2018

Noninvasive Collection of Saliva in Panthera leo : Creation and Validation of a Novel Technique for Health Assessment in Captive African Lions (Panthera leo)

Elizabeth Sgambelluri
Antioch University, New England

ANTIOCH UNIVERSITY
NEW ENGLAND

Department of Environmental Studies

THESIS COMMITTEE PAGE

The undersigned have examined the thesis entitled:

Noninvasive Collection of Saliva in *Panthera leo*
Creation and Validation of a Novel Technique for Health Assessment in Captive African Lions (*Panthera leo*)

presented by: ***Elizabeth Sgambelluri***

candidate for the degree of Master of Science, and hereby certify that it is accepted.*

Committee chair name: ***Beth Kaplin, Ph.D.***

Title/Affiliation: ***Antioch University New England, Environmental Studies Department***

Committee member name: ***Lisabeth Willey, Ph.D.***

Title/Affiliation: ***Antioch University New England, Environmental Studies Department***

Committee member name: ***Michelle Kneeland, DVM***

Title/Affiliation: ***Biodiversity Research Institute, Wildlife Health Program Director***

Date Approved by all committee members:

*Signatures are on file with the Registrar's Office at Antioch University New England.

Noninvasive Collection of Saliva in *Panthera leo*

Creation and Validation of a Novel Technique for Health Assessment in Captive African Lions (*Panthera leo*)

Elizabeth Sgambelluri, *M.S., PGCert., B.S*

Master's Candidate, Conservation Biology

Department of Environmental Studies

May 6, 2018

Under the Supervision of:

Beth Kaplin, PhD., *Thesis Advisor*, Antioch University New England

Lisabeth Willey, PhD., Antioch University New England

Michelle Kneeland, DVM, Biodiversity Research Institute

Table of Contents

List of Figures

List of Tables

ACKNOWLEDGEMENTS

I would like to thank all the individuals that contributed to this study and provided mountains of support. First and foremost, I would like to thank my thesis advisor, Beth Kaplin, and committee for their enduring support, guidance, and stress relief. This group of wonderful human beings helped keep my head above water during the most trying of times in this study and were extremely motivational throughout the process. In particular, I would like to thank Lisabeth Willey for always being there when I was having a meltdown, or simply needed reassurance in my own potential. You all helped fuel this study, but more importantly you all helped me to stay confident in my potential as a researcher and devotion to my aspirations.

A huge thanks is rightly due to my family, whom have always supported my academic endeavors and dreams for the future. Thank you to my amazing father, Richard Sgambelluri, who constructed the beautiful outer shell of the device in his free time. I cannot express in words how grateful I am, and how appreciative I am for all the help and advice you provided regarding device design and construction. I also am forever grateful for your confidence in me, and the comedic relief you provided. To my sister, Jessica, and my mother, Carol: thank you for listening to me whenever I hit a roadblock or was at my maximal frustration threshold. Thank you for keeping me grounded, motivated, and bright minded. Thank you also to Figaro, my cat, for the snuggle breaks and gentle reminders when it was time to stop for dinner.

To Tristan Anderson, a recent senior from the Engineering department at Keene State College. This amazing, brilliant minded individual enabled what had started as a vision and drawing on a piece of paper to become reality. I cannot thank him enough for his dedication to this project, like the time he looked up the average teeth measurements and jaw dimensions for lions

to help guide the dimensions of the grating. He took the time out of his extremely busy schedule to help design and build the internal collection structure of this device. Tristan, if it was not for you, I would probably still be sitting in my basement with plastic funnels and duct tape! I wish you the best for whatever you pursue in the future, and know that any engineering firm is blessed to have you on their team. Thank you for your dedication, your sense of wonder, your contagious enthusiasm, and work ethic.

Thank you to the staff – specifically Adrienne Whiteley – and community at Rosamond-Gifford Zoo, for permission to work with your two lions and your support in this project. This community served a crucial role in this research, providing the means to test the device in a captive setting. Your willingness to support this project, and support novel research when many others did not, truly meant the world and such should not go unnoticed.

Lastly, I would like to thank all of the individuals that contributed financially to the GoFundMe page that supported the entirety of expenses for this project. Your faith in this project was inspirational and served as a huge proponent in allowing this research to occur. I would also like to give thanks to all of the members of the Antioch community, faculty and colleagues alike, as well as my boyfriend Travis Smith, who offered reassurance, self-confidence, support, guidance, and – perhaps most importantly – many laughs.

ABSTRACT

Very little research exists concerning direct correlations between alterations in stress stimuli and direct effects on carnivore physiology. Many studies have researched and documented what factors correlate to increased stress levels in predator species. Lacking, however, are studies observing the specific physiologic influences of increased glucocorticoid concentrations in those individuals. Furthermore, methods for monitoring and assessing health or immune function in many carnivore species often involve invasive techniques, such as physical restraint, blood sampling, and chemical immobilization. This study aimed to create, evaluate, and describe a new device for noninvasive collection of saliva for health assessment. The device itself was constructed on a "bucket in a bucket" concept, wherein saliva was deposited into a smaller, inner container and attractants housed in the larger, outer container. Two attractants, catnip extract and bear bait, were placed inside the device for elicitation of salivation and passive drooling response. Efficacy of the device, as well as evidence regarding its accuracy and safety, were evaluated through testing trials conducted at the Rosamond-Gifford Zoo in Syracuse, NY (USA) using two captive lions. Success was determined and scored using several metrics, including the ability to elicit behaviors, whether or not lion(s) approached the device, and whether or not saliva was collected. Three trials were conducted, with two of the three yielding a success value greater than three (maximal score being four). Saliva was successfully deposited and collected in the device during the first trial. Urine and hair samples were also collected using the device. The results obtained substantiate that such can be utilized in collection of saliva. Moreover, they highlight the potential of this device in reforming or providing an alternative approach to current techniques, while providing a springboard for the use of saliva in wildlife health assessment.

Disclosure: Provisional patent pending for device (Ser. No 62/720,961) by Wendy W. Koba, Esq. (see Appendix 5)

CHAPTER 1

INTRODUCTION

Stress responses are controlled by the hypothalamo-pituitary-adrenal axis (HPA). This axis comprises the hypothalamus, pituitary gland, and adrenal gland (Chrousos, 2013). When vertebrates are exposed to stress factors, large amounts of glucocorticoids (GC) are released into the body system (Busch, 2009). In mammals, the glucocorticoid hormone released is cortisol. The degree or magnitude of the GC response differs according to stimuli and duration of exposure (Sapolsky, 2002; Romero, 2004; Busch, 2009). A stress response is a natural process; it is the mechanism through which animals evolved for survival. However, that applies only to short term. Chronic exposure to stress stimuli causes overproductions of glucocorticoids that can actually impair health (Randall, 2012).

