frame (between 5 and 8 samples per mean). The data are oriented around parturition (Day 0); mating and conception occurred at an average of –203 d.
A. PdG (pregnanediol-3-glucuronide); mean values for *n* = 8 pregnancies.
B. Pg-diol (5β-pregnane-3α,20α-diol 3HS:BSA); mean values for *n* = 7 pregnancies. A nadir is noted at mid-gestation for both profiles, followed by a marked increase through the second half of gestation until parturition.

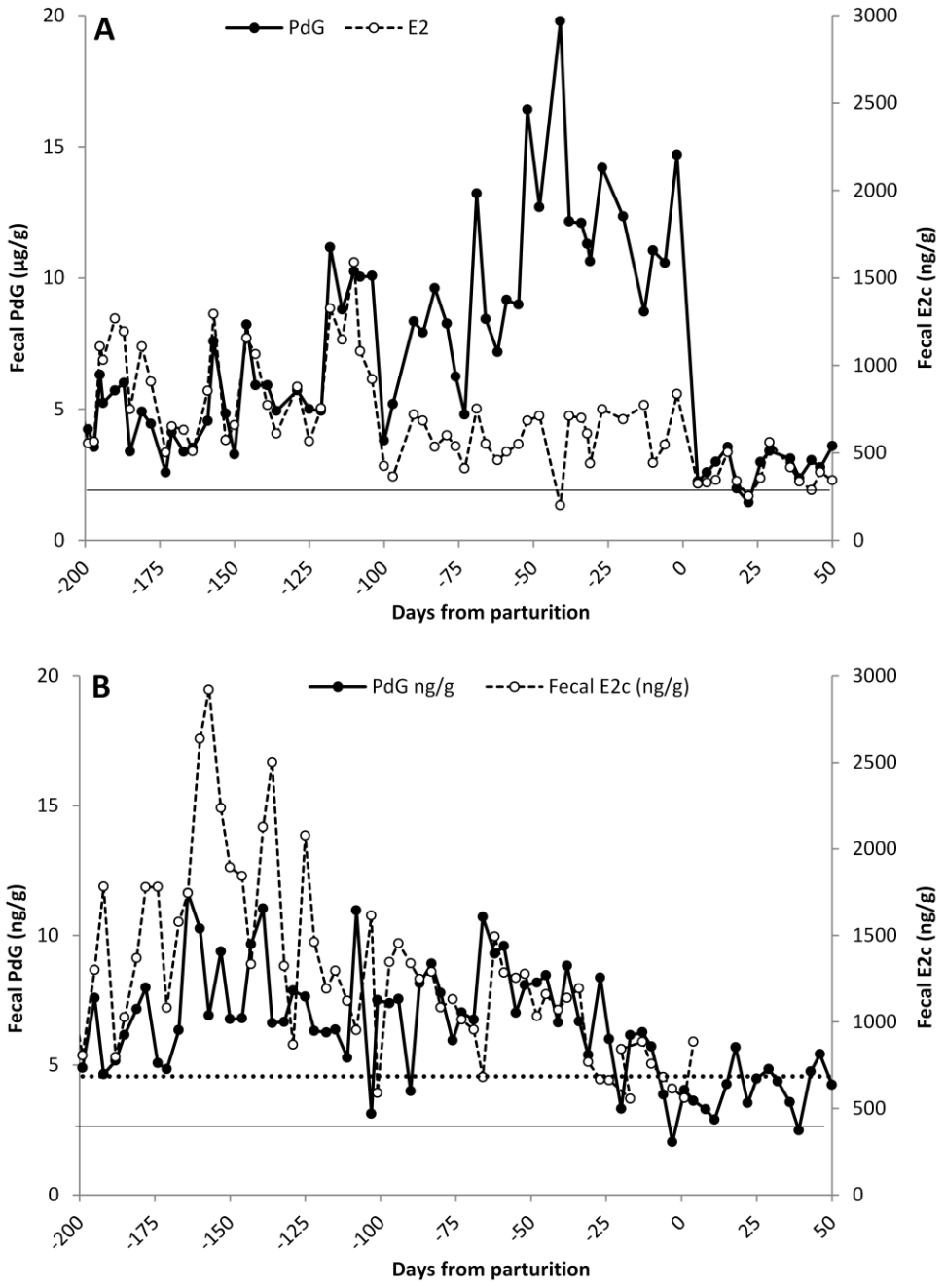


Fig. 5-3 - Individual profiles for fecal PdG (pregnanediol-3-glucuronide; ●) and E2c (Estradiol 17β R0008; ○) immunoreactivity for two pygmy hippos.

The data are oriented around parturition (Day 0).
A. Example of a profile that demonstrates a gradual increase in progestogen metabolite concentrations after conception with a visible decline around mid-gestation (–100 d) followed by a substantial increase during the second half of gestation and a subsequent return to baseline shortly after parturition. Several estrogen metabolite peaks are also evident during the first half of gestation. The baseline for both PdG and E2c are indicated by the solid horizontal line. Gestation was 199 days.
B. Example of a pregnancy profile that does not demonstrate clear patterns in progestogen metabolites. Estrogen metabolite peaks are again noted during the first half of gestation. The baseline for PdG is indicated by a solid horizontal line; the baseline for E2c is indicated by a dotted horizontal line. Gestation was 210 days.

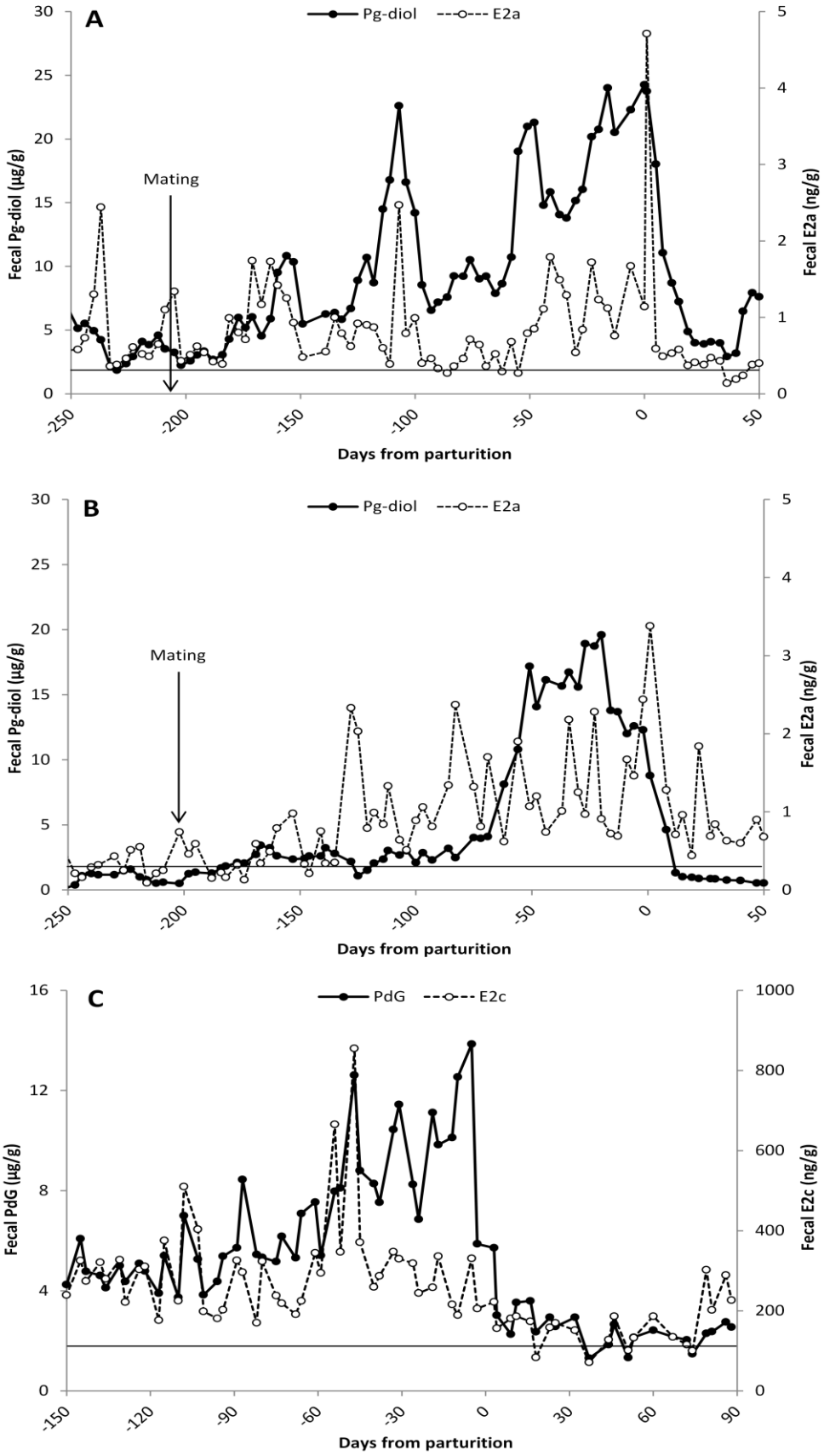


Fig. 5-4 - Individual profiles for fecal progestogen and estrogen metabolites during gestation for three pygmy hippos.

Fig. 5–4. Con't: The baseline for both progestogen and estrogen metabolites is indicated by the horizontal line; the data are oriented around parturition (Day 0).

A. Pg-diol (pregnanediol-3-glucuronide; ●) and E2a (estradiol-17β-OH 17-HS:BSA; ○); progestogen metabolite concentrations remain above baseline from early gestation until parturition. An estrogen metabolite peak is noted at the time of mating (consistent with estrus), at parturition, and at various points throughout gestation, A peak occurs just prior to mid-gestation (–107 d) that temporally correlates with a marked decline in progestogen metabolites.

B. Pg-diol (pregnanediol-3-glucuronide; ●) and E2a (estradiol-17β-OH 17-HS:BSA; ○); progestogen metabolite concentrations do not significantly increase above baseline until the second half of gestation. Estrogen metabolite peaks are again noted at the time of mating, throughout gestation including just before parturition, and approximately 22 days post-partum, indicative of post-partum estrus.

C. PdG (pregnanediol-3-glucuronide R13904; ●) and E2c (Estradiol 17β R0008; ○); progestogen metabolite concentrations begin to rise at mid-gestation (–101 d) and remain elevated through parturition followed by a return to baseline by 9 days post-partum. Estrogen metabolite concentrations fluctuate throughout gestation with several prominent peaks, including one just prior to mid-gestation (–108 d).

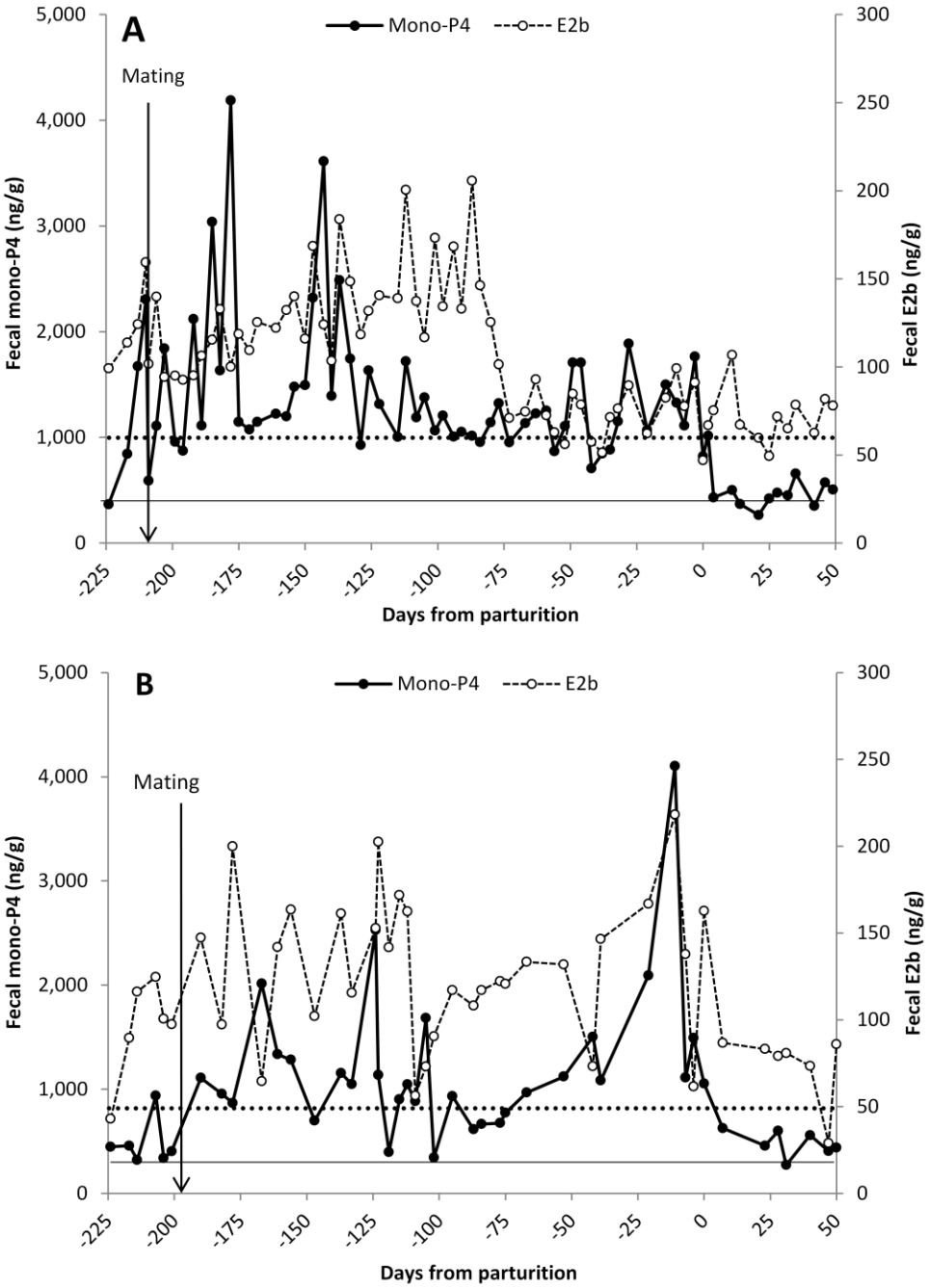


Fig. 5-5 - Individual profiles for fecal mono-P4 (Quidel clone 425; ●) and E2b (estradiol-17β R4972; ○) immunoreactivity for two pygmy hippos before, during and after gestation.

The data are oriented around parturition (Day 0). The baseline for mono-P4 is indicated by a solid horizontal line and for E2b by a dotted horizontal line.

A. Progestogen metabolite concentrations rise above baseline immediately after mating with several initial peaks and remain elevated until parturition followed by an immediate return to baseline. Estrogen metabolite concentrations are already well above baseline at the time of mating and continue to increase until mid-gestation (–90 d) followed by a rapid decline and fluctuating lower levels until parturition. Gestation was 210 days.

B. Progestogen metabolite concentrations also begin to rise above baseline immediately after mating but return to baseline at mid-gestation (–125 d and –100 d), then remain elevated until parturition followed by a return to baseline within 25 days. Estrogen metabolite concentrations fluctuate throughout gestation but generally remain above baseline with a peak just before (–11 d) and at parturition (Day 0). Gestation was 200 days.

Estrogen values varied considerably during gestation, but some trends were evident. There was no significant difference between overall mean pregnant and non-pregnant concentrations of estrogen metabolites for E2a (n = 16 hippos, P = 0.319) whereas for E2b and E2c, overall concentrations were significantly higher during pregnancy than in non-pregnant hippos (n = 10 hippos, $P < 0.001$). There was evidence of follicular activity as demonstrated by clear peaks in estrogen metabolite concentrations, especially during the first half of gestation, with a final peak just before or at the time of parturition in several females (e.g. Fig. 5-4a). In other females, clear peaks were not discernable, but values remained above baseline for extended periods during gestation, although in some cases the sampling interval was greater than twice weekly (e.g. Fig. 5-5b) and thus may not constitute a true representation of estrogen metabolite patterns. An estrogen metabolite peak prior to mid-gestation was evident in 4 of the 5 females with E2a data that spanned the mid-gestation time frame (e.g. Fig. 5-4a). For E2c pregnancy profiles, a similar peak just prior to mid-gestation was noted for 3 of 7 females (e.g. Fig. 5-4c). Only two pregnancy profiles were analyzed with E2b and neither showed a mid-gestation peak (Fig. 5-5).

The first post-partum estrus, demonstrated by an estrogen metabolite peak indicative of follicular activity (e.g. Fig. 5-4b), was observed from 22 to 92 days (mean 40 ± 21 days) after parturition with viable calves (n = 10). However, only seven of these first post-partum follicular peaks were followed by a luteal phase indicative of ovulation. It is unknown whether these were fertile cycles as the male was always kept separate from the female and calf. For two females, the first post-partum estrogen metabolite peak was followed by an additional period of lactational anestrus (61 and 99 days); the other eight females continued to cycle after the first post-partum estrus event. Hormonal evidence of estrous cycling resumed well before the calf was weaned for all 10 females. Two calves experienced perinatal mortality; in these cases estrus and mating occurred at 14 and 16 days post-partum and neither female conceived. However, calving intervals from studbook data indicate that the female is able to conceive on the first post-partum estrus, within one month of parturition, when the calf experiences neonatal mortality (Steck 2016).

5.4.4 REPRODUCTIVE PATTERNS – ESTROUS CYCLE

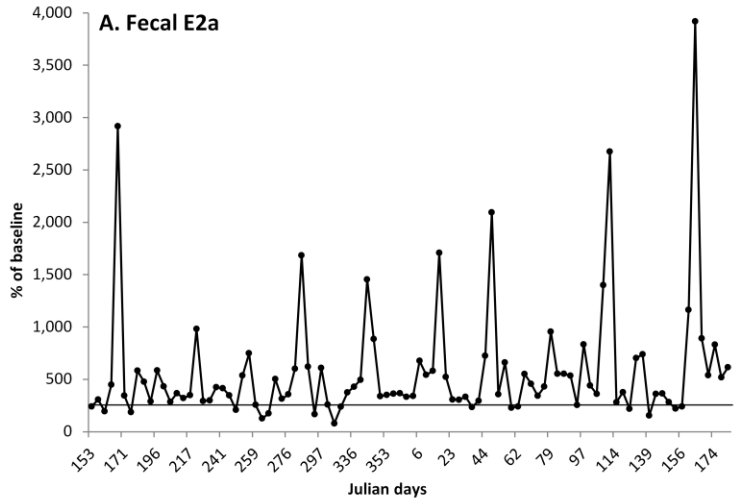
Mean (± SD) values for nadir and peak luteal concentrations of progestogens and peak follicular concentrations of estrogens are presented in Table 5.1. The overall mean estrous cycle length based on the interval between follicular peaks was 31.8 ± 7.4 days (*n* = 185 cycles; *n* = 20 hippos). Peak estrogen metabolite levels compared to baseline varied considerably between and among individual hippos. The overall mean estrous cycle length based on the interval between luteal phases was 30.9 ± 7.3 days (*n* = 287 cycles, *n* = 30 hippos). The overall mean estrous cycle length based on behavioral observations was 32.4 ± 6.3 days (*n* = 42 cycles, *n* = 13 hippos). There was hormonal evidence of estrous cycling during all months of the year. Consecutive, year-round estrous cycles were observed for 13 females (age range 3 to 37 years), 10 from temperate climates where the animals were housed indoors during the colder months and three from sub-tropical climates where they were housed outdoors year-round. For the remaining 23 females in this study these trends could not be assessed, either because a portion of the data was collected during gestation and lactation, or the sampling time frame(s) did not include a contiguous 12-month period.

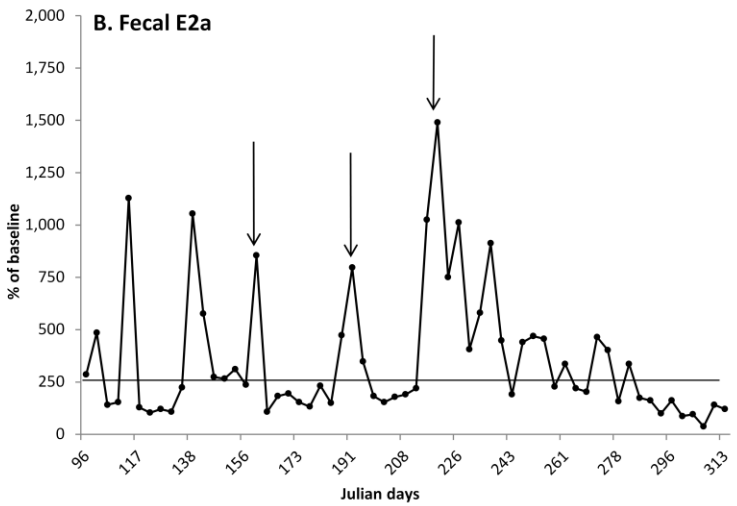
Follicular estrogen metabolite peaks or luteal progestogen metabolite nadirs were associated with observed estrous behavior in 31/46 cases (67%) when behavioral estrus was reported by the husbandry staff (e.g. Fig. 5-6b). In the other 15 cases, behavioral estrus was reported but hormone metabolite levels did not indicate estrus (e.g. Fig. 5-6c). Behavioral estrus was not reported for the majority of physiologic estrous cycles demonstrated by follicular (139/185) or luteal phases (241/287). In most cases, we were unable to determine if this disagreement was due to a lack or reporting or an actual absence of behavioral signs of estrus because behavioral data were not provided for most females (e.g. Fig. 5-6d). However, for some females, the husbandry staff ensured us they had not noted any signs of behavioral estrus yet patterns for estrogen and/or progestogen metabolites indicated clear estrous cycling (e.g. Fig. 5-6a).

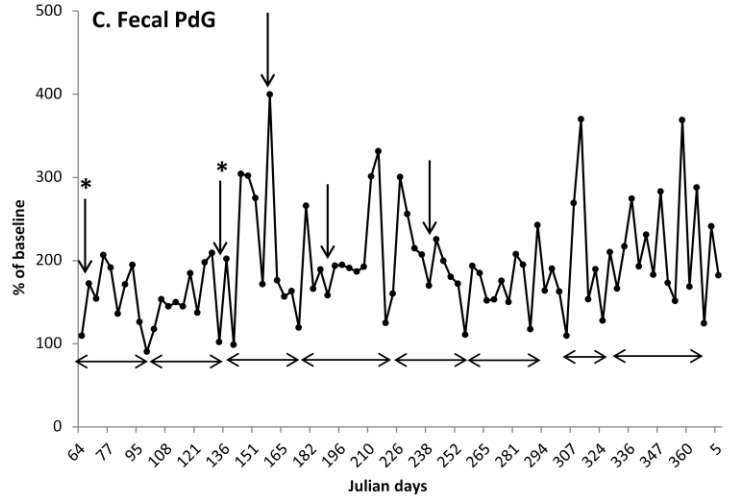
Our study included four hippos aged 30 to 38 years at the start of the sample collection period; all four showed evidence of either intermittent or continuous estrous cycling. There were three

juvenile hippos aged 2 to 2.5 yrs at the start of the study period and each showed evidence of intermittent estrous cycling from the age of three onward. Only five adult females were housed at a facility without a male; the remaining females were housed at least adjacent to a male and in many cases the male was continuously together with the female (Appendix IV). In all five females housed in complete absence of a male, there was clear evidence of estrous cycling as indicated by luteal phases (e.g. Fig. 5-6d).

There was marked individual variation among hormonal patterns for non-pregnant, non-lactating, sexually mature females (n = 26 hippos). Seventeen of these animals exhibited near-continuous estrous cycling throughout the study period. The remainder exhibited variable periods of acyclicity (8/26); one adult female (age 7 yrs) did not show any patterns consistent with estrous cycling throughout the 11-month sampling period and neither behavioral estrus nor mating was observed. There were several instances where estrogen metabolite peaks did not temporally match progestogen metabolite nadirs. Additionally, a clear luteal phase was not consistently detected after every follicular phase and some luteal phases were not preceeded by a follicular phase.


A. Fecal E2a
% of baseline
Julian days


B. Fecal E2a
% of baseline
Julian days


C. Fecal PdG
% of baseline
Julian days

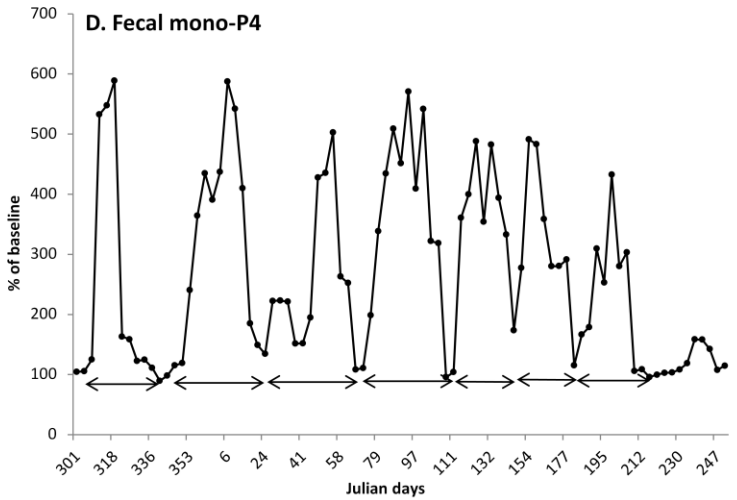

D. Fecal mono-P4
% of baseline
Julian days

Fig. 5-6 - Representative profiles from individual pygmy hippos demonstrating estrous cycles via peak estrogen metabolite concentrations or nadir progestogen metabolite concentrations.

Data are presented as percentage of baseline (100%).The horizontal line indicates 250% baseline (2.5-fold increase) for estrogen metabolites; vertical arrows denote behavioral estrus events; horizontal arrows denote a luteal phase.
A. Estrous cycling indicated by peaks in E2a (estradiol-17β-OH 17-HS:BSA) immunoreactivity; there was no behavioral evidence of estrus during the sampling period.
B. Estrous cycling indicated by peaks in E2a immunoreactivity; there are three points where behavioral estrus corresponds with an estrogen peak.
C. Luteal phases indicated by successive nadirs in PdG (pregnanediol-3-glucuronide R13904) immunoreactivity; there are two behavioral/hormonal matches (arrow with *) and the remaining behavioral estrus events do not correlate with a luteal nadir.
D. Luteal phases indicated by nadirs in mono-P4 (Quidel clone 425) immunoreactivity; no data were provided concerning behavioral estrus. This female was housed at a zoo without a male; the data support spontaneous ovulation in the absence of a male.

5.5 DISCUSSION

We have demonstrated biological relevance for a number of EIAs for measuring fecal metabolites of estrogen and progestogens in pygmy hippos and have employed these techniques to characterize several important aspects of female reproductive biology.

5.5.1 GESTATION AND THE POST-PARTUM PERIOD

Pregnancy diagnosis was possible from mid-gestation onward (approximately 100 days before parturition) using an EIA that cross-reacts with 5β-pregnanediol metabolites of progesterone (Pg-diol, PdG). Progestogen metabolite concentrations significantly exceeded peak luteal values throughout the second half of gestation, in contrast with the first half of gestation where values fluctuated considerably and were similar to luteal values. It is therefore necessary to analyze fecal samples for at least four months after an observed mating event to avoid a false negative pregnancy diagnosis. With the mono-P4 EIA, progestogen metabolite concentrations did not rise above peak luteal levels at any point during gestation and only exhibited a sustained rise above baseline in one female, so this assay cannot be used to diagnose pregnancy in pygmy hippos. This finding is in contrast to the common hippo, where mono-P4 immunoreactivity was elevated throughout gestation at levels significantly greater than during luteal phases (Graham et al. 2002).

We noted a general trend of increasing estrogen metabolite concentrations during the second half of gestation, but there was considerable variation between individuals. Mean estrogen immunoreactivity during gestation was not significantly different to non-pregnant values for E2a, but was significantly higher during gestation for E2b and E2c. This discrepancy may be due to these EIAs cross-reacting with different metabolites of estrogen (see Appendix V), or because the majority of these metabolites are excreted via the urinary versus fecal route. Based on our results, fecal estrogen metabolites cannot be used to reliably diagnose pregnancy in the pygmy hippo.

In many species the feto-placental unit produces a large quantity of estrogens and marked elevations are noted during gestation (Lasley & Kirkpatrick 1991). Pregnancy diagnosis via serum or urinary estrogen levels is possible in the Baird's (*Tapirus bairdii*), lowland (*Tapirus terrestris*) and Malayan (*Tapirus indicus*) tapir from mid-gestation onward when concentrations increase steadily until parturition (Brown et al. 1994a; Pukazhenthi et al. 2013). Other ungulate species where fecal estrogen metabolites have been used for pregnancy determination include the Przewalski's horse (*Equus przewalskii*), Hartmann's zebra (*Equus zebra*), Nubian ibex (*Capra nubiana*), African forest buffalo (*Syncerus caffer nanus*), yak (*Bos mutus*), musk ox (*Ovibos moschatus*), sable antelope (*Hippotragus niger*), Dama gazelle (*Nanger dama*), giant anteater (*Myrmecophaga tridactyla*) and red river hog (*Potamochoerus porcus*) (Bamberg et al. 1991; Berger et al. 2006; Lasley & Kirkpatrick 1991; Patzl et al. 1998; Schwarzenberger et al. 1996a). However, in other species, including the common hippo (n = 1), greater one-horned rhinoceros (*Rhinoceros unicornis*), black rhinoceros (*Diceros bicornis*), okapi (*Okapia johnstoni*) and giraffe (*Giraffa camelopardalis*), fecal estrogen metabolite concentrations do not increase significantly during gestation and cannot be used to diagnose pregnancy (Safar-Hermann et al. 1987; Schwarzenberger et al. 2000, 1996b). These species likely also generate some estrogens from the feto-placental unit during gestation, but either the levels parallel follicular phase concentrations or the majority of estrogen metabolites are excreted via the urinary versus fecal route.

The decrease in progestogen metabolite concentrations around mid-gestation followed by a marked increase above luteal values until parturition suggests a change in the dynamics of progestogen secretion. There are two possible explanations for these patterns, although both need verification as a priori hypotheses. First, the follicular activity noted during gestation for several pygmy hippos in our study, especially during the first 100 days, may indicate the formation of accessory corpora lutea (CLs) to support the pregnancy until the luteal-placental shift occurs, similar to the domestic mare (Blanchard et al. 2003). A fluctuating pattern in serum estrogen concentrations during the first half of gestation, possibly indicative of accessory follicle formation, has also been observed in some lowland tapirs (Pukazhenthi et al. 2013).

Progestogen metabolite profiles during the initial part of gestation in the Malayan and Baird's tapir also exhibited a biphasic pattern and may similarly be associated with the development of accessory CLs to support pregnancy (Brown et al. 1994a; Pukazhenthi et al. 2013). Concentrations of progestogen metabolites in pygmy hippos also oscillated between baseline and peak luteal levels during the first half of gestation. These findings warrant additional research to determine if the hormone metabolite patterns we noted are associated with accessory CL formation.

Second, the marked increase in progestogens in the second half of pregnancy may be indicative of a luteal-placental shift. This phenomenon has been documented via non-invasive endocrine monitoring in a number of other large mammalian species, including the mare (Schwarzenberger et al. 1991). Another well-studied example is the black rhino, where fecal progestogen metabolites remain at luteal phase concentrations during the initial part of pregnancy but increase dramatically between three and five months after conception, signifying a luteal-placental shift (Brown et al. 1997; Schwarzenberger et al. 1993). Czekala and Callison (1996) found similar trends using salivary progestogen analysis, with the shift occurring around five to six months after conception. A similar shift also takes place approximately five months after conception in the greater one-horned rhino (Schwarzenberger et al. 2000) and between two and four months after conception in the white rhinoceros (*Ceratotherium simum*; Patton et al. 1999; Schwarzenberger et al. 1998). Other species with a presumed luteal-placental shift, as indicated by luteal progestogen metabolite concentrations during the first part of gestation followed by a significant and sustained increase above luteal concentrations include the Alaskan moose (*Alces alces gigas*; Brown et al. 1997), southern three-banded armadillo (*Tolypeutes matacus*; Howell-Stephens et al. 2013), wood bison (*Bison bison athabascae*; Othen 1997), giant anteater (Patzl et al. 1998), red river hog (Berger et al. 2006), and Baird's tapir (Brown et al. 1994a).

There were 10 females in our study with viable calves and sufficient post-partum data to determine timing of the first estrous cycle; seven of these females had hormonal evidence of an

estrous cycle approximately 30 days after calving. Other species known to experience a 'foal heat' approximately within three to four weeks post-partum include the domestic horse (Blanchard and Varner 1993), white rhino (Hildebrandt et al. 2007), black rhino (Berkeley et al. 1997; Brown et al. 2001; Schwarzenberger et al. 1993), okapi (Schwarzenberger et al. 1999), and Baird's tapir (Brown et al. 1994a). Although weaning age in the wild is unknown, if the female can become pregnant approximately one month after parturition and carry a second calf during lactation, then weaning in wild pygmy hippos may occur around eight months of age (one month post-partum estrus followed by a seven-month gestation). Calving intervals from studbook data indicate that the female is able to conceive on the first post-partum estrus, within one month of parturition, when the calf experiences neonatal mortality (Steck 2016). In the common hippo, lactational anestrus averaged 34 weeks; however it was not always observed and was inconsistent between and among individuals, possibly indicating the influence of external factors, such as photoperiod or body condition, on the timing of the first post-partum estrus (Graham et al. 2002).

5.5.2 ESTROUS CYCLE

All of the estrogen metabolite EIAs we used in this study detected patterns indicative of follicular development. Similarly, all three progestogen assays (Pg-diol, PdG, mono-P4) demonstrated hormone metabolite patterns consistent with luteal activity. However, the mono-P4 EIA most clearly demonstrated luteal phases with minimal background fluctuations. Mean estrous cycle length was 31.8 days based on follicular peaks and 30.9 days based on luteal nadirs, comparable to the 32.4 days based on behavioral observations in this study and slightly shorter than the mean cycle length of 35.3 days reported for the common hippo (Graham et al. 2002). Cycle length ranged from 14 to 49 days and exhibited considerable variability both between and among individual animals.

For animals with longer cycles, our biweekly sampling protocol may have missed some estrogen peaks indicating follicular development. Although we were able to identify 185 follicular phases with this protocol, we could not determine the precise duration of these phases

as the majority were represented by a single estrogen metabolite peak. However, the follicular phase likely spans at least three to four days or else biweekly sampling would not have detected the associated endocrine patterns. Nadir luteal concentrations may likewise have been missed with biweekly sampling as the progestogen metabolite nadir was typically also short, with only one or two points at or near baseline before the next luteal phase.

On the other hand, the pygmy hippo may actually exhibit both short and long cycles, similar to the Malayan tapir (Pukazhenthi et al. 2013), the white rhino (Brown et al. 2001; Patton et al. 1999; Schwarzenberger et al. 1998) and the black rhino (Edwards et al. 2015; Garnier et al. 2002). The only previous reproductive hormone study for the pygmy hippo reported an average cycle length of 26 days based on salivary progestogen metabolites; the data were for a total of 10 cycles from two females (Dathe & Kuckelkorn 1989). However, these authors noted hormonal oscillations every nine days, leading them to hypothesize a normal cycle length of 18, 27, or 36 days.