The notion of health relates to the vitality/vigor of an individual and/or population, and is tied directly to production of glycoprotein molecules called immunoglobulins (French, 2012). Immunoglobulins function in production of antibodies within body systems. Mammalian systems rely on these antibodies, as well as a specialized suite of immune proteins called lymphocytes, for detection and defense against deleterious pathogens. Studies confirm, however, that cortisol and other glucorticoids inhibit this protein, consequently suppressing the body's immune system, increasing susceptibility to infections (Hansen, 2014), and exacerbating severity of infections and inflammation.

Various external and internal factors – such as competition, predation, and habitat change – influence physiological systems in large carnivores. There is a dearth, however, in research regarding how alterations in stress stimuli specifically could be detrimental to individual physiology and population health. In the following section I will review the existing research on

sources of stress in wildlife, focusing on African predator species, and propose several sources of evidence to explore the immunological impacts of stress.

CHAPTER 2

LITERATURE REVIEW

This review addresses the link between stress and immunosuppression, as well as the deficit in noninvasive methods for assessing health. Recent evidence from human medicine and laboratory studies suggests that stress influences neuroendorcrine mechanisms that catalyze immunosuppression (Dohms *et al.,* 1991; Glaser *et al.,* 1987; Råberg *et al.,* 1998; Roth, 1985). In the sections that follow I provide background on two major stressors cited for African predators, how stress influences body systems, and both human and wildlife based evidence for stress induced immunosuppression. This section is organized into two principal parts: (1) major stressors in African predators, and (2) evidence of stress induced immunosuppression.

Major Stressors for Wild / Free-Range African Predators

A **stressor** is any adverse factor (biotic or abiotic) that disrupts normal body system processes (Sapolsky, 2002). When exposed to stress stimuli, individuals will exhibit **stress responses**, which are various behavioral and/or hormonal changes evolutionary adapted for survival and re-establishment of internal homeostasis (Aich *et al.,* 2009; Young *et al.,* 2004). The adrenal gland, part of the endocrine system, governs this response through release of two hormones: the core releases epinephrine, or adrenaline, whereas glucocorticoids are expelled by the adrenal cortex (Sapolsky, 2002; Busch, 2009).

Anthropogenic Stressors

Over the past 20 years, populations of African lions have been reduced by at least 30 percent (Rabinowitz, 2010), and current population size is estimated somewhere within the range of 16,500

to 30,000 individuals. This estimate is half of that quantified during the 1950s (Hayward *et al.,* 2005). The demise of this predator has largely been driven by anthropogenic activities such as poaching. Increased human development has aggravated fatal competition with hyena populations, as well as disease due to population overlap with other species (i.e. olive baboons and African wild dogs). Between 1993 and 1997, over one thousand lions were estimated to have died from outbreaks of canine distemper, a disease transmitted from wild dog species through spotted hyena populations (Hayward *et al.,* 2007).

Anthropogenic activities have often served as sources of wildlife stress. In one study, stress responses in lions were negatively correlated with proximity to human settlements (Creel *et al.,* 2013). In this study, glucocorticoid levels were measured through immunoassay surveys on lion fecal samples to evaluate stress levels. Results indicated that lions within human settlement study zones had a 25% increase in fecal glucocorticoid concentration, as compared to those measured from lions inhabiting conservation areas (Creel *et al.,* 2013). As distance from settlement zones increased, glucocorticoid metabolite concentrations within fecal samples declined by 22% per kilometer (Creel *et al.,* 2013). Glucocorticoid concentrations also increased if lions were unable to move away from settlement zones (Creel *et al.,* 2013). Observations on movement patterns, through direct observation and radio telemetry, revealed that lions shift occupancy and utilize other habitats to evade human interaction (Creel *et al.,* 2013). Analogous findings were established in a similar study with spotted hyenas inhabiting the Masai Mara National Reserve in Kenya. Fecal glucocorticoids from both males and females in four social groups were used to delineate major stressors (Van Meter *et al.,* 2009). Interestingly, ecological stressors such as climate factors and prey availability did not influence stress levels. Instead significant increases in glucocorticoid concentrations correlated with increases in density of human populations bordering the

conservation area (Van Meter *et al.,* 2009). Samples further revealed that agricultural practices augmented stress levels in hyenas (Van Meter *et al.,* 2009). All of these findings provide strong evidence towards anthropogenic activities as major stressors.

Interspecific Competition

Interspecific killing

Accounting for roughly 70% of mortalities in some carnivore populations (Palomares *et al.,* 1999), interspecific killing typically involves large carnivorous predators purposefully hunting and eliminating smaller carnivores (Palomares *et al.,* 1999). Interspecific killing can be observed within Africa's large carnivore guild, where repeated incidents of deleterious interactions between lions, hyenas, cheetahs, and African wild dogs have been documented (Caro *et al.,* 2003; Creel *et al.,* 1996; Laurenson 1995; Palomares *et al.,* 1999).

Lions and hyenas compete with each other for food due to high dietary overlap (Mills, 2005; Trinkle, 2005). While both are adept hunters, they also act as opportunists or scavengers. Spotted hyenas are notorious for stealing carcasses acquired by lions. In Uganda, studies from Rwenzori National Park have indicated that 33% of lion kills were stolen by hyenas (Palomares *et al.,* 1999; Trinkle, 2005) through the use of mobbing behavior. This behavior strategy causes hyenas to aggressively attack and taunt lions in an attempt to drive them away (Hayward *et al.,* 2007; Palomares *et al.,* 1999). In Saviti, Botswana, lionesses were mobbed at a kill site and chased up trees by hyenas (Joubert, 1992; Nat Geo Wild, 2015). Instances such as this are fueled by food competition. However, accounts of hostile interactions and randomized attacks between both species have been documented that are not food related. Lionesses whose young were killed by hyenas act most aggressively towards hyenas (Joubert, 1992). In the Serengeti, male lions have

sought and killed female matriarch hyenas when food was not concerned (Joubert, 1992; Nat Geo Wild, 2015). Approximately 71% of hyena mortality was linked to lion predation within the Etosha National Park (Trinkle, 2005).

There may be another purpose for hyena mob behavior: offspring protection. Though the individual fitness is at risk, by engaging in aggressive behavior that may drive away predator species, a social group's inclusive fitness is preserved by protecting the offspring (kin selection view) (Trinkle *et al.,* 2005). This notion arises from observation that all mobbing events documented in Ngorongoro Crater where in close proximity to hyena dens housing pups (Trinkle *et al.,* 2005). In these particular instances, mobbing not only drove away lions, but lions avoided those regions for several weeks (Trinkle *et al.,* 2005).

Similar dispersion patterns of these two species occurred in Addo Elephant National Park, South Africa. When lion populations traveled to and inhabited northern regions of the park area, hyenas would move divergently to inhabit southern regions (Hayward, 2007). Such behavior is reflective of avoidance techniques described in the following section.

Avoidance behavior

Species react in different ways to counteract interspecific competition or predation, and often display adaptive behaviors. In the savannahs of Africa, many of the carnivore guild species – lions, hyenas, cheetahs, leopards, and African wild dogs – have modified behavioral patterns or altered movement in order to avoid interaction with other predators (Valeix *et al.,* 2012). Some species migrate to new habitats to avoid confrontations (Palomares *et al.,* 1999).

Cheetahs illustrate excellent examples of such predator induced adaptations. Interspecific competition by lions serves as a large limiting agent in cheetah densities (Kelly *et al.,* 1998;

Laurenson, 1995; Palomares *et al.,* 1999). Studies have documented negative correlations between cheetah reproductive outputs and proximity to lions – the greater the proximity to lions, the lower the reproductive success of cheetah individuals (Kelly *et al.,* 1998; Palomares *et al.,* 1999). In the Serengeti plains, this interaction contributed huge impacts on the population dynamics of cheetahs, specifically, cub mortality dramatically increased with lion predation (Kelly *et al.,* 1998; Woodroffe *et al.,* pg 165). In the Serengeti, lions accounted for 70% of overall cub mortality in cheetah populations (Kelly *et al.,* 1998; Laurenson, 1995). By adapting avoidance strategies and modifying activity patterns, cheetahs are able to maintain or increase relative fitness (Palomares *et al.,* 1999; Ritchie, 2009). Research in Kruger National Park showed that cheetahs modified hunting periods under pressures of interspecific competition. They changed to hunting during mid-day hours to avoid kleptoparasitism by lions and hyenas (Palomares *et al.,* 1998).

Adjustments in activity patterns to avoid larger predators can also be observed in African wild dogs (Creel *et al.,* 1996). In Hluhluwe-imfolozi National Park, South Africa, African wild dogs deliberately altered movement patterns and modified hunt times to avoid lions (Darnell, 2014). As referenced previously, altered habitat use patterns by lions and hyenas in Addo Park, South Africa, could be the results of avoidance behaviors. Several lionesses were observed to alter activity patterns to become completely diurnal, with peak hunts occurring around sunrise (Hayward, 2007). Such behavioral changes are believed to represent an avoidance method in response to mobbing by spotted hyenas (Hayward, 2007).

Major Stressors for Captive African Predators

Discussions concerning the ethics and efficacy of maintaining captive populations are often controversial, largely in regards to animal welfare. The purpose of captive environments, such as those within zoological settings, is three-fold: (1) to provide a means of preserving threatened or

endangered species, (2) rehabilitation of orphaned or ill individuals, and (3) to increase public education of species conservation. Some additionally strive to maintain healthy populations that will ultimately be reintroduced into the wild. Unfortunately, the reality of the situation is that many captive environments fail to provide the optimal conditions required for species flourishment (Sajjad *et al.,* 2011), thus serving more as a detriment rather than benefit in scope. Such failures include lack of natural habitat, confined enclosures, noise levels, and overloaded human exposure.

Maintenance of healthy and sustainable captive populations is largely thwarted by stress, and thus serves as the principle concern for such organizations (Conforti *et al.,* 2012). Many stressors stem from those failures described previously. However, the type and number of stressors vary across species and captive setting. For captive felids, the primary stressors are chemical restraint, translocation, and inadequate enclosures. Clouded leopards that were housed in confined, size restricted enclosures exhibited higher fecal corticoid metabolites (FCMs) than those in large enclosures (Wielebnowski *et al.,* 2002). For captive cheetahs, translocation and public exposure serve as the primary stressors. Individuals moved "on-exhibit" (housed in an enclosure space for public viewing) were 20 times more likely to have elevated corticoid levels that were at least two standard deviations from their baseline, compared to those that were "off-exhibit" (Franklin, 2014; Munson *et al.,* 2005; Terio *et al.,* 2004; Wells *et al.,* 2004). These elevated levels were not transient in nature; in fact, these levels remained elevated on average for 30 days (Franklin, 2014; Wells *et al*., 2004). A study by Nogueira et al (1997) found a strong correlation between stress (increased cortisol level) and restraint in *Panthera onca* and *Felis pardalis*. Felines that were physically restrained exhibited plasma cortisol levels that were, on average, four to ten times greater than those that were not restrained (333 ± 47 nmol/l compared to 35 nmol/l and 87 ± 16 nmol/l; Nogueira *et al.,* 1997).

Numerous studies document behaviors believed to be representative of stress – known as "stereotypies" – in many captive taxa (Vaz *et al.,* 2017). Such behaviors include pacing, head bobbing, anorexia, variations in skull morphology (Duckler, 1998), and excessive grooming. However, such do not serve as a definitive indicator of increased stress (such could rather be a factor of boredom), and fail to provide explicit quantitative measure of variations in stress level.

The behavioral adaptations previously described all stem from avoidance of or exposure to a deleterious factor. While these factors trigger behavioral modifications or adaptations to minimize costs and risks (Adamo *et al.,* 2013), they also trigger a stress response. Exposure and adaptation to stress factors impacts body system functions in several ways. In small doses slight alterations in body systems may occur, though innocuous short term (Adamo *et al.,* 2013). What happens, however, when African predators are exposed to multiple stress sources on a chronic basis?

Evidence of Stress Induced Immunosuppression

Stress Physiology in Humans

In humans, stress responses are known to suppress immune function and pathogen defense (Sapolsky, 2002; Chrousos, 2013). This occurs largely through impairment of immunoglobulin production. Immunoglobulins function in the production of antibodies within body systems. There are five types found in all organisms: IgA, IgG, IgM, IgE, and IgD. Important to this study are IgA, as such is indicative of both immune health and susceptibility. IgA antibodies protect body surfaces that are exposed to foreign substances, and assists with immune function of mucosal membranes. They are found in the intestinal tract, breathing passageways (e.g. nose and mouth), and blood (eBioscience, 2015).

Chronic stress not only compromises health through immunosuppression, but through impairment of multiple organ systems. For example, overproduction of glucocorticoids catalyzes muscle mass attrition and bone marrow loss through calcium reductions (Young *et al.,* 2004). Cortisols can act as atherogenics, meaning they increase plaque in both coronary and cerebral arteries (Dr. Sgambelluri, MD). Thus, stress increases risk for hypertension, osteoporosis, and Cushing's syndrome (Chrousos, 2013; Young *et al.,* 2004) in humans. If these health detriments are present in humans, there is a possibility that similar reactions manifest in other mammalian species.

Evidence in Free-Ranging African Carnivore Populations

There is some evidence that stress in African carnivores correlates with immunosuppression and disease susceptibility. Infections are typically asymptomatic and common to African carnivore species (Williams *et al.,* 2014; Goller, 2011). However, they can be pathogenic under certain circumstances (e.g. unnatural hosts or habitat degradation) (Williams *et al.,* 2014; Goller, 2011). For example, *Babesia leo,* a form of babesiosis in lions that is usually tolerated, will become pathogenic if an individual is immunocompromised (Williams *et al.,* 2014). Another similar example is with the protozoan Hepatozoon (Williams *et al.,* 2014; Goller, 2011). Adults of many African carnivorous species, such as spotted hyenas, display high prevalence (>90%) of this infection with no clinical signs (Williams *et al.,* 2014; Goller, 2011). However, Hepatozoon was the cause of mortality in 18% of infected juvenile spotted hyenas. (Aguirre *et al.,* 2012; Williams *et al.,* 2014). I believe it may be a function of stress induced by sibling rivalry.