Our ability to detect estrous cycles has allowed us to determine several aspects of the basic reproductive biology of the female pygmy hippo under managed care. We noted hormonal evidence of estrous cycling between 2.5 and 3 yrs of age for the three juvenile females, indicative that puberty occurs within this time frame. This is slightly earlier than the 3 to 4 yrs of age reported for the onset of puberty in common hippos based on non-invasive endocrine monitoring (Wheaton et al. 2006). Our data also indicate that females continue to exhibit hormonal evidence of estrous cycles well into their 30s. Behavioral estrus and mating was observed for one aged (>30 years) pygmy hippo on several occasions and hormone profiles also provided evidence of estrous cycles; however, she never conceived. Within the overall Studbook population, the oldest multiparous female to successfully reproduce was 37 years old at the time of conception, but generally reproduction in females is uncommon beyond the age of 30 (Steck 2016).

In accordance with the Studbook (Steck 2016), and similar to the common hippo (Graham et al. 2002), our results also indicate the pygmy hippo to be non-seasonally polyestrous in both

subtropical as well as temperate climates. Additionally, similar to domestic ruminants (Evans 2003), pygmy hippos in the managed population exhibit spontaneous ovulation, as demonstrated by clear hormonal evidence of estrous cycles in females housed in facilities without a male. There are several environmental factors that can influence reproductive physiology and seasonality of the estrous cycle, most importantly photoperiod, temperature, resource availability, and rainfall. In general, species whose natural environment provides a relatively constant level of these elements will reproduce year-round, whereas species endemic to habitats with marked variability of one or several of these elements are seasonal breeders (Bronson 1985; Zerbe et al. 2012). Although we have no comparative information from wild populations, the ecologic life history traits of the pygmy hippo largely support non-seasonally polyestrous reproduction. The species is endemic to a very limited region of tropical, equatorial West African rainforest where photoperiod and temperature remain relatively constant throughout the year; however, there is a seasonal difference in rainfall (Critical Ecosystem Partnership Fund, 2000). During the wet season there are marked habitat changes prompted by cyclical flooding during the rains, up to 6000mm annually in some regions. It is unknown if these fluctuations in rainfall patterns place any limitations on the resources necessary for rearing pygmy hippo calves.

Several other ungulate species endemic to tropical, equatorial habitats also exhibit non-seasonally polyestrous patterns and spontaneous ovulation in wild populations, including the lesser mouse deer (*Tragulus javanicus*), native to Indonesia and Malaysia, and the white-lipped (*Tayassu pecari*) and collared (*Pecari tajacu*) peccary, both native to Central and tropical South America (Gottdenker & Bodmer 1998; Kusuda et al. 2013). Warthogs (*Phacochoerus africanus*) are non-seasonal breeders in equatorial regions but farther from the equator farrowing is synchronized with the end of the dry season (Berger et al. 2006). Common hippos exhibit year-round estrous cycling in equatorial regions of Africa, but there is a peak of calving events during the rainy season in areas farther from the equator with more variable rainfall, temperature, and photoperiod (Laws & Clough 1966; Marshall & Sayer 1976; Smuts & Whyte 1981).

5.5.3 GENERAL DISCUSSION

Interestingly, the mono-P4 EIA was able to detect luteal phases but not pregnancy. This assay predominately cross-reacts with 20-oxo-progestogen metabolites, so its spectrum of detection is notably different to the Pg-diol and PdG EIAs that primarily detect metabolites with 20α-hydroxyl groups (Appendix V). 20-oxo-progestogen metabolites predominate in the feces of many species (Schwarzenberger, Möstl, et al. 1996), and EIAs that cross-react with the C20-oxo group have proven useful for monitoring reproductive activity in a number of artiodactylid and perissodactylid ungulates including the common hippo, moose (*Alces alces*), elk (*Cervus elaphus*), African elephant (*Loxodonta africana*), sable antelope, okapi, and black and white rhino (Graham et al. 2001).

PdG immunoreactivity during gestation in the common hippo exhibited a pattern very similar to the pygmy hippo, with levels fluctuating until mid-gestation followed by a marked increase and sustained elevation until parturition (Smith et al. 2000). Graham et al. (2002) hypothesized that the PdG EIA in the common hippo reflects progestogens produced by the feto-placental unit rather than from the CL because metabolite levels didn't increase until the second half of gestation and there were no clear increases associated with the luteal phase following ovulation. In order for two EIAs with distinctly different cross-reactivities to accurately reflect two separate phases of gestation, namely the post-ovulatory CL and the feto-placental unit, the predominant progestogens produced by each must be structurally dissimilar. This phenomenon has most clearly been demonstrated in the mare where Δ4 progestogens are produced in the first part of gestation by the primary and accessory CLs. Thereafter, an increase in 5α-dihydroprogesterone (a 5α-reduced pregnane) marks the luteo-placental shift, and metabolites of 5α-reduced pregnanes, produced by the feto-placental unit, predominate in mid to late gestation (Legacki et al. 2016). A similar transition in the principal type of progestogen produced by the CL versus the feto-placental unit in pygmy hippos may explain why Pg-diol and PdG immunoreactivity increased in the second half of gestation and mono-P4 did not, remaining at or below luteal levels. However, all three of these EIAs were all reflective of luteal

phases, indicating that Pg-diol and PdG must cross-react with some of the metabolites from the post-ovulatory CL as well as those produced by the fetal-placental unit.

Temporally-associated estrus behavior was not reported for the majority of estrous cycles identified via endocrine monitoring. Unfortunately, in most cases we were unable to determine if the lack of behavior data reflected a true absence of behavioral estrus or simply a failure to notice and/or report these behaviors. However, for some females we confirmed with the husbandry staff that behavioral estrus was definitively not observed when our data clearly supported estrous cycling. Dathe and Kuckelkorn (1989) also noted a lack of behavioral signs during periods of physiologic estrus for the two females in their study. These findings indicate that behavioral evaluation, even when performed by experienced zoo staff, is not an infallible method of estrus detection. A comparable phenomenon of 'silent' estrus has been reported in other species, for example the greater one-horned rhino (Stoops et al. 2014) and the Baird's tapir (Brown et al. 1994a), further emphasizing the value of endocrine monitoring as an important tool to guide breeding programs for endangered species. Additionally, data from two sets of females (n = 4 hippos) at the same zoo (n = 2 zoos) suggest that pygmy hippos housed in close proximity possibly synchronize their cycles as follicular activity exhibited temporal association on a number of occasions. Thus, behavioral signs of estrus may be more difficult to identify in subdominant females when multiple females are housed together or in close proximity.

When behavioral signs of estrus are evident, they are likely to be a true indication of physiologic estrus as the majority (67%) of behavioral reports temporally coincided with an estrogen metabolite peak or progestogen metabolite nadir. Thus, husbandry staff should be consistently trained in recognizing and correctly classifying these behaviors, which include male vocalization, increased activity level, and direct interest in the female; females may exhibit a deep, audible breathing pattern (Dathe & Kuckelkorn 1989; Lang 1975). Potential explanations for the 33% of cases where estrus-associated behaviors were reported but expected endocrine patterns were not detected include incorrect classification of behaviors or sampling frequency.

The lack of an estrogen metabolite peak before all luteal phases could also be due to the biweekly sampling schedule occasionally missing these peaks. Reproductive behavior and mating in the absence of endocrine evidence of estrus has been reported in the white rhino (Patton et al. 1999) and was noted for one female in our study.

There were occasional estrogen metabolite peaks during luteal phases; these may be indicative of follicular waves similar to the patterns seen in many domestic animals (Evans 2003), wood bison (Othen 1997) and several species of non-domestic felid (Brown 2011; Graham et al. 1995). We also intermittently noted temporally coinciding estrogen and progestogen metabolite peaks; this finding may represent secretion of progestogens from a non-ovulatory follicle undergoing atresia. Some individuals had periods of regular estrous cycling with clear follicular peaks and luteal phases followed by periods where physiologically relevant patterns were not discernable. This phenomenon may represent periods of normal cyclicity interspersed with irregular ovarian activity, possibly associated with environmental factors, captivity-associated stress (Mason 2010), underlying reproductive pathology, or persistent luteal activity, as recently characterized in the mare (Santos et al. 2015).

Overall, estrous cycle patterns exhibited considerable variability both among and between individuals. A similar phenomenon has been reported for other pachyderms, including elephants and rhinoceroses, with potential links to social stress and other captivity-related factors (Edwards et al. 2015; Schwarzenberger & Brown 2013). Certain husbandry variables, including diet, body condition, and social grouping might also influence estrous cycling and reproduction in pygmy hippos.

Diet can affect the assessment of fecal hormone metabolites in two major ways. First, hippo dung is largely comprised of undigested, fibrous vegetation and moisture content varied considerably in our study animals. Studies have shown that biologically inert material, including fibrous vegetation, within fecal samples can result in inconsistent measurement of hormone metabolites via EIA (Brown et al. 1994b; Ganswindt et al. 2012; Goymann 2012; Wasser et al. 1993). Lyophilization of samples prior to extraction may help detect lower-concentrations and

measure hormone metabolites more consistently overall. However, analysis of fecal hormone metabolites using wet extraction methods has been successfully performed in the common hippo, a species with very similar fecal composition to the pygmy hippo (Graham et al. 2002; Smith et al. 2000). Phytoestrogens represent a second important dietary influence on reproductive hormone levels, especially in herbivores. Estrogenic compounds in some plants species and in soy-based pelleted feeds can cause significant reproductive abnormalities in domestic ruminants, even when present at low levels (Adams 1995). The potential influence of dietary estrogenic compounds on reproduction in pygmy hippos requires further investigation.

Diet also has a direct influence on body condition, and a number of pygmy hippos in zoological collections are overweight (Flacke et al. 2015). In women, reproductive problems associated with obesity include early pregnancy loss and stillbirth, reduced fertility, prolonged estrous cycles, anovulation, and polycystic ovarian syndrome (Brewer & Balen 2010; Davies 2006; Lim & Cheng 2011; Rachoń & Teede 2010). In Asian elephants (*Elephas maximus*), obesity has been proposed as a primary cause of reproductive problems in captivity, including a high incidence of stillbirth (Taylor & Poole 1998). In zoo-housed black rhinos, higher body condition may play a role in reduced reproductive success for some females (Edwards et al. 2015). Overweight pygmy hippos may also experience impaired reproductive function.

Direct and indirect (i.e., camera trap photos, footprint tracking) observation of wild pygmy hippos has repeatedly demonstrated that these animals are predominately solitary (Bülow 1987; Collen et al. 2011; Conway 2013; Hentschel 1990; van Heukelum 2011). Separate housing therefore more closely reflects this species' natural biology and accordingly the husbandry manual recommends keeping the male and female separate except during estrus and mating (von Houwald et al. 2007). However, logistical constraints often preclude separate housing and many zoos keep their pygmy hippo pair together continuously (see Appendix IV). Abnormal social structure in zoo environments has been linked to reproductive abnormalities in several species. Female cheetahs (*Acinonyx jubatus*) are naturally solitary and when housed with other females will exhibit lower estrogen concentrations and abnormal estrous cycles; however,

females resume normal estrous cycling and associated reproductive behaviors when housed on their own (Brown 2011; Wielebnowski et al. 2002). In the African elephant, a species with a complex matriarchal social structure, unstable and isolated social structure were correlated with abnormal estrous cycling among females housed in North American zoos (Brown et al. 2016). The red river hog lives in large coed groups of 30 to 50 animals in the wild but is often housed in pairs or small, same-sex groups in captivity; abnormal estrous cycling has been documented in females housed together in the absence of a male (Jarboe et al. 2015). Chronic stress is hypothesized to be the primary link between unnatural social structure and resulting reproductive abnormalities and has been shown to negatively influence reproductive parameters in dairy cows (Walker et al. 2008), domestic ewes (Pierce et al. 2009) and male cheetah (Terio et al. 2004). Poor reproductive success and abnormal estrous cycling in zoo-born white rhinos is hypothesized to be associated with stress and undetermined captivity-associated factors (Brown et al. 2001; Carlstead & Brown 2005; Patton et al. 1999; Schwarzenberger & Brown 2013; van der Goot et al. 2015). These findings raise concern that chronic stress may also potentially influence reproduction in pygmy hippos.

5.5.4 FUTURE RESEARCH AND RECOMMENDATIONS

Additional investigations are warranted to address a number of unanswered questions concerning the reproductive biology of the female pygmy hippo. We recommend performing ultrasound examination in conjunction with endocrine monitoring to correlate reproductive events with fecal hormone metabolite profiles and to further validate physiologic relevance. Ultrasound can also be used to determine if additional CLs are formed during gestation and to identify the timing of CL regression as an indication of luteal-placental shift. Further research is also needed to establish if there is more than one distinct estrous cycle length in the pygmy hippo, if longer periods of luteal activity are abnormal, and if the failure of some pairs to reproduce despite endocrine evidence of (often irregular) ovarian activity is indicative of underlying reproductive pathology as has been documented for the elephant and white rhino (Hermes et al. 2004, 2006). Additionally, future studies can employ the techniques we have

described for monitoring reproductive hormones to investigate potential links between zoo-specific husbandry variables, obesity, chronic stress, and reproduction in pygmy hippos.

From a technical standpoint, more frequent sample collection may facilitate identification of follicular and luteal patterns, and lyophilization may improve consistency and accuracy of the EIAs, particularly for studies involving animals from more than one zoological facility. Saliva sampling, piloted in two pygmy hippos by Dathe and Kuckelkorn (1989), has been successfully employed for endocrine monitoring in numerous other wildlife species (Czekala & Callison 1996; Dathe et al. 1992; Heintz et al. 2011; Illera et al. 2014) and further research may demonstrate it to be a valuable tool in pygmy hippos as well. Determining the biochemical structure of immunoreactive metabolites of estrogen and progestogen in the feces using high performance liquid chromatography (HPLC) will help further refine which EIAs most accurately reflect reproductive hormones for this species. Recently, some laboratories are quantifying steroid hormones using mass spectrometry with improved results compared to EIA analysis (Handelsman & Wartofsky 2013); future application of this methodology to pygmy hippo samples could be most instructive. Radio-label studies would also be useful to determine what percentage of reproductive hormone metabolites are excreted via the urinary and fecal route and to more precisely determine lag time. However, this approach is not always practical or safe, and is therefore often not justified in rare and endangered species. Thus, non-invasive endocrine monitoring paired with ultrasound validation of physiologic events is the next-best approach for tracking reproductive events.

5.6 CONCLUSIONS

This landmark collaborative study sets a significant milestone in elucidating reproductive endocrinology of the female pygmy hippo and provides essential data for future research. We describe endocrine patterns during pregnancy, lactation and the estrous cycle and have identified several EIAs with biological relevance for non-invasively monitoring reproductive hormone metabolites in the feces. Additionally, we have identified two progestogen metabolite

EIAs (Pg-diol & PdG) that can diagnose pregnancy in the second half of gestation. We have also established that the pygmy hippo under managed care is a spontaneous ovulator and a non-seasonally polyestrous species. The length of the estrous cycle as determined by endocrine analysis was slightly shorter than the length reported from behavioral observations. Results were repeatable and consistent between laboratories using slightly different EIAs and techniques. Our data provide a vital resource for further investigations of reproductive physiology in the pygmy hippo and demonstrate how non-invasive endocrine monitoring can be a valuable tool for characterizing normal biology. These same technologies can potentially help diagnose reproductive abnormalities and improve future breeding management of this endangered species.

5.7 ACKNOWLEDGEMENTS

We would like to express our gratitude to all of the institutions that participated in this multi-year study: Aalborg Zoo; Arnhem Royal Burger's Zoo; Baton Rouge Zoo; Bristol Zoo; Chicago Zoological Society, Brookfield Zoo; Colchester Zoo; Dierenpark Wissel, Epe; Dvůr Králové Zoo Safari; Edinburgh Zoo; Gaia Zoo, Kerkrade; Gladys Porter Zoo, Brownsville; Jackson Zoo; Louisville Zoo; Marwell Zoo; Omaha's Henry Doorly Zoo and Aquarium; Paris Zoological Park; Parken Zoo, Eskilstuna; Rotterdam Zoo; Rum Creek Center for Conservation of Tropical Ungulates; Tampa's Lowry Park Zoo; Zoo Basel; Zoo Berlin; Zoo Miami; and ZooParc Overloon. Our special appreciation goes out to the husbandry staff that collected the samples; without their help and dedication we would not have been able to conduct this research. We also thank Cayman Adams, Sarah Allred, Kim Daly-Crews, Thijs van den Houten, Saleha Khan, Lara Metrione, Erich Möstl, Paige Pickering and Kayla Weller for technical support.

Initial financial support for MP was provided by Dr. Mervyn Jacobson; additional support was provided by Zoo Basel, Givskud Zoo, Zodiac Zoos, and the Royal Zoological Society of Scotland. Financial support for GF was provided by the American Association of Zoo Veterinarians Wild Animal Health Fund; the Center for Conservation of Tropical Ungulates;

Omaha's Henry Doorly Zoo and Aquarium; the University of Western Australia (UWA), Convocation Postgraduate Research Travel Award; UWA Postgraduate Student's Association Fieldwork and Data Collection Award; and a UWA Graduate Research School PhD Completion Scholarship.

5.8 LITERATURE CITED

Adams, N.R., 1995. Detection of the effects of phytoestrogens on sheep and cattle. J. Anim. Sci. 73, 1509–1515.

Bamberg, E., Möstl, E., Patzl, M., King, G.J., 1991. Pregnancy diagnosis by enzyme immunoassay of estrogens in feces from nondomestic species. J. Zoo Wildl. Med. 22, 73–77.

Barnes, S.A., Teare, J.A., Staaden, S., Metrione, L., Penfold, L.M., 2016. Characterization and manipulation of reproductive cycles in the jaguar (*Panthera onca*). Gen. Comp. Endocrinol. 225, 95–103.

Berger, E.M., Leus, K., Vercammen, P., Schwarzenberger, F., 2006. Faecal steroid metabolites for non-invasive assessment of reproduction in common warthogs (*Phacochoerus africanus*), red river hogs (*Potamochoerus porcus*) and babirusa (*Babyrousa babyrussa*). Anim. Reprod. Sci. 91, 155–171.

Berkeley, E. V., Kirkpatrick, J.F., Schaffer, N.E., Bryant, W.M., Threlfall, W.R., 1997. Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the black rhinoceros (*Diceros bicornis*). Zoo Biol. 16, 121–132.

Blanchard, T.L., Varner, D.D., 1993. Uterine involution and post-partum breeding, in: McKinnon, A.O., Voss, J.L. (Eds.), Equine Reproduction. Lea and Febiger, Philadelphia, pp. 622–625.

Blanchard, T.L., Varner, D.D., Schumacher, J., Love, C.C., Brinsko, S.P., Rigby, S.L., 2003. Pregnancy: physiology and diagnosis, in: E.M. Fathman (Ed.), Manual of Equine Reproduction. Mosby, St. Louis, pp. 69–76.

Brewer, C.J., Balen, A.H., 2010. The adverse effects of obesity on conception and implantation. Reproduction 140, 347–364.

Bronson, F.H., 1985. Mammalian reproduction: an ecological perspective. Biol. Reprod. 32, 1–26.

Brown, J.L., 2011. Female reproductive cycles of wild female felids. Anim. Reprod. Sci. 124, 155–162.

Brown, J.L., Bellem, A.C., Fouraker, M., Wildt, D.E., Roth, T.L., 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol. 20, 463–486.

Brown, J.L., Citino, S.B., Shaw, J., Miller, C., 1994a. Endocrine profiles during the estrous cycle and pregnancy in the Baird's Tapir (*Tapirus bairdii*). Zoo Biol. 13, 107–117.

Brown, J.L., Paris, S., Prado-Oviedo, N., Meehan, C., Hogan, J.N., Morfeld, K., Carlstead, K., 2016. Reproductive health assessment of female elephants in North American zoos and association of husbandry practices with reproductive dysfunction in African elephants (*Loxodonta africana*). PLoS One 11, e0145673. doi:10.1371/journal.pone.0145673

Brown, J.L., Wasser, S.K., Wildt, D.E., Graham, L.H., 1994b. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol. Reprod. 51, 776–786.

Brown, J.L., Wasser, S.K., Wildt, D.E., Graham, L.H., Monfort, S.L., 1997. Faecal steroid analysis for monitoring ovarian and testicular function in diverse wild carnivore, primate, and ungulate species. J. Mamm. Biol. 62, 27–31.

Bülow, W., 1987. Untersuchungen am Zwergflußpferd, Choeropsis liberiensis im Azagny - Nationalpark, Elfenbeinküste. Diplomarbeit, Zoologischen Institut Braunschweig.

Carlstead, K., Brown, J.L., 2005. Relationships between patterns of fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. Zoo Biol. 24, 215–232.

Critical Ecosystem Partnership Fund, 2000. Ecosystem Profile - Upper Guinean Forest Ecosystem of the Guinean Forests of West Africa Biodiversity Hotspot.

Collen, B., Howard, R., Konie, J., Daniel, O., Rist, J., 2011. Field surveys for the endangered pygmy hippopotamus Choeropsis liberiensis in Sapo National Park, Liberia. Oryx 45, 35–37.

Conway, A.L., 2013. Conservation of the Pygmy Hippopotamus (*Choeropsis liberiensis*) in Sierra Leone, West Africa. PhD Thesis, University of Georgia, Athens.

Czekala, N.M., Callison, L., 1996. Pregnancy diagnosis in the black rhinoceros (*Diceros bicornis*) by salivary hormone analysis. Zoo Biol. 15, 37–44.

Dathe, H.H., Kuckelkorn, B., 1989. Progesteronnachweis in Sekreten des Zwergflußpferdes (*Choeropsis liberiensis* Morton, 1844). Der Zool. Garten NF 59, 201–208.

Dathe, H.H., Kuckelkorn, B., Minnemann, D., 1992. Salivary cortisol assessment for stress detection in the Asian elephant (*Elephas maximus*): A pilot study. Zoo Biol. 11, 285–289.

Davies, M.J., 2006. Evidence for effects of weight on reproduction in women. Reprod. Biomed. Online 12, 552–561.

Edwards, K.L., Shultz, S., Pilgrim, M., Walker, S.L., 2015. Irregular ovarian activity, body condition and behavioural differences are associated with reproductive success in female eastern black rhinoceros (*Diceros bicornis michaeli*). Gen. Comp. Endocrinol. 214, 186–194.

Evans, A.C.O., 2003. Characteristics of ovarian follicle development in domestic animals. Reprod. Domest. Anim. 38, 240–246.

Flacke, G.L., Chambers, B.K., Martin, G.B., Paris, M.C.J., 2015. The pygmy hippopotamus *Choeropsis liberiensis* (Morton, 1849): Bringing to light research priorities for the largely forgotten, smaller hippo species. Der Zool. Garten NF 84, 234–265.

Ganswindt, A., Muilwijk, C., Engelkes, M., Muenscher, S., Bertschinger, H., Paris, M., Palme, R., Cameron, E.Z., Bennett, N.C., Dalerum, F., 2012. Validation of noninvasive monitoring of adrenocortical endocrine activity in ground-feeding aardwolves (*Proteles cristata*): exemplifying the influence of consumption of inorganic material for fecal steroid analysis. Physiol. Biochem. Zool. 85, 194–199.

Garnier, J.N., Holt, W. V., Watson, P.F., 2002. Non-invasive assessment of oestrous cycles and evaluation of reproductive seasonality in the female wild black rhinoceros (*Diceros bicornis minor*). Reproduction 123, 877–889.

Gottdenker, N., Bodmer, R.E., 1998. Reproduction and productivity of white-lipped and collared peccaries in the Peruvian Amazon. J. Zool. 245, 423–430.

Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods Ecol. Evol. 3, 757–765.

Graham, L., Schwarzenberger, F., Möstl, E., Galama, W., Savage, A., 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biol. 20, 227–236.

Graham, L.H., Goodrowe, K.L., Raeside, J.I., Liptrap, R.M., 1995. Non-invasive monitoring of ovarian function in several felid species by measurement of fecal estradiol-17β and progestins. Zoo Biol. 14, 223–237.

Graham, L.H., Reid, K., Webster, T., Richards, M., Joseph, S., 2002. Endocrine patterns associated with reproduction in the Nile hippopotamus (*Hippopotamus amphibius*) as assessed by fecal progestagen analysis. Gen. Comp. Endocrinol. 128, 74–81.

Handelsman, D.J., Wartofsky, L., 2013. Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism. J. Clin. Endocrinol. Metab. 98, 3971–3973.

Heintz, M.R., Santymire, R.M., Parr, L.A., Lonsdorf, E. V., 2011. Validation of a cortisol enzyme immunoassay and characterization of salivary cortisol circadian rhythm in chimpanzees (*Pan troglodytes*). Am. J. Primatol. 73, 903–908.

Hentschel, K.M., 1990. Untersuchung zu Status, Ökologie und Erhaltung des Zwergflusspferdes (*Choeropsis liberiensis*) in der Elfenbeinküste. Doktorand Dissertation, Technischen Universität Carolo-Wilhelmina, Braunschweig.

Hermes, R., Hildebrandt, T.B., Göritz, F., 2004. Reproductive problems directly attributable to long-term captivity–asymmetric reproductive aging. Anim. Reprod. Sci. 82–83, 49–60.

Hermes, R., Hildebrandt, T.B., Walzer, C., Göritz, F., Patton, M.L., Silinski, S., Anderson, M.J., Reid, C.E., Wibbelt, G., Tomasova, K., Schwarzenberger, F., 2006. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). Theriogenology 65, 1492–1515.

Hildebrandt, T.B., Hermes, R., Walzer, C., Sós, E., Molnar, V., Mezösi, L., Schnorrenberg, A., Silinski, S., Streich, J., Schwarzenberger, F., Göritz, F., 2007. Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation. Theriogenology 67, 1473–1484.

Howell-Stephens, J., Bernier, D., Brown, J.S., Mulkerin, D., Santymire, R.M., 2013. Using non-invasive methods to characterize gonadal hormonal patterns of southern three-banded armadillos (*Tolypeutes matacus*) housed in North American zoos. Anim. Reprod. Sci. 138, 314–323.

Illera, J.-C., Silván, G., Cáceres, S., Carbonell, M.-D., Gerique, C., Martínez-Fernández, L., Munro, C., Casares, M., 2014. Assessment of ovarian cycles in the African elephant (*Loxodonta africana*) by measurement of salivary progesterone metabolites. Zoo Biol. 33, 245–249.

Isaac, N.J.B., Turvey, S.T., Collen, B., Waterman, C., Baillie, J.E.M., 2007. Mammals on the EDGE: Conservation priorities based on threat and phylogeny. PLoS One 2, e296. doi:10.1371/journal.pone.0000296

Jarboe, M., Adams, C., Bahr, J.M., Penfold, L., Newell-Fugate, A.E., 2015. Boar urine pheromone exposure modifies estrous cycle length and regularity in same-sex housed female red river hogs (*Potamochoerus porcus*), in: Proceedings of the 5th International Society of Wildilfe Endocrinology Conference. Berlin, Germany, p. 10.

Kusuda, S., Adachi, I., Fujioka, K., Nakamura, M., Amano-Hanzawa, N., Goto, N., Furuhashi, S., Doi, O., 2013. Reproductive characteristics of female lesser mouse deers (*Tragulus javanicus*) based on fecal progestagens and breeding records. Anim. Reprod. Sci. 137, 69–73.

Lang, E.M., 1975. Das Zwergflußpferd. A. Ziemsen Verlag, DDR, Wittenberg Lutherstadt.

Lasley, B.L., Kirkpatrick, J.F., 1991. Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids. J. Zoo Wildl. Med. 22, 23–31.

Laws, R.M., Clough, G., 1966. Observations on reproduction in the hippopotamus (*Hippopotamus amphibius* LINN). Symp. Zool. Soc. London 15, 117–140.

Legacki, E.L., Scholtz, E.L., Ball, B.A., Stanley, S.D., Berger, T., Conley, A.J., 2016. The dynamic steroid landscape of equine pregnancy mapped by mass spectrometry. Reproduction 151, 421–430.

Lim, C.E.D., Cheng, N.C.L., 2011. Obesity and reproduction. J. Aust. Tradit. Med. Soc. 17, 143–145.

Mallon, D., Wightman, C., De Ornellas, P., Ransom, C., 2011. Conservation Strategy for the Pygmy Hippopotamus. IUCN Species Survival Commission, Gland, Switzerland & Cambridge, UK.

Marshall, P.J., Sayer, J.A., 1976. Population ecology and response to cropping of a hippopotamus population in eastern Zambia. J. Appl. Ecol. 13, 391–403.

Mason, G.J., 2010. Species differences in responses to captivity: Stress, welfare and the comparative method. Trends Ecol. Evol. 25, 713–721.

Metrione, L.C., Norton, T.M., Beetem, D., Penfold, L.M., 2008. Seasonal reproductive characteristics of female and male Jackson's hartebeest (*Alcelaphus buselaphus jacksoni*). Theriogenology 70, 871–879.

Munro, C.J., Stabenfeldt, G.H., Cragun, J.R., Addiego, L.A., Overstreet, J.W., Lasley, B.L., 1991. Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin. Chem. 37, 838–844.

Othen, L.S., 1997. Reproductive endocrinology of wood bison during estrus synchronization, superovulation and pregnancy. University of Guelph.

Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused 14C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63.

Patton, M.L., Swaisgood, R.R., Czekala, N.M., White, A.M., Fetter, G.A., Montagne, J.P., Rieches, R.G., Lance, V.A., 1999. Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biol. 18, 111–127.

Patzl, M., Schwarzenberger, F., Osmann, C., Bamberg, E., Bartmann, W., 1998. Monitoring ovarian cycle and pregnancy in the giant anteater (*Myrmecophaga tridactyla*) by faecal progestagen and oestrogen analysis. Anim. Reprod. Sci. 53, 209–219.

Pierce, B.N., Clarke, I.J., Turner, A.I., Rivalland, E.T.A., Tilbrook, A.J., 2009. Cortisol disrupts the ability of estradiol-17β to induce the LH surge in ovariectomized ewes. Domest. Anim. Endocrinol. 36, 202–208.

Pukazhenthi, B., Quse, V., Hoyer, M., van Engeldorp Gastelaars, H., Sanjur, O., Brown, J.L., 2013. A review of the reproductive biology and breeding management of tapirs. Integr. Zool. 8, 18–34.

Rachoń, D., Teede, H., 2010. Ovarian function and obesity - interrelationship, impact on women's reproductive lifespan and treatment options. Mol. Cell. Endocrinol. 316, 172–179.

Ransom, C., Robinson, P.T., Collen, B., 2015. *Choeropsis liberiensis*. The IUCN Red List of Threatened Species 2015: e.T10032A18567171 http://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T10032A18567171.en. (accessed 22.12.2015).

Safar-Hermann, N., Ismail, M.N., Choi, H.S., Möstl, E., Bamberg, E., 1987. Pregnancy diagnosis in zoo animals by estrogen determination in feces. Zoo Biol. 6, 189–193.

Santos, V.G., Bettencourt, E.M. V., Ginther, O.J., 2015. Long-term characteristics of idiopathic persistent corpus luteum in the mare. Theriogenology 84, 242–251.

Schwarzenberger, F., 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. Int. Zoo Yearb. 41, 52–74.

Schwarzenberger, F., Brown, J.L., 2013. Hormone monitoring: An important tool for the breeding management of wildlife species. Wien. Tierarztl. Monatsschr. 100, 209–225.

Schwarzenberger, F., Francke, R., Göltenboth, R., 1993. Concentrations of faecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*). J. Reprod. Fertil. 98, 285–291.

Schwarzenberger, F., Möstl, E., Bamberg, E., Pammer, J., Schmehlik, O., 1991. Concentrations of progestagens and oestrogens in the faeces of pregnant Lipizzan, trotter and thoroughbred mares. J. Reprod. Fertil. Suppl. 44, 489–499.

Schwarzenberger, F., Möstl, E., Palme, R., Bamberg, E., 1996a. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim. Reprod. Sci. 42, 515–526.

Schwarzenberger, F., Rietschel, W., Matern, B., Schaftenaar, W., Bircher, P., Van Puijenbroeck, B., Leus, K., 1999. Noninvasive reproductive monitoring in the okapi (*Okapia johnstoni*). J. Zoo Wildl. Med. 30, 497–503.

Schwarzenberger, F., Rietschel, W., Vahala, J., Holeckova, D., Thomas, P., Maltzan, J., Baumgartner, K., Schaftenaar, W., 2000. Fecal progesterone, estrogen, and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros, *Rhinoceros unicornis*. Gen. Comp. Endocrinol. 119, 300–307.

Schwarzenberger, F., Tomášová, K., Holečková, D., Matern, B., Möstl, E., 1996b. Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol. 15, 159–171.

Schwarzenberger, F., Walzer, C., Tomasova, K., Vahala, J., Meister, J., Goodrowe, K.L., Zima, J., Strauß, G., Lynch, M., 1998. Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*). Anim. Reprod. Sci. 53, 173–190.

Smith, T.E., Richards, M., Joseph, S., Savage, A., 2000. Endocrine determinants of pregnancy in the Nile hippopotamus (*Hippopotamus amphibius*), in: Proceedings of the 2nd Annual Symposium on Zoo Research. Paignton Zoo Environmental Park, Paignton, Devon, United Kingdom, pp. 187–190.

Smuts, G.L., Whyte, I.J., 1981. Relationships between reproduction and environment in the hippopotamus *Hippopotamus amphibius* in the Kruger National Park. Koedoe 24, 169–185.

Steck, B. (Ed.), 2016. International Studbook for the Pygmy Hippopotamus 2015, 22nd ed. Zoo Basel, Switzerland, Basel.

Stoops, M.A., West, G.D., Roth, T.L., Lung, N.P., 2014. Use of urinary biomarkers of ovarian function and altrenogest supplementation to enhance captive breeding success in the Indian rhinoceros (*Rhinoceros unicornis*). Zoo Biol. 33, 83–88.

Taylor, V.J., Poole, T.B., 1998. Captive breeding and infant mortality in Asian elephants: A comparison between twenty western zoos and three eastern elephant centers. Zoo Biol. 17, 311–332.

Terio, K.A., Marker, L., Munson, L., 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. J. Wildl. Dis. 40, 259–266.

van der Goot, A.C., Martin, G.B., Millar, R.P., Paris, M.C.J., Ganswindt, A., 2015. Profiling patterns of fecal 20-oxopregnane concentrations during ovarian cycles in free-ranging southern white rhinoceros (*Ceratotherium simum simum*). Anim. Reprod. Sci. 161, 89–95.

van Heukelum, M., 2011. In search of the elusive Pygmy Hippo; Establishment of methods to determine population structure of Pygmy Hippos in Taï National Park, and assessment of their role in seed dispersal. MSc Thesis, Wageningen University.

von Houwald, F., Macdonald, A.A., Pagan, O., Steck, B. (Eds.), 2007. Husbandry Guidelines for the Pygmy Hippopotamus (*Hexaprotodon liberiensis*). Zoo Basel, Switzerland, Basel.

Walker, S.L., Smith, R.F., Jones, D.N., Routly, J.E., Dobson, H., 2008. Chronic stress, hormone profiles and estrus intensity in dairy cattle. Horm. Behav. 53, 493–501.

Walker, S.L., Waddell, W.T., Goodrowe, K.L., 2002. Reproductive endocrine patterns in captive female and male red wolves (*Canis rufus*) assessed by fecal and serum hormone analysis. Zoo Biol. 21, 321–335.