There is also evidence that stress may increase susceptibility to parasitic infections. Hookworms are common extracellular gastrointestinal parasites of mammals. Spotted hyenas are known to be infected with two hookworm species called *Cystoisospora* and *Ancylostoma* (East *et*

al., 2013). Lactating females were found to have higher infections of these hookworms than non-lactating females (East *et al.,* 2013). Furthermore, infections also increased in females nursing twins rather than singletons (East *et al.,* 2013).

Cheetahs provide an interesting example of how stress may correlate with immunosuppression. A study observing stress impacts on captive breed cheetahs indicted that feline panleukopesvirus (FPV) and canine parvovirus were most prevalent in these individuals during times of stress (Munson *et al.,* 2004). It also showed that even with vaccinations, captive populations were highly susceptible to infections (Munson *et al.,* 2004) - indicated by the consistently elevated corticoid levels under unmodified captive care (Munson *et al.,* 2005; Munson *et al.,* 2004).

The negative impacts of stress induced immunosuppression extend beyond an individual scope, in that it can harm the health of the population. Stress induced disease from intensive handling and vaccination against canine distemper caused massive declines in wild dog populations between 1965 and 1991 (Goller, 2011). *Mycobacterium bovis,* a strain of tuberculosis or bovine virus, can go into dormancy and later be reactivated in a host (Drewe *et al.,* 2009). In the Kalahari, dormant bovine viruses were reactivated in male meerkats due to stress from aggressive relations and finding mates (Drewe, 2009; Drewe *et al.,* 2009). This event was responsible for the extinction of four social groups between 1995 and 2005.

Evidence in Captive Populations

Evidence of immunosuppression becomes increasingly apparent in captive populations. Captive cheetah populations often fail to thrive, exhibiting increased disease prevalence and diminished reproductive success (Terio *et al.,* 2004). Interestingly, many of the diseases that

manifest in captive populations are either absent or avirulent in free ranging individuals (Terio *et al.,* 2004). Furthermore, captive individuals exhibit atypical immune responses to common pathogens, divulged by recurrent viral infections and high mortality associated with degenerative and/or inflammatory diseases (i.e., glomerulosclerosis and amyloidosis). In 2004, Terio et al. used fecal corticoid metabolites to evaluate degrees of stress between captive and free ranging cheetah populations. Baseline corticoid levels were significantly higher in the fecal samples of captive individuals then free-range (Terio *et al.,* 2004). Samples from midsagittal region of adrenal glands indicated that corticomedullary ratios were significantly larger in captive individuals then wild, with prolonged elevations in corticosteroid concentrations (Munson *et al.,* 2005). A study by Munson et al. (2005) showed the presence of severe, multiple organ lesions in captive cheetahs with elevated plasma cortisol levels – dissimilar to the wild counterparts. A large majority of the individuals with these lesions also presented cardiac fibrosis, splenic lymphoid depletion, and glomerulosclerosis (Munson *et al.,* 2005). All findings were correlated with significant elevations in adrenal cortical levels, indicating a high prevalence of adrenal cortical hyperplasia in the captive populations (Munson *et al.,* 2005). These findings provide concrete evidence for the physiologic influences of stress on the health of these individuals.

Discrepancies in disease virulence between captive and free-range populations are also apparent in African lions (*Panthera leo*). In the wild, many lions are carriers for different subtypes of feline immunodeficiency virus (FIV), though the lentiviral strains are relatively nonpathogenic or seemingly latent (Hoover *et al.,* 1991; Roelke *et al.,* 2009). Synonymous with trends reflected in cheetah populations, pathogenesis of FIV specific to African lions (FIV-Ple or LLV) will alter under certain conditions (Roelke *et al.,* 2009). For example, the lentivirus will become

pathogenic/virulent during periods of environmental stress, in captive populations, or when the individual has an underlying immunologic or neurologic disorder.

Current Methods for Health Assessment in Wildlife

Modern methods of health assessment in both captive and wild populations vary, depending on the specific variables targeted by the research for analysis. For evaluation of stress, most studies now employ the use of fecal samples and metabolites; a preferable, noninvasive alternative. When it comes to monitoring and assessing health or immune function in carnivore species, however, the techniques employed are far more invasive. These techniques include physical restraint, blood sampling, and chemical immobilization. While these practices enable acquisition of body fluids for health examination, they carry a high degree of risk for both the researcher and the animal.

The majority of the time blood is collected and used for health analysis. However, this is not the only medium that yields information relative to the health of an animal. Both urine and saliva can provide a wealth of information, ranging from health indicators to reproductive function to genetics (Chiappin *et al.,* 2007; Laudenslager *et al.,* 2006). Two variables in particular that can be evaluated through saliva as health measures are secretory immunoglobin A (IgA) and C-Reactive protein (CRP) levels. Secretory immunoglobulin A (SIgA) is a type of IgA antibody found in mucous secretions that prevents pathogen invasion in oral, lung, and gut systems. C-Reactive Protein (CRP) is a protein produced in the liver that indicates inflammation.

Again, a significant amount of information can be gleaned from saliva and/or urine. However, at the current time, obtaining these secretions is difficult. Collection of urine in the wild is more of a serendipitous event, usually only occurring when the animal is actively followed by

the researcher or research team. When it is collected, only small amounts or swabs are obtained; never a fresh catch. Furthermore, noninvasive methods for retrieving saliva and/or urine from wildlife populations are absent. With saliva, the only method that exists are oral swabs. Thus to obtain saliva from an animal, it must either be physically restrained or immobilized.

Summary

As stated previously, very little research exists concerning direct correlations between alterations in stress stimuli and direct effects on carnivore physiology. Many studies have researched and documented what factors correlate to increased stress levels in predator species (Adamo et al., 2013; Creel et al., 1996; Hayward, 2007; Kelly et al., 1998; Palomares et al., 1999; Van Meter et al., 2009). Lacking, however, are studies observing the specific immunological impacts of increased glucocorticoid concentrations on those individuals (Martin *et al.,* 2011), as well as noninvasive methods for assessing health in wild felid and canid species. The research described thus far demonstrates that stress influences certain physiological systems, particularly movement or behavior, of African carnivores (Adamo et al., 2013; Creel et al., 1996; Hayward, 2007; Kelly et al., 1998; Palomares et al., 1999; Van Meter et al., 2009). More importantly, evidence from human subjects reveals high degrees of, or chronic exposure to, stress augments immunosuppression (Chrousos, 2013; Sapolsky, 2002; Young et al., 2004), with some evidence indicative of similar trends in wildlife (Drewe et al., 2009; East et al., 2013; Goller, 2011; Munson et al., 2004). The findings that some infections become pathogenic under stress, and several disease outbreaks coincide with stress events, demonstrates that high levels of stress could negatively impact health and individual fitness.

The current methods employed for assessing health in wildlife populations – both captive and wild – are highly invasive. This is concerning as such techniques burden a high level of risk to both the animal and the researcher. Even more concerning, however, is the impact these techniques may have on the health of an animal, particularly if exposure to stress events augment immunosuppression. If stress does in fact jeopardize an animal's immune function, it is imperative that noninvasive techniques for health assessment be devised and employed in current practice.

CHAPTER 3

CREATION OF A NOVEL TECHNIQUE FOR SALIVA COLLECTION

Faunal species are exposed to various sources of stress, whether from environmental change, competition, or habitat degradation. These stressors influence physiological systems largely in terms of behavioral modifications. However, based on current and historic evidence in human medicine, it seems plausible to consider immunological impacts of stress as well, particularly if stressors are burdened on a chronic basis.

There is a current deficit in research and knowledge regarding stress and immunosuppression, and how alterations in stress stimuli could detriment individual and population health. While there is evidence of stress induced immunosuppression in wildlife, there have been little to no studies to date specifically identifying this relationship, nor studies assessing the immunologic impacts directly caused by a predetermined stressor (Buchanan, 2000; Martin *et al.,* 2011). Such research is extremely important, as it can provide information about how incessant exposure to stress stimuli may impact population health in the future.

There is also a deficit in regard to noninvasive techniques for wildlife health assessment. Currently, methods for collecting body fluids to be used in health assessment are nonexistent. The absence of noninvasive techniques for saliva, as well as urine, collection is likely attributed to the fact that it is difficult, if not impossible, to predict where these body fluids will be secreted by individual of interest. Furthermore, there are currently no methods existent in the literature that enable a controlled collection of body fluids for evaluation, and none that do not require the presence of the researcher nor physical interaction with the study individual.

GOALS AND OBJECTIVES

The goal of this research was to devise a novel technique for saliva collection in African lions (*Panthera leo*), while providing a new way to assess wildlife health without requiring immobilization or invasive procedures (and the resulting stress). Of particular interest to this study, is its potential for use in evaluating stress-induced immunosuppression. This goal was achieved by designing a device, and evaluating the efficacy of a device for efficient and effective saliva collection from lions.

METHODS

Study Location and Individuals

Three 20-30-minute trials were conducted between the months of August and October 2017 using captive lions at the Rosamond-Gifford Zoo in Syracuse, New York. During these trials, samples were collected and device efficacy tested. Testing dates were contingent on both the availability of zoo personnel and personal availability. Two of the three trials (trials #1 and #3) were conducted in the morning hours immediately following the morning feeding. The other trial (trial #2) was conducted in the late afternoon after their evening feeding. All three were conducted in the "off exhibit" enclosure – that which was inaccessible for public viewing. Such enabled a more controlled test setting by avoiding potential disturbances from zoo visitors, as well as enabled better observation and/or monitoring of behavior.

Two lions at the Rosamond Gifford Zoo were used for this study; one male and one female. Both subjects were adults between 15 to 16 years of age. They were obtained by the zoo separately; they are not related. Physical, external examination of each individual yielded healthy body and coat condition, normal activity, and no apparent abnormalities (in both behavior and physical condition).

As stated previously, all three trials were conducted in the enclosure that was off-exhibit. This was large, caged area underground where the lions would sleep, eat, and be housed during colder weather. The entire enclosure was composed of four conjoined enclosures, separated from the other by a grated wall with a sliding door. The doors could be opened or closed by zoo personnel outside of the enclosure. Several staggered horizontal platforms were suspended inside the enclosure to provide enrichment for the lions. A large hammock was installed at the far end,

and various other enrichment toys were scattered throughout the enclosure. Ambient temperature in the enclosure ranged from was on the cooler side, ranging between an estimated 64 to 73 degrees Fahrenheit.

Experimental Design and Data Collection

Use of non-invasive methods, though potentially more costly and time consumptive, prevents artificial increases in stress levels. Though I did not test whether or not my presence alone elicited a stress response, by enforcing non-invasive techniques the burden of my presence should be minimal.

Saliva Collection

Salivary samples were collected as whole saliva from passive drool samples using a "bucket in a bucket" device (Figure 1). The device was comprised of two stainless steel buckets: a larger outer

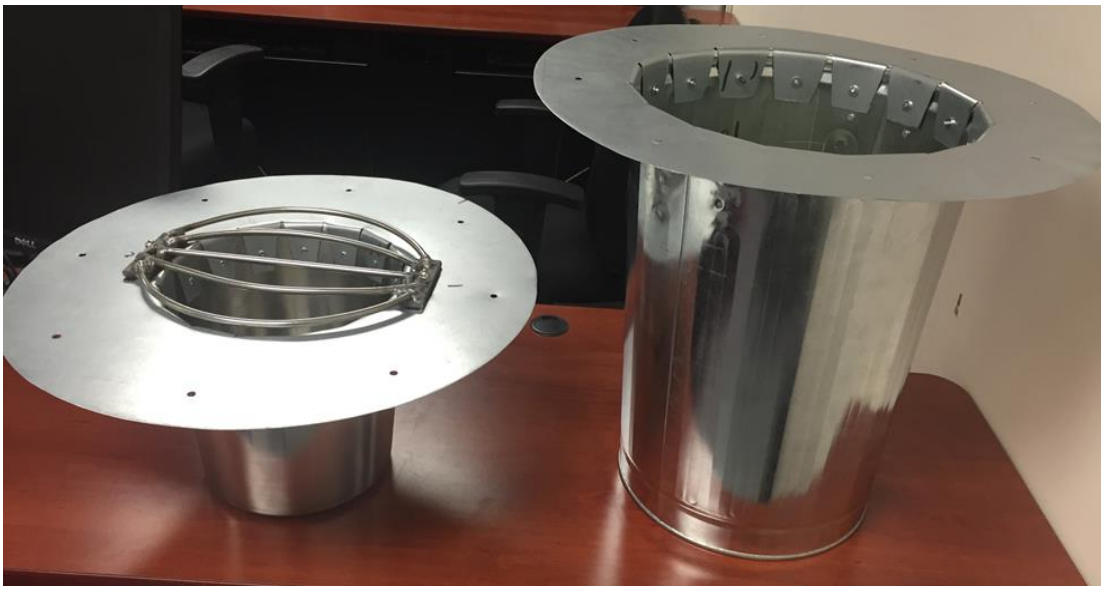


Figure 1. The two buckets comprising the "bucket in a bucket" collection device. *Left:* smaller, inner bucket serving as collection basin for drooled saliva. A grate, that can be removed when deposited saliva is being collected, covers opening. *Right:* larger, outer bucket housing ice and scent wicks. Bolts and acorn nuts hold buckets together, while also securing device to wooden housing. (Sgambelluri, 2017)

bucket and a smaller inner bucket. The outer bucket served as a holding chamber for ice and the attractant. Use of ice in the outer bucket facilitated cooling of the sample to prevent bacterial growth. Sponges soaked in attractants (see *Attractants* section) were housed in the bottom of the outer bucket. Several holes in the wall of the smaller bucket (inner bucket; held within larger bucket) enabled the scent to waft through from outer to inner bucket so that it may be detected. The inner bucket served as the collection chamber for drooled saliva, which was then collected for testing. Several metal rods that grate the mouth of the smaller bucket facilitated licking and thus salivation.