Wasser, S.K., Thomas, R., Nair, P.P., Guidry, C., Southers, J., Lucas, J., Wildt, D.E., Monfort, S.L., 1993. Effects of dietary fibre on faecal steroid measurements in baboons (*Papio cynocephalus cynocephalus*). J. Reprod. Fertil. 97, 569–574.

Wheaton, C.J., Joseph, S., Reid, K., Webster, T., Richards, M., Savage, A., 2006. Body weight as an effective tool for determination of onset of puberty in captive female Nile hippopotami (*Hippopotamus amphibious*). Zoo Biol. 25, 59–71.

Wielebnowski, N.C., Ziegler, K., Wildt, D.E., Lukas, J., Brown, J.L., 2002. Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). Anim. Conserv. 5, 291–301.

Zerbe, P., Clauss, M., Codron, D., Bingaman Lackey, L., Rensch, E., Streich, J.W., Hatt, J.M., Müller, D.W.H., 2012. Reproductive seasonality in captive wild ruminants: Implications for biogeographical adaptation, photoperiodic control, and life history. Biol. Rev. 87, 965–990.

Chapter 6 NON-INVASIVE MONITORING OF FECAL GLUCOCORTICOID AND ANDROGEN METABOLITES IN PYGMY HIPPOPOTAMUS (*CHOEROPSIS LIBERIENSIS*)

A Pygmy Hippo Pair – Center for Conservation of Tropical Ungulates

There is an eagle in me that wants to soar,
and there is a hippopotamus in me
that wants to wallow in the mud.

–Carl Sandburg

Non-invasive Monitoring of Fecal Glucocorticoid and Androgen Metabolites in Pygmy Hippopotamus (*Choeropsis liberiensis*)

Gabriella L. Flacke [a,b,*], Linda M. Penfold [c], Franz Schwarzenberger [d], Graeme B. Martin [a], and Monique C. J. Paris [a,b,e,f]

[a] *School of Animal Biology, University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia*

[b] *Institute for Breeding Rare and Endangered African Mammals (IBREAM), Edinburgh EH3 6AT, United Kingdom*

[c] *South East Zoo Alliance for Reproduction & Conservation,* 581705 White Oak Road, Yulee, FL 32097, USA

[d] *Department of Biomedical Sciences, Unit of Physiology, Pathophysiology and Experimental Endocrinology, University of Veterinary Medicine (Vetmeduni Vienna), Veterinärplatz 1, 1210 Vienna, Austria*

[e] *Mammal Research Institute and Centre for Neuroendocrinology, University of Pretoria, Department of Zoology and Entomology, Pretoria 0084, South Africa*

[f] *College of Public Health, Veterinary and Medical Sciences, James Cook University, Townsville 4811, Australia*

Corresponding author: Gabriella L. Flacke, DVM, MVSc; School of Animal Biology, M092; The University of Western Australia; Crawley WA 6009, Australia; Email: gflacke@grs.uwa.edu.au

6.1 ABSTRACT

The pygmy hippopotamus (*Choeropsis liberiensis*) is endangered in the wild and we have limited information concerning this species' reproduction and well-being under managed care. We therefore developed non-invasive methods for characterizing androgen profiles and glucocorticoid activity in this species. Our objectives were to: 1) identify enzyme immunoassays (EIAs) for measuring metabolites of cortisol and testosterone in pygmy hippo feces that produce biologically relevant data; 2) investigate the relationship between the fecal metabolites of cortisol and testosterone, particularly with respect to possible cross-reactions; 3) test for seasonality of androgen metabolite concentrations in males; 4) determine if there is a difference in androgen metabolites concentrations between proven breeding males and adult males that have not reproduced; and 5) to test if gonadal activity in adult males is influenced by housing (indoor versus outdoor) and latitude. Hormone metabolite concentrations generated by a corticosterone assay (CJM006, Coralie Munro, University of California, Davis, CA, USA) were significantly correlated with testosterone metabolite concentrations (C196, Arbor Assays, Ann Arbor, MI, USA) for both males and females, so the corticosterone assay could not be used to assess glucocorticoid metabolites in pygmy hippos. However, a group-specific EIA exhibiting cross-reactivity with 11,17-dioxoandrostane (DOA) metabolites of cortisol clearly reflected adrenocortical activity (response to ACTH challenge) in both males and females. The testosterone metabolite assay also produced biologically coherent data: adult males exhibited the highest androgen metabolite concentrations, followed by adult females and juvenile males, and proven breeding males had higher concentrations than unproven males. There were significant differences in mean concentrations among seasons for adult males overall, with higher values in spring and summer than in fall and winter. Adult males housed outdoors year-round in subtropical climates exhibited higher mean androgen concentrations than males in temperate climates that were housed indoors year-round or in colder weather. We can now non-invasively monitor gonadal activity in male pygmy hippos and adrenocortical activity in both sexes. Our findings provide valuable information for future studies investigating relationships between fecal androgen metabolites and reproductive health in pygmy hippos, and for exploring

the dynamics of short and long-term stress and associated welfare implications in this species. Finally, our results emphasize the importance of biological validation of EIAs used to measure fecal hormone metabolites and demonstrate the potential for cross-reactivity between assays to confound results.

Keywords: ACTH challenge, cortisol, pygmy hippo, stress, testosterone

6.2 INTRODUCTION

The pygmy hippopotamus (*Choeropsis liberiensis*) – hereafter referred to as pygmy hippo – is classified as Endangered by the International Union for the Conservation of Nature (Ransom et al. 2015) and is identified by Programme EDGE (www.edgeofexistence.org) as a priority for conservation action, ranked 21st worldwide among mammals (Isaac et al. 2007). The first Conservation Strategy Action Plan for this species was developed by the IUCN Pygmy Hippo Specialist Group in 2010 (Mallon et al. 2011) and one of several research priorities was to describe basic reproductive biology for both sexes. There is no information concerning reproduction in wild pygmy hippos, but some general aspects are known from animals under managed care (Flacke et al. 2015). For example, both males and females reach sexual maturity between three and four years of age and can remain reproductively active into their third decade. The species is assumed to be a non-seasonal breeder because births occur throughout the year in both northern and southern hemispheres (Steck 2016). Numerous breeding pairs at several zoological facilities worldwide have failed to reproduce and others have repeatedly experienced perinatal calf mortality or stillbirth (Steck 2016). These issues have limited the success of captive breeding programs and have the potential to reduce genetic diversity of the managed population in the long-term.

In a recent review, Mason (2010) identifies a substantial number of species that experience behavioral abnormalities and low reproductive success in comparison to their wild counterparts, at least partially due to captivity-associated stress. There are several specific examples of impaired reproductive success being linked to stress in captivity, including the cheetah (*Acinonyx jubatus*), where wild males have higher testosterone levels than captive males, and the Humboldt penguin (*Spheniscus humboldti*), where wild, undisturbed breeding colonies have approximately double the chick output of their conspecifics in zoo breeding colonies (Mason 2010). Poor reproductive success and abnormal cycling in zoo-born white rhinos (*Ceratotherium simum*) is hypothesized to be secondary to chronic stress and other, yet undetermined, captivity-associated factors (Brown et al. 2001; Carlstead & Brown 2005; Patton

et al. 1999; van der Goot et al. 2015). Lower rates of glucocorticoid production in free-ranging animals compared to their captive conspecifics has been demonstrated for several species, including the cheetah (Terio et al. 2004) and the African wild dog (*Lycaon pictus*; Van der Weyde et al. 2016). Although there is no reported behavioral evidence of chronic stress in pygmy hippos, these observations from other species suggest that investigations concerning its potential influence on reproductive health and welfare are warranted.

The adverse effects of chronic stress on reproductive health are associated with the increased production of glucocorticoids and their resulting impact on the hypothalamic-pituitary-gonadal axis (Moberg 2000; Rivier and Rivest 1991; Sapolsky 1992; Tilbrook et al. 2000; Wingfield and Sapolsky 2003). Negative outcomes include abnormal expression of sexual behavior, irregular ovulatory patterns, and inhibited production of gonadal hormones. It is therefore essential to monitor objectively both stress and reproductive parameters in wildlife species under managed care, especially for endangered species breeding programs, principally because it is challenging to mimic natural environmental, dietary, and social conditions in captivity. However, we must be able to assess these parameters without handling and restraint because repeated blood sampling is not practical in most species and the sampling itself often induces a stress response, especially if anesthesia is required.

An attractive option for evaluating both stress and reproduction without disrupting normal behavior is the non-invasive monitoring of metabolites of glucocorticoids (as an indicator of stress) and gonadal steroids (as an indicator of reproductive function) in the feces using enzyme immunoassays (EIAs). These assays were originally established to quantify native hormones in serum or urine and exhibit varying degrees of cross-reactivity with the multitude of hormone metabolites excreted in the feces. Although some EIAs have been developed with antibodies to species-specific fecal metabolites, financial and logistical limitations make this approach impractical in a broader conservation context given the sheer number of taxa that need attention. It is therefore more common to test the relevance of several EIAs for demonstrating biologically appropriate patterns.

However, because even closely related species produce a unique repertoire of hormone metabolites of unknown cross-reactivity with an assay designed to measure the native steroid, it is essential to demonstrate biological relevance of an EIA for measuring the metabolites of the hormone of interest (Touma & Palme 2005). Many factors can cause variation in hormone metabolism and excretion among species and individuals, including age, sex, diet, digestive physiology, degree of enterohepatic circulation, and microbial flora in the gastrointestinal tract (Goymann 2012; Millspaugh & Washburn 2004; Palme et al. 2005; Touma et al. 2003). Thus, EIAs measuring native hormones often show limited cross-reactivity with the diverse spectrum of metabolites, particularly in herbivores compared to carnivores due to longer gastrointestinal transit times and more extensive hormone metabolism during foregut and hindgut fermentation. To address this issue, several laboratories have developed group-specific EIAs that cross-react with groups of hormone metabolites of similar chemical structure, thereby increasing assay sensitivity for detecting a larger number of compounds. There are many species where group-specific EIAs exhibit superior biologically relevance for assessing adrenocortical activity via fecal monitoring, including the chimpanzee (*Pan troglodytes*; Heistermann et al. 2006), the Western lowland gorilla (*Gorilla gorilla gorilla*; Shutt et al. 2012), the African elephant (*Loxodonta africana*; Ganswindt et al. 2003), the African buffalo (*Syncerus caffer*; Ganswindt et al. 2012) and domestic pigs, horses, sheep and cows (Möstl et al. 1999, 2002).

Additionally, because the native glucocorticoids (cortisol, corticosterone) and reproductive steroids (testosterone, estrogen, progesterone) are derived from the same parent hormone, the metabolites of these hormones can also have relatively similar structures. For example, the majority of testosterone metabolites and some glucocorticoid metabolites exhibit a common androstane structure that only differs by one functional group (Appendix VI; Ganswindt et al. 2003). Consequently, EIAs used to assess fecal glucocorticoid metabolites could exhibit cross-reactivity with metabolites of gonadal hormones, especially androgens, complicating interpretation of endocrine patterns. This phenomenon was clearly demonstrated for the male dog (Schatz & Palme 2001) and the male African elephant (Ganswindt et al. 2003).

Biological relevance for EIAs assessing gonadal steroids is demonstrated by correlating endocrine patterns with reproductive status – for example a higher level of androgen metabolites for an adult breeding male compared to a juvenile or a female. The most common approach for demonstrating biological relevance for measurement of cortisol metabolites is an adrenal corticotropic hormone (ACTH) challenge, where release of glucocorticoids from the adrenal cortex is stimulated by injection of synthetic ACTH. It is also possible to evaluate glucocorticoid metabolites before and after a known stressful event, such as anesthesia (e.g. Shutt et al. 2012) or translocation (e.g. Franceschini et al. 2008). If the assay is predominately measuring fecal metabolites of cortisol it should demonstrate a marked increase in concentrations subsequent to the treatment, in accordance with gastrointestinal transit time for the species, followed by a return to baseline.

Non-invasive methods for quantifying glucocorticoid metabolites and characterizing male endocrine patterns have not yet been established for the pygmy hippo. Thus, the first objective of the present study was to identify EIAs that demonstrate biological relevance for measuring immunoreactive metabolites of glucocorticoids and androgens in pygmy hippo feces, particularly with respect to possible cross-reactions. Additional aims included examining patterns in androgen metabolites for males throughout the year to test for seasonality, to determine if there is a difference in circulating testosterone levels between proven breeding males and adult males that have not reproduced, and to test if gonadal activity is influenced by environmental factors, specifically indoor versus outdoor housing and geographic location. Our study is expected to provide valuable data for future research investigating the potential influence of certain husbandry variables on stress, welfare, and reproductive health in pygmy hippos under managed care.

6.3 MATERIALS AND METHODS

6.3.1 *ANIMALS AND SAMPLE COLLECTION*

Twelve male and four female pygmy hippos from 12 North American zoological institutions were included in this study. Ten of the males and all of the females were sexually mature (≥ 3 years) at the time sampling commenced (Appendix VII). Fresh fecal samples for assessment of androgen metabolites were collected once weekly for 1 year and stored frozen at −20°C until extraction and analysis via EIA. Additional fecal sample collection for biological validation of an EIA to assess glucocorticoid metabolites is described below.

6.3.2 *STABILITY OF HORMONE METABOLITES IN FECAL SAMPLES*

Age of the sample has the potential to influence hormone concentrations and not all samples could be collected immediately after defecation. To test the rate of degradation for androgen and glucocorticoid hormone metabolites in the feces, we collected fresh samples immediately after defecation from one male and one female. We homogenized each sample, separated it into equal-sized parts, and froze one subsample at 0, 4, 8, 12, 16, 20, and 24 h after defecation. Between defecation and freezing, subsamples were stored outside on a concrete surface under conditions of ambient temperature and daylight in Omaha, Nebraska from 10:00 am the 21st of May until 10:00 am on the 22nd of May, 2014. Conditions were partly cloudy, there was no rainfall, and temperatures ranged from 17°C during the night to 26°C during the day. Subsamples were analyzed using both an androgen metabolite and a glucocorticoid metabolite EIA (described below).

6.3.3 *GASTROINTESTINAL TRANSIT TIME*

The lag time between patterns of steroid secretion into the blood and subsequent metabolite excretion in the feces is correlated with gastrointestinal transit time (Schwarzenberger, Möstl, et al. 1996; Whitten et al. 1998). We determined the transit time for one male and one female

pygmy hippo housed at the same facility and fed the same diet. We used an easily identifiable fecal marker (glitter; Sulyn Industries, Coral Springs, Florida, USA) mixed with grain and recorded the time from ingestion until the first and last passage of glitter in the feces.

6.3.4 ACTH CHALLENGE

We conducted an ACTH challenge in four adult male and three adult female pygmy hippos; one additional adult female was used as a control. The control hippo was previously trained to accept routine veterinary intervention, including injections, while receiving a food reward and was therefore not expected to find the injection process itself stressful. Initially, three males and two females received an injection of 50 IU (approximately 0.2 IU/kg) short-acting synthetic ACTH (Cosyntropin 0.25 IU/mL, Sandoz Inc., Princeton, NJ, USA). Due to lack of clear and consistent results with the short-acting product, we performed an additional ACTH challenge for one male and one female using a higher dose rate and a longer-acting product. These two hippos received an injection of 250 IU (approximately 1.0 IU/kg) sustained-release synthetic ACTH (Corticotropin 80 IU/mL, Wedgewood Pharmacy, Swedesboro, NJ, USA). The control female was injected with an equal volume of 0.9% saline solution. Injections were given intramuscularly behind the ear, where the subcutaneous fat layer is thinnest, using an 18 gauge, 1.5 inch needle. Fecal samples were collected for 3 to 4 days before and 7 to 10 days after the ACTH or saline injection and stored at −20 °C until analysis. For the first 72 h after injection, all fecal material produced by the animal was collected; on all other days of the sampling period only a single fresh sample was collected.

6.3.5 FECAL HORMONE EXTRACTION

Fecal hormone metabolites were extracted using methods previously described by Metrione et al. (2008) with the following modifications. Briefly, after manual homogenization of the sample, ~0.5 g wet fecal material was mixed with 4 mL methanol (reagent grade, Fisher Scientific, Fair Lawn, NJ, USA) and 1 mL reverse osmosis-purified water. Samples were then shaken in a Glas-Col Large Capacity Mixer (Glas-Col LLC, Terre Haute, IN, USA) for 20 min at 90 rpm followed by centrifugation for 10 min at 3100 rpm. Supernatant (200μL) was

transferred to a duplicate set of 12 mm x 75 mm tubes (Perfector Scientific, Atascadero, CA, USA) for each sample. One set was stored at −20 °C until analysis; the second set was evaporated to dryness before subsequent freezing at −20 °C.

6.3.6 *ENZYME IMMUNOASSAYS (EIA) & VALIDATION*

Fecal extracts were analyzed in two separate laboratories to maximize the number of EIAs available for evaluating glucocorticoid metabolites. Italicized terms are the terms we have used to refer to the individual hormone assays throughout the study. Cross-reactivities for all of the assays are provided in Appendix VIII. All samples, controls, and standards were assayed in duplicate.

Lab A (SEZARC, Yulee, FL, USA) used a polyclonal cortisol antibody (*cortisol*: R4866, Coralie Munro, University of California, Davis, CA, USA), a polyclonal corticosterone antibody (*CC*: CJM006, Coralie Munro, University of California, Davis, CA, USA), and a testosterone antibody (*Testo*: C196, Arbor Assays, Ann Arbor, MI, USA). A double-antibody protocol was used with the following specific dilutions: (1) antibody was diluted in assay buffer (0.1 M $NaPO_4$, 150 mM NaCl, 0.1% BSA) at 1:25,000 for cortisol and CC, and 1:50 for Testo; (2) HRP conjugate was diluted in assay buffer at 1:100,000 for cortisol, 1:150,000 for CC, and 1:50 for Testo (C197, Arbor Assays, Ann Arbor, MI, USA); (3) standards were 3.9–1000 pg/well for cortisol and CC, and 2.0–1000 pg/well for Testo (C198, Arbor Assays, Ann Arbor, MI, USA).

A pool of randomly selected sample extracts was prepared for both males and females. Parallel displacement curves were generated by comparing serial dilutions of these pooled extracts (cortisol, CC and Testo; 1:2−1:1024) with known standards for each hormone. Samples and standards were diluted in assay buffer and the optimal dilution yielding approximately 50% binding was as follows for each assay: cortisol, 1:10 male and female (sample % binding = 454 + 118 [standard % binding], $r^2 = 0.6548$, $F_{1,11} = 10.4$, $P = 0.003$); CC, 1:50 male and female (sample % binding = 859 + 67.5 [standard % binding], $r^2 = 0.691$, $F_{1,11} = 12.3$, $P = 0.002$); and

Testo, 1:50 for male and female (sample % binding = 0.249 + 0.003 [standard % binding], r^2 = 0.795, $F_{1,9}$ = 17.4, P < 0.001).

Microtiter plates (96-well; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were coated with 150 µL of 0.010 mg/mL goat anti-rabbit IgG (Arbor Assay, Ann Arbor, MI, USA) and incubated overnight at room temperature (approximately 20°C). Plates were then washed with 0.008% Tween 20 (400 µL in 5 L RO water; Sigma Aldrich, St. Louis, MO, USA) wash buffer to remove unbound antibody. Subsequently, 250µL of blocking buffer (Catalog # X-109; Arbor Assays, Ann Arbor, MI, USA) was added to each well and plates were again incubated at room temperature for an additional 12 h. Finally, the buffer was removed and plates were dried, packed into airtight containers, and stored at 4°C.

For the assays, 50 µL of standards, controls and diluted fecal extracts were added to each well followed by 50 µL of HRP-conjugate solution and finally 50 µL of antibody. After incubation for 2 h at room temperature, plates were washed four times with 300 µL of 0.008% Tween 20 wash buffer and 100 µL color substrate solution (ABTS; 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt, Sigma Aldrich, St. Louis, MO, USA) was added to each well. Plates were read at 405 nm using an optical density plate reader (Dynex Technologies, Chantilly, VA, USA) when the zero standard reached the target optical density of 1.00. The intra- and inter-assay coefficients of variation for all assays were < 10% (average 2.27%) and < 20% (average 5.22%), respectively. The minimum assay sensitivities, determined at 90–95% binding, were 3.17 pg/well for cortisol, 3.90 pg/well for CC, and 2.3 pg/well for Testo.

There was no evidence of matrix interference for Testo, as demonstrated by the recovery of 108% (at 1:50) of pooled fecal extract added to hormone standards (2.0–1000 pg/well; $F_{1,17}$ = 20,270, $y = 1.0295x + 0.0006$, r^2 = 0.9998, P < 0.001). The cortisol and CC EIAs were not biologically validated for assessing fecal glucocorticoid metabolites. Thus, recovery was not

evaluated and neither EIA was investigated further at Lab A. Data are reported as ng/g fecal wet weight for cortisol, CC and Testo.

Lab B (Vienna, Austria) received the duplicate set of dried, frozen fecal extracts and reconstituted each sample with 200µL 80% reagent grade methanol. Lab B analyzed these aliquots using previously performed group-specific EIAs with rabbit-origin polyclonal antibodies against the following: *i*) cortisol (*CORT*: 4-pregnene-11β,17α,21-triol-3,20-dione, Palme and Möstl 1997); *ii*) corticosterone (*CCST*: 4-pregnene-11β,21-diol-3,20-dione, Palme and Möstl 1997); *iii*) *5α-3β,11β-diol-CM* (5α-pregnane-3β,11β,21-triol-20-one-CMO:BSA, Touma et al. 2003); *iv*) 11β-hydroxy-etiocholanolone (*3α,11ß-dihydroxy-CM*; 5β-androstane-3α,11β-diol-17-one-CMO:BSA, Frigerio et al. 2004); *v*) 11-oxo-etiocholanolone-I (*11,17-DOA*; 5β-androstane-11,17-dione-3-HS:BSA, Palme and Möstl 1997); *vi*) 11-oxo-etiocholanolone-II (*3α,11-oxo-CM*; 5β-androstane-3α-ol-11-one-17-CMO:BSA, Möstl et al. 2002). The intra- and inter-assay coefficients of variation for all assays were < 10% and < 15%, respectively. The detection limits were 1.5 pg/well for CORT; 2.0 pg/well for CCST; 0.8 pg/well for 5α-3β-11β-diol-CM; 2.0 pg/well for 3α,11ß-dihydroxy-CM; 3.0 pg/well for 11,17-DOA; and 3.0 pg/well for 3α,11-oxo-CM. Serial dilutions of fecal extracts yielded a displacement curve parallel to the standard curve for all four EIAs. There was no evidence of matrix interference, demonstrated by significant recovery of pooled fecal extract added to the standards. Data are reported as ng/g fecal wet weight for CORT, CCST, 5α-3β-11β-diol-CM , 3α,11ß-dihydroxy-CM, 11,17-DOA and 3α,11-oxo-CM.

6.3.7 DATA ANALYSIS

6.3.7.1 STABILITY OF HORMONE METABOLITES IN FECAL SAMPLES

We calculated the percentage change in androgen (Testo) and glucocorticoid (CC) metabolite concentrations within the fecal samples relative to time 0 for each time point of subsample freezing. An increase or decrease of more than 10% from the value at time 0 was considered a significant change and indicative of hormone degradation.

6.3.7.2 ACTH CHALLENGE

We calculated baseline concentrations separately for each of the seven pygmy hippos for each glucocorticoid EIA using the mean of values for the samples collected before ACTH injection. All concentrations for pre- and post-ACTH challenge or saline control were subsequently calculated as a percent of each animal's baseline. We then compared results for the eight glucocorticoid metabolite EIAs to determine which assay exhibited the most biologically relevant pattern for a stress response after ACTH challenge, and a lack of stress response for the saline control.

6.3.7.3 FECAL ANDROGEN METABOLITES

As baseline values, we used an average of the lowest 10% of the samples for each pygmy hippo. Peak values were calculated as a percentage of each hippo's baseline. Androgen metabolite concentrations were then plotted over time for the duration of the sampling period. We first used Shapiro-Wilk tests for normality and then used paired *t*-tests to compare mean androgen metabolite concentrations between adult males (n = 10 hippos; n = 631 samples) and females (n = 4 hippos; n = 166 samples) and for adult versus juvenile (n = 2 hippos; n = 96 samples) males. One-way ANOVA was used to compare mean concentrations between adult males overall, followed by paired *t*-tests to compare mean concentrations for adult proven breeding males (n = 7 hippos; n = 392 samples) with those for adult males that have not reproduced despite having access to a breeding-age female (n = 2 hippos; n = 163 samples). Additionally, the data were grouped by northern hemisphere season as follows: spring (March, April, May); summer (June, July, August); fall (September, October, November); winter (December, January, February) and paired *t*-tests (2 groups) or one-way ANOVA (> 2 groups) was used to compare seasons for adult male pygmy hippos. Finally, mean androgen metabolite concentrations were compared between adult males housed outside year-round in subtropical climates (n = 5 hippos; n = 279 samples) and adult males in temperate climates that are housed indoors either year-round or in colder weather (n = 5 hippos; n = 352 samples). For all statistical analyses, values of $P < 0.05$ were considered significant.

6.4 RESULTS

6.4.1 STABILITY OF HORMONE METABOLITES IN FECAL SAMPLES

In the fecal samples that were left at ambient outdoor temperature (17–26°C), concentrations of glucocorticoid metabolites (CC) did not change by more than 10% from starting concentrations until 24 h after defecation (Fig. 6-1). Thus, samples collected up to 20 h post-defecation for analysis with the CC EIA will be representative of concentrations at time 0 if ambient conditions are similar. For androgen metabolites (Testo), some degradation was noted at 4 h post-defection for the male (Fig. 6-1a) and 8 h post-defecation for the female (Fig. 6-1b). However, androgen metabolite concentrations did not differ by more than 20% from time 0 concentrations until 24 h post-defecation for both the male and female. It is therefore ideal to collect fecal samples for androgen metabolite analysis using the Testo EIA within 4 h post defecation; however, samples collected up to 20 h post-defecation will still be representative of concentrations at time 0 (again, under similar ambient conditions).

6.4.2 GASTROINTESTINAL TRANSIT TIME

The time between consumption of the fecal marker and the first passage of glitter in the feces was 20 h for the female and 30 h for the male. Peak glitter excretion occurred between 30 and 46 h for the female and between 48 to 70 h for the male. Thus, gastrointestinal transit time for pygmy hippos can vary between animals fed the same diet, but ranges from one to three days. Hormone metabolite levels measured in the feces are therefore likely to represent endocrine events that occurred in the previous 24–48 h.

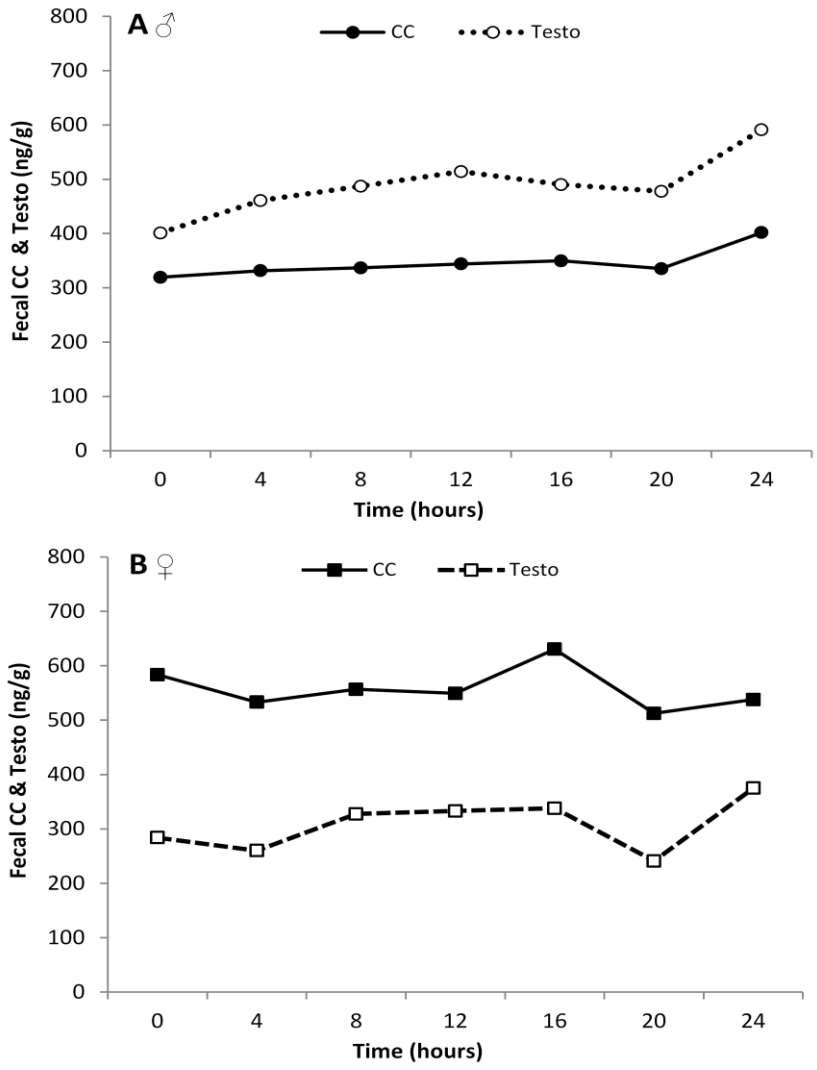


Fig. 6-1 - Glucocorticoid (CC, R4859; ●) and androgen (Testo, C196; ○) metabolite concentrations over a 24 hour period post-defecation for one male **(A)** and one female **(B)** pygmy hippo.

6.4.3 ACTH CHALLENGE

Samples from the ACTH challenge were initially analyzed in Lab A. For both males (n = 4) and females (n = 3), neither the cortisol nor the CC assay demonstrated biologically relevant patterns (increased concentration followed by return to baseline after injection of short- or long-acting ACTH) that would suggest the assays could be used to detect a stress response. Additionally, immunoreactivity and profile patterns for the Testo EIA closely paralleled those for the CC EIA for both male and female hippos (Fig. 6-2), and the concentrations of the hormone metabolites as measured by these two EIAs exhibited significant positive correlation for both males (Pearson's r = 0.736, $P < 0.001$) and females (Pearson's r = 0.583, $P < 0.001$).

These results suggest that the Testo and CC EIAs cross-react with a similar spectrum of hormone metabolites in pygmy hippo feces, thereby confounding results.

Due to suspected cross-reactivity between the CC and Testo EIAs and the difficulty of biological interpretation for both cortisol and CC, pre- and post-ACTH challenge samples were subsequently analyzed in Lab B using six additional glucocorticoid metabolite assays. A comparison of these six EIAs is shown in Fig. 6-3. The 11,17-DOA assay demonstrated the most logical pattern post-ACTH challenge. For long-acting ACTH, there was a marked increase in glucocorticoid metabolite concentrations above baseline 1 to 2 days post-injection followed by a return to baseline within 3 to 4 days post-injection. The male exhibited a longer duration of response than the female, but the peak concentration for both animals was similar (1660 and 1670% above pre-injection baseline). For short-acting ACTH, there was also an increase in 11,17-DOA concentrations above baseline on the day after injection with a return to baseline the following day (e.g. Fig. 6-3c). As expected, the magnitude and duration of response was much less pronounced for short-acting than long-acting ACTH, but patterns were otherwise similar. Of all the assays tested, including cortisol and CC in Lab A, the 11,17-DOA assay most accurately reflects glucocorticoid metabolite concentrations in the feces of pygmy hippos.

Pre-injection baseline values for 11,17-DOA ranged from 1.7 to 23.6 ng/g with a mean of 9.3 ng/g for all hippos (n = 8), including the saline control. For long-acting ACTH, post-injection peaks were more than 1600% above baseline and were significantly different to pre-injection concentrations for both the male (137.5 ng/g; $P < 0.01$) and female (96.3 ng/g; $P < 0.01$). Peak concentrations were reached approximately 24 h post-injection for the female and 48 hours post-injection for the male (Fig. 6-4), consistent with the shorter gastrointestinal transit time observed for the female. Similarly, concentrations returned to pre-injection baseline values by 72 h post-injection for the female and 96-h post-injection for the male. For the saline control, post-injection 11,17-DOA concentrations did not differ significantly from pre-injection concentrations (P = 0.937). Four hippos (2 ♂, 2 ♀) also showed a less substantial, shorter

increase in androgen metabolite concentrations following ACTH challenge (e.g. Fig. 6-5, ♀), whereas three hippos (2 ♂, 1 ♀) did not (e.g. Fig. 6-5, ♂).

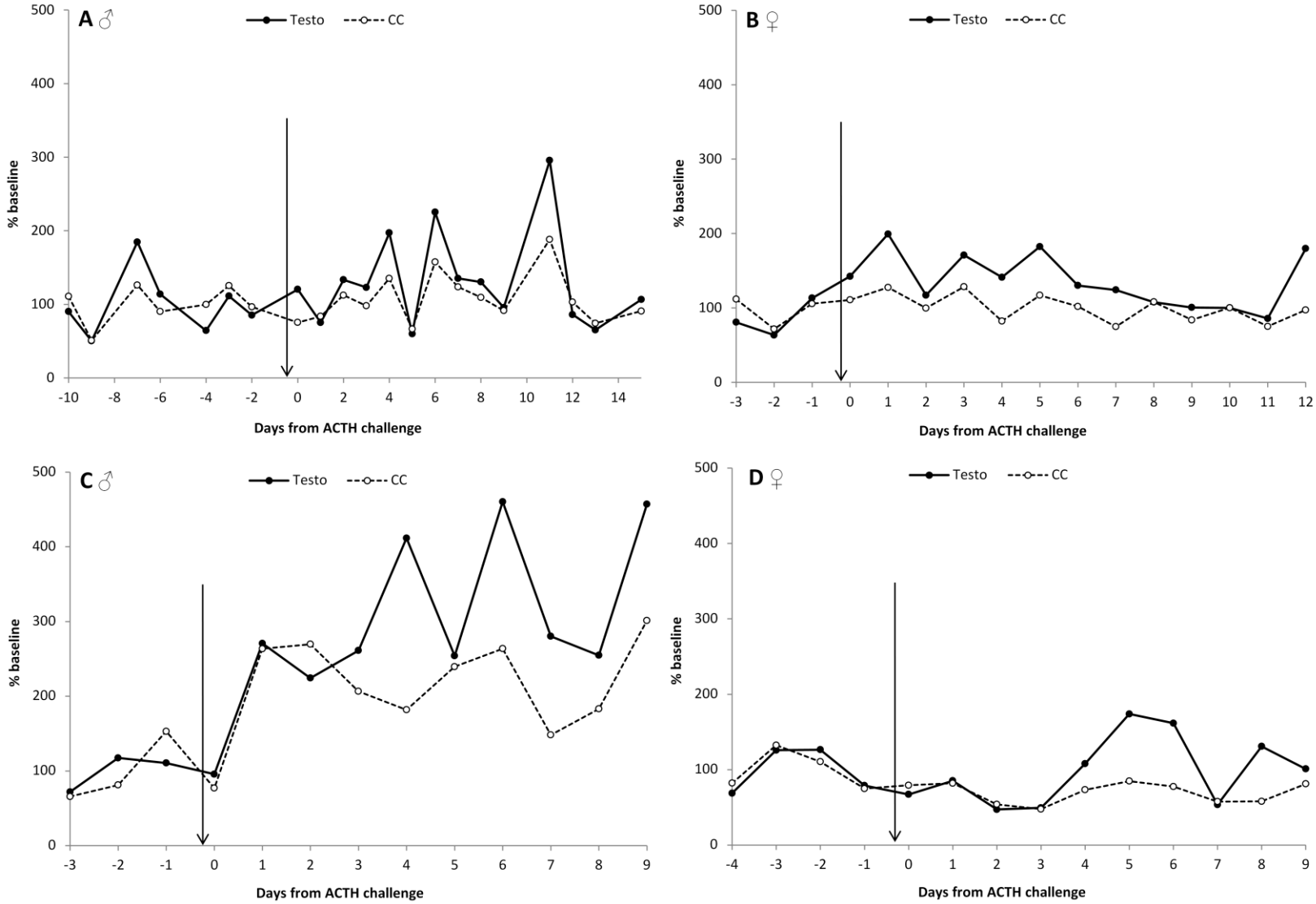

A ♂
Testo
CC
% baseline
Days from ACTH challenge
B ♀
Testo
CC
% baseline
Days from ACTH challenge
C ♂
Testo
CC
% baseline
Days from ACTH challenge
D ♀
Testo
CC
% baseline
Days from ACTH challenge

Fig. 6-2 - Changes in concentrations of CC (R4859; ○) and Testo (C196; ●), presented as a percentage of baseline, pre- and post-ACTH challenge in four pygmy hippos.