The entire contraption was housed in a reinforced wooden box (Appendix 2, Figure 3). This provided another layer of protection for the device, as well as enabled secure attachment to the enclosure wall. Two durable ratchet straps with industrial grade polyester webbing (breaking weight 1,200 lbs.) were run through four slits made in the back wall of the box. The straps were then drawn through the grated enclosure wall, and tightened on the enclosure exterior. This enabled the box to be secured to the wall of the enclosure, while keeping the straps completely out of reach by the lions. Furthermore, zoo personnel could easily and safely access the ratchet for tightening without having to enter the enclosure.

Samples were retrieved within 30 minutes of collection to avoid bacterial growth. Addition of ice in the outer bucket would provide a slightly more flexible timeline in case the retrieval could not occur within the 30-minute timeframe. Deposited saliva was collected using 1 to 5 cc (1 – 5 mL) syringes (Bstean syringes; model #: SY-10) with blunt tipped needles (14, 16, and 18 gauge), which were then capped and labeled according to collection protocol (Appendix). Those allocated for biological validations were stored in a cooler (short term storage for four hours) until they could be placed for long term storage in a freezer.

Attractants

Two attractants were used and tested in this study: (1) catnip extract and (2) bear bait. Catnip extract (SynergyLabs Xtreme Catnip concentrated catnip spray; model: FG00004) was employed to elicit a licking response and salivation from the lions – as found in catnip study by Hill et al. (1975). The bear bait (Moultrie Bear Magnet® Liquid Bear Attractant; bacon flavored) served as a secondary attractant, playing more of a role in motivating a lion to approach the device. Ability of such to provide such functions were derived from whether or not a lion approached the device, whether drooling was observed, and observation of the behaviors displayed by the lions in the presence of the device.

Four kitchen sponges were placed in two airlocked Pyrex containers (two sponges per container) filled with a specific attractant; one containing catnip extract, and the other filled with bear bait. Concentration of attractant during time trial could be mediated by number of sponges soaked and used; the more soaked sponges, the higher the concentration of attractant. The sponges were soaked in each attractant, separately, for at least 15 hours prior to time trial.

During the trials, sponges were moved to aluminum baking trays, and then placed in the outer bucket. The aluminum trays prevented the attractants from reacting with the metal bucket itself, as well as enable addition of extra liquid attractant if desired. Sponges were replaced for each trial.

Sample Retrieval and Storage

Deposited saliva was collected following the procedure detailed in Appendix 1 using 1 to 5 mL capped syringes, depending on the amount of saliva visible. Each syringe contained a cryolabel indicating sample number, date of collection (mnth/day/yr), and gender (M / F). Sample number

was constructed based on a self designed system. Each sample began with the abbreviated zoo name (i.e. RGZ for Rosamond Gifford Zoo). This provided information on the location of the sample. This acronym was then followed with either M or F, depending on which lion deposited the sample. Since there were only two lions at this zoo, simply M or F was sufficient. Had there been several individuals of the same gender, a number assigned to each individual (i.e. 1 or 2) would have followed. A line of code contiaining 00 followed by the sample number for that individual followed RGZM or RGZF text.

Information was also transcribed to an Excel sheet for each collection/sample. This information included: sample number (matching that on syringe), date of collection, collection time (military time), gender, approximate amount of saliva collected (mL), internal basin temperature at time of collection (according to reading on digital thermometer), ambient temperature of enclosure, general zoo activity (busy, moderate, quiet), and outdoor weather. Base and walls of basins, as well as rods of grating, were wiped dry with paper towels between each collection to prevent mixing of saliva or contamination. Scent sponges were replaced for each trial.

Collected samples were then stored in a cryofreezer until time of laboratory analysis. At that time, the frozen saliva samples were thawed and then centrifuged at 3000 rpm for 15 minutes to remove mucins and particulate matter (Salimetrics, 2015). Samples were then left out after centrifugation until they reached room temperature. Once at room temperature, the samples were pipetted into dilution tubes for 1) Corticosterone, 2) SIgA, and 3) C-Reactive Protein (CRP). Any remaining saliva was re-frozen. The salivary samples in the SIgA and CRP tubes were used for biological validation of these two test variables (Salimetrics, 2015).

Data Analysis

Method / Device Success

Efficacy and success were evaluated using several metrics listed in Appendix 3. The immediate success of the device was ranked on a numeric scale based on the behavior or reaction exhibited by the lion(s) towards the device. This method was employed to account for the fact that the actual degree of success was dependent on the degree of behaviors elicited. The behaviors were categorized as either being level 1 or level 2 behaviors. Level one behaviors comprised those associated with general interaction with the device. Such behaviors included investigation, sniffing, and playing with box, as well as pawing ground next to the device. Level two behaviors, on the other hand, pertain to responsive behaviors. In other words, these were the behaviors exhibited in response to the device, often following level one behavior(s). Such behaviors deemed as level two included scent marking (rubbing of head, flank, or hind limbs) on device, scent marking on enclosure, urine spraying, salivation/drooling, and licking.

In general, there were four predominant determinants of success value: (1) whether or not the lion(s) approached the device, (2) whether or not the lion(s) interacted with the device (level 1 behavior), (3) whether or not the device elicited a level 2 behavior, and (4) whether or not any saliva was collected in the basin. Degree of success was then ranked accordingly (Table 1). Approach refers simply to the lion walking up to the device, not necessarily interacting with it; it does not involve level one or level two behaviors. Interaction or investigation differs from approach in that the lion made a direct and explicit effort to interact with the box, rather than just looking at it. In general, it involved the lion standing at the device for more than five seconds and exhibition of an investigative behavior, such as sniffing.

Table 1. Degrees of success yielded according to trial outcome, and the determinants founding each evaluation. Failure to approach device is reported as a 0 – no success.

Degree of Success	**Description of Value Determinants**
1	lion(s) approached device, but failed to interact with the device any further than the initial approach
2	lion(s) approached and interacted with the device (elicitation of level 1 behaviors). No elicitation of saliva or scent marking (failed to elicit level 2 behaviors).
3	lion(s) approached and interacted with the device (elicitation of level 1 behaviors). Successfully elicited salivation or scent marking (level 2 behaviors).
4	All prior success values were displayed, and actual collection of saliva in the basin occurred.