Time of injection is denoted by the arrow.
A) Male and **B)** female; short-acting ACTH. A rise in glucocorticoid metabolites (CC) followed by return to baseline is not discernable. Patterns in androgen (Testo) and glucocorticoid (CC) metabolite profiles mirror each other.
C) Male and **D)** female; long-acting ACTH. For the male, concentrations for both Testo and CC increase from baseline immediately after ACTH challenge, but they do not return to baseline within 9 days post-injection. Again, patterns are very similar for both EIAs. For the female, neither Testo nor CC concentrations increase from baseline after ACTH challenge while patterns for both EIAs are again very similar.

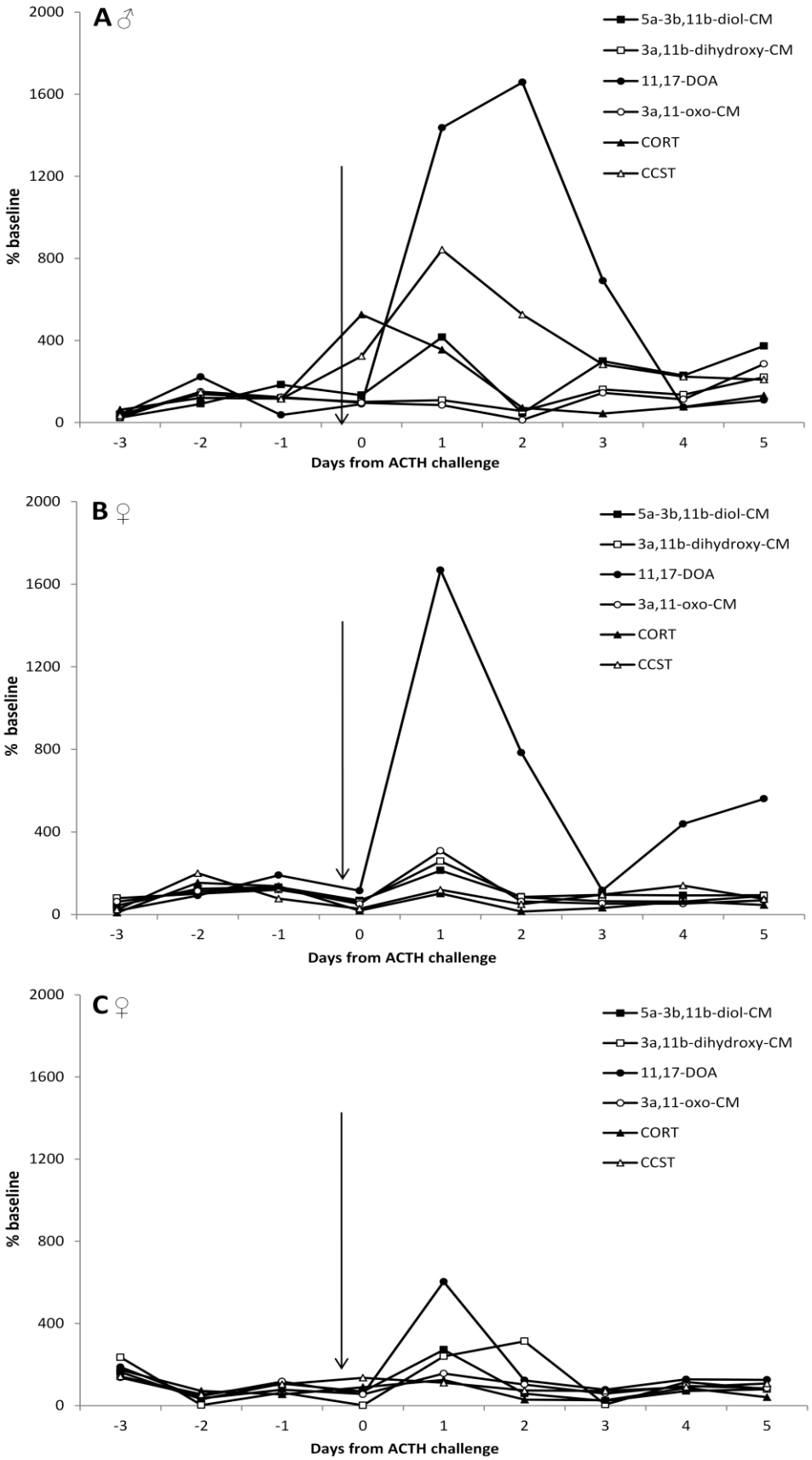


Fig. 6-3 - Glucocorticoid metabolite profiles for thee pygmy hippos, pre- and post-ACTH challenge, analyzed with six EIAs: **i)** 5α-3β,11β-diol-CM; **ii)** 3α,11β-dihydroxy-CM; **iii)** 11,17-DOA; **iv)** 3α,11-oxo-CM; **v)** CORT; **vi)** CCST.

Fig. 6–3 Con't. Time of injection is denoted by the arrow. The 11,17-DOA assay shows the most biologically relevant response to ACTH and detected a physiologic stressor in all cases.
A) Male, long-acting ACTH; 11,17-DOA concentrations peak at more than 1600% above pre-injection baseline and remain elevated for 3 days before returning to baseline.
B) Female, long-acting ACTH; 11,17-DOA concentrations also peak at more than 1600% above pre-injection baseline and remain elevated for 2 days before returning to baseline.
C) Female, short-acting ACTH; 11,17-DOA concentrations peak at 600% above pre-injection baseline and return to baseline the following day.

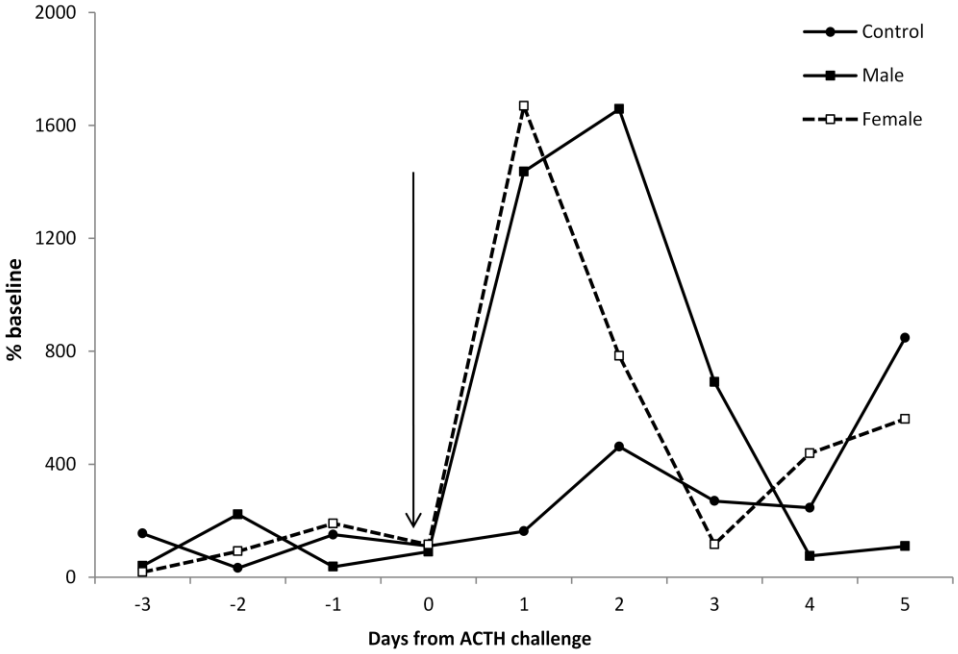


Fig. 6-4 - Changes in concentrations of 11,17-DOA in pygmy hippos pre- and post-ACTH challenge with long-acting ACTH (or sterile saline as a control).

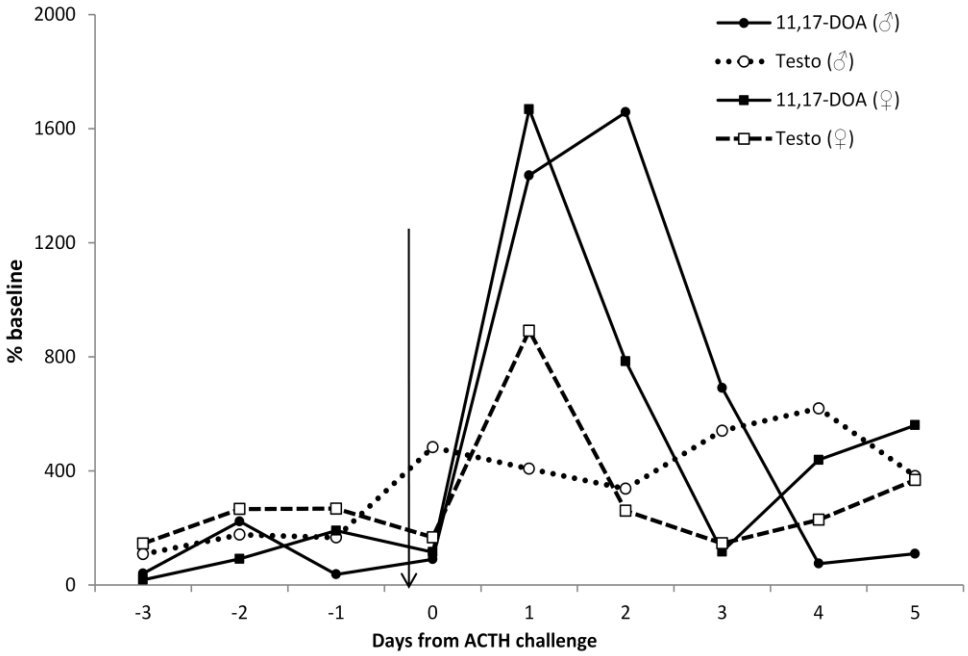


Fig. 6-5 - Changes in concentrations of 11,17-DOA (solid symbols) and Testo (open symbols) in pygmy hippos pre- and post-ACTH challenge with long-acting ACTH.

Time of injection is denoted by the arrow. The male (circles) did not exhibit an increase in androgen metabolites post-ACTH challenge, indicating that the 11,17-DOA assay is cross-reacting with glucocorticoid metabolites and the Testo assay is not. The female (squares) showed a modest increase in androgen metabolites post-ACTH challenge; the response was less pronounced and more transient than for glucocorticoid metabolites and may be reflective of adrenal androgens.

6.4.4 *REPRODUCTIVE PATTERNS – FECAL ANDROGEN METABOLITES*

To demonstrate biological relevance for the Testo EIA, we compared metabolite concentrations for a proven breeding male, a geriatric male (aged 40 years) held at a facility without a female and presumed to be reproductively senescent, and two juvenile males that were 1.5 years old at the start of the sampling period (Fig. 6-6). Additionally, we compared mean concentrations for the 10 adult males in our study with the four adult females and the two juvenile males Table 6.1. Overall mean concentrations of androgen metabolites were significantly higher for adult males (470 ng/g) than for juvenile males (147 ng/g; $P < 0.001$) or adult females (270 ng/g; $P < 0.001$). In most mammals, adult males have higher circulating testosterone levels than juveniles or females, and consequently would also be expected to have higher fecal androgen metabolite concentrations. Therefore, the Testo EIA was considered biologically relevant for assessing androgen metabolites in pygmy hippo feces.

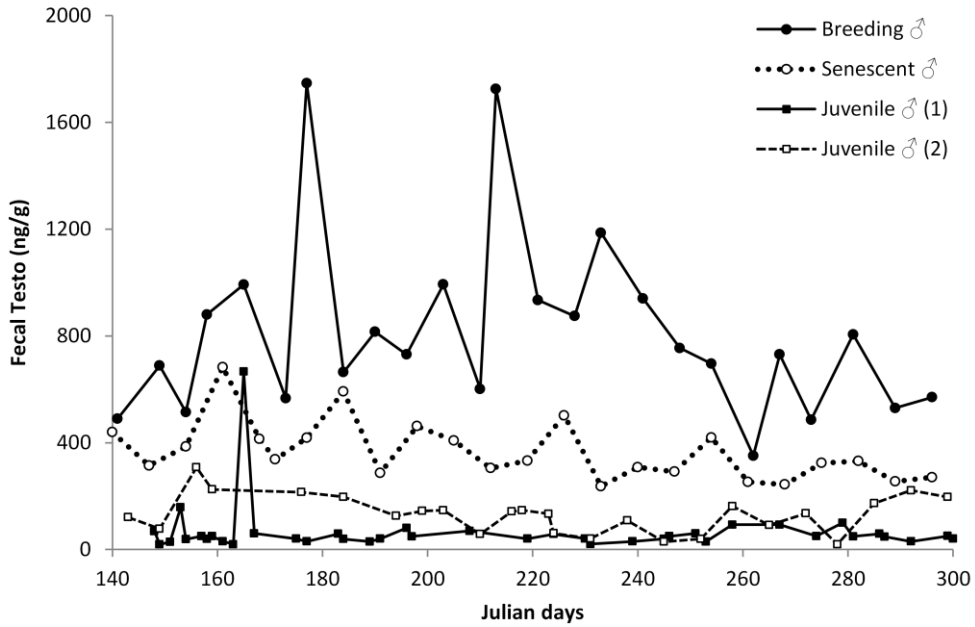


Fig. 6-6 - Androgen metabolite (Testo; C196) concentrations in an adult breeding male, a reproductively senescent male, and two juvenile male pygmy hippos.

The breeding male exhibits the highest concentrations, while the two juvenile males both exhibit relatively low androgen metabolite levels. The senescent male, housed at a facility without a female, exhibits intermediate androgen metabolite concentrations. These results demonstrate biological relevance for the Testo EIA.

Table 6.1 - Mean (± SD) fecal androgen metabolite concentrations in pygmy hippos.

The data are presented individually for each hippo as well as grouped for adult males, juvenile males, and adult females. Unproven adult males are marked with a (*).

Studbook No	Sex	Age (years)	Mean fecal androgens (ng/g)	Overall
460	♂	40+	358 ± 103	
880	♂	23	289 ± 124	
902	♂	20.5	674 ± 471	
919*	♂	20	276 ± 155	
996	♂	17	607 ± 450	adult ♂ (*n* = 10)
1053	♂	14.5	746 ± 380	Mean 470 ± 205
1093*	♂	12.5	372 ± 234	Range 69–3146
1135	♂	10.5	335 ± 171	
1241	♂	6	267 ± 86	
1359	♂	3	771 ± 591	
1422	♂	1.5	228 ± 135	juvenile ♂ (*n* = 2)
1423	♂	1.5	51 ± 26	Mean 147 ± 114 Range 19–666
931	♀	19	281 ± 105	
1063	♀	14	268 ± 60	adult ♀ (*n* = 4)
1147	♀	10	242 ± 206	Mean 270 ± 21 Range 20–1094
1392	♀	3.5	290 ± 215	

Baseline concentrations of androgen metabolites ranged between 95–322 ng/g for the 10 adult males and between 56–123 ng/g for adult females. Peak values for adult males ranged between 280–1860% above baseline. Androgen metabolite peaks were noted throughout the year. Baseline was 24 ng/g and 41 ng/g for the two juvenile males. One of the two juveniles (Studbook No. 1423; Fig. 6-6, #1) was still housed with his dam during the entire sampling period, whereas the other (Studbook No. 1422; Fig. 6-6, #2) was moved to a zoo with a breeding-age adult female on Julian Day 197. This male's androgen metabolite concentrations remained low (< 100 ng/g) until Day 285; during the subsequent six months they slowly increased to reach ~ 600 ng/g by the end of the sampling period.

There was a significant difference in mean androgen metabolite concentrations between individual adult males (F = 24.1, df = 9, P < 0.001). Additionally, mean concentrations were significantly higher in proven breeding males (474 ng/g) than in adult males that have not reproduced (341 ng/g; P < 0.001). Mean androgen metabolite concentrations also differed significantly between seasons (F = 6.64, df = 3, P < 0.001) for adult males (Fig. 6-7). Mean values in spring (538 ng/g) were significantly higher than in fall (422 ng/g; P = 0.002) and winter (389 ng/g; P < 0.001). Mean values in summer (543 ng/g) were also significantly higher than in fall (P = 0.003) and winter (P < 0 .001). There was no difference between mean values in spring and summer (P = 0.913) or fall and winter (P = 0.323). Adult males housed outdoors year-round in subtropical climates (Appendix VII) had significantly higher mean androgen metabolite concentrations (554 ng/g) than adult males in temperate-climates that were housed indoors year-round or in the winter months (412 ng/g, P < 0.001).

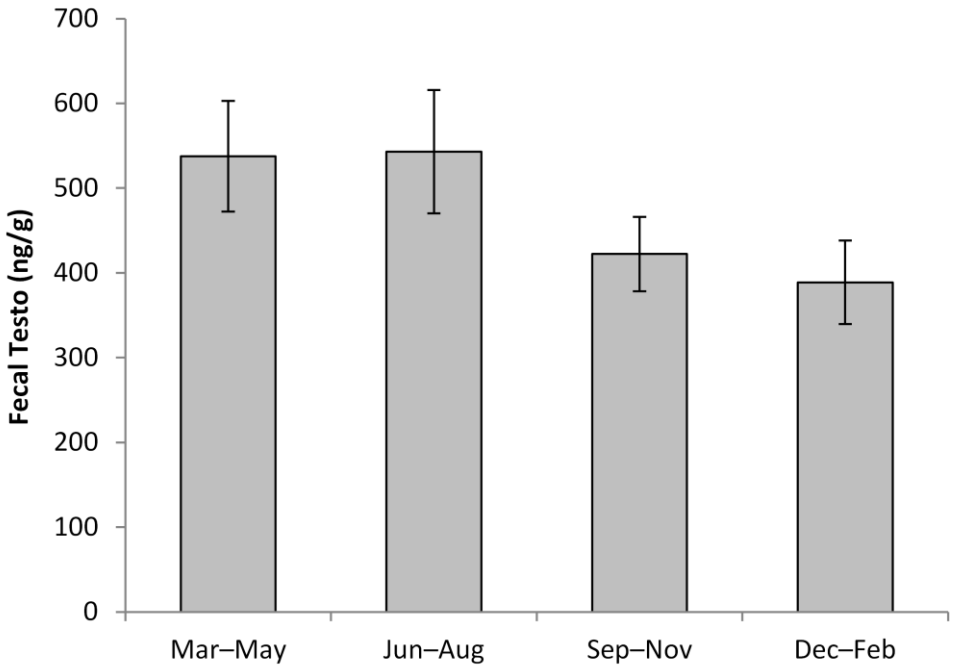


Fig. 6-7 - Changes with season in mean concentrations of androgen metabolites (Testo; C196) in adult male pygmy hippos (n = 10).

Concentrations in spring (Mar–May) and summer (Jun–Aug) are significantly higher than concentrations in fall (Sep–Nov) and winter (Dec–Jan). Error bars represent 95% confidence intervals of the mean.

6.5 DISCUSSION

6.5.1 *FECAL GLUCOCORTICOID METABOLITES AND ACTH CHALLENGE*

For the measurement of glucocorticoid metabolites in feces, the group-specific antibody exhibiting cross-reactivity with 11,17-dioxoandrostane (DOA) metabolites of cortisol clearly reflects adrenocortical activity in both male and female pygmy hippos. This study emphasizes the need to validate the biological relevance of an EIA for each hormone under investigation, especially in males where androgen and glucocorticoid metabolites may exhibit considerable cross-reactivity, and highlights the need for judicious interpretation of results. This is particularly relevant when using an ACTH challenge to induce a physiological response because ACTH can stimulate not only the zona fasciculata and glucocorticoid production but the entire adrenal cortex, resulting in a concurrent increase in circulating adrenal androgens (dehydroepiandrosterone) from the zona reticularis. Cross-reaction of an EIA with adrenal androgens must be considered when adrenal stimulation results in an increase in fecal androgens. In this study there was significant cross-reactivity between CC and Testo for both males and females, although the correlation was stronger in males. Additionally, two male and two female pygmy hippos also showed a marginal increase in androgen metabolite concentrations following ACTH challenge (two males and one female did not). These responses were more transient and less-pronounced than post-injection increases in glucocorticoid metabolites and suggest that a portion of the androgen metabolites measured by the Testo EIA could be of adrenal origin.

This study also verifies the advantage of using a long-acting ACTH product for challenge studies, especially in herbivores. Although a glucocorticoid response was also noted with the short-acting product, it was noticeably dampened in duration and amplitude, and, in some hippos, it was not discernible. Short-acting ACTH products have been used to induce a temporary adrenocortical response in several other species but, with the exception of the white tailed deer (*Odocoileus virginianus*), these studies were primarily conducted in carnivores and

primates and the ACTH was often administered intravenously, not intramuscularly, for more immediate effect (Heintz et al. 2011; Heistermann et al. 2006; Schatz & Palme 2001; Shepherdson et al. 2013; Washburn & Millspaugh 2002; Young et al. 2004). Carnivores and some omnivores have a markedly shorter gastrointestinal transit time, and they also tend to defecate only once or twice per day, so a single fecal sample is more likely to reflect a transient stress response. However, in species with large fecal mass and frequent defecation (e.g. larger herbivores), it is recommended to use a sustained-release, long-acting ACTH product so short-term changes in adrenal status are not diluted within the voluminous gastrointestinal tract (Wasser et al. 2000).

There was clear variation among individuals in response to ACTH challenge, particularly for the male and female injected with the long-acting product, reflecting the link between gastrointestinal transit time and the excretion of hormone metabolites in the feces. The delay to initial, peak, and cessation of glitter excretion was shorter in the female than in the male, and the initial peak in glucocorticoid metabolite concentrations as well as the return to baseline values occurred earliest in the female. Marked differences between sexes and individuals in excretion lag time, baseline cortisol levels, and the magnitude of stress response, has been highlighted for several other mammalian species, including the clouded leopard (*Neofelis nebulosi*), the spotted hyena (*Crocuta crocuta*), the brown hyena (*Hyaena brunnea*), the cheetah, the southern elephant seal (*Mirounga leonina*), and the African elephant (Engelhard et al. 2002; Ganswindt et al. 2003; Hulsman et al. 2011; Ludwig et al. 2013; van Jaarsfeld and Skinner 1992; Wielebnowski et al. 2002). Some species, such as the domestic mouse, also exhibit sex differences in the metabolism and chemical structure of glucocorticoids (Touma et al. 2003). It is therefore essential to recognize the potential for significant temporal and concentration differences between individuals when using non-invasive endocrine monitoring to assess baseline glucocorticoids and to characterize the magnitude of a stress response.

Individual variation in both baseline glucocorticoid levels and degree of stress response is linked to many factors, including genetics and variation in the experience of the individuals in

their pre-natal, post-natal and adult life stages, all of which can affect temperament (Moberg 2000; Cockrem 2013). Additional factors influencing the secretion of glucocorticoids and the excretion of associated metabolites include: the magnitude of the stress response, diet, body condition, metabolic rate, reproductive status, season, social environment and rank (Creel et al. 2013; Jachowski et al. 2015; Millspaugh & Washburn 2004; Palme et al. 2005). The male and female pygmy hippo injected with long-acting ACTH in this study were the same age, housed at the same facility, fed the same diet, of comparably body condition, exposed to the same environmental factors, and neither was in a breeding situation. The difference in temporal response to the same stressor may therefore be linked to sex, but only two individuals were observed so further research is necessary to determine if there are consistent sex differences.

It is important to note that neither the cortisol nor the CC EIAs demonstrated biological relevance for measuring glucocorticoids in pygmy hippos. Assays using a corticosterone antibody have been biologically validated for a large number of diverse mammalian species, as have assays that measure native cortisol, particularly for carnivores and some primates (Keay et al. 2006; Young et al. 2004). However, the digestive physiology of herbivores results in more extensive metabolism of native hormones by bacterial flora in the gastrointestinal tract (Möstl et al. 1999), especially in foregut fermenters like ruminants, camelids, and hippos. Additionally, herbivores tend to have slower gastrointestinal transit times compared to similarly sized carnivores or omnivores, offering more time for hormone metabolism before excretion.

High pressure liquid chromatography (HPLC) analysis has demonstrated cortisol metabolites with an 11,17-DOA structure in the feces of a number of other species including cattle, sheep, horses, pigs, guinea pigs, African elephants, African buffalo, okapi (*Okapi johnstoni*), roe deer (*Capreolus capreolus*), chamois (*Rupicapra rupicapra*), banded mongoose (*Mungos mungo*), long-tailed macaques (*Macaca fascicularis*), common marmosets (*Callithrix jacchus*), chimpanzees, and domestic dogs and cats (Bahr et al. 2000; Dehnhard et al. 2001; Ganswindt et al. 2012; Hadinger et al. 2015; Laver et al. 2012; Möstl et al. 1999; Palme et al. 1998; Schatz and Palme 2001; Schwarzenberger et al. 1998; Stead et al. 2000). Additionally, the biological

relevance of this EIA for measuring a physiologic stress response has been established for most of these species, and studies investigating seasonal, environmental, and disease-associated variations in stress levels have been conducted in free-ranging populations of chamois and banded mongoose (Hadinger et al. 2015; Laver et al. 2012). Thus, this EIA may prove to be a valuable tool for monitoring stress in wild pygmy hippos, particularly in response to anthropogenic environmental changes as a result of extensive logging, mining, and bush-meat hunting in their West African rainforest habitat (Collen et al. 2011; Hoppe-Dominik et al. 2011; Lindsell et al. 2011; Norris et al. 2010).

6.5.2 MALE REPRODUCTIVE PATTERNS

The Testo EIA used for measuring androgen metabolite concentrations in pygmy hippo feces in the present study produces data that are biologically coherent. Adult males exhibited the highest Testo values, followed by adult females and juvenile males, and proven breeding males had significantly higher concentrations than unproven males, although the sample size for unproven adult males was limited. The Testo EIA was developed for measuring native testosterone and also exhibits cross-reactivity with dihydrotestosterone (35%), although cross-reactions with other androgen metabolites have not been assessed (Appendix VIII). HPLC analysis has shown the presence of native testosterone in the feces of a number of ungulate species, including the Chinese goral (*Naemorhedus griseus*), the plains bison (*Bison bison bison*) and the black (*Diceros bicornis*) and white rhinoceros, as well as in the spotted hyena (Brown et al. 2001; Dloniak et al. 2004; Khonmee et al. 2014; Kretzschmar et al. 2004; Mooring et al. 2004). For the goral, native testosterone constituted about 25% of the androgens present in the dung (Khonmee et al. 2014). We did not perform HPLC in this study, but the validity of the Testo assay for assessing androgen activity in the pygmy hippo suggests this species also excretes a percentage of un-metabolized testosterone in the feces.

There was a considerable variation among the 10 individual adult males in androgen metabolite profiles, both in patterns and concentrations. However, their ages ranged from three to more than 40 years, and each male was housed at a different zoological facility, fed a different diet,

and subjected to different environmental and husbandry conditions. With so many confounding factors, we cannot formulate hypotheses to explain the variation, but it is clear that an individual's hormone patterns must be interpreted within the context of that animal's baseline, not a population-wide mean.

Even though juvenile males consistently exhibited lower androgen metabolite concentrations than adults, there was overlap between nadir concentrations for adults and the upper end of the range for juveniles. There was also overlap in the range of concentrations for adult males and females. Thus, a single point-in-time measurement of androgen metabolites in pygmy hippo feces with the Testo EIA cannot be use used to distinguish a male from a female or an adult from a juvenile, limiting the applicability of this assay to field studies when the identity of the individual animals is unknown.

For one juvenile male, there was evidence of increasing androgen concentrations three months after being introduced to a breeding-age female even though the animal was only 19 months old at the time of introduction. Testo immunoreactivity continued to steadily increase from that point forward until the end of the sampling period, peaking at 1400% of baseline when the animal was 26 months old. This trend suggests the onset of puberty and impending sexual maturity in this young male, possibly triggered by the presence of a breeding-age female.

Of particular interest is the observation that androgen metabolite concentrations were higher in the eight proven breeding males than in the two males that had not successfully reproduced, although one of these two males wasn't provided with many opportunities to breed with the female because of agonistic interactions and concerns for animal safety. We were unable to correlate the androgen metabolite concentrations with other measures of male reproductive potential because testicular size and ejaculate quality were not addressed in this study. Information concerning reproductive endocrinology between proven and unproven breeding males is limited for non-domestic species and the patterns are variable. For the maned wolf (*Chrysocyon brachyurus*), captive males with proven fertility had a higher proportion of structurally normal sperm than unproven males, and spermatogenesis showed a positive

correlation with testosterone metabolite concentrations (Comizzoli et al. 2009). For eight species of endemic wild felids housed in Latin American zoos, proven breeders had significantly larger testicular size, semen volumes, and higher circulating testosterone concentrations than unproven conspecifics (Swanson et al. 2003). On the other hand, there was no difference in testicular size, semen quality, or circulating testosterone concentrations in proven versus unproven male cheetah in the North American zoo population (Wildt et al. 1993). Similarly, in a study investigating captive black and white rhinos in North American zoos, there was no significant difference in androgen metabolite concentrations between proven and unproven breeding males (Brown et al. 2001). However, for the critically endangered eastern black rhinoceros (*Diceros bicornis michaeli*) within European zoos, males that had sired calves also had significantly higher concentrations of fecal androgen metabolites than unproven males (Edwards et al. 2015). Further research investigating semen characteristics in conjunction with testosterone levels is needed to establish if these variables are correlated in male pygmy hippos and if lower fecal androgen metabolite concentrations are associated with reduced reproductive success in this species.

The seasonal differences in androgen metabolite concentrations are also of interest given this species is non-seasonally polyestrus in captivity (see Chapter 5) and births occur in all months of the year (Steck 2016). Reproductive seasonality is generally dictated by photoperiod and resource availability, although ambient temperature and rainfall can also play a role due to their influence on resource availability and body condition. In general, species whose natural environment provides a relatively constant level of these elements will reproduce year-round, whereas species endemic to habitats with marked variability are seasonal breeders (Bronson 1985). We have no comparable information for wild populations of pygmy hippos, but their ecologic life history traits largely support year-round reproduction: the species is endemic to a very limited region of tropical, equatorial West African rainforest where temperature and light cycle remain relatively constant throughout the year, although there is seasonal variation in rainfall (Critical Ecosystem Partnership Fund 2000; Lindsell et al. 2011; Verschuren 1983). It is

unknown if fluctuations in rainfall patterns place any limitations on food availability or other environmental resources necessary for reproduction.

Under human care, wildlife does not experience resource limitations so only photoperiod and ambient temperature have the potential to influence seasonality of reproduction. In a review of 110 ruminant species housed in temperate-climate zoos worldwide, all of the species with non-seasonal reproduction in the wild were endemic to tropical climates and latitudes, leading the authors to conclude that latitude (in its relationship to day length) is the most important factor influencing reproductive seasonality (Zerbe et al. 2012). Additionally, 99 of these 110 species exhibited conserved reproductive patterns between the wild and captivity, leading the authors to conclude that for most species, seasonality of reproductive patterns is a genetically determined (Zerbe et al. 2012). Similar findings have been reported for domestic ruminants from subtropical and temperate latitudes (Delgadillo et al. 2004; Martin et al. 1999). A limited number of studies have examined circulating testosterone concentrations in other *ex situ* populations of species endemic to tropical latitudes, but in all cases seasonal differences were not detected. Androgen concentrations the male fishing cat (*Prionailurus viverrinus*), a species endemic to tropical and subtropical Southeast Asia, and a non-seasonal breeder in captivity, exhibited similar concentrations of fecal androgens throughout the year in North American zoos, regardless of photoperiod (Santymire et al. 2011). Similarly, Brown et al. (2001) reported consistent androgen patterns across seasons for black and white rhinos in North American zoos; both species are native to tropical and sub-tropical regions in Africa. Consistent with these findings, Hermes et al. (2005) demonstrated consistent semen production throughout the year in zoo-housed white rhinos. There was also no difference in testosterone levels across seasons for zoo-housed three-banded armadillos (*Tolypeutes matacus*), a species is native to tropical and subtropical regions of South America (Howell-Stephens et al. 2013).

Although most species endemic to tropical latitudes have non-seasonal birth patterns, this relationship does not necessarily dictate that testosterone levels remain constant throughout the year. Indeed, while the ‘Challenge Hypothesis’ predicts that testosterone concentrations are

positively correlated with breeding season, among other reproductive parameters, the researchers who offered this hypothesis also reported that testosterone levels are not always indicative of reproductive state and temporal patterns can vary markedly among individuals (Wingfield et al. 1990). Because reproduction in captive pygmy hippos is non-seasonal, circulating testosterone levels must be sufficient for spermatogenesis in all seasons of the year, even when they are lower in the fall and winter. Without comparative data from wild pygmy hippos, we cannot hypothesize that seasonality of androgen concentrations is reflective of normal biology for this species. It may be that more variable day length in non-tropical latitudes influences testosterone production but not spermatogenesis in pygmy hippos.

Other factors potentially influencing circulating testosterone levels in pygmy hippos include body condition and ambient temperature. We do not have body weight data across seasons for all of the males in the study; however, ambient temperature does change throughout the year in all of the locations where these males were housed, although the variability is less distinct in sub-tropical latitudes. Two of the males in this study were housed indoors year-round in climate-controlled rainforest habitats with both natural (skylights) and artificial light; presumably these hippos experienced negligible fluctuations in ambient temperature but were subject to variations in day length. Three males were housed outdoors except during cold weather, and five were housed outdoors year-round. The latter eight hippos would have experienced alterations in ambient temperature.