Statistical Analysis

Variable data from each trial were compiled and organized using Microsoft Excel Software (V. 2017). This included documentation of trial demographics (i.e. time, date, and duration), as well as success determinants (i.e. level 1 behaviors, level 2 behaviors, time to approach device, deposition of samples, amount collected). Such records were generated for each trial and individual separately. Ethograms and time budget plots were generated using BORIS software program, as well as calculation of trial statistics.

RESULTS

A total of three time trials were conducted to evaluate device efficacy. Trial #1 and #2 were both conducted on September 17th, 2017. Trial #3 occurrred on October 1st, 2017. Both the first and third trials were conducted early in the morning immediately following morning feeding. The second trial was conducted during the evening of the first trial day.

Two of the three trials yielded a success rating of greater than or equal to three; trials one and three (Table 6). The first trial yielded a success rating of four for the male and three for the female, with a mean success value of 3.5 (Table 6). No success was achieved in the second trial for either individual, yielding an overall success value of zero for that trial (Table 6). The third trial yielded a success value of three for the male and one for the female, for a mean success value of two (Table 6). In general, a higher success in elicitation of behavioral responses and actual collection of saliva was observed with the male.

Of note, ice was not used in any of the trials, since such were tailored strictly to 20-30 minute periods. Thus, the potential for bacterial growth prior to collection was not of concern.

Trial #1 Results and Observations

During the first trial, the male approached the device nine minutes after entering the enclosure (Figure 3). Four level one behaviors were exhibited within the 20 minute trial. These were (1) investigation, (2) sniffing of box, (3) pawing at box or ground next to box, and (4) playing with the box (Table 6). The device held strong when played with by the male lion, and no damages occurred. Level two behaviors observed included salivation/drooling along corners of mouth, and rubbing sides of face (cheeks) against box (Table 6). This type of head rubbing serves as scent marking. Although actual drooling of saliva into the device was not observed, pooled saliva was

present at the bottom of the basin following termination of trial. Approximately 0.1 cc (100 µL) of saliva was retrieved. Observation of level one behaviors, elicitation of level two behaviors, and the collection of saliva yielded this trial – for the male individually – as extremely successful, earning the highest success value of four. Furthermore, the amount of saliva collected exceeded the minimum amount required for conduction of ELISAs – about 10-20 µL – indicating the device was successful in collecting adequate sized samples for salivary analyses.

Approach by the female occurred approximately 10 minutes after entry into enclosure (Figure 3). Both level one and two behaviors were observed. Level one behaviors included (1) investigation of box, (2) sniffing, and (3) pawing ground next to box (Table 6). Frequent head ru vn bbing – scent marking and level two behavior - occurred, though such was performed on the enclosure rather than the box (Figure 3, Table 3). Several behaviors were of particular interest, and appeared to be a direct response to the device. These such behaviors – which are discussed further in the following chapter – included frequent head rubbing on enclosure, frequent sneezing fits, and pawing at ground next to box. Since no saliva was collected, but both level one and two behaviors were observed, this individual trial for the female was ranked as a success value of three. Overall success for this trial, or the mean value between scores for male and female, was a 3.5.

Time Budget

Behaviors in both individuals were allocated between stationary, vocalization, and grooming (Figure 2, Table 3). For the male, 70% of total trial time was spent in stationary behavior – either standing or resting/laying down – comprising a total duration of approximately 1345 seconds for stationary (frequency = 10; Table 3). This was followed by vocalization, which comprised 13.8% of the trial period for a total duration of 265 seconds (frequency of 4), and grooming activities, comprising 9.9% of the total trial with a total duration of 191 seconds (frequency of 4) (Table 3).

In total, device specific behaviors constituted 8.5% of the total trial period (*0.3% play, 3.6% investigation of box, 0.7% roll, 3.9% scent marking*) (Table 3), for total dhuration of approximately 163.5 seconds.

Synonymous with the male, the majority of trial period for the female was spent in stationary behavior (Figure 2). Such comprised 73% of total trial for a total duration of approximately 1400 seconds (frequency = 18; Table 3). Also synonymous with the male, vocalization was the second largest time contributive behavior for the female, constituting over 9% of the trial period (total duration of approximately 184 seconds) (Table 3). Such is expected, as most vocalization events occur as a call and response between individuals of a small group or