The higher androgen concentrations in male pygmy hippos house outdoors year-round may therefore be due to the lower seasonal variability in both temperature and day length at sub-tropical latitudes (Brownsville, Texas – 25.9°N; Dade City, Florida – 28.3°N; Miami, Florida – 25.8°N; Punta Gorda, Florida – 27.0°N; Tampa, Florida – 27.9°N); compared to more temperate latitudes (Baton Rouge, Louisiana – 30.5°N; Chicago, Illinois – 41.9°N; Louisville, Kentucky – 38.3°N; Oklahoma City, Oklahoma – 35.0°N; Omaha, Nebraska – 41.3°N). Again, it is unknown if these differences in androgen concentrations are correlated to differences in spermatogenesis and fertility. Research investigating ejaculate characteristics throughout the

year for proven and unproven males in different climatic zones would be necessary to investigate potential relationships between fertility and circulating androgens. The two previous studies examining ejaculates from ten pygmy hippos in European zoos did not investigate possible relationships with season or between proven and unproven males (Saragusty et al., 2010a, 2012).

In contrast to the pygmy hippo, there have been a number of previous studies investigating male reproductive biology in the common hippo (*Hippopotamus amphibius*) for both wild and captive populations (Kayanja 1989; Laws & Clough 1966; Macdonald 2007; Pazzoto Alves et al. 2016; Saragusty et al. 2010b). This heftier and more gregarious species lives in large herds with a dominant bull and inhabits riverine savannah throughout sub-Saharan Africa. Despite being semi-seasonal breeders with a peak in calving during the rainy season, males are sexually active and can fertilize a female at any time of the year (Laws & Clough 1966). Similarly, males do not exhibit seasonal variation in the size of their reproductive organs, including accessory sex glands (Kayanja 1989), but circulating testosterone levels across seasons have not been studied. Although the anatomical features of the reproductive tract and semen characteristics are likely conserved between common and pygmy hippos (Macdonald 2007), these two species differ considerably in their ecology, social structure and life history traits and therefore may also exhibit significant differences in their reproductive strategies and associated endocrine patterns.

6.5.3 FUTURE RESEARCH AND RECOMMENDATIONS

Further research is necessary to investigate potential links between zoo-specific husbandry variables, chronic stress, and reproductive patterns in male pygmy hippos. However, a number of additional questions need to be addressed before the information described in this study can be applied for population-wide assessments. First, additional studies investigating gastrointestinal transit time in a larger number of male and female animals will further elucidate if there are sex differences in the lag time for hormone excretion. Radio-label studies would also be useful to determine what percentage of glucocorticoid and androgen metabolites are excreted via the urinary and fecal route and to more precisely determine lag time for each metabolite.

However, this approach is not always practical or logistically possible, especially in rare, endangered species. The degradation of hormone metabolites in feces over time should also be determined for the 11,17-DOA assay as it was for CC and Testo because the timing of degradation can vary markedly depending on hormone chemistry, diet, ambient temperature and humidity. Previous studies utilizing the 11,17-DOA to assess glucocorticoid metabolites have shown greater than 20% degradation within 1 to 4 h after defecation for domestic cattle, horses, pigs and African buffalo (Ganswindt et al. 2012; Möstl et al. 1999). Additionally, before conducting research to investigate baseline endocrine profiles and stress responses for individual animals, it is essential to determine if there are diel fluctuations in excretion of glucocorticoid metabolites linked to circadian alterations in hormone secretion. This phenomenon is often less pronounced in larger herbivores and ruminants than in carnivores and smaller species with relatively faster metabolic rates and shorter gastrointestinal transit times (Millspaugh & Washburn 2004; Touma & Palme 2005). However, it is essential to understand these technical limitations to ensure sampling protocols for future studies are designed in a manner that limits bias and inconsistencies in hormone measurements.

Determining the biochemical structure of fecal cortisol and testosterone metabolites via HPLC will help further refine which EIAs most accurately measure metabolites of these hormones with minimal cross-reactivity. The biological relevance of the 11,17-DOA assay could be further validated by determining if it also can detect a response to a natural stressor versus a pharmacological stimulus – for example, after anesthesia or after transfer between zoological facilities. Similarly, an increase in androgen metabolites after administration of luteinizing hormone would provide additional evidence that the Testo assay is quantifying gonadal or adrenal androgens.

Further investigations are also indicated to address a number of questions concerning the reproductive biology of the male pygmy hippo, including whether the seasonal trend in testosterone levels applies to the *ex situ* population in general. Investigating hormone concentrations in conjunction with other measures of reproductive function, primarily ejaculate

characteristics and mating behavior, would also be of interest. However, collecting semen samples requires general anesthesia and may not be logistically possible or ideal from an animal welfare perspective when multiple samples are needed over a longer time frame. Determining reproductive patterns for wild pygmy hippos by means of non-invasive endocrine monitoring would offer a useful comparison but presents its own set of logistical challenges, including being able to identify individuals.

6.6 CONCLUSIONS

Measurement of hormone metabolites in fecal samples using immunoassays is a valuable tool for monitoring stress and reproduction in both free-ranging and captive wildlife due to the relative ease of sample collection. This study characterized reproductive endocrine patterns in the male pygmy hippo and validated an EIA for non-invasively measuring glucocorticoid metabolites in the feces for both sexes. We are now equipped to investigate the dynamics of stress in this species, and its potential effects on reproductive health and wellbeing. Such data can be used as part of an integrative management strategy and help to optimize welfare for pygmy hippos in the *ex situ* population. Overall, it is essential to interpret results within a context of the limitations and assumptions inherent to the methods for endocrine assessment used in this study.

6.7 ACKNOWLEDGEMENTS

We would like to express our gratitude to all of the institutions that participated in study: Baton Rouge Zoo; Giraffe Ranch; Gladys Porter Zoo, Brownsville; Jackson Zoo; Louisville Zoo; Lincoln Park Zoo, Chicago; Lowry Park Zoo, Tampa; Oklahoma City Zoo; Omaha's Henry Doorly Zoo and Aquarium; Rum Creek Center for Conservation of Tropical Ungulates; and Zoo Miami. Our special appreciation goes out to the husbandry staff that collected the samples; without their help and dedication we would not have been able to conduct this research. We also thank Cayman Adams, Sarah Allred, Kim Daly-Crews, Saleha Khan, Lara Metrione, Paige

Pickering and Kayla Weller for technical support. Financial support for GF was provided by the American Association of Zoo Veterinarians Wild Animal Health Fund; the Center for Conservation of Tropical Ungulates; Omaha's Henry Doorly Zoo and Aquarium; the University of Western Australia (UWA), Convocation Postgraduate Research Travel Award; UWA Postgraduate Student's Association Fieldwork and Data Collection Award; and a UWA Graduate Research School PhD Completion Scholarship.

6.8 LITERATURE CITED

Bahr, N.I., Palme, R., Möhle, U., Hodges, J.K., Heistermann, M., 2000. Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. Gen. Comp. Endocrinol. 117, 427–438.

Bronson, F.H., 1985. Mammalian reproduction: an ecological perspective. Biol. Reprod. 32, 1–26.

Brown, J.L., Bellem, A.C., Fouraker, M., Wildt, D.E., Roth, T.L., 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol. 20, 463–486.

Carlstead, K., Brown, J.L., 2005. Relationships between patterns of fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. Zoo Biol. 24, 215–232.

Critical Ecosystem Partnership Fund, 2000. Ecosystem Profile - Upper Guinean Forest Ecosystem of the Guinean Forests of West Africa Biodiversity Hotspot.

Cockrem, J.F., 2013. Individual variation in glucocorticoid stress responses in animals. Gen. Comp. Endocrinol. 181, 45–58.

Collen, B., Howard, R., Konie, J., Daniel, O., Rist, J., 2011. Field surveys for the endangered pygmy hippopotamus *Choeropsis liberiensis* in Sapo National Park, Liberia. Oryx 45, 35–37.

Comizzoli, P., Crosier, A.E., Songsasen, N., Szykman Gunther, M., Howard, J.G., Wildt, D.E., 2009. Advances in reproductive science for wild carnivore conservation. Reprod. Domest. Anim. 44, 47–52.

Creel, S., Dantzer, B., Goymann, W., Rubenstein, D.R., 2013. The ecology of stress: Effects of the social environment. Funct. Ecol. 27, 66–80.

Dehnhard, M., Clauss, M., Lechner-Doll, M., Meyer, H.H.D., Palme, R., 2001. Noninvasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. Gen. Comp. Endocrinol. 123, 111–120.

Delgadillo, J.A., Fitz-Rodríguez, G., Duarte, G., Véliz, F.G., Carrillo, E., Flores, J.A., Vielma, J., Hernandez, H., Malpaux, B., 2004. Management of photoperiod to control caprine reproduction in the subtropics. Reprod. Fertil. Dev. 16, 471–478.

Dloniak, S.M., French, J.A., Place, N.J., Weldele, M.L., Glickman, S.E., Holekamp, K.E., 2004. Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*). Gen. Comp. Endocrinol. 135, 51–61.

Edwards, K.L., Shultz, S., Pilgrim, M., Walker, S.L., 2015. Male reproductive success is correlated with testosterone in the eastern black rhinoceros (*Diceros bicornis michaeli*). Gen. Comp. Endocrinol. 213, 40–49.

Engelhard, G.H., Brasseur, S.M.J.M., Hall, A.J., Burton, H.R., Reijnders, P.J.H., 2002. Adrenocortical responsiveness in southern elephant seal mothers and pups during lactation and the effect of scientific handling. J. Comp. Physiol. B 172, 315–328.

Flacke, G.L., Chambers, B.K., Martin, G.B., Paris, M.C.J., 2015. The pygmy hippopotamus *Choeropsis liberiensis* (Morton, 1849): Bringing to light research priorities for the largely forgotten, smaller hippo species. Der Zool. Garten NF 84, 234–265.

Franceschini, M.D., Rubenstein, D.I., Low, B., Romero, L.M., 2008. Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. Anim. Conserv. 11, 263–269.

Frigerio, D., Dittami, J., Möstl, E., Kotrschal, K., 2004. Excreted corticosterone metabolites co-vary with ambient temperature and air pressure in male Greylag geese (*Anser anser*). Gen. Comp. Endocrinol. 137, 29–36.

Ganswindt, A., Palme, R., Heistermann, M., Borragan, S., Hodges, J.K., 2003. Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. Gen. Comp. Endocrinol. 134, 156–166.

Ganswindt, A., Tordiffe, A.S.W., Stam, E., Howitt, M.J., Jori, F., 2012. Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis. African Zool. 47, 261–269.

Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods Ecol. Evol. 3, 757–765.

Hadinger, U., Haymerle, A., Knauer, F., Schwarzenberger, F., Walzer, C., 2015. Faecal cortisol metabolites to assess stress in wildlife: Evaluation of a field method in free-ranging chamois. Methods Ecol. Evol. 6, 1349–1357.

Heintz, M.R., Santymire, R.M., Parr, L.A., Lonsdorf, E. V., 2011. Validation of a cortisol enzyme immunoassay and characterization of salivary cortisol circadian rhythm in chimpanzees (*Pan troglodytes*). Am. J. Primatol. 73, 903–908.

Heistermann, M., Palme, R., Ganswindt, A., 2006. Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis. Am. J. Primatol. 68, 257–273.

Hermes, R., Hildebrandt, T.B., Blottner, S., Walzer, C., Silinski, S., Patton, M.L., Wibbelt, G., Schwarzenberger, F., Göritz, F., 2005. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum*, *C.s. cottoni*): Evaluation of male genital tract morphology and semen quality before and after cryopreservation. Theriogenology 63, 219–238.

Hoppe-Dominik, B., Kühl, H.S., Radl, G., Fischer, F., 2011. Long-term monitoring of large rainforest mammals in the Biosphere Reserve of Taï National Park, Cote d'Ivoire. Afr. J. Ecol. 49, 450–458.

Howell-Stephens, J., Bernier, D., Brown, J.S., Mulkerin, D., Santymire, R.M., 2013. Using non-invasive methods to characterize gonadal hormonal patterns of southern three-banded armadillos (*Tolypeutes matacus*) housed in North American zoos. Anim. Reprod. Sci. 138, 314–323.

Hulsman, A., Dalerum, F., Ganswindt, A., Muenscher, S., Bertschinger, H.J., Paris, M., 2011. Non-invasive monitoring of glucocorticoid metabolites in brown hyaena (*Hyaena brunnea*) feces. Zoo Biol. 30, 451–458.

Isaac, N.J.B., Turvey, S.T., Collen, B., Waterman, C., Baillie, J.E.M., 2007. Mammals on the EDGE: Conservation priorities based on threat and phylogeny. PLoS One 2, e296. doi:10.1371/journal.pone.0000296

Jachowski, D.S., Washburn, B.E., Millspaugh, J.J., 2015. Revisiting the importance of accounting for seasonal and diel rhythms in fecal stress hormone studies. Wildl. Soc. Bull. 39, 738–745. doi:10.1002/wsb.592

Kayanja, F.I.B., 1989. The reproductive biology of the male hippopotamus. Symp. Zool. Soc. London 61, 181–196.

Keay, J.M., Singh, J., Gaunt, M.C., Kaur, T., 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: A literature review. J. Zoo Wildl. Med. 37, 234–244.

Khonmee, J., Brown, J.L., Rojanasthien, S., Thumasanukul, D., Kongphoemphun, A., Siriaroonrat, B., Tipkantha, W., Punyapornwithaya, V., Thitaram, C., 2014. Seasonality of fecal androgen and glucocorticoid metabolite excretion in male goral (*Naemorhedus griseus*) in Thailand. Anim. Reprod. Sci. 146, 70–78.

Kretzschmar, P., Ganslo\u00dfer, U., Dehnhard, M., 2004. Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (*Ceratotherium simum simum*). Horm. Behav. 45, 1–9.

Laver, P.N., Ganswindt, A., Ganswindt, S.B., Alexander, K.A., 2012. Non-invasive monitoring of glucocorticoid metabolites in banded mongooses (*Mungos mungo*) in response to physiological and biological challenges. Gen. Comp. Endocrinol. 179, 178–183.

Laws, R.M., Clough, G., 1966. Observations on reproduction in the hippopotamus (*Hippopotamus amphibius* LINN). Symp. Zool. Soc. London 15, 117–140.

Lindsell, J.A., Klop, E., Siaka, A.M., 2011. The impact of civil war on forest wildlife in West Africa: mammals in Gola Forest, Sierra Leone. Oryx 45, 69–77.

Ludwig, C., Wachter, B., Silinski-Mehr, S., Ganswindt, A., Bertschinger, H., Hofer, H., Dehnhard, M., 2013. Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*). Gen. Comp. Endocrinol. 180, 15–23.

Macdonald, A.A., 2007. The Reproductive Biology of the Pigmy Hippopotamus (*Choeropsis liberiensis*) with comparative observation on the Common Hippopotamus (*Hippopotamus amphibious*), in: von Houwald, F., Macdonald, A.A., Pagan, O., Steck, B. (Eds.), Husbandry Guidelines for the Pygmy Hippopotamus (*Hexaprotodon liberiensis*). Zoo Basel, Switzerland, Basel, pp. 86–100.

Mallon, D., Wightman, C., De Ornellas, P., Ransom, C., 2011. Conservation Strategy for the Pygmy Hippopotamus. IUCN Species Survival Commission, Gland, Switzerland & Cambridge, UK.

Martin, G.B., Tjondronegoro, S., Boukhliq, R., Blackberry, M.A., Briegel, J.R., Blache, D., Fisher, J.A., Adams, N.R., 1999. Determinants of the annual pattern of reproduction in mature male Merino and Suffolk sheep: modification of endogenous rhythms by photoperiod. Reprod. Fertil. Dev. 11, 355–366.

Mason, G.J., 2010. Species differences in responses to captivity: Stress, welfare and the comparative method. Trends Ecol. Evol. 25, 713–721.

Metrione, L.C., Norton, T.M., Beetem, D., Penfold, L.M., 2008. Seasonal reproductive characteristics of female and male Jackson's hartebeest (*Alcelaphus buselaphus jacksoni*). Theriogenology 70, 871–879.

Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen. Comp. Endocrinol. 138, 189–199.

Moberg, G.P, 2000. Biological response to stress: Implications for animal welfare, in Moberg, G.P., Mench, J. A. (Eds.). The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, New York, pp. 1–21.

Mooring, M.S., Patton, M.L., Lance, V.A., Hall, B.M., Schaad, E.W., Fortin, S.S., Jella, J.E., McPeak, K.M., 2004. Fecal androgens of bison bulls during the rut. Horm. Behav. 46, 392–398.

Möstl, E., Maggs, J.L., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–139.

Möstl, E., Messmann, S., Bagu, E., Robia, C., Palme, R., 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. Zentralbl. Veterinarmed. A 46, 621–631.

Norris, K., Asase, A., Collen, B., Gockowksi, J., Mason, J., Phalan, B., Wade, A., 2010. Biodiversity in a forest-agriculture mosaic – The changing face of West African rainforests. Biol. Conserv. 143, 2341–2350.

Palme, R., Möstl, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int. J. Mamm. Biol. Suppl. II 62, 192–197.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Ann. N. Y. Acad. Sci. 1040, 162–171.

Palme, R., Robia, C., Messmann, S., Möstl, E., 1998. Measuring faecal cortisol metabolites: a non-invasive tool to evaluate adrenocortical activity in mammals. Adv. Ethol. 33, 27.

Patton, M.L., Swaisgood, R.R., Czekala, N.M., White, A.M., Fetter, G.A., Montagne, J.P., Rieches, R.G., Lance, V.A., 1999. Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biol. 18, 111–127.

Pazzoto Alves, I., Diniz Garcia, S., Magnani Grassi, T.L., Ribeiro de Araújo Rocha, G., Franciscato, D.A., Kipper, B.H., Burkhardt de Koivisto, M., 2016. Epididymal spermatozoa from *Hippopotamus amphibious*. Anim. Reprod. Sci. 169, 110–111.

Ransom, C., Robinson, P.T., Collen, B., 2015. *Choeropsis liberiensis*. IUCN Red List Threat. Species 2015. doi:e.T10032A18567171. Accessed 22.12.2015.

Rivier, C., Rivest, S., 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biol. Reprod. 45, 523–532.

Santymire, R.M., Brown, J.L., Stewart, R.A., Santymire, R.C., Wildt, D.E., Howard, J., 2011. Reproductive gonadal steroidogenic activity in the fishing cat (*Prionailurus viverrinus*) assessed by fecal steroid analyses. Anim. Reprod. Sci. 128, 60–72.

Sapolsky, R.M., 1992. Neuroendocrinology of the stress response, in: Becker, J.B., Breedlove, S.M., Crews, D. (Eds.), Behavioral Endocrinology. MIT Press, Cambridge, pp. 287–324.

Saragusty, J., Hermes, R., Hofer, H., Bouts, T., Göritz, F., Hildebrandt, T.B., 2012. Male pygmy hippopotamus influence offspring sex ratio. Nat. Commun. 3, 1–5.

Saragusty, J., Hildebrandt, T.B., Bouts, T., Göritz, F., Hermes, R., 2010a. Collection and preservation of pygmy hippopotamus (*Choeropsis liberiensis*) semen. Theriogenology 74, 652–657.

Saragusty, J., Walzer, C., Petit, T., Stalder, G., Horowitz, I., Hermes, R., 2010b. Cooling and freezing of epididymal sperm in the common hippopotamus (*Hippopotamus amphibius*). Theriogenology 74, 1256–1263.

Schatz, S., Palme, R., 2001. Measurement of faecal cortisol metabolites in cats and dogs: A non-invasive method for evaluating adrenocortical function. Vet. Res. Commun. 25, 271–287.

Schwarzenberger, F., Kolter, L., Zimmerman, W., Rietschel, W., Matern, B., Birher, P., Leus, K., 1998. Faecal cortisol metabolite measurement in the okapi (*Okapi johnstoni*). Adv. Ethol. 33, 28.

Schwarzenberger, F., Möstl, E., Palme, R., Bamberg, E., 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim. Reprod. Sci. 42, 515–526.

Shepherdson, D., Lewis, K.D., Carlstead, K., Bauman, J., Perrin, N., 2013. Individual and environmental factors associated with stereotypic behavior and fecal glucocorticoid metabolite levels in zoo housed polar bears. Appl. Anim. Behav. Sci. 147, 268–277.

Shutt, K., Setchell, J.M., Heistermann, M., 2012. Non-invasive monitoring of physiological stress in the Western lowland gorilla (*Gorilla gorilla gorilla*): validation of a fecal glucocorticoid assay and methods for practical application in the field. Gen. Comp. Endocrinol. 179, 167–177.

Stead, S.K., Meltzer, D.G., Palme, R., 2000. The measurement of glucocorticoid concentrations in the serum and faeces of captive African elephants (*Loxodonta africana*) after ACTH stimulation. J. S. Afr. Vet. Assoc. 71, 192–196.

Steck, B. (Ed.), 2016. International Studbook for the Pygmy Hippopotamus 2015, 22nd ed. Zoo Basel, Switzerland, Basel.

Swanson, W.F., Johnson, W.E., Cambre, R.C., Citino, S.B., Quigley, K.B., Brousset, D.M., Morais, R.N., Moreira, N., O'Brien, S.J., Wildt, D.E., 2003. Reproductive status of endemic felid species in Latin American zoos and implications for ex situ conservation. Zoo Biol. 22, 421–441.

Terio, K.A., Marker, L., Munson, L., 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. J. Wildl. Dis. 40, 259–266.

Tilbrook, A.J., Turner, A.I., Clarke, I.J., 2000. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. Rev. Reprod. 5, 105–113.

Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann. N. Y. Acad. Sci. 1046, 54–74.

Touma, C., Sachser, N., Möstl, E., Palme, R., 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen. Comp. Endocrinol. 130, 267–278.

van der Goot, A.C., Martin, G.B., Millar, R.P., Paris, M.C.J., Ganswindt, A., 2015. Profiling patterns of fecal 20-oxopregnane concentrations during ovarian cycles in free-ranging southern white rhinoceros (*Ceratotherium simum simum*). Anim. Reprod. Sci. 161, 89–95.

Van der Weyde, L.K., Martin, G.B., Paris, M.C.J., 2016. Monitoring stress in captive and free-ranging African wild dogs (*Lycaon pictus*) using faecal glucocorticoid metabolites. Gen. Comp. Endocrinol. 226, 50–55.

van Jaarsveld, A.S., Skinner, J.D., 1992. Adrenocortical responsiveness to immobilization stress in spotted hyenas (*Crocuta crocuta*). Comp. Biochem. Phys. A 103(1), 73–79.

Verschuren, J., 1983. Conservation of Tropical Rain Forest in Liberia - Recommendations for Wildlife Conservation and National Parks. Gland, Switzerland.

Washburn, B.E., Millspaugh, J.J., 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. Gen. Comp. Endocrinol. 127, 217–222.

Wasser, S.K., Hunt, K.E., Brown, J.L., Cooper, K., Crockett, C.M., Bechert, U., Millspaugh, J.J., Larson, S., Monfort, S.L., 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen. Comp. Endocrinol. 120, 260–275. doi:10.1006/gcen.2000.7557

Whitten, P.L., Brockman, D.K., Stavisky, R.C., 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearb. Phys. Anthropol. 41, 1–23.

Wielebnowski, N.C., Fletchall, N., Carlstead, K., Busso, J.M., Brown, J.L., 2002. Noninvasive assessment of adrenal activity associated with husbandry and behavioral factors in the North American clouded leopard population. Zoo Biol. 21, 77–98.

Wildt, D.E., Brown, J.L., Bush, M., Barone, M.A., Cooper, K.A., Grisham, J., Howard, J.G., 1993. Reproductive status of cheetahs (*Acinonyx jubatus*) in North American zoos: The benefits of physiological surveys for strategic planning. Zoo Biol. 12, 45–80.

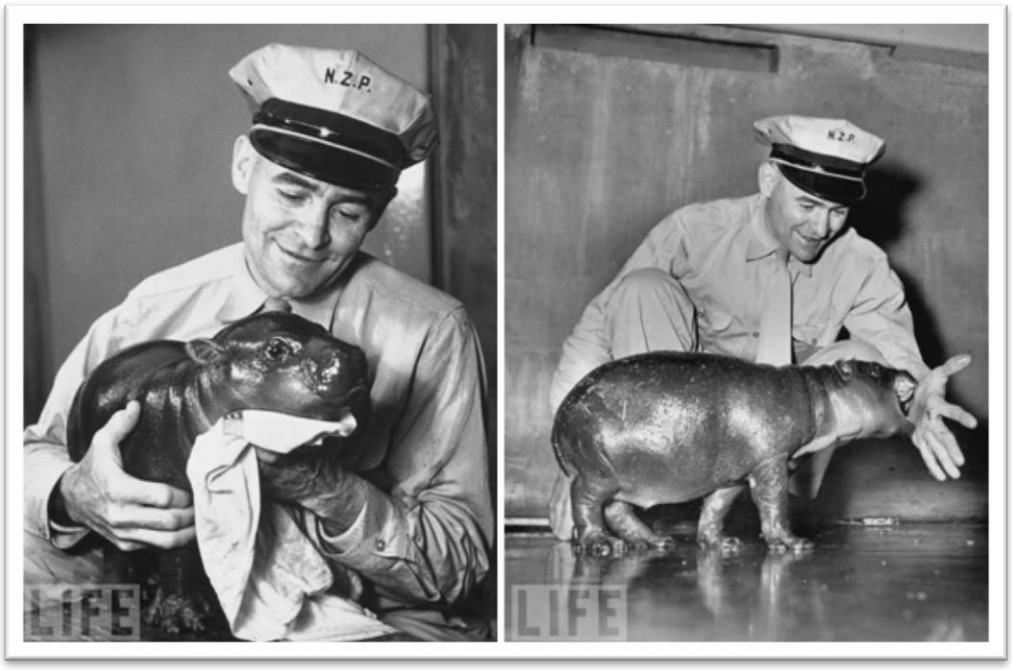
Wingfield, J.C., Hegner, R.E., Dufty Jr., A.M., Ball, G.F., 1990. The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136, 829–846.

Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: When and how. J. Neuroendocrinol. 15, 711–724.

Young, K.M., Walker, S.L., Lanthier, C., Waddell, W.T., Monfort, S.L., Brown, J.L., 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. Gen. Comp. Endocrinol. 137, 148–165.

Zerbe, P., Clauss, M., Codron, D., Bingaman Lackey, L., Rensch, E., Streich, J.W., Hatt, J.M., Müller, D.W.H., 2012. Reproductive seasonality in captive wild ruminants: Implications for biogeographical adaptation, photoperiodic control, and life history. Biol. Rev. 87, 965–990.

Chapter 7 GENERAL CONCLUSIONS



Pygmy Hippo Calf 'Gumdrop,' National Zoo, Washington, D.C.

The one thing to remember about an adventure
is that if it turns out the way you expect it to,
it has not been an adventure at all.

–Kim Fay, 'The Map of Lost Memories'

7.1 OVERVIEW

The overall aim of the research presented in this thesis was to expand our understanding of the pygmy hippopotamus to help optimize health and welfare for this species under managed care, and to develop a foundation of data and tools necessary for future research. A systematic literature review, including extensive translation from older German-language documents, has provided access to diverse historical and previously unpublished information. A population-wide mortality survey identified several trends and pathologic conditions of potential concern for pygmy hippos in zoological collections and thus provides a critical resource for clinical veterinarians. A detailed examination of the prevalence and demographics of polycystic kidney disease (PKD) has revealed it to be unexpectedly common. Although the condition can significantly impact the health of individual animals, PKD does not appear to pose a threat to the long-term viability of *ex situ* populations. A longitudinal assessment of gonadal hormone patterns in both males and females using non-invasive techniques has provided new information about the reproductive biology of pygmy hippos. Finally, ACTH challenge tests have revealed an enzyme immunoassay that can measure glucocorticoid metabolites of biological relevance in pygmy hippo dung, so we are now equipped to investigate the dynamics of stress and potential correlations with husbandry practices, reproduction, health and welfare.

Sound scientific management and breeding of pygmy hippos in the *ex situ* population is imperative given that this endangered species lives in a diminishing, fragmented habitat in West Africa, that there are no accurate estimates for wild population numbers, and that so little is known about their wild conspecifics. However, many logistical, financial, and sometimes ethical challenges remain. Moreover, if we are successful in reducing juvenile mortality and improving reproductive capacity, then space constraints in zoos must also be considered. A greater understanding of reproductive biology and how pygmy hippos respond to potential stressors in the captive environment will therefore be useful for optimizing management and husbandry of this species in the zoological community.

7.2 Overall Conclusions and Recommendations

The mortality review unveiled a number of pathological conditions previously unreported for the pygmy hippo, and our findings lead us to caution veterinary clinicians that comorbidities often contribute to or exacerbate the clinical presentation of disease conditions in adult and geriatric pygmy hippos. We also identified a number of areas where morbidity and mortality can potentially be reduced through husbandry modifications and veterinary procedures. The majority of pygmy hippos in captivity are overweight, so maintaining an age-appropriate body condition is recommended using the scoring system presented in Appendix II. Although EMCV was not a common cause of mortality, the most common presentation was acute death in the absence of clinical signs, making this viral disease a primary concern. Opportunistic serological screening is recommended to help characterize the epidemiology of EMCV in this species, especially in endemic areas. Mortality rates for neonatal pygmy hippos have declined somewhat as husbandry and management have improved over the last century but are still higher than desirable, suggesting a need for further optimization of zoo practices in the perinatal period. The underlying causes for most perinatal mortalities are poorly understood, as are the reasons for the higher mortality rate in males during this time frame; both phenomena limit the success of captive breeding efforts for this endangered species.

PKD was diagnosed in 37% of adult and geriatric pygmy hippos. Although this condition does not appear to limit fecundity or longevity, it does pose a clinical problem for the health and welfare of the affected animal. The data support a possible X-linked dominant inheritance pattern, but further demographic analysis is necessary for a definite conclusion. Given the high prevalence and potential for severe secondary complications, we urge clinicians to perform ultrasound examinations when possible and, if PKD is diagnosed, to monitor renal function and screen for secondary urinary tract infections via serial serum chemistry and urinalysis. If PKD is diagnosed at necropsy or via ultrasound, the approximate number and distribution of cysts should always be reported because this will help differentiate PKD from simple renal cysts and

the data could be used to develop a grading system. Systematic, comprehensive necropsies are essential, preferably using the standardized protocol available from the EEP/SSP, so that negative findings are always included in addition to pathology. This information must be reported to the International Studbook keeper in a timely manner. Due to our limited understanding of inheritance patterns and other potential contributing factors, we cannot recommend breeding only unaffected pairs as this strategy might not reduce PKD prevalence but would significantly diminish genetic diversity over time. Despite a projected increase in captive population size over time ($\lambda > 1.0$), we must strive to maintain genetic diversity and minimize inbreeding, especially since importation of new genetic material from wild populations is unlikely in the foreseeable future. Continued reassessment of population viability over time is also recommended to account for ongoing changes in demographic parameters.

We identified several immunoassays (EIAs) that could provide non-invasive, biologically relevant measures of female reproductive hormone metabolites for the pygmy hippo. These assays were used to characterize, for the first time, endocrine patterns during pregnancy, lactation and the estrous cycle. The PdG and Pg-diol assays can be used to diagnose pregnancy in the second half of gestation. Samples should also be taken prior to breeding to establish a baseline levels because they vary among females. A monoclonal progesterone EIA (mono-P4) did not clearly demonstrate pregnancy but was effective for identifying luteal phases and therefore could be used to monitor estrous cycles. Estrous cycle patterns can also be assessed by detecting follicular phases using several EIAs that measure estrogen metabolites. We now know that the pygmy hippo under managed care is a spontaneous ovulator and non-seasonally polyestrous. The length of the estrous cycle as determined by endocrine analysis was slightly shorter than the length reported from behavioral observations. We recommend performing ultrasound examination in conjunction with endocrine monitoring to correlate reproductive events with fecal hormone metabolite profiles. Ultrasound can also be used to determine if additional corpora lutea (CLs) are formed during gestation and to identify the timing of CL regression as an indication of luteal-placental shift.

Metabolites of glucocorticoids and androgens exhibited cross-reactivity when using an EIA that measures corticosterone. However, a testosterone EIA produced biologically relevant data for measuring androgen metabolites in the feces and can be used in future studies to assess gonadal activity in male pygmy hippos. Additionally, an EIA that cross-reacts with fecal cortisol metabolites with an 11,17-dioxoandrostane (DOA) structure was able to detect a stress response after ACTH challenge in both sexes. This EIA can be used in future studies to monitor stress in pygmy hippos. However, it is first necessary to determine the degradation rate of hormone metabolites in feces for the 11,17-DOA assay and to establish whether there are diel fluctuations in fecal excretion of glucocorticoid metabolites in this species. An understanding of these technical limitations is essential if sampling protocols for future studies are to limit bias and inconsistencies in hormone measurements. High performance liquid chromatography (HPLC) may help further refine which EIAs are most biologically relevant for measuring metabolites of both reproductive hormones and glucocorticoids in the feces because patterns and concentrations varied widely between individuals.

7.3 FUTURE DIRECTIONS

Overall, the material presented in this thesis is expected to provide an essential framework for future studies with pygmy hippos, including the potential influences of anthropogenic stress on wild populations, and the possible relationships among captivity-associated stressors, current husbandry practices, reproduction, health and welfare under managed care. We can never perfectly replicate the natural ecological environment for wildlife species held in zoological institutions, but our desire to optimize their wellbeing under our care should never fade. We now have an important foundation for additional research in the several key areas to further improve husbandry and welfare, leading to the following recommendations:

- Performing a systematic analysis of potential risk factors associated with stillbirth and early mortality.
- Determining if maternal stress or obesity are correlated with reduced calf survival

- Further investigation of the potential heritability mechanisms and clinical significance of PKD.
- Examining the potential influence of external factors, including diet, hormones, and microbial pathogens, in the development of PKD.
- Developing a database of baseline renal function tests as a tool to guide clinical decisions in animals affected by PKD.
- Generating a standardized grading system, akin to what is used for PKD diagnosis in humans, as an objective guideline for assessing severity.
- Banking renal tissue for potential future identification of genetic markers.
- Clarifying underlying reasons for the lack of breeding success experienced by many zoos.
- Assessing the potential for captivity-induced stress to alter normal physiology, behavior, reproduction, or contribute to disease processes.
- Investigating potential correlations between zoo-specific husbandry variables, obesity, chronic stress, and reproduction in pygmy hippos.
- Determining if there are clear sex differences in gastrointestinal transit time (and hence lag time for hormone excretion).
- Establishing if there is more than one distinct estrous cycle length in the pygmy hippo, and if longer periods of luteal activity are indicative of reproductive pathology.
- Examining seasonal trends in androgen metabolites and determining if these are correlated with other measures of male reproductive function, including semen quality and breeding status (proven or unproven).

Although not within the scope of this thesis, a number of important questions concerning the wild population remain unanswered. As we address these questions, we can apply the resulting information to optimize *ex situ* husbandry and to determine if the patterns observed in the managed population reflect normal biology, particularly the female-biased sex ratio and the high prevalence of PKD. As outlined in the *Conservation Strategy for the Pygmy Hippopotamus*, prospective research is necessary to establish a variety of ecologic and physiologic parameters

for the wild population, ranging from morphometrics and reproductive physiology to habitat utilization, feeding strategies, and behavioral ecology and population demographics. The non-invasive methods for assessing hormonal activity described in this thesis can be used to evaluate reproductive patterns and monitor longitudinal trends in stress levels for the wild pygmy hippo, particularly in response to the ongoing and widespread anthropogenic land-use changes throughout its habitat. Ultimately, we must apply the information presented in this thesis together with data from wild populations to guide integrative conservation strategies and secure the persistence of this unique and enigmatic species in perpetuity. It would be disheartening to imagine the rainforests of West Africa without the pygmy hippo meandering along with quiet footsteps, forever a ghost in the forest.

BIBLIOGRAPHY

Adams NR. 1995. Detection of the effects of phytoestrogens on sheep and cattle. J Anim Sci. 73:1509–1515.

Adler B, de la Peña Moctezuma A. 2010. Leptospira and leptospirosis. Vet Microbiol. 140:287–296.

Alford BT, Burkhart RL, Johnson WP. 1974. Etorphine and diprenorphine as immobilizing and reversing agents in captive and free-ranging mammals. J Am Vet Med Assoc. 164:702–705.

Atkins A, Backues K. 2005. Serologic survey for encephalomyocarditis virus in zoological institutions throughout the United States and Canada. In: Proceedings of the American Association of Zoo Veterinarians (AAZV), American Association of Wildlife Veterinarians (AAWV), Association of Zoos Aquariums (AZA) / Nutrition Advisory Group (NAG) Joint Conference. Omaha, Nebraska; p. 284.

Backues KA. 2008. Encephalomyocarditis virus infection in zoo animals. In: Fowler ME, Miller RE, editors. Zoo and Wild Animal Medicine. 6th ed. St. Louis, Missouri: Saunders Elsevier; p. 75–78.

Bahr NI, Palme R, Möhle U, Hodges JK, Heistermann M. 2000. Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. Gen Comp Endocrinol. 117:427–438.

Bamberg E, Möstl E, Patzl M, King GJ. 1991. Pregnancy diagnosis by enzyme immunoassay of estrogens in feces from nondomestic species. J Zoo Wildl Med. 22:73–77.

Barnes SA, Teare JA, Staaden S, Metrione L, Penfold LM. 2016. Characterization and manipulation of reproductive cycles in the jaguar (*Panthera onca*). Gen Comp Endocrinol. 225:95–103.

Benirschke K. 2007. East African River Hippopotamus & Pygmy Hippopotamus. In: Comparative Placentation. Available from: http://placentation.ucsd.edu/hippofs.htm

Berenbaum F, Eymard F, Houard X. 2013. Osteoarthritis, inflammation and obesity. Curr Opin Rheumatol. 25:114–118.

Berger EM, Leus K, Vercammen P, Schwarzenberger F. 2006. Faecal steroid metabolites for non-invasive assessment of reproduction in common warthogs (*Phacochoerus africanus*), red river hogs (*Potamochoerus porcus*) and babirusa (*Babyrousa babyrussa*). Anim Reprod Sci. 91:155–171.

Berkeley E V., Kirkpatrick JF, Schaffer NE, Bryant WM, Threlfall WR. 1997. Serum and fecal steroid analysis of ovulation, pregnancy, and parturition in the black rhinoceros (*Diceros bicornis*). Zoo Biol. 16:121–132.

Biller DS, DiBartola SP, Eaton KA, Pflueger S, Wellman ML, Radin MJ. 1996. Inheritance of polycystic kidney disease in Persian cats. J Hered. 87:1–5.

Blaszkiewitz B. 1983. Haltung und Zucht des Zwergflußpferdes (*Choeropsis liberiensis* Morton 1849) im Zoologischen Garten Berlin. Bongo. 7:71–78.

Boever WJ. 1978. Artiodactyla. In: Fowler ME, editor. Zoo and Wild Animal Medicine. Philadelphia, Pennsylvania: W.B. Saunders Company; p. 771–815.

Boisserie J-R. 2005. The phylogeny and taxonomy of Hippopotamidae (Mammalia: Artiodactyla): a review based on morphology and cladistic analysis. Zool J Linn Soc. 143:1–26.

Boisserie J-R, Lihoreau F, Brunet M. 2005. Origins of Hippopotamidae (Mammalia, Cetartiodactyla): towards resolution. Zool Scr. 34:119–143.

Boonyarittichaikij R. 2010. Studying the effect of factors that potentially influence the sex ratio of captive pygmy hippopotamus (*Choeropsis liberiensis*). MSc Thesis, Utrecht University.

Bouts T, Hermes R, Gasthuys F, Saragusty J, Taylor P, Routh A, Hildebrandt TB. 2012. Medetomidine-ketamine-isoflurane anaesthesia in pygmy hippopotami (*Choeropsis liberiensis*) - a case series. Vet Anaesth Analg. 39:111–118.

Bouts T, Vordermeier M, Flach E, Routh A. 2009. Positive skin and serologic test results of diagnostic assays for bovine tuberculosis and subsequent isolation of Mycobacterium interjectum in a pygmy hippopotamus (*Hexaprotodon liberiensis*). J Zoo Wildl Med. 40:536–542.

Brewer CJ, Balen AH. 2010. The adverse effects of obesity on conception and implantation. Reproduction. 140:347–364.

Bronson FH. 1985. Mammalian reproduction: an ecological perspective. Biol Reprod. 32:1–26.

Brown JL. 2011. Female reproductive cycles of wild female felids. Anim Reprod Sci. 124:155–162.

Brown JL, Bellem AC, Fouraker M, Wildt DE, Roth TL. 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol. 20:463–486.

Brown JL, Citino SB, Shaw J, Miller C. 1994. Endocrine profiles during the estrous cycle and pregnancy in the Baird's Tapir (*Tapirus bairdii*). Zoo Biol. 13:107–117.

Brown JL, Paris S, Prado-Oviedo N, Meehan C, Hogan JN, Morfeld K, Carlstead K. 2016. Reproductive health assessment of female elephants in North American zoos and association of husbandry practices with reproductive dysfunction in African elephants (*Loxodonta africana*). PLoS One. 11:e0145673.

Brown JL, Wasser SK, Wildt DE, Graham LH. 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol Reprod. 51:776–786.

Brown JL, Wasser SK, Wildt DE, Graham LH, Monfort SL. 1997. Faecal steroid analysis for monitoring ovarian and testicular function in diverse wild carnivore, primate, and ungulate species. J Mamm Biol. 62:27–31.

Bülow W. 1987. Untersuchungen am Zwergflußpferd, Choeropsis liberiensis im Azagny - Nationalpark, Elfenbeinküste. Diplomarbeit: Zoologischen Institut Braunschweig.

Bush M, Lemken R, Moore JA. 1972. Prolapsed uterus in a pygmy hippopotamus. J Am Vet Med Assoc. 140:651.

Büttikofer J. 1890. Reisebilder aus Liberia, Bd. 2. Leiden: E. J. Brill.

Canelli E, Luppi A, Lavazza A, Lelli D, Sozzi E, Martin AMM, Gelmetti D, Pascotto E, Sandri C, Magnone W, Cordioli P. 2010. Encephalomyocarditis virus infection in an Italian zoo. Virol J. 7:1–7.

Carlstead K, Brown JL. 2005. Relationships between patterns of fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. Zoo Biol. 24:215–232.

Caughley G. 1977. Analysis of vertebrate populations. New York, New York: John Wiley & Sons.

Critical Ecosystem Partnership Fund. 2000. Ecosystem Profile - Upper Guinean Forest Ecosystem of the Guinean Forests of West Africa Biodiversity Hotspot.

Chapman HC. 1894. Notes on *Choeropsis liberiensis* (Morton). Proc Acad Nat Sci Philadelphia.:185–187.

Chen JH, Martin-Gronert MS, Tarry-Adkins J, Ozanne SE. 2009. Maternal protein restriction affects postnatal growth and the expression of key proteins involved in lifespan regulation in mice. PLoS One. 4:e4950. doi: 10.1371/journal.pone.0004950.

Clark AB. 1978. Sex ratio and local resource competition in a prosimian primate. Science (80-). 201:163–165.

Clauss M, Jürgen Streich W, Schwarm A, Ortmann S, Hummel J. 2007. The relationship of food intake and ingesta passage predicts feeding ecology in two different megaherbivore groups. Oikos. 116:209–216.

Clauss M, Schwarm A, Ortmann S, Alber D, Flach EJ, Kühne R, Hummel J, Streich WJ, Hofer H. 2004. Intake, ingesta retention, particle size distribution and digestibility in the Hippopotamidae. Comp Biochem Physiol Part A. 139:449–459.

Clyde VL, Wallace RS, Pocknell AM. 1998. Dermatitis caused by group G beta-hemolytic Streptococcus in Nile hippos (*Hippopotamus amphibius*). In: Proceedings of the American Association of Zoo Veterinarians (AAZV) and American Association of Wildlife Veterinarians (AAWV) Joint Conference. Omaha, Nebraska; p. 221–225.

Cockrem JF. 2013. Individual variation in glucocorticoid stress responses in animals. Gen Comp Endocrinol. 181:45–58.

Cohrs P. 1952. Protozoen als Ursache wiederholter Fehlgeburten beim Zwergflußpferd (*Choeropsis liberiensis*). Der Zool Garten NF. 19:192–195.

Collen B, Howard R, Konie J, Daniel O, Rist J. 2011. Field surveys for the endangered pygmy hippopotamus *Choeropsis liberiensis* in Sapo National Park, Liberia. Oryx. 45:35–37.

Comizzoli P, Crosier AE, Songsasen N, Szykman Gunther M, Howard JG, Wildt DE. 2009. Advances in reproductive science for wild carnivore conservation. Reprod Domest Anim. 44:47–52.

Conway AL. 2013. Conservation of the Pygmy Hippopotamus (*Choeropsis liberiensis*) in Sierra Leone, West Africa. PhD Thesis: University of Georgia, Athens.

Coryndon SC. 1977. The taxonomy and nomenclature of the Hippopotamidae (Mammalia, Artiodactyla) and a description of a two new fossil species. Proc R Netherlands Acad Arts Sci B. 80:61–88.

Cowley BD, Gudapaty S, Kraybill AL, Barash BD, Harding MA, Calvet JP, Gattone VH. 1993. Autosomal-dominant polycystic kidney disease in the rat. Kidney Int. 43:522–534.

Cracknell JM, Stidworthy M, Holliman A. 2011. Leptospirosis in a pygmy hippopotamus (*Choeropsis liberiensis*). In: Proceedings of the American Association of Zoo Veterinarians (AAZV) Annual Conference. Kansas City, Missouri; p. 35–37.

Crandall LS. 1964. Hippopotamuses. In: The Management of Wild Mammals in Captivity. Chicago, Illinois: The University of Chicago Press; p. 530–543.

Creel S, Dantzer B, Goymann W, Rubenstein DR. 2013. The ecology of stress: Effects of the social environment. Funct Ecol. 27:66–80.

Cresswell JA, Campbell OMR, De Silva MJ, Filippi V. 2012. Effect of maternal obesity on neonatal death in sub-Saharan Africa: Multivariable analysis of 27 national datasets. Lancet. 380:1325–1330.

Czekala NM, Callison L. 1996. Pregnancy diagnosis in the black rhinoceros (*Diceros bicornis*) by salivary hormone analysis. Zoo Biol. 15:37–44.

Dathe HH, Kuckelkorn B. 1989. Progesteronnachweis in Sekreten des Zwergflußpferdes (*Choeropsis liberiensis* Morton, 1844). Der Zool Garten NF. 59:201–208.

Dathe HH, Kuckelkorn B, Minnemann D. 1992. Salivary cortisol assessment for stress detection in the Asian elephant (*Elephas maximus*): A pilot study. Zoo Biol. 11:285–289.

Davies MJ. 2006. Evidence for effects of weight on reproduction in women. Reprod Biomed Online. 12:552–561.

Dehnhard M, Clauss M, Lechner-Doll M, Meyer HHD, Palme R. 2001. Noninvasive monitoring of adrenocortical activity in roe deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. Gen Comp Endocrinol. 123:111–120.

Delgadillo JA, Fitz-Rodríguez G, Duarte G, Véliz FG, Carrillo E, Flores JA, Vielma J, Hernandez H, Malpaux B. 2004. Management of photoperiod to control caprine reproduction in the subtropics. Reprod Fertil Dev. 16:471–478.

Dittrich L. 1976. Age of sexual maturity in the hippopotamus. Int Zoo Yearb. 16:171–173.

Dloniak SM, French JA, Place NJ, Weldele ML, Glickman SE, Holekamp KE. 2004. Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*). Gen Comp Endocrinol. 135:51–61.

Ebert TA. 1999. Plant and Animal Populations: Methods in Demography. San Diego, California: San Diego State University Academic Press.

Eddie C, Maher M, Groome C. 2010. Population Analysis and Breeding and Transfer Plan - Pygmy Hippopotamus (*Choeropis liberiensis liberiensis*) Species Survival Plan. Chicago, Illinois.

Edwards KL, Shultz S, Pilgrim M, Walker SL. 2015a. Irregular ovarian activity, body condition and behavioural differences are associated with reproductive success in female eastern black rhinoceros (*Diceros bicornis michaeli*). Gen Comp Endocrinol. 214:186–194.

Edwards KL, Shultz S, Pilgrim M, Walker SL. 2015b. Male reproductive success is correlated with testosterone in the eastern black rhinoceros (*Diceros bicornis michaeli*). Gen Comp Endocrinol. 213:40–49.

Endo H, Sasaki M, Kogiku H, Hayashi Y, Komiya T, Narushima E, Arishima K, Yamamoto M. 2001. Anatomy and histology of the stomach in a newborn pygmy hippopotamus (*Choeropsis liberiensis*). Mammal Study. 26:53–60.

Engelhard GH, Brasseur SMJM, Hall AJ, Burton HR, Reijnders PJH. 2002. Adrenocortical responsiveness in southern elephant seal mothers and pups during lactation and the effect of scientific handling. J Comp Physiol B. 172:315–328.

Eshuis H. 2011. Habitat preference and activity pattern of the pygmy hippopotamus analyzed by camera trapping and GIS. MSc Thesis: Wageningen University.

Eulenberger K. 1995. Flußpferde. In: Göltenboth R, Klös HG, editors. Krankheiten der Zoo- und Wildtiere. Berlin: Blackwell Wissenschafts-Verlag; p. 246–255.

Evans ACO. 2003. Characteristics of ovarian follicle development in domestic animals. Reprod Domest Anim. 38:240–246.

Fábián L. 1976. Darmpech-Obstipation („Fohlenkolik“) bei neugeborenem Zwergflußpferd, *Choeropsis liberiensis*. Der Zool Garten NF. 46:452–454.

Falconer DS, Mackay TFC. 1996. Introduction to quantitative genetics. Essex, United Kingdom: Longman Group Ltd.

Fisher RE, Scott KM, Naples VL. 2007. Forelimb myology of the pygmy hippopotamus (*Choeropsis liberiensis*). Anat Rec. 290:673–693.

Flach EJ, Furrokh IK, Thornton SM, Smith J, Parkyn JP, Campbell EJ. 1998. Caesarean section in a pygmy hippopotamus (*Choeropsis liberiensis*) and the management of the wound. Vet Rec. 143:611–613.

Flacke GL, Chambers BK, Martin GB, Paris MCJ. 2015. The pygmy hippopotamus *Choeropsis liberiensis* (Morton, 1849): Bringing to light research priorities for the largely forgotten, smaller hippo species. Der Zool Garten NF. 84:234–265.

Flower WH. 1887. On the pygmy hippopotamus of Liberia, *Hippopotamus liberiensis* (Morton), and its claims to distinct generic rank. Proc Zool Soc London.:612–614.

Föllmi J, Steiger A, Walzer C, Robert N, Geissbühler U, Doherr MG, Wenker C. 2007. A scoring system to evaluate physical condition and quality of life in geriatric zoo mammals. Anim Welf. 16:309–318.

Fox CW, Bush ML, Wallin WG. 2003. Maternal age affects offspring lifespan of the seed beetle, *Callosobruchus maculatus*. Funct Ecol. 17:811–820.

Franceschini MD, Rubenstein DI, Low B, Romero LM. 2008. Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy’s zebra. Anim Conserv. 11:263–269.

Franz W, Heymann H, Zscheile D. 1978. Immobilisierung und Nabelbruchoperation beim Zwergflusspferd (*Choeropsis liberiensis*). Erkrankungen der Zootiere Verhandlungsbericht. 20:197–200.

Frias AE, Morgan TK, Evans AE, Rasanen J, Oh KY, Thornburg KL, Grove KL. 2011. Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. Endocrinology. 152:2456–2464.

Frigerio D, Dittami J, Möstl E, Kotrschal K. 2004. Excreted corticosterone metabolites co-vary with ambient temperature and air pressure in male Greylag geese (*Anser anser*). Gen Comp Endocrinol. 137:29–36.

Gabow PA. 1993. Autosomal dominant polycystic kidney disease. N Engl J Med. 329:332–342.

Ganswindt A, Muilwijk C, Engelkes M, Muenscher S, Bertschinger H, Paris M, Palme R, Cameron EZ, Bennett NC, Dalerum F. 2012. Validation of noninvasive monitoring of adrenocortical endocrine activity in ground-feeding aardwolves (*Proteles cristata*): exemplifying the influence of consumption of inorganic material for fecal steroid analysis. Physiol Biochem Zool. 85:194–199.

Ganswindt A, Palme R, Heistermann M, Borragan S, Hodges JK. 2003. Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. Gen Comp Endocrinol. 134:156–166.

Ganswindt A, Tordiffe ASW, Stam E, Howitt MJ, Jori F. 2012. Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis. African Zool. 47:261–269.

Garnier JN, Holt W V., Watson PF. 2002. Non-invasive assessment of oestrous cycles and evaluation of reproductive seasonality in the female wild black rhinoceros (*Diceros bicornis minor*). Reproduction. 123:877–889.

Gaskin JM, Andresen TL, Olsen JH, Schobert EE, Buesse D, Lynch JD, Walsh M, Citino S, Murphy D. 1987. Encephalomyocarditis in zoo animals: Recent experiences with the disease and vaccination. In: Proceedings of the International Conference on Zoological and Avian Medicine. Oahu, Hawaii; p. 491.

Gaskin JM, Jorge MA, Simpson CF, Lewis AL, Olson JH, Schobert EE, Wollenman EP, Marlowe C, Curtis MM. 1980. The tragedy of encephalomyocarditis virus infection in zoological parks of Florida. In: Proceedings of the American Association of Zoo Veterinarians (AAZV) Annual Conference. Washington, D.C.; p. 1–7.

Geisler JH, Uhen MD. 2003. Morphological support for a close relationship between hippos and whales. J Vertebr Paleontol. 23:991–996.

Gilmour AR, Gogel BJ, Cullis BR, Thompson R. 2009. ASReml user guide release 3.0. Hemel Hempstead, United Kingdom: VSN International Ltd. Available from www.vsni.co.uk.

Gippoliti S, Leoni A. 1999. The pygmy hippopotamus at Rome Zoological Garden. Int Zoo News. 46:335–339.

Gottdenker N, Bodmer RE. 1998. Reproduction and productivity of white-lipped and collared peccaries in the Peruvian Amazon. J Zool. 245:423–430.

Goymann W. 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods Ecol Evol. 3:757–765.

Graf Z. 1981. Über den Verlust eines Zwergflußpferdes im Budapester Zoo. Erkrankungen der Zootiere Verhandlungsbericht. 23:389–390.

Graham L, Schwarzenberger F, Möstl E, Galama W, Savage A. 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biol. 20:227–236.

Graham LH, Goodrowe KL, Raeside JI, Liptrap RM. 1995. Non-invasive monitoring of ovarian function in several felid species by measurement of fecal estradiol-17β and progestins. Zoo Biol. 14:223–237.

Graham LH, Reid K, Webster T, Richards M, Joseph S. 2002. Endocrine patterns associated with reproduction in the Nile hippopotamus (*Hippopotamus amphibius*) as assessed by fecal progestagen analysis. Gen Comp Endocrinol. 128:74–81.

Grantham JJ. 2008. Autosomal dominant polycystic kidney disease. N Engl J Med. 349:1477–1485.

Gray CW, Bush RM. 1974. Malocclusion in a pygmy hippopotamus. In: National Zoological Park 18-Month Report. Washington, D.C.: Smithsonian Institution Press; p. 41.

Greed GR. 1983. Husbandry and breeding of the pigmy hippopotamus (*Choeropsis liberiensis*). In: Proceedings Symposium 7, Association of British Wild Animal Keepers; p. 10–23.

Hadinger U, Haymerle A, Knauer F, Schwarzenberger F, Walzer C. 2015. Faecal cortisol metabolites to assess stress in wildlife: Evaluation of a field method in free-ranging chamois. Methods Ecol Evol. 6:1349–1357.

Handelsman DJ, Wartofsky L. 2013. Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism. J Clin Endocrinol Metab. 98:3971–3973.

Harcourt-Brown FM. 2007. The progressive syndrome of acquired dental disease in rabbits. J Exot Pet Med. 16:146–157.

Hashimoto K, Saikawa Y, Nakata M. 2007. Studies on the red sweat of the *Hippopotamus amphibius*. Pure Appl Chem. 79:507–517.

Hediger H. 1946. Die Baseler Zwergflußpferd-Zucht. Zool Garten Basel. 74:23–29.

Hegner B. 1967. Zur Morphologie des Auges von *Choeropsis liberiensis* und *Hippopotamus amphibius* (Mammalia, Artiodactyla, Hippopotamidae). Acta Zool. 48:59–85.

Heintz MR, Santymire RM, Parr LA, Lonsdorf E V. 2011. Validation of a cortisol enzyme immunoassay and characterization of salivary cortisol circadian rhythm in chimpanzees (*Pan troglodytes*). Am J Primatol. 73:903–908.

Heistermann M, Palme R, Ganswindt A. 2006. Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis. Am J Primatol. 68:257–273.

Helmick KE, Rush EM, Ogburn AL, Trupkiewicz JG, Garner M. 2007. Dermatopathy in captive hippopotamus (*Hippopotamus amphibius*). In: Proceedings of the American Association of Zoo Veterinarians (AAZV), American Association of Wildlife Veterinarians (AAWV) and Association of Zoos and Aquariums (AZA) Joint Conference. Knoxville, Tennessee; p. 92.

Hentschel KM. 1990. Untersuchung zu Status, Ökologie und Erhaltung des Zwergflusspferdes (*Choeropsis liberiensis*) in der Elfenbeinküste. Doktorand Dissertation: Technischen Universität Carolo-Wilhelmina, Braunschweig.

Hermes R, Hildebrandt TB, Blottner S, Walzer C, Silinski S, Patton ML, Wibbelt G, Schwarzenberger F, Göritz F. 2005. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*): Evaluation of male genital tract morphology and semen quality before and after cryopreservation. Theriogenology. 63:219–238.

Hermes R, Hildebrandt TB, Göritz F. 2004. Reproductive problems directly attributable to long-term captivity–asymmetric reproductive aging. Anim Reprod Sci. 82–83:49–60.

Hermes R, Hildebrandt TB, Walzer C, Göritz F, Patton ML, Silinski S, Anderson MJ, Reid CE, Wibbelt G, Tomasova K, Schwarzenberger F. 2006. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). Theriogenology. 65:1492–1515.

Heslop IRP. 1944. The pigmy hippopotamus. Field. 183:588.

Heuschele WP, Doyle LG, Hooker PA, Gottling KL, Kawanabe PS. 1982. Current status of some important viruses of domestic ruminants in captive wild ruminants in the USA. In: Proceedings of the American Association of Zoo Veterinarians (AAZV) Annual Conference. New Orleans, Louisiana; p. 94–121.

Hildebrandt TB, Göritz F. 1999. Use of Ultrasonography in Zoo Animals. In: Fowler ME, Miller RE, editors. Zoo and Wild Animal Medicine. 4th ed. Philadelphia, Pennsylvania: W.B. Saunders Company; p. 41–54.

Hildebrandt TB, Hermes R, Walzer C, Sós E, Molnar V, Mezösi L, Schnorrenberg A, Silinski S, Streich J, Schwarzenberger F, Göritz F. 2007. Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation. Theriogenology. 67:1473–1484.

Hillers A, Muana AM. 2010. Pygmy Hippo Conservation Project Within the "Across the River - A Transboundary Peace Park for Sierra Leone and Liberia" Project (ARTP). Basel, Switzerland: Zoo Basel.

Hope K, Deem SL. 2006. Retrospective study of morbidity and mortality of captive jaguars (*Panthera onca*) in North America: 1982 – 2002. Zoo Biol. 25:501–512.

Hoppe-Dominik B, Kühl HS, Radl G, Fischer F. 2011. Long-term monitoring of large rainforest mammals in the Biosphere Reserve of Taï National Park, Cote d'Ivoire. Afr J Ecol. 49:450–458.

Hornaday WT. 1912. Our pygmy hippopotami. New York Zool Soc Bull. 16(52):877–879.

Hornaday WT. 1920. Birth of a pygmy hippopotamus. New York Zool Soc Bull. 23:11–13.

Howell-Stephens J, Bernier D, Brown JS, Mulkerin D, Santymire RM. 2013. Using non-invasive methods to characterize gonadal hormonal patterns of southern three-banded armadillos (*Tolypeutes matacus*) housed in North American zoos. Anim Reprod Sci. 138:314–323.

Hulsman A, Dalerum F, Ganswindt A, Muenscher S, Bertschinger HJ, Paris M. 2011. Non-invasive monitoring of glucocorticoid metabolites in brown hyaena (*Hyaena brunnea*) feces. Zoo Biol. 30:451–458.

Hunter P, Swanepoel SP, Esterhuysen JJ, Raath JP, Bengis RG, van der Lugt JJ. 1998. The efficacy of an experimental oil-adjuvanted encephalomyocarditis vaccine in elephants, mice and pigs. Vaccine. 16:55–61.

Igarashi P, Somlo S. 2007. Polycystic kidney disease. J Am Soc Nephrol. 18:1371–1373.

Illera J-C, Silván G, Cáceres S, Carbonell M-D, Gerique C, Martínez-Fernández L, Munro C, Casares M. 2014. Assessment of ovarian cycles in the African elephant (*Loxodonta africana*) by measurement of salivary progesterone metabolites. Zoo Biol. 33:245–249.

Isaac NJB, Turvey ST, Collen B, Waterman C, Baillie JEM. 2007. Mammals on the EDGE: Conservation priorities based on threat and phylogeny. PLoS One. 2:e296. doi: 10.1371/journal.pone.0000296.

Jachowski DS, Washburn BE, Millspaugh JJ. 2015. Revisiting the importance of accounting for seasonal and diel rhythms in fecal stress hormone studies. Wildl Soc Bull. 39:738–745.

James WH. 1996. Evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. J Theor Biol. 180:271–286.

Jann P, Ward PI. 1999. Maternal effects and their consequences for offspring fitness in the yellow dung fly. Funct Ecol. 13:51–58.

Jarboe M, Adams C, Bahr JM, Penfold L, Newell-Fugate AE. 2015. Boar urine pheromone exposure modifies estrous cycle length and regularity in same-sex housed female red river hogs (*Potamochoerus porcus*). In: Proceedings of the 5th International Society of Wildlife Endocrinology Conference. Berlin, Germany; p. 10.

Jarofke D. 1993. Hippopotamidae (Hippopotamus). In: Fowler ME, editor. Zoo and Wild Animal Medicine, Current Therapy. 3rd ed. Philadelphia, Pennsylvania: W.B. Saunders Company; p. 522–525.

Jarofke D, Klös HG. 1982. Immobilisierung und Krankheiten von Zwergflusspferden: Auswertung einer Umfrage bei mehr als 100 Zoologischen Gärten. Erkrankungen der Zootiere Verhandlungsbericht. 24:361–374.

Johnston NW. 2002. Atraumatic malocclusion in two pygmy hippos (*Choeropsis liberiensis*). J Vet Dent. 19:144–147.

Kawamura H, Hibino S, Nakamura A, Hashikawa H, Tamamura F. 1996. A comparison between etorphine hydrochloride and a combination of xylazine hydrochloride and ketamine hydrochloride for the immobilization of pygmy hippopotamus, *Choeropsis liberiensis*. J Japanese Assoc Zool Gard Aquariums. 37:113–116.

Kayanja FIB. 1989. The reproductive biology of the male hippopotamus. Symp Zool Soc London. 61:181–196.

Keay JM, Singh J, Gaunt MC, Kaur T. 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: A literature review. J Zoo Wildl Med. 37:234–244.

Kersey DC, Dehnhard M. 2014. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. Gen Comp Endocrinol. 203:296–306.

Khonmee J, Brown JL, Rojanasthien S, Thumasanukul D, Kongphoemphun A, Siriaroonrat B, Tipkantha W, Punyapornwithaya V, Thitaram C. 2014. Seasonality of fecal androgen and glucocorticoid metabolite excretion in male goral (*Naemorhedus griseus*) in Thailand. Anim Reprod Sci. 146:70–78.

Kilburn JJ, Murphy DP, Titus M, Payton ME, Backues KA. 2011. Vaccination of llamas, *Llama glama*, with an experimental killed encephalomyocarditis virus vaccine. J Zoo Wildl Med. 42:65–68.

Knief U, Hemmrich-Stanisak G, Wittig M, Franke A, Griffith SC, Kempenaers B, Forstmeier W. 2015. Quantifying realized inbreeding in wild and captive animal populations. Heredity. 114:397–403.

Korpelainen H. 1999. Genetic maternal effects on human life span through the inheritance of mitochondrial DNA. Hum Hered. 49:183–185.

Krackow S. 1995. Potential mechanisms for sex ratio adjustment in mammals and birds. Biol Rev. 70:225–241.

Kranz KR. 1982. A note on the structure of tail hairs from a pygmy hippopotamus (*Choeropsis liberiensis*). Zoo Biol. 1:237–241.

Krebs CJ. 1999. Estimation of Survival Rates. In: Krebs CJ, editor. Ecological Methodology. 2nd ed. Menlo Park, CA: Addison Wesley Educational Publishers, Inc; p. 499–539.

Kreeger TJ, Arnemo JM. 2002. Handbook of Wildlife Chemical Immobilization, International Edition. Fort Collins, Colorado: Wildlife Pharmaceuticals, Inc.

Kretzschmar P, Gansloßer U, Dehnhard M. 2004. Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (*Ceratotherium simum simum*). Horm Behav. 45:1–9.

Kristensen J, Vestergaard M, Wisborg K, Kesmodel U, Secher NJ. 2005. Pre-pregnancy weight and the risk of stillbirth and neonatal death. BJOG An Int J Obstet Gynaecol. 112:403–408.

Kumar R, Singh J, Husni MM. 1990. Anaesthesia and repair of a ventral hernia in a pygmy hippopotamus (*Choeropsis liberiensis*). Indian Vet J. 67:166–167.

Kusuda S, Adachi I, Fujioka K, Nakamura M, Amano-Hanzawa N, Goto N, Furuhashi S, Doi O. 2013. Reproductive characteristics of female lesser mouse deers (*Tragulus javanicus*) based on fecal progestagens and breeding records. Anim Reprod Sci. 137:69–73.

Laflamme DP. 2012. Obesity in dogs and cats: What is wrong with being fat? J Anim Sci.:1653–1662.

Lamglait B, Joris A, Romey A, Bakkali-Kassimi L, Lemberger K. 2015. Fatal encephalomyocarditis virus infection in an African savanna elephant (*Loxodonta africana*) in a French zoo. J Zoo Wildl Med. 46:393–396.

Lane EA, Hyde TS. 1973. Effect of maternal stress on fertility and sex ratio: A pilot study with rats. J Abnorm Psychol. 82:78–80.

Lang EM. 1968. Das Zwergflußpferd. In: Grzimek B, editor. Grzimek's Tierleb - Enzyklopädie des Tierreiches, Bd 13. Zürich: Kindler Verlag AG; p. 118–120.

Lang EM. 1975. Das Zwergflußpferd. Wittenberg Lutherstadt: A. Ziemsen Verlag, DDR.

Langer P. 1975. Macroscopic anatomy of the stomach of Hippopotamidae Gray, 1821. Zentralblatt für Veterinärmedizin C. 4:334–359.

Lasley BL, Kirkpatrick JF. 1991. Monitoring ovarian function in captive and free-ranging wildlife by means of urinary and fecal steroids. J Zoo Wildl Med. 22:23–31.

Laver PN, Ganswindt A, Ganswindt SB, Alexander KA. 2012. Non-invasive monitoring of glucocorticoid metabolites in banded mongooses (*Mungos mungo*) in response to physiological and biological challenges. Gen Comp Endocrinol. 179:178–183.

Laws RM. 1984. Hippopotamuses. In: Macdonald D, editor. The Encyclopaedia of Mammals. New York, New York: Facts on File, Inc.; p. 506–511.

Laws RM, Clough G. 1966. Observations on reproduction in the hippopotamus (*Hippopotamus amphibius* LINN). Symp Zool Soc London. 15:117–140.

Legacki EL, Scholtz EL, Ball BA, Stanley SD, Berger T, Conley AJ. 2016. The dynamic steroid landscape of equine pregnancy mapped by mass spectrometry. Reproduction. 151:421–430.

Legendre LFJ. 2002. Malocclusions in guinea pigs, chinchillas and rabbits. Can Vet J. 43:385–390.

Leidy J. 1853. On the osteology of the head of hippopotamus, and a description of the osteological characters of a new Genus of Hippopotamidæ. J Acad Nat Sci Philadelphia. 2:207–224.

Leong KM, Terrell SP, Savage A. 2004. Causes of mortality in captive cotton-top tamarins (*Saguinus oedipus*). Zoo Biol. 23:127–137.

Leutenegger M. 1978. Pygmy hippopotamus *Choeropsis liberiensis* births in captivity. Int Zoo Yearb. 18:234.

Lewison R, Oliver W. (IUCN SSC Hippo Specialist Subgroup). 2008. *Choeropsis liberiensis*. IUCN 2012; IUCN Red List of Threatened Species. Version 2012.1. Available from www.iucnredlist.org. Accessed 19.09.2012.

Li M, Sloboda DM, Vickers MH. 2011. Maternal obesity and developmental programming of metabolic disorders in offspring: Evidence from animal models. Exp Diabetes Res. Article ID:9 pages. doi: 10.1155/2011/592408.

Lim CED, Cheng NCL. 2011. Obesity and reproduction. J Aust Tradit Med Soc. 17:143–145.

Lindau K-H. 1982. Hippopotamuses. In: Göltenboth R, Jarofke D, editors. Handbook of Zoo Medicine. New York: Van Nostrand Reinhold Company; p. 216–223.

Lindsell JA, Klop E, Siaka AM. 2011. The impact of civil war on forest wildlife in West Africa: mammals in Gola Forest, Sierra Leone. Oryx. 45:69–77.

Lintzenich BA, Ward AM. 1997. Hay and Pellet Ratios: Considerations in Feeding Ungulates. In: Nutrition Advisory Group Handbook; p. Fact Sheet 006. Available from: http://www.nagonline.net/Home/Site Map.htm

Lochte T. 1951. Untersuchungen an Haaren eines neugeborenen Nilpferdes und eines Zwergflußpferdes. Der Zool Garten NF. 18:119–124.

Ludwig C, Wachter B, Silinski-Mehr S, Ganswindt A, Bertschinger H, Hofer H, Dehnhard M. 2013. Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*). Gen Comp Endocrinol. 180:15–23.

Lynch M, Ennis R. 1983. Resource availability, maternal effects, and longevity. Exp Gerontol. 18:147–165.

Macalister A. 1873. The anatomy of *Choeropsis liberiensis*. Proc R Irish Acad. 2:494–500.

Macdonald AA. 2007. The Reproductive Biology of the Pigmy Hippopotamus (*Choeropsis liberiensis*) with comparative observation on the Common Hippopotamus (*Hippopotamus amphibious*). In: von Houwald F, Macdonald AA, Pagan O, Steck B, editors. Husbandry Guidelines for the Pygmy Hippopotamus (*Hexaprotodon liberiensis*). Basel: Zoo Basel, Switzerland; p. 86–100.

Macdonald AA, Bosma AA. 1985. Notes on placentation in the Suina. Placenta. 6:83–91.

Macdonald AA, Hartman W. 1983. Comparative and functional morphology of the stomach in the adult and newborn pigmy hippopotamus (*Choeropsis liberiensis*). J Morphol. 177:269–276.

Mallon D, Wightman C, De Ornellas P, Ransom C. 2011. Conservation Strategy for the Pygmy Hippopotamus. Gland, Switzerland & Cambridge, UK: IUCN Species Survival Commission.

Maluf NSR. 1978. Anatomy of the kidneys of a newly born pigmy hippopotamus (*Choeropsis liberiensis* Morton). Zentralblatt für Veterinärmedizin C. 7:28–48.

Maluf NSR. 1994. Renal anatomy of the pigmy hippopotamus (*Choeropsis liberiensis*): An overview. Zentralblatt für Veterinärmedizin C. 23:189–204.

Manton VJA, Jones PM. 1971. Whipsnade Park Report 1970. The Zoological Society of London, Scientific Report, 1969–1971, 553.

Marshall PJ, Sayer JA. 1976. Population ecology and response to cropping of a hippopotamus population in eastern Zambia. J Appl Ecol. 13:391–403.

Marshall WG, Bockstahler BA, Hulse DA, Carmichael S. 2009. A review of osteoarthritis and obesity: Current understanding of the relationship and benefit of obesity treatment and prevention in the dog. Vet Comp Orthop Traumatol. 22:339–345.

Martin GB, Tjondronegoro S, Boukhliq R, Blackberry MA, Briegel JR, Blache D, Fisher JA, Adams NR. 1999. Determinants of the annual pattern of reproduction in mature male Merino and Suffolk sheep: modification of endogenous rhythms by photoperiod. Reprod Fertil Dev. 11:355–366.

Mason GJ. 2010. Species differences in responses to captivity: Stress, welfare and the comparative method. Trends Ecol Evol. 25:713–721.

Masters N, Franklinos L, Feltrer Y, Pocknell A, Bolt D, Smith S, Molenaar FM. 2014. Successful chemotherapy of an oral anaplastic sarcoma in a pygmy hippopotamus (*Hexaprotodon liberiensis*). In: Proceedings of the International Conference on Diseases of Zoo and Wild Animals. Warsaw, Poland; p. 78.

Mbaya AW, Aliyu MM, Nwosu O, Ibrahim UI. 2008. Captive wild animals as potential reservoirs of haemo and ectoparasitic infections of man and domestic animals in the arid-region of Northeastern Nigeria. Vet Arh. 78:429–440.

McCurdy P, Sangster C, Lindsay S, Vogelnest L. 2014. Acute lymphoblastic leukemia in a pygmy hippopotamus (*Hexaprotodon liberiensis*). J Zoo Wildl Med. 45:906–910.

McLelland DJ, Kirkland PD, Rose KA, Dixon RJ, Smith N. 2005. Serologic responses of Barbary sheep (*Ammotragus lervia*), Indian antelope (*Antilope cervicapra*), wallaroos (*Macropus robustus*), and chimpanzees (*Pan troglodytes*) to an inactivated encephalomyocarditis virus vaccine. J Zoo Wildl Med. 36:69–73.

Metrione LC, Norton TM, Beetem D, Penfold LM. 2008. Seasonal reproductive characteristics of female and male Jackson's hartebeest (*Alcelaphus buselaphus jacksoni*). Theriogenology. 70:871–879.

Miller M. 2007. Hippopotami. In: West G, Heard D, Caulkett N, editors. Zoo Animal and Wildlife Immobilization and Anesthesia. Ames, Iowa: Blackwell Publishing; p. 579–584.

Miller MA. 2003. Hippopotamidae (Hippopotamus). In: Fowler ME, Miller RE, editors. Zoo and Wild Animal Medicine. 5th ed. St. Louis, Missouri: Saunders Elsevier; p. 602–612.

Miller M, Fleming GJ, Citino SB, Hofmeyr M. 2014. Hippopotamidae. In: West G, Heard D, Caulkett N, editors. Zoo Animal and Wildlife Immobilization and Anesthesia. 2nd ed. Ames, Iowa: John Wiley & Sons, Inc.; p. 787–795.

Miller RE, Boever WJ. 1983. Repair of a rectal stricture and prolapse in a pygmy hippopotamus (*Choeropsis liberiensis*). J Zoo Wildl Med. 14:63–66.

Millspaugh JJ, Washburn BE. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen Comp Endocrinol. 138:189–199.

Moberg GP. 2000. Biological response to stress: Implications for animal welfare. In: Moberg GP, Mench JA. editors. The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. New York, New York: CABI Publishing; p. 1–21.

Mooring MS, Patton ML, Lance VA, Hall BM, Schaad EW, Fortin SS, Jella JE, McPeak KM. 2004. Fecal androgens of bison bulls during the rut. Horm Behav. 46:392–398.

Morgan KN, Tromborg CT. 2007. Sources of stress in captivity. Appl Anim Behav Sci. 102:262–302.

Morris PJ, Bicknese B, Janssen D, Loudis B, Shima A, Sutherland-Smith M, Young L. 2001. Chemical restraint of juvenile east African river hippopotamus (*Hippopotamus amphibius kiboko*) at the San Diego Zoo. In: Heard D, editor. Zoological Restraint and Anesthesia. Ithica, New York: International Veterinary Information Systems (www.ivis.org); p. 10–14.

Morton SG. 1844. On a supposed new species of hippopotamus. Proc Acad Nat Sci Philadelphia. 2:14–17.

Möstl E, Maggs JL, Schrötter G, Besenfelder U, Palme R. 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet Res Commun. 26:127–139.

Möstl E, Messmann S, Bagu E, Robia C, Palme R. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. Zentralbl Veterinarmed A. 46:621–631.

Mousseau TA, Dingle H. 1991. Maternal effects in insect life histories. Annu Rev Entomol. 36:511–534.

Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstreet JW, Lasley BL. 1991. Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin Chem. 37:838–844.

Nees S, Schade B, Clauss M, Steinmetz HW, Ehrensperger F, Steck B, Hatt J-M. 2009. Polycystic kidney disease in the pygmy hippopotamus (*Hexaprotodon liberiensis*). J Zoo Wildl Med. 40:529–535.

Nielsen AWN, van Dreumel T, Crawshaw G, Dutton CJ, Hollamby SR, Pastor AR, Flacke GL, Smith DA. 2015. Haemorrhagic stroke in a pygmy hippopotamus. In: Proceedings of the International Conference on Diseases of Zoo and Wild Animals. Barcelona, Spain: Leibniz Institute for Zoo and Wildlife Research (IZW) & European Association of Zoo and Wildlife Veterinarians (EAZWV); p. 190.

Nohr EA, Vaeth M, Bech BH, Henriksen TB, Cnattingius S, Olsen J. 2007. Maternal obesity and neonatal mortality according to subtypes of preterm birth. Obstet Gynecol. 110:1083–1090.

Nomura O, Yasue H. 1999. Genetic relationships among hippopotamus, whales, and bovine based on SINE insertion analysis. Mamm Genome. 10:526–527.

Norris K, Asase A, Collen B, Gockowksi J, Mason J, Phalan B, Wade A. 2010. Biodiversity in a forest-agriculture mosaic – The changing face of West African rainforests. Biol Conserv. 143:2341–2350.

O'Leary CA, Mackay BM, Malik R, Edmondston JE, Robinson WF, Huxtable CR. 1999. Polycystic kidney disease in bull terriers: an autosomal dominant inherited disorder. Aust Vet J. 77:361–366.

Osakwe ME, Meduna AJ, Kigbu EE, Ishaya PD. 1988. Management of pigmy hippopotamus and West African manatee in Jos Wildlife Park. Niger F. 53:175–178.

Osorio JE, Hubbard GB, Soike KF, Girard M, van der Werf S, Moulin J-C, Palmenberg AC. 1996. Protection of non-murine mammals against encephalomyocarditis virus using a genetically engineered Mengo virus. Vaccine. 14:155–161.

Othen LS. 1997. Reproductive endocrinology of wood bison during estrus synchronization, superovulation and pregnancy. MSc Thesis: University of Guelph.

Pacifici M, Santini L, Di Marco M, Baisero D, Francucci L, Grottolo Marasini G, Visconti P, Rondinini C. 2013. Generation length for mammals. Nat Conserv. 5:87–94.

Palme R, Fischer P, Schildorfer H, Ismail MN. 1996. Excretion of infused 14C-steroid hormones via faeces and urine in domestic livestock. Anim Reprod Sci. 43:43–63.

Palme R, Möstl E. 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int J Mamm Biol Suppl II. 62:192–197.

Palme R, Rettenbacher S, Touma C, El-Bahr SM, Möstl E. 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Ann N Y Acad Sci. 1040:162–171.

Palme R, Robia C, Messmann S, Möstl E. 1998. Measuring faecal cortisol metabolites: a non-invasive tool to evaluate adrenocortical activity in mammals. Adv Ethol. 33:27.

Paris M, Millar R, Colenbrander B, Schwarzenberger F. 2008. Non-invasive assessment of female reproductive physiology in the pygmy hippopotamus (*Choeropsis liberiensis*). In: Proc 16th Int Congr Anim Reprod. Budapest, Hungary; p. 17.

Paris MCJ, Mastromonaco GF, Paris DBBP, Krisher RL. 2007. A perspective on the role of emerging technologies for the propagation of companion animals, non-domestic and endangered species. Reprod Fertil Dev. 19:iii–vii.

Partridge J. 1983. The management of the pygmy hippopotamus (*Choeropsis liberiensis*) at Bristol Zoo. Int Zoo News. 30:28–41.

Patton ML, Swaisgood RR, Czekala NM, White AM, Fetter GA, Montagne JP, Rieches RG, Lance VA. 1999. Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biol. 18:111–127.

Patzl M, Schwarzenberger F, Osmann C, Bamberg E, Bartmann W. 1998. Monitoring ovarian cycle and pregnancy in the giant anteater (*Myrmecophaga tridactyla*) by faecal progestagen and oestrogen analysis. Anim Reprod Sci. 53:209–219.

Pazzoto Alves I, Diniz Garcia S, Magnani Grassi TL, Ribeiro de Araújo Rocha G, Franciscato DA, Kipper BH, Burkhardt de Koivisto M. 2016. Epididymal spermatozoa from *Hippopotamus amphibious*. Anim Reprod Sci. 169:110–111.

Pearce PC, Gustavo C, Gulland F, Knight J. 1985. Immobilization of a pygmy hippopotamus (*Choeropsis liberiensis*). J Zoo Anim Med. 16:104–106.

Penfold LM, Powell D, Traylor-Holzer K, Asa CS. 2014. "Use it or lose it": characterization, implications, and mitigation of female infertility in captive wildlife. Zoo Biol. 33:20–28.

Peters DJ, Breuning MH. 2001. Autosomal dominant polycystic kidney disease: modification of disease progression. Lancet. 358:1439–1444.

Pienaar U de V., van Wyk P, Fairall N. 1966. An experimental cropping scheme of hippopotami in the Letaba River of the Kruger National Park. Koedoe. 9:1–33.

Pierce BN, Clarke IJ, Turner AI, Rivalland ETA, Tilbrook AJ. 2009. Cortisol disrupts the ability of estradiol-17β to induce the LH surge in ovariectomized ewes. Domest Anim Endocrinol. 36:202–208.

Pilleri G. 1962. Zur Anatomie des Gehirnes von *Choeropsis liberiensis* Morton (Mammalia, Artriodactyla). Acta Zool. 43:229–245.

Pocock RI. 1923. The external characteristics of the pigmy hippopotamus (*Choeropsis liberiensis*) and of the Suidæ and Camelidæ. Proc Zool Soc London. 35:531–549.

Pratt NC, Lisk RD. 1989. Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (*Mesocricetus auratus*). J Reprod Fertil. 87:763–769.

Pukazhenthi B, Quse V, Hoyer M, van Engeldorp Gastelaars H, Sanjur O, Brown JL. 2013. A review of the reproductive biology and breeding management of tapirs. Integr Zool. 8:18–34.

Pukazhenthi BS, Wildt DE. 2004. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod Fertil Dev. 16:33–46.

Rachoń D, Teede H. 2010. Ovarian function and obesity - interrelationship, impact on women's reproductive lifespan and treatment options. Mol Cell Endocrinol. 316:172–179.

Rahn P. 1978. On housing the pygmy hippopotamus in pairs: a survey of zoo practice. Int Zoo Yearb. 18:187–190.

Ransom C, Robinson PT, Collen B. 2015. Choeropsis liberiensis. IUCN Red List Threat Species 2015. Available from: http://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T10032A18567171.en. Accessed 22.12.2015.

Raymond JT, Eaton KA, Montali RJ. 2000. A disease in captive pygmy hippopotamuses (*Choeropsis liberiensis liberiensis*) anatomically resembling polycystic kidney disease. In: Proceedings of the American Association of Zoo Veterinarians (AAZV) and International Association for Aquatic Animal Medicine (IAAAM) Joint Conference. New Orleans, Louisiana; p. 302.

Reddacliff LA, Kirkland PD, Hartley WJ, Reece RL. 1997. Encephalomyocarditis virus infections in an Australian zoo. J Zoo Wildl Med. 28:153–157.

Reifinger VM, Kübber-Heiss A, Linhart P. 1997. Streptokokkenseptikämie und Candidiasis bei einem sieben Tage alten Flusspferd (*Hippopotamus amphibius*) mit Missbildung der grossen herznahen Gefässe. Erkrankungen der Zootiere Verhandlungsbericht. 38:395.

Renshaw G. 1904. The Pigmy Hippopotamus. In: Nat Hist Essays. London, UK: Sherratt & Hughes; p. 113–125.

Rey MS. 2013. Feline hereditary and congenital kidney diseases. IVIS Vet Focus. 23:10–16.

Rivier C, Rivest S. 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biol Reprod. 45:523–532.

Robinson PT. 1970. The Status of the Pygmy Hippopotamus and Other Wildlife in West Africa. MSc Thesis: Michigan State University.

Robinson PT. 2013. *Choeropsis liberiensis* Pygmy Hippopotamus. In: Kingdon J, Hoffman M, editors. Mamm Africa, Vol VI - Pigs, Hippopotamuses, Deer Bovids. London, UK: Bloomsbury; p. 80–83.

Rosenfeld CS, Roberts RM. 2004. Maternal diet and other factors affecting offspring sex ratio: A review. Biol Reprod. 71:1063–1070.

Roth HH. 1962. Mitteilung über die Zwergflußpferdzucht in Gelsenkirchen. Der Zool Garten NF. 26:327–331.

Roth HH, Hoppe-Dominik B, Mühlenberg M, Steinhauer-Burkart B, Fischer F. 2004. Distribution and status of the hippopotamids in the Ivory Coast. Afr J Ecol. 39:211–224.

Safar-Hermann N, Ismail MN, Choi HS, Möstl E, Bamberg E. 1987. Pregnancy diagnosis in zoo animals by estrogen determination in feces. Zoo Biol. 6:189–193.

Santos VG, Bettencourt EM V., Ginther OJ. 2015. Long-term characteristics of idiopathic persistent corpus luteum in the mare. Theriogenology. 84:242–251.

Santymire RM, Brown JL, Stewart RA, Santymire RC, Wildt DE, Howard J. 2011. Reproductive gonadal steroidogenic activity in the fishing cat (*Prionailurus viverrinus*) assessed by fecal steroid analyses. Anim Reprod Sci. 128:60–72.

Sapolsky RM. 1992. Neuroendocrinology of the stress response. In: Becker JB, Breedlove SM, Crews D, editors. Behavioral Endocrinology. Cambridge, Massachusetts: MIT Press; p. 287–324.

Saragusty J, Hermes R, Hofer H, Bouts T, Göritz F, Hildebrandt TB. 2012. Male pygmy hippopotamus influence offspring sex ratio. Nat Commun. 3:1–5.

Saragusty J, Hildebrandt TB, Bouts T, Göritz F, Hermes R. 2010. Collection and preservation of pygmy hippopotamus (*Choeropsis liberiensis*) semen. Theriogenology. 74:652–657.

Saragusty J, Walzer C, Petit T, Stalder G, Horowitz I, Hermes R. 2010. Cooling and freezing of epididymal sperm in the common hippopotamus (*Hippopotamus amphibius*). Theriogenology. 74:1256–1263.

Sayer JA, Rakha AM. 1974. The age of puberty of the hippopotamus (*Hippopotamus amphibius* Linn.) in the Luangwa River in eastern Zambia. East African Wildl J. 12:227–232.

Schanberger A, Weinhardt D. 1997. Management of pygmy hippopotamus (*Choeropsis liberiensis*) at the Houston Zoological Gardens. In: American Zoological Association (AZA) Regional Conference Proceedings. Bethesda, Maryland: AZA; p. 377–381.

Schatz S, Palme R. 2001. Measurement of faecal cortisol metabolites in cats and dogs: A non-invasive method for evaluating adrenocortical function. Vet Res Commun. 25:271–287.

Schomburgk H. 1912. On the trail of the pygmy hippo – an account of the Hagenbeck expedition to Liberia. New York Zool Soc Bull. 16:880–884.

Schomburgk H. 1913. Das Zwergflußpferd, eine zoologische Neuheit. Kosm Handweiser für Naturfreunde. 2:62–65.

Schubert T. 2004. Haltung von Zwergflusspferden. In: Puschmann W, editor. Zootierhaltung. Frankfurt, Germany: Verlag Harri Deutsch; p. 636–639.

Schulze W. 1955. Nephritis beim Zwergflußpferd. Der Zool Garten NF. 21:188.

Schwarm A, Ortmann S, Hofer H, Streich WJ, Flach EJ, Kühne R, Hummel J, Castell JC, Clauss M. 2006. Digestion studies in captive Hippopotamidae: a group of large ungulates with an unusually low metabolic rate. J Anim Physiol Anim Nutr. 90:300–308.

Schwarm A, Ortmann S, Wolf C, Jürgen Streich W, Clauss M. 2008. Excretion patterns of fluid and different sized particle passage markers in banteng (*Bos javanicus*) and pygmy hippopotamus (*Hexaprotodon liberiensis*): two functionally different foregut fermenters. Comp Biochem Physiol Part A. 150:32–39.

Schwarm A, Ortmann S, Wolf C, Jürgen Streich W, Clauss M. 2009. More efficient mastication allows increasing intake without compromising digestibility or necessitating a larger gut: comparative feeding trials in banteng (*Bos javanicus*) and pygmy hippopotamus (*Hexaprotodon liberiensis*). Comp Biochem Physiol Part A. 152:504–512.

Schwarzenberger F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. Int Zoo Yearb. 41:52–74.

Schwarzenberger F, Brown JL. 2013. Hormone monitoring: An important tool for the breeding management of wildlife species. Wien Tierarztl Monatsschr. 100:209–225.

Schwarzenberger F, Francke R, Göltenboth R. 1993. Concentrations of faecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*). J Reprod Fertil. 98:285–291.

Schwarzenberger F, Kolter L, Zimmerman W, Rietschel W, Matern B, Birher P, Leus K. 1998. Faecal cortisol metabolite measurement in the okapi (*Okapi johnstoni*). Adv Ethol. 33:28.

Schwarzenberger F, Möstl E, Bamberg E, Pammer J, Schmehlik O. 1991. Concentrations of progestagens and oestrogens in the faeces of pregnant Lipizzan, trotter and thoroughbred mares. J Reprod Fertil Suppl. 44:489–499.

Schwarzenberger F, Möstl E, Palme R, Bamberg E. 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim Reprod Sci. 42:515–526.

Schwarzenberger F, Rietschel W, Matern B, Schaftenaar W, Bircher P, Van Puijenbroeck B, Leus K. 1999. Noninvasive reproductive monitoring in the okapi (*Okapia johnstoni*). J Zoo Wildl Med. 30:497–503.

Schwarzenberger F, Rietschel W, Vahala J, Holeckova D, Thomas P, Maltzan J, Baumgartner K, Schaftenaar W. 2000. Fecal progesterone, estrogen, and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros, *Rhinoceros unicornis*. Gen Comp Endocrinol. 119:300–307.

Schwarzenberger F, Tomášová K, Holečková D, Matern B, Möstl E. 1996. Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol. 15:159–171.

Schwarzenberger F, Walzer C, Tomasova K, Vahala J, Meister J, Goodrowe KL, Zima J, Strauß G, Lynch M. 1998. Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*). Anim Reprod Sci. 53:173–190.

Sclater PL. 1873. Remarks on the Liberian hippopotamus. Proc Zool Soc London.:434.

Seaman JT, Finnie EP. 1987. Acute myocarditis in a captive African elephant (*Loxodonta africana*). J Wildl Dis. 23:170–171.

Senn H, O'Donoghue P, McEwing R, Ogden R. 2014. Hundreds of SNPs for the Endangered pygmy hippopotamus. Conserv Genet Resour. 6:535–538.

Shepherdson D, Lewis KD, Carlstead K, Bauman J, Perrin N. 2013. Individual and environmental factors associated with stereotypic behavior and fecal glucocorticoid metabolite levels in zoo housed polar bears. Appl Anim Behav Sci. 147:268–277.

Shimamura M, Yasue H, Ohshima K, Abe H, Kato H, Kishiro T, Goto M, Munechika I, Okada N. 1997. Molecular evidence from retroposons that whales form a clade within even-toed ungulates. Nature. 388:666–670.

Shutt K, Setchell JM, Heistermann M. 2012. Non-invasive monitoring of physiological stress in the Western lowland gorilla (*Gorilla gorilla gorilla*): validation of a fecal glucocorticoid assay and methods for practical application in the field. Gen Comp Endocrinol. 179:167–177.

Smith TE, Richards M, Joseph S, Savage A. 2000. Endocrine determinants of pregnancy in the Nile hippopotamus (*Hippopotamus amphibius*). In: Proc 2nd Annu Symp Zoo Res. Paignton Zoo Environmental Park, Paignton, Devon, United Kingdom; p. 187–190.

Smuts GL, Whyte IJ. 1981. Relationships between reproduction and environment in the hippopotamus *Hippopotamus amphibius* in the Kruger National Park. Koedoe. 24:169–185.

Soares JF, Pereira H, Desta FS, Sandouka M, Macasero W. 2015. Causes of mortality of captive Arabian gazelles (*Gazella arabica*) at King Khalid Wildlife Research Centre, Kingdom of Saudi Arabia, from 1988 to 2011. J Zoo Wildl Med. 46:1–8.

Spriggs M, Reeder C. 2012. Treatment of vasculitis and dermatitis in a 59-yr-old Nile hippopotamus (*Hippopotamus amphibius*). J Zoo Wildl Med. 43:652–656.

Stead SK, Meltzer DG, Palme R. 2000. The measurement of glucocorticoid concentrations in the serum and faeces of captive African elephants (*Loxodonta africana*) after ACTH stimulation. J S Afr Vet Assoc. 71:192–196.

Steck B, editor. 2012. Pygmy Hippopotamus *Choeropsis liberiensis* (Morton, 1844) International Studbook 2011. 18th ed. Basel: Zoo Basel, Switzerland.

Steck B, editor. 2014. Pygmy Hippopotamus *Choeropsis liberiensis* (Morton, 1844) International Studbook 2013. 20th ed. Basel: Zoo Basel, Switzerland.

Steck B, editor. 2015. Pygmy Hippopotamus *Choeropsis liberiensis* (Morton, 1844) International Studbook 2014. 21st ed. Basel: Zoo Basel, Switzerland.

Steck B, editor. 2016. International Studbook for the Pygmy Hippopotamus 2015. 22nd ed. Basel: Zoo Basel, Switzerland.

Steinmetz H. 1937. Beobachtungen über die Entwicklung junger Zwergflußpferde im Zoologischen Garten Berlin. Der Zool Garten NF. 9:255–263.

Stoops MA, West GD, Roth TL, Lung NP. 2014. Use of urinary biomarkers of ovarian function and altrenogest supplementation to enhance captive breeding success in the Indian rhinoceros (*Rhinoceros unicornis*). Zoo Biol. 33:83–88.

Stroman HR, Slaughter LM. 1972. The care and breeding of the pygmy hippopotamus. Int Zoo Yearb. 12:126–131.

Stubben CJ, Milligan BG. 2007. Estimating and analyzing demographic models using the popbio package in R. J Stat Softw. 22:11.

Swanson WF, Johnson WE, Cambre RC, Citino SB, Quigley KB, Brousset DM, Morais RN, Moreira N, O'Brien SJ, Wildt DE. 2003. Reproductive status of endemic felid species in Latin American zoos and implications for ex situ conservation. Zoo Biol. 22:421–441.

Taylor D, Greenwood A. 1986. Hippopotamidae (Hippopotamus). In: Fowler ME, editor. Zoo and Wild Animal Medicine. 2nd ed. Philadelphia, Pennsylvania: W.B. Saunders Company; p. 967–969.

Taylor VJ, Poole TB. 1998. Captive breeding and infant mortality in Asian elephants: A comparison between twenty western zoos and three eastern elephant centers. Zoo Biol. 17:311–332.

Terio KA, Marker L, Munson L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. J Wildl Dis. 40:259–266.

Thompson SD. 2002. North American Regional Studbook for the Pygmy Hippopotamus (*Hexaprotodon liberiensis*). Brown E, editor. Chicago, Illinois: Lincoln Park Zoo.

Thompson SD, Ryan S. 2001. AZA Pygmy Hippopotamus Husbandry Manual. Chicago, Illinois: Lincoln Park Zoo.

Thomson GR, Bengis RG, Brown CC. 2001. Picornavirus infections. In: Williams ES, Barker IK, editors. Infectious Diseases of Wild Mammals. 3rd ed. Ames, Iowa: Iowa State University Press; p. 119–130.

Tilbrook AJ, Turner AI, Clarke IJ. 2000. Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. Rev Reprod. 5:105–113.

Torres VE, Harris PC. 2007. Polycystic kidney disease: genes, proteins, animal models, disease mechanisms and therapeutic opportunities. J Intern Med. 261:17–31.

Torres VE, Harris PC, Pirson Y. 2007. Autosomal dominant polycystic kidney disease. Lancet. 369:1287–1301.

Touma C, Palme R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann N Y Acad Sci. 1046:54–74.

Touma C, Sachser N, Möstl E, Palme R. 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocrinol. 130:267–278.

Trivers RL, Willard DE. 1973. Natural selection of parental ability to vary the sex ratio of offspring. Science (80-). 179:90–92.

United Nations Environment Programme, World Conservation Monitoring Centre. 2011. Taï National Park, Côte d'Ivoire. Cambridge, United Kingdom: UNEP-WCMC.

Ursing BM, Arnason U. 1998. Analyses of mitochondrial genomes strongly support a hippopotamus-whale clade. Proc R Soc London B. 265:2251–2255.

Verschuren J. 1983. Conservation of Tropical Rain Forest in Liberia - Recommendations for Wildlife Conservation and National Parks. Gland, Switzerland.

Walker EP. 1964. Hippopotamuses. In: Mammals of the World, Vol. 2. Baltimore, Maryland: The Johns Hopkins Press; p. 1367–1370.

Walker SL, Smith RF, Jones DN, Routly JE, Dobson H. 2008. Chronic stress, hormone profiles and estrus intensity in dairy cattle. Horm Behav. 53:493–501.

Walker SL, Waddell WT, Goodrowe KL. 2002. Reproductive endocrine patterns in captive female and male red wolves (*Canis rufus*) assessed by fecal and serum hormone analysis. Zoo Biol. 21:321–335.

Walzer C, Stalder G. 2014. Hippopotamidae (Hippopotamus). In: Miller RE, Fowler ME, editors. Fowler's Zoo and Wild Animal Medicine. 8th ed. Philadelphia, Pennsylvania: Saunders Elsevier; p. 584–592.

Washburn BE, Millspaugh JJ. 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. Gen Comp Endocrinol. 127:217–222.

Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, Bechert U, Millspaugh JJ, Larson S, Monfort SL. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. Gen Comp Endocrinol. 120:260–275.

Wasser SK, Thomas R, Nair PP, Guidry C, Southers J, Lucas J, Wildt DE, Monfort SL. 1993. Effects of dietary fibre on faecal steroid measurements in baboons (*Papio cynocephalus cynocephalus*). J Reprod Fertil. 97:569–574.

Wells SK, Gutter AE, Soike KF, Baskin GB. 1989. Encephalomyocarditis virus : Epizootic in a zoological collection. J Zoo Wildl Med. 20:291–296.

Weston EM. 2000. A new species of hippopotamus *Hexaprotodon lothagamensis* (Mammalia: Hippopotamidae) from the late Miocene of Kenya. J Vertebr Paleontol. 20:177–185.

Weston EM. 2003. Evolution of ontogeny in the hippopotamus skull: using allometry to dissect developmental change. Biol J Linn Soc. 80:625–638.

Weston HS, Fagella AM, Burt L, Crowley K, Moore T. 1996. Immobilization of a pygmy hippopotamus (*Choeropsis liberiensis*) for the removal of an oral mass. In: Proceedings of the American Association of Zoo Veterinarians (AAZV) Annual Conference. Puerto Vallarta, Mexico; p. 576–581.

Van den Bergh HK. 1971. Can the pygmy hippopotamus, *Choeropsis liberiensis* (Morton), look through its open mouth? Der Zool Garten NF. 40:167–171.

van der Goot AC, Martin GB, Millar RP, Paris MCJ, Ganswindt A. 2015. Profiling patterns of fecal 20-oxopregnane concentrations during ovarian cycles in free-ranging southern white rhinoceros (*Ceratotherium simum simum*). Anim Reprod Sci. 161:89–95.

Van der Weyde LK, Martin GB, Paris MCJ. 2016. Monitoring stress in captive and free-ranging African wild dogs (*Lycaon pictus*) using faecal glucocorticoid metabolites. Gen Comp Endocrinol. 226:50–55.

van Heukelum M. 2011. In search of the elusive Pygmy Hippo; Establishment of methods to determine population structure of Pygmy Hippos in Taï National Park, and assessment of their role in seed dispersal. MSc Thesis: Wageningen University.

van Jaarsveld AS, Skinner JD. 1992. Adrenocortical responsiveness to immobilization stress in spotted hyenas (*Crocuta crocuta*). Comp Biochem Phys A 103(1):73–79.

von Houwald F, Macdonald AA, Pagan O, Steck B, editors. 2007. Husbandry Guidelines for the Pygmy Hippopotamus (*Hexaprotodon liberiensis*). Basel: Zoo Basel, Switzerland.

Wheaton CJ, Joseph S, Reid K, Webster T, Richards M, Savage A. 2006. Body weight as an effective tool for determination of onset of puberty in captive female Nile hippopotami (*Hippopotamus amphibious*). Zoo Biol. 25:59–71.

Whitten PL, Brockman DK, Stavisky RC. 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearb Phys Anthropol. 41:1–23.

Wielebnowski N. 1996. Reassessing the relationship between juvenile mortality and genetic monomorphorism in captive cheetahs. Zoo Biol. 15:353–369.

Wielebnowski NC, Fletchall N, Carlstead K, Busso JM, Brown JL. 2002. Noninvasive assessment of adrenal activity associated with husbandry and behavioral factors in the North American clouded leopard population. Zoo Biol. 21:77–98.

Wielebnowski NC, Ziegler K, Wildt DE, Lukas J, Brown JL. 2002. Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). Anim Conserv. 5:291–301.

Wildt DE, Brown JL, Bush M, Barone MA, Cooper KA, Grisham J, Howard JG. 1993. Reproductive status of cheetahs (*Acinonyx jubatus*) in North American zoos: The benefits of physiological surveys for strategic planning. Zoo Biol. 12:45–80.

Wilson PD. 2004. Polycystic kidney disease. N Engl J Med. 350:151–164.

Wingfield JC, Hegner RE, Dufty Jr. AM, Ball GF. 1990. The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am Nat. 136:829–846.

Wingfield JC, Sapolsky RM. 2003. Reproduction and resistance to stress: When and how. J Neuroendocrinol. 15:711–724.

Wings O, Hatt J-M, Schwarm A, Clauss M. 2008. Gastroliths in a pygmy hippopotamus (*Hexaprotodon liberiensis* Morton 1844). Senckenb Biol. 88:345–348.

Young KM, Walker SL, Lanthier C, Waddell WT, Monfort SL, Brown JL. 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. Gen Comp Endocrinol. 137:148–165.

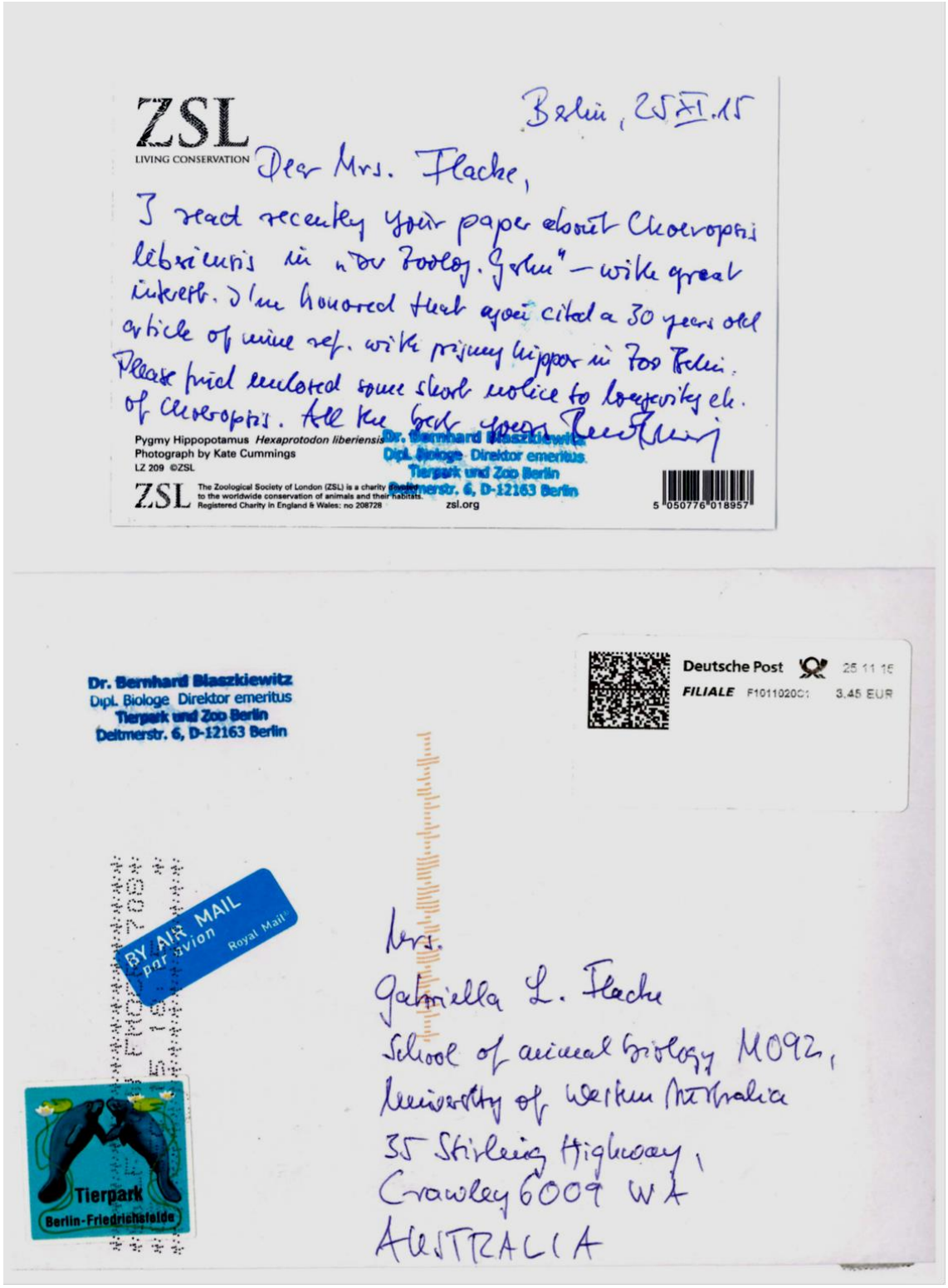
Zerbe P, Clauss M, Codron D, Bingaman Lackey L, Rensch E, Streich JW, Hatt JM, Müller DWH. 2012. Reproductive seasonality in captive wild ruminants: Implications for biogeographical adaptation, photoperiodic control, and life history. Biol Rev. 87:965–990.

Zerres K, Rudnik-Schöneborn S, Steinkamm C, Becker J, Mücher G. 1998. Autosomal recessive polycystic kidney disease. J Mol Med. 76:303–309.

Zschokke S. 2002. Distorted sex ratio at birth in the captive pygmy hippopotamus, *Hexaprotodon liberiensis*. J Mammal. 83:674–681.

Zschokke S, Steck B. 2001. Tragzeit und Geburtsgewicht beim Zwergflusspferd, *Hexaprotodon liberiensis*. Der Zool Garten NF. 71:57–61.

Appendix I (CHAPTER 2) – LETTER FROM DR. BLASZKIEWITZ, TIERPARK BERLIN



ZSL
LIVING CONSERVATION

Berlin, 25.11.15

Dear Mrs. Flache,

I read recently your paper about Choeropsis liberiensis in "der Zoolog. Garten" – with great interest. I'm honored that again cited a 30 years old article of mine ref. with primary keeper in Zoo Berlin. Please find enclosed some short notice to longevity etc. of Choeropsis. All the best yours

Dr. Bernhard Blaszkiewitz
Dipl. Biologe Direktor emeritus
Tierpark und Zoo Berlin
Deltmerstr. 6, D-12163 Berlin

Pygmy Hippopotamus *Hexaprotodon liberiensis*
Photograph by Kate Cummings
LZ 209 ©ZSL

ZSL The Zoological Society of London (ZSL) is a charity devoted to the worldwide conservation of animals and their habitats. Registered Charity in England & Wales: no 208728
zsl.org

5 050776 018957

Deutsche Post FILIALE F10110200 25.11.15 3.45 EUR

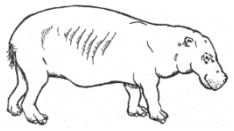
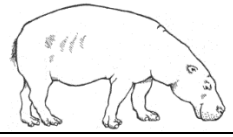
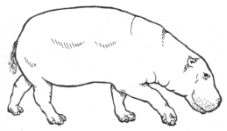
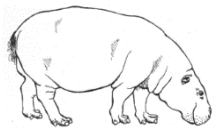
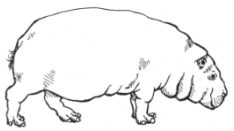
Dr. Bernhard Blaszkiewitz
Dipl. Biologe Direktor emeritus
Tierpark und Zoo Berlin
Deltmerstr. 6, D-12163 Berlin

BY AIR MAIL par avion Royal Mail

Tierpark Berlin-Friedrichsfelde

Mrs.
Gabriella L. Flache
School of animal biology M092,
University of Western Australia
35 Stirling Highway,
Crawley 6009 WA
AUSTRALIA

Appendix II (CHAPTER 3) – BODY CONDITION SCORE CHART

SCORE	1. Emaciated	2. Underweight	3. Ideal	4. Overweight	5. Obese
					
Head & Neck	• Loose skin sagging around the neck • Sunken, hollow appearance to facial structures	• Thin, narrow neck • Reduced subcutaneous tissue around facial features	• Neck has minimal ventral skin folds • Neck narrower than back of head when viewed from above • Face appears full	• Visible rolls of skin and fat at ventral neck behind angle of jaw. • Neck similar diameter to back of head; no tapering apparent	• Rolls of fat prominent and full on dorsal and ventral neck • Neck is much thicker in diameter than the head
Ribs	• Individual ribs prominent	• Rib cage is apparent but individual ribs not discernable	• Rib cage is not visible, but ribs can still be felt underneath	• Rib cage is not visible, ribs cannot be felt	• Thick fat cover, rib cage is not visible and ribs cannot be felt
Back & Scapula	• Dorsal spinous process of vertebrae visible and prominent along the back • Top of scapula visible • Generalized lack of subcutaneous fat	• Dorsal spinous processes are not visible but can be readily palpated • Top of scapula not readily visible but palpable	• Dorsal spinous processes are not visible and can only be palpated with firm pressure • Top of scapula not visible or palpable	• Dorsal spinous processes not palpable • Scapula not apparent or palpable • Skin rolls noticeable behind the elbow	• The area directly above the spine is indented between layers of back fat on both sides along the length of the spine • Fat rolls quite apparent behind the elbow
Abdomen	• Abdomen tucked up in appearance • Loose skin may be noticeable at ventral aspect	• Abdomen slightly tucked; belly not rounded	• Abdomen full but not sagging; belly slightly rounded	• Abdomen very full; moderate sagging • Skin starts to fold or roll above fore and rear limbs at the elbow/knee	• Abdomen very rounded and noticeably sagging • Fat rolls prominent above the fore and rear limbs at the elbow/knee
Flank	• Flank area sunken and narrow when viewed from above • Transverse spinous processes readily apparent	• Flank area is slightly hollow but not sunken • Edges of the transverse spinal processes visible but not prominent	• Flank is full • Edges of the transverse spinal process not visible but palpable with firm pressure	• Flank appears full and rounded • Edges of transverse spinal process not visible or palpable	• Flank very full and rounded, flank area not discernable
Pelvis &Tail Base	• Pelvic bones are very prominent • Deep, sunken cavity around the tail base (viewed from behind)	• Pelvic bones are visible but covered • The tail base is covered but flat (viewed from behind)	• Pelvic bones not visible, and are only palpable with firm pressure • Tail base full but not rounded	• Pelvic area rounded, pelvis cannot be palpated • Tail base rounded, set within prominent fat	• Pelvic area very rounded, hippo becomes barrel-shaped • Tail base bulging, set deeply in surrounding fat

Appendix III (CHAPTER 4) – LIFE TABLE COMPONENT DATA

Age	407 deceased ♀s			222 living ♀s			
(yrs)	px	mx	Ex	px	mx	fxl	pfxl
0	0.547912	0.000000	14.57	0.743333	0.000000	12	12.00
1	0.905830	0.000000	24.26	0.986547	0.000000	7	8.92
2	0.945545	0.004950	25.18	0.985227	0.000000	14	8.80
3	0.958115	0.000000	25.07	0.983852	0.000000	9	8.67
4	0.956284	0.014104	24.62	0.981243	0.027778	11	8.53
5	0.982857	0.045714	24.20	0.978495	0.080361	8	8.37
6	0.970930	0.127907	23.11	0.976801	0.100385	9	8.19
7	0.970060	0.135216	22.27	0.975000	0.085526	7	8.00
8	0.950617	0.083833	21.43	0.973077	0.062069	11	7.80
9	0.948052	0.121708	20.99	0.971014	0.138664	6	7.59
10	0.958904	0.134116	20.58	0.968792	0.098289	6	7.37
11	0.928571	0.126736	19.92	0.967787	0.152303	6	7.14
12	0.976923	0.153115	19.88	0.965268	0.130707	3	6.91
13	0.968504	0.125984	18.82	0.962519	0.097345	6	6.67
14	0.959350	0.094154	17.90	0.961059	0.113664	6	6.42
15	0.949153	0.111542	17.12	0.959481	0.079208	6	6.17
16	0.973214	0.162161	16.48	0.956081	0.073684	9	5.92
17	0.935780	0.131413	15.41	0.955830	0.123035	5	5.66
18	0.911765	0.142951	14.90	0.951941	0.081247	4	5.41
19	0.956989	0.129032	14.74	0.951456	0.059494	7	5.15
20	0.932584	0.175067	13.86	0.948980	0.108300	4	4.90
21	0.951807	0.139530	13.29	0.948387	0.106061	7	4.65
22	0.936709	0.159253	12.41	0.945578	0.111542	4	4.41
23	0.891892	0.088932	11.68	0.942446	0.054545	7	4.17
24	0.939394	0.151515	11.48	0.941476	0.125000	5	3.93
25	0.935484	0.083258	10.65	0.940541	0.069767	3	3.70
26	0.913793	0.130707	9.82	0.936782	0.075000	4	3.48
27	0.943396	0.132075	9.15	0.935583	0.000000	5	3.26
28	0.880000	0.020000	8.14	0.934426	0.064516	1	3.05
29	0.886364	0.068182	7.61	0.929825	0.100000	5	2.85

Age	407 deceased ♀s			222 living ♀s			
(yrs)	**px**	**mx**	**Ex**	**px**	**mx**	**fxl**	**pfxl**
30	0.846154	0.051282	6.96	0.932075	0.000000	4	2.65
31	0.969697	0.030303	6.55	0.927126	0.000000	4	2.47
32	0.781250	0.000000	5.22	0.925764	0.058824	3	2.29
33	0.720000	0.040000	4.90	0.924528	0.000000	1	2.12
34	0.833333	0.000000	4.92	0.923469	0.000000	2	1.96
35	0.733333	0.000000	4.20	0.917127	0.181818	1	1.81
36	0.909091	0.000000	3.86	0.921687	0.000000	1	1.66
37	0.500000	0.000000	2.65	0.915033	0.000000	1	1.53
38	0.600000	0.000000	2.80	0.914286	0.000000	5	1.40
39	0.666667	0.000000	2.50	0.914062	0.000000	1	1.28
40	0.500000	0.000000	1.75	0.905983	0.000000	0	1.17
41	0.000000	0.000000	1.00	0.915094	0.000000	1	1.06
42	0.000000	0.000000	0.00	0.000000	0.000000	1	0.97
43	0.000000	0.000000	0.00	0.000000	0.000000	0	0.00
λ (pulse)	0.985966			1.011313			

All p_x and m_x values were calculated using the birth pulse model. These data were then converted using Ebert's (1999, pp. 89–91) formulas to a birth flow model. For birth flow, *xl* represents age (in years); *fxl* is the actual age distribution, or frequency of individuals in each age class; and *pfxl* is the age distribution transformed by a factor of *p*, or the Caughley correction factor (Caughley, 1977), for each age class such that the resulting survival curve exhibits a constant rather than irregular decline over time.

Appendix IV (CHAPTER 5) – FEMALE PYGMY HIPPO DEMOGRAPHICS

Studbook (SB) number, demographics, sampling date range (Lab A, B or C), and reproductive events for 36 female pygmy hippos (*Choeropsis liberiensis*) from European and North American zoological institutions. Estrus behavior and mating are based on husbandry staff observations and were not reported for some study animals.

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
438	Betty	38	No	–	–	21 May 2014	–	–	–	Suspected post-reproductive
						26 Apr 2015				
441	Paula	30	Yes☼	14 Sep 2006	07 Oct 2007	–	14 Nov 2007	04 Apr 2007	–	
				04 Oct 2007	24 Aug 2009		17 Sep 2008	15 Apr 2008		
							01 Dec 2008	16 Jun 2008		
							06 Mar 2009	19 Jul 2008		
								03 Dec 2008		
								11 Jan 2009		

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
444	Agnes	38	No	–	–	14 Apr 2014	–	–	–	Suspected post-reproductive
						16 Apr 2015				
454	Adele	37	No	–	–	20 May 2014	–	–	–	Suspected post-reproductive
						21 May 2015				
548	Tana	27	Yes	06 Apr 2007	–	–	08 Jun 2007	–	–	
				13 Jan 2009			10 Jul 2007			
							08 Aug 2007			
626	Lise	23	Yes☼	14 Dec 2006	–	–	22 Jan 2007	12 Dec 2006	26 Nov 2006[b]	
				13 Aug 2007			03 Mar 2007			
							03 Apr 2007			
							10 May 2007			

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
626	Lise (con't)	23	Yes☼				11 Jun 2007 21 Jul 2007 24 Aug 2007			
687	Lalla	21	Yes[Δ]	02 Oct 2006 15 Sep 2008	–	–	–	2-3 Oct 2006[a]	20 Jul 2006[b] 23 Apr 2007	
758	Obesa	26	No	–	–	20 May 2014 21 May 2015	15 Jan 2015 17-19 Feb 2015	–	–	
772	Anais	19	Yes	05 Feb 2007 05 Oct 2007	–	–	–	–	05 Jun 2007	
817	Tootsie	24	Yes☼	–	–	14 Apr 2014 16 Apr 2015	–	–	23 Mar 2014	Housed with an adult male from 21 Aug 2014 to 15 Jan 2015

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
864	Kelsey	22	No	–	–	05 Mar 2014	13 Mar 2014 14 May 2014 07 Jun 2014 03 Jul 2014 26 Aug 2014 18 Mar 2015	–	–	Housed separately from but adjacent to an adult male
904	Nelie	13	No	13 Oct 2006 20 Feb 2009	–	–	–	–	–	
913	Leah	13	Yes☼	–	28 Oct 2007 03 May 2009	–	–	–	–	Male arrived 21 May 2008

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
931	Hope	19	Yes[Δ]	–	–	05 May 2014	–	–	28 Aug 2014	
						04 May 2015				
938	Wendy	13	Yes[☼]	–	25 Mar 2008	–	17 Feb 2009	25 Apr 2008[a]	11 Nov 2008	
					23 May 2009		21 Mar 2009			
941	Sabine	11	Yes[☼]	10 Oct 2006	–	–	–	–	–	
				06 Dec 2007						
993	Debby	9	Yes[Δ]	05 Sep 2006	08 Oct 2007	–	22 Sep 2006	24 Oct 2006[a]	17 May 2007	
				05 Oct 2007	02 Apr 2009			22 May 2008[a]	18 Dec 2008	
1028	Monica	8	Yes	02 Nov 2006	–	–	15 Apr 2008	01 Mar 2009[a]	11 Dec 2006	
				22 Jan 2009			09 May 2008		19 Sep 2009	
							02 Jun 2008			

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
1028	Monica (con't)	8	Yes				19 Aug 2008			
							31 Oct 2008			
							03 Dec 2008			
1033	Torpedo	8	Yes[Δ]	25 Jul 2006	–	–	25 Sep 2006	17 Aug 2007[a]	17 Feb 2006	
				13 Nov 2009			30 Jan 2007	15 Apr 2009[a]	06 Mar 2008	
									28 Oct 2009	
1063	Chomel	14	Yes[Δ]	–	–	20 May 2014	–	22 May 2014[a]	07 Dec 2014[b]	
						18 May 2015		21-22 Dec 2014	01 Oct 2015	
								03-05 Feb 2015		
								07-09 Mar 2015		
								15 Mar 2015[a]		

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
1068	Venus	7	Yes☼	21 Mar 2007	25 Oct 2007	–	27 May 2007	05 May 2009[a]	27 Nov 2009	Inconsistent sampling
				29 Sep 2007	03 Aug 2009		02 Jul 2007			
							22 Oct 2008			
							14 Apr 2009			
1078	Tanquey	7	Yes	06 Apr 2007	–	–	24 Apr 2007	30 Nov 07	–	
				13 Jan 2009			25 May 2007			
							22 Jun 2007			
							27 Jul 2007			
							24 Aug 2007			
							11 Sep 2007			
							25 Oct 2007			

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
1082	Bagatelle	5	Yes☼	10 Oct 2006 30 Dec 2008	06 Jan 2009 10 Sep 2009	–	15 Mar 2009 28 Apr 2009 23 Aug 2009	28 May 2009	–	
1096	Haley	13	Yes☼	–	–	14 Apr 2014 16 Apr 2015	–	03 Nov 2014[a]	30 May 2015	Housed with an adult male from 10 July 2014 to 04 Nov 2015
1143	Fitri	10	Yes[Δ]	–	–	28 Apr 2014 27 Apr 2015	–	–	12 Oct 2014	Mating and conception approximately 21 Mar 2014
1147	Clover	10	Yes[Δ]	–	–	29 May 2014 28 May 2015	–	13-14 Oct 2014	24 Jun 2015	Mating and conception approximately 01 Dec 2014
1148	Krakunia	3	Yes☼	–	28 Oct 2007 03 May 2009	–	–	–	–	Male arrived 21 May 2008

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
1169	Violet	9	Yes☼	–	–	14 Apr 2014 16 Apr 2015	–	15-16 Apr 2014[a]	12 Nov 2014	
1178	Kabibi	9	Yes☼	–	–	14 Apr 2014 16 Apr 2015	–	–	20 Aug 2014	Mating and conception approximately01 Feb 2014
1182	Ashaki	5	Yes☼	19 Apr 2011 25 Nov 2013	–	–	–	25 Aug 2013[a]	18 Mar 2014	
1185	Ellen	2	Yes[Δ]	30 Nov 2006 12 Oct 2007	18 Oct 2007 09 Jul 2009	–	–	20 Nov 2008[a]	09 Jun 2009	Inconsistent sampling
1194	Tahiti	2	No	30 May 2008 13 Jan 2009	–	–	–	–	–	Juvenile

SB No.	Name	Age* (years)	Housed with a ♂	Lab A	Lab B	Lab C	Estrus behavior	Mating	Births[§]	Notes
1201	Sirana	2	No	–	25 Mar 2008 17 Feb 2009	–	–	–	–	Inconsistent sampling, juvenile
1208	Isoke	7	Yes[Δ]	–	–	02 Jun 2014 23 May 2015	–	–	–	
1283	Clementine	5	Yes[Δ]	–	–	25 Jul 2014 06 Aug 2015	31 Oct 2014	–	–	Housed with a juvenile male from 04 Sept 2014
1338	Asali	3	No	–	–	05 Mar 2014 31 Oct 2014	13 Mar 2014 11 May 2014 07 Jun 2014 27 Aug 2014 26-28 Sep 2014	–	–	Housed separately from but adjacent to an adult male

Appendix IV. Table Notes

* Age at the beginning of the study period

[§] Reported for 12 months before, during and 12 months after the study period for each hippo

[☼] Only put with a male for breeding or during estrus

[Δ] Housed together with a male when not with a calf

[a] Conceived after mating

[b] Stillbirth or neonatal mortality

Appendix V (CHAPTER 5) – EIA CROSS REACTIVITIES

	% Cross Reactivity							
	Estradiol-17β-OH	Estradiol 17β R4972	Estradiol 17β R0008	5α-pregnane-3β-ol-20-one	Monoclonal progesterone quidel clone 425	Polyclonal progesterone R4849	5β-pregnane-3α,20α-diol 3HS:BSA	Pregnanediol-3-glucuronide R13904
Cross-Reacting Steroids	***E2a***	***E2b***	***E2c***	***20-oxo-pregnane***	***Mono-P4***	***Poly-P4***	***Pg-diol***	***PdG***
Estrone	100.0	3.3	0.73	< 0.10	–	< 0.01	–	–
Estrone sulfate	–	< 0.01	< 0.01	–	–		–	–
Estradiol 17α	19.0	–	–	–	–	–	–	–
Estradiol 17β	70.0	100.00	100.0	–	–	< 0.01	–	0.04
Estriol	129.0	–	–	–	–	–	–	–
1,3,5(10),7-estratetraen-3-ol-17-one	87.0	–	–	–	–	–	–	–
1,3,5(10),7-estratetraen-3,17β-diol	20.0	–	–	–	–	–	–	–
Progesterone	–	0.80	< 0.01	100.0	100.0	100.0	–	0.20
Pregnenolone	–	–	–	–	–	0.12	–	–
11α-hydroxy-progesterone	–	–	–	–	–	40.0	–	–
17α-hydroxy-progesterone	–	–	–	–	–	0.38	–	–
20α-hydroxy-progesterone	–	–	–	<0.10	–	0.13	150.0	44.8
20β-hydroxy-progesterone	–	–	–	<0.10	–	0.13	2.1	3.2
4-pregnen-3α-ol-20-one	–	–	–	20.0	188.0	–	–	–
4-pregnen-3β-ol-20-one	–	–	–	68.0	172.0	–	–	–
4-pregnen-11α-ol-3,20 dione	–	–	–	–	147.0	–	–	–
4-pregnen-11β-ol-3,20 dione	–	–	–	–	2.7	–	–	–

	% Cross Reactivity							
	Estradiol-17β-OH	Estradiol 17β R4972	Estradiol 17β R0008	5α-pregnane-3β-ol-20-one	Monoclonal progesterone quidel clone 425	Polyclonal progesterone R4849	5β-pregnane-3α,20α-diol 3HS:BSA	Pregnanediol-3-glucuronide R13904
Cross-Reacting Steroids	***E2a***	***E2b***	***E2c***	***20-oxo-pregnane***	***Mono-P4***	***Poly-P4***	***Pg-diol***	***PdG***
5-pregnen-3β, 20α-diol	–	–	–	–		–	16.6	–
5α-pregnane-3,20-dione	–	–	–	75.0	55.0	12.2	–	–
5α-pregnane-3α-ol-20-one	–	–	–	8.0	64.0	–	–	–
5α-pregnane-3β-ol-20-one	–	–	–	102.0	94.0	–	–	–
5α-pregnane-20α-ol-3-one	–	–	–	–	–	–	50.0	–
5α-pregnane-3α, 20α-diol	–	–	–	< 0.01	–	–	24.0	–
5α-pregnane-3β, 20α-diol	–	–	–	–	–	–	55.6	–
5α-pregnane-3α, 20β-diol	–	–	–	< 0.01	< 0.01	–	–	–
Pregnanediol	–	–	–	< 0.01	< 0.01	–	100.0	–
Pregnanediol-3-glucuronide	–	–	–	–	–	< 0.01	–	100.0
5β-pregnane-3,20-dione	–	–	–	151.0	8.0	–	–	–
5β-pregnane-3α-ol-20-one	–	–	–	20.0	2.5	–	–	–
5β-pregnane-3β-ol-20-one	–	–	–	36.0	12.5	–	–	–
5β-pregnane-20α-ol-3-one	–	–	–	–	–	–	176.0	–
5β-pregnane-3β, 20α-diol	–	–	–	–	–	–	100.0	–
Androstenedione	–	1.00	–	–	< 0.01	–	–	–
Testosterone	–	1.00	< 0.01	–	–	< 0.01	–	0.20
Cortisol	–	< 0.01	< 0.01	< 0.01	–	< 0.04	–	0.06
Corticosterone	–	< 0.01	< 0.01	–	< 0.01	–	–	–

Appendix VI (Chapter 6) – Cortisol and Testosterone Metabolites

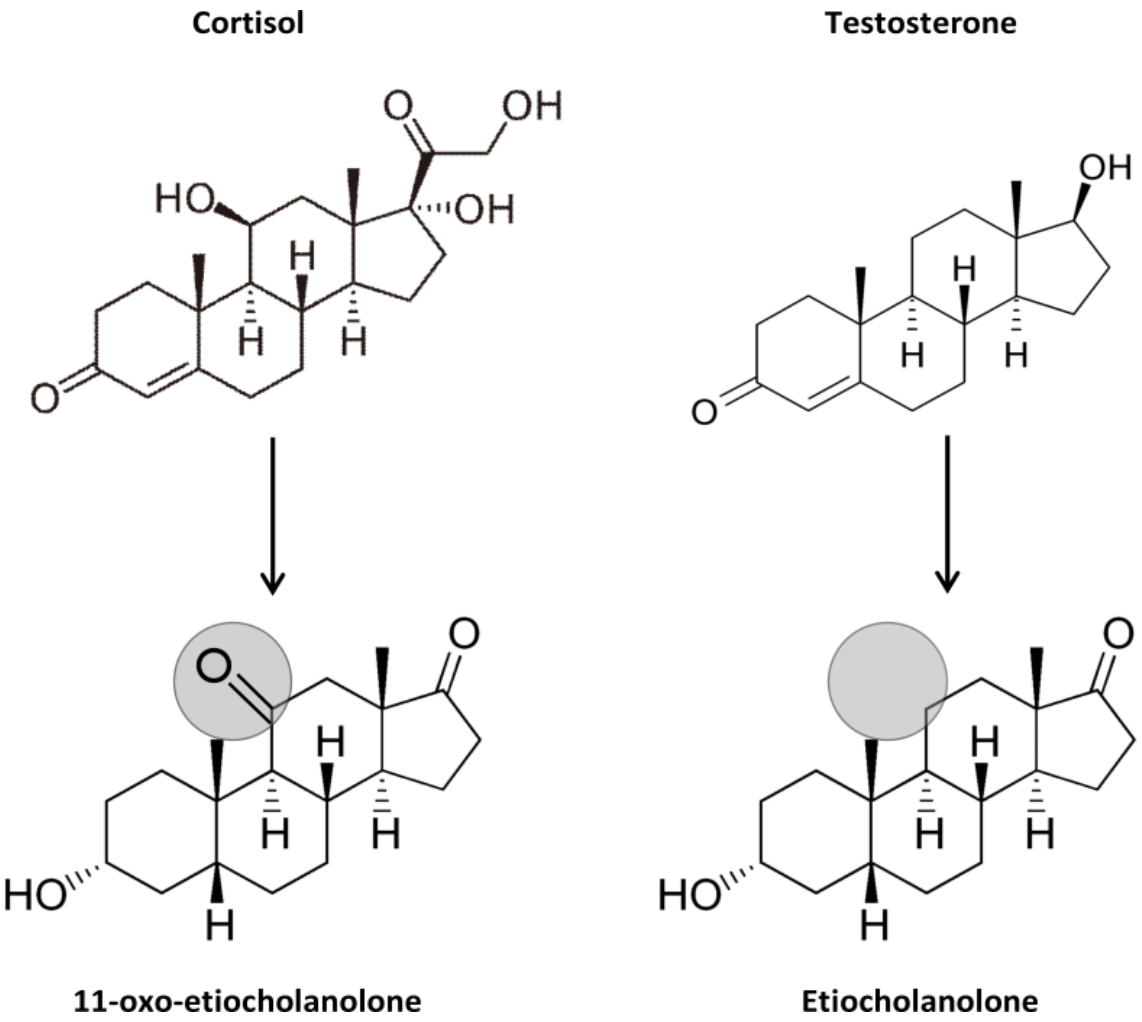


Figure from Ganswindt et al. 2003: Structural formula of 11-oxo-etiocholanolone and etiocholanolone, resenting typical metabolites of cortisol and testosterone, respectively. The grey area indicates the position C11, which bears an oxygen in case of glucocorticoid, but not androgen metabolites.

Appendix VII (CHAPTER 6) – PYGMY HIPPO DEMOGRAPHICS

Studbook (SB) number, demographics and sampling date range for 4 female and 12 male pygmy hippos (*Choeropsis liberiensis*)

SB No.	Sex	Age* (years)	Date of Birth	Zoo	Climate	Sample Date Range	ACTH Challenge	Notes
460	M	40	Unknown	Oklahoma	Temperate	18 Mar 2014 to 19 Mar 2015	–	Proven breeding male; wild-caught in 1977, age is an estimate
880	M	23	13-Jan-91	Omaha	Temperate	20 May 2014 to 20 May 2015	24-May-14	Proven breeding male
902	M	20.5	16-Sep-93	Louisville	Temperate	05 May 2014 to 04 May 2015	08-May-14	Proven breeding male
919	M	20	25-Jun-94	Baton Rouge	Temperate	02 June 2014 to 31 May 2015	–	
931	F	19	25-Feb-95	Louisville	Temperate	05 May 2014 to 04 May 2015	08-May-14	Saline control for ACTH challenge
996	M	17	23-Mar-97	Giraffe Ranch	Subtropical	15 Apr 2014 to 23 Dec 2014	–	Proven breeding male
1053	M	14.5	02-Sep-99	Miami	Subtropical	05 Mar 2014 to 24 Mar 2015	–	Proven breeding male
1063	F	14	01-Mar-00	Omaha	Temperate	20 May 2014 to 20 May 2015	24-May-14	
1093	M	12.5	01-Dec-01	Chicago Lincoln Park	Temperate	19 May 2014 to 14 May 2015	29-May-14	

SB No.	Sex	Age* (years)	Date of Birth	Zoo	Climate	Sample Date Range	ACTH Challenge	Notes
1135	M	10.5	16-Nov-03	Brownsville	Subtropical	28 May 2014 to 28 May 2015	–	Proven breeding male
1147	F	10	17-Mar-04	Brownsville	Subtropical	28 May 2014 to 28 May 2015	04-Jun-14	
1241	M	6	20-Mar-08	Lowry Tampa	Subtropical	28 Apr 2014 to 27 Apr 2015	–	Proven breeding male
1359	M	3	19-May-11	Rum Creek	Subtropical	14 Apr 2014 to 16 Apr 2015	10-Sep-15	
1392	F	3.5	25-Mar-12	Rum Creek	Subtropical	07 Sept 2015 to 19 Sept 2015	10-Sep-15	
1422	M	1.5	22-Feb-13	Jackson	Temperate	08 Aug 2014 to 11 Aug 2015	–	Juvenile
1423	M	1.5	22-Feb-13	Omaha	Temperate	28 May 2014 to 27 Oct 2014	–	Juvenile; transferred to another zoo 30 Oct 2014 and sample collection was not continued

*Age at the beginning of the study period

Appendix VIII (CHAPTER 6) – EIA CROSS REACTIVITIES

Cross-reactivities for the nine EIAs used to assess androgen and/or glucocorticoid metabolites in pygmy hippos.

Cross-Reacting Steroids	**Cortisol R4866**	**Corticosterone CJM006**	**Testosterone**	**CORT**	**CCST**	**5α-3β,11β-diol-CM**	**3α,11β-dihydroxy-CM**	**11,17-DOA**	**3α,11-oxo-CM**
Androstenedione	0.1	–	–	–	–	< 1.0	–	–	–
11-ketoandrosterone	–	–	–	–	–	–	< 1.0	–	
Dehydroepiandrosterone	0.1	–	–	–	–	< 1.0	–	–	–
Testosterone	0.1	0.64	100	–	–	–	–	–	–
Dihydrotestosterone	–	–	35.4	–	–	< 1.0	–	–	–
Etiocholanolone				–	–		< 1.0	–	< 1.0
11-ketoetiocholanolone	–	–	–	–	–	–	3.5	–	–
11β-hydroxyetiocholanolone	–	–	–	–	–	–	100	–	–
5α-androstane-3,11,17-trione	–	–	–	< 0.01	< 0.01	–	–	14.7	–
5α-androstane-3α-ol-11,17-dione	–	–	–	< 0.01	< 0.01	–	–	5.7	–
5α-androstane-3β,11β-diol-17-one	–	–	–	–	–	230	–	–	–
5α-androstane-3β,17β-diol	–	–	–	–	–	< 1.0	–	–	–
5α-androstane-3β-ol-11,17-dione	–	–	–	< 0.01	< 0.01	–	–	6.7	–
5α-androstane-3β-ol-17-one	–	–	–	–	–	< 1.0	–	–	–
5β-androstane-3,11,17-trione	–	–	–	< 0.01	< 0.01	–	–	84	1.2
5β-androstane-3α,11β-diol-17-one	–	–	–	< 0.01	< 0.01	–	–	0.6	3.3

Cross-Reacting Steroids	Cortisol R4866	Corticosterone CJM006	Testosterone	CORT	CCST	5α-3β,11β-diol-CM	3α,11β-dihydroxy-CM	11,17-DOA	3α,11-oxo-CM
5β-androstane-3α-ol-11,17-dione	–	–	–	< 0.01	< 0.01	–	–	100	100
5β-androstane-3β,17β-diol	–	–	–	–	–	< 1.0	–	–	–
5β-androstane-3β-ol-17-one	–	–	–	–	–	< 1.0	–	–	–
5α-pregnane-3α,11β,17α,21-tetrol-20-	–	–	–	0.8	0.15	–	–	< 0.01	–
5α-pregnane-3β,11β,17α,21-tetrol-20-	–	–	–	–	–	45	–	–	–
5α-pregnane-3β,11β,20β,21-tetrol	–	–	–	–	–	110	–	–	–
5α-pregnane-3β,11β,21-triol-20-one	–	–	–	–	–	100	–	–	–
5α-pregnane-3β-ol-20-one	–	–	–	–	–	< 1.0	–	–	–
5α-pregnane-11β,17α,21-triol-3,20-	–	–	–	4.6	< 0.01	–	–	< 0.01	–
5β-pregnane-3α-11β,17α,21-tetrol-20-	–	–	–	0.1	0.20	–	–	< 0.01	–
5β-pregnane-3α-11β,21-triol-20-one	–	–	–	< 0.01	0.25	–	20.0	< 0.01	< 1.0
5β-pregnane-3α-11β-diol-20-one	–	–	–	–	–	–	14.6	–	< 1.0
5β-pregnane-3α-ol-11,20-dione	–	–	–	–	–	–	–	–	37
5β-pregnane-3β-ol-20-one	–	–	–	–	–	< 1.0	< 1.0	–	–
Pregnanediol	–	–	–	–	–	–	< 1.0	–	< 1.0
Pregnenolone	0.1	–	–	–	–	–	–	–	–
17α-hydroxypregnenolone	0.1	–	–	–	–	–	–	–	–
Progesterone	0.2	2.65	0.02	–	–	< 1.0	–	–	–
17α-hydroxyprogesterone	0.2	–	–	–	–	–	–	–	–

Cross-Reacting Steroids	Cortisol R4866	Corticosterone CJM006	Testosterone	CORT	CCST	5α-3β,11β-diol-CM	3α,11β-dihydroxy-CM	11,17-DOA	3α,11-oxo-CM
Estradiol-17β	0.1	< 0.01	< 0.01	–	–	–	–	–	–
Estriol	0.1	–	–	–	–	–	–	–	–
Estrone	0.1	–	–	–	–	–	–	–	–
Cortisol	100	0.23	< 0.01	100	5.0	< 1.0	–	< 0.01	–
5β-dihydrocortisol	–	–	–	–	–	–	< 1.0	–	< 1.0
11-deoxycortisol	0.2	0.03	–	–	–	–	–	–	–
Tetrahydrocortisol	–	–	–	–	–	–	< 1.0	–	< 1.0
Cortisone	5.0	< 0.01	< 0.01	–	–	< 1.0	–	–	–
21-deoxycortisone	0.5	–	–	–	–	–	–	–	–
Corticosterone	0.7	100	< 0.01	6.2	100	< 1.0	–	< 0.01	–
Desoxycorticosterone	0.3	14.25	–	–	–	< 1.0	–	–	–
Tetrahydrocorticosterone	–	0.90	–	–	–	< 1.0	–	–	–
Prednisolone	9.9	0.07	–	–	–	–	–	–	–
Prednisone	6.3	< 0.01	–	–	–	–	–	–	–