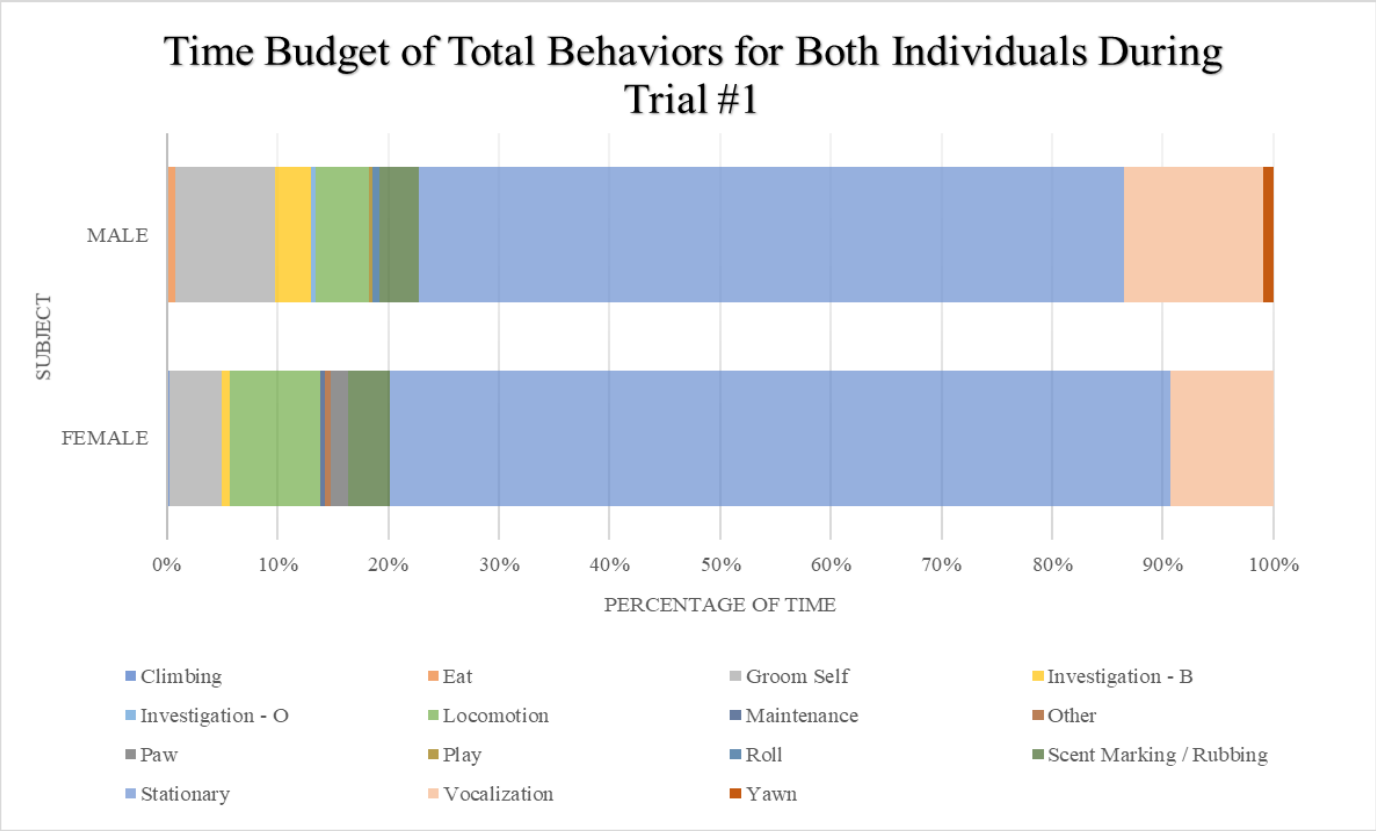


Figure 2. Percentage of time spent per activity throughout the entire 20 minutes of trial one. Activity time percentages are displayed for both individuals; male (*top*) and female (*bottom*). Ranking of behaviors do not correspond to first occurrence in trial. Each color correlates to a different behavior or grouping of behaviors.

pride, as a means to strengthen family bonds and/or secure territory. Of all the vocalization events documented, there was only one instance where the female did not join the male in chorus. Though not the greatest contributer for time, the most frequent behavior displayed by the female was locomotion – observed 21 times (Table 3). A significant amount of scent marking behavior was observed, often enacted as facial scent marking or head rubbing on the enclosure grating (Figure 3). A total of nine different scent marking events were observed for the female, often followed or paired with grooming activities and short sneezing fits (Table 3, Figure 3). A significantly smaller amount of time was allotted to device specific behaviors; roughly 6.3% of the total trial period, with a total duration of 121 seconds. Device specific behaviors for the female included investigation of box (0.8%), pawing (1.6%), and scent marking/head rubbing (3.9%) (Table 3).

Table 2. Ethogram of all recorded and/or major behaviors for captive African lions, with operational definitions. Behavioral measures grouped according to their assumed "type"; *solitary, ambulatory, food, social, aggression/territorial*, and *miscellaneous*. Of note: urination and urine spraying are two different behaviors. Urination involves the lion squatting to dispel urine, and typically no external stimulus is involved. Urine spraying, on the other hand, represents scent marking and/or territorial behavior. Unlike urination, the animal is standing with tail erect in air. A jet of urine, measuring up to or over 1 meter in length, is expelled onto a vertical object. Tail will often quiver during such, and the animal may be seen hindlimb scraping.

Type of Behavior	**Behavior**	**Code**	**Description of Behavior**
Solitary	Sleep	S	Animal assumes species-specific position for sleep; stays in one place and is not alert to environmental changes
	Stationary	R	Animal stays in one place, but may be roused easily by environmental changes; may be either laying down (R-L) or standing (R-S); eyes may be open or closed
	Yawn	Y	Common usage
	Groom Self	GS	Animal engages in washing its fur, scratching, and/or grooming activities using its tongue and forelimbs
	Play	P	Animal engages in solitary play-based interactions (exhibits no intention to harm) with enrichment toys or with box
	Investigation	I-B; I-O	Animal engages in solitary investigation of box (I-B) or other object (I-O) in enclosure
	Head Shake	HS	Animal shakes head from side to side
	Maintenance	M	Animal urinates (in squatting position) or defecates
Ambulatory	Locomotion	L	Animal moves from one location to another in a nonrepetitive manner; with purpose (i.e. walk, trotting)
	Pacing	PC	Repetitive movement between two locations, with no apparent purpose
	Climbing	CL	Animal ascends and/or descends an object or structure
	Hindlimb Scraping	HH	Animal scrapes hind feet backwards along ground surface, shuffling between feet
	Roll	RL	Animal moves onto its back and exposes belly. Playful intention
	Pawing	PW	Animal pats at ground surface using forelimb, with no clear purpose; only one paw used and claws are retracted
Food Related	Eat	E	Animal consumes food
	Drink	D	Animal consumes water
Social	Communal Groom / Ear Suckling	CG	Animal engages in washing fur, grooming, or ear suckling of another individual in environment
	Play	GP	Animal engages in interactions with other individuals that may involve locomotion, climb, manipulating objects, or other activities that show a relationship between two or more interacting animals (i.e. wrestling)
	Contact	CO	Animal comes in physical contact with another individual
	Vocalization	V	Animal engages in non-aggressive vocal activities (i.e. roaring, mewing, chatting)
Aggressive or Territorial	Fight	F	Animal engages in aggressive physical contact with another animal in its environment (i.e. biting, hit, slap, chase, snarl)
	Scent Marking / Rubbing	SM	Animal rubs glands near tail, legs, or on face against surface or object.
	Urine Spray	US	Jet of urine released onto vertical surface or object while standing. Tail is raised vertically, and may quiver.
Misc	Other	O	Animal engages in behavior not explicitly detailed or identified above

Table 3. Time budget summaries for male *(top)* and female *(bottom)* African lions during trial #1, at Rosamond-Gifford Zoo. Frequency represents the number of times a behavioral event was observed. Total duration represents cumulative time (in seconds) allocated to a specific behavior. Such valuation was utilized to evaluate the percentage of total trial period spent per behavior. Average duration and inter-event intervals (IEI) reported as mean ± SD. There were no inter-event intervals for those behaviors occurring a single time (frequency of 1), and thus IEI means for such were reported as NA. Time trial was conducted on September 17, 2017 for a total time of 20 min (1200 sec). Data constructed using BORIS software (Friard et al., 2016). (Sgambelluri, 2018)

Behavior	Frequency	Total duration (s)	Duration Mean (s)	IEI Mean (s)	% of total length
Investigation - O	1	9.10	9.1	NA	0.5
Stationary	10	1345.50	134.55 ± 139.31	49.72 ± 42.37	70.1
Yawn	3	19.00	6.33 ± 2.31	307 ± 74.95	1
Groom Self	4	191.00	47.75 ± 47.82	323 ± 254.21	9.9
Play	1	5.00	5	NA	0.3
Investigation - B	4	69.20	17.3 ± 20.86	240.93 ± 274.67	3.6
Locomotion	13	102.59	7.89 ± 7.5	132.62 ± 179.77	5.3
Climbing	1	2.00	2	NA	0.1
Roll	1	14.10	14.1	NA	0.7
Eat	1	14.00	14	NA	0.7
Vocalization	4	265.10	66.28 ± 11.76	192.63 ± 148.66	13.8
Scent Marking / Rubbing	4	75.20	18.8 ± 16.0	185.27 ± 308.86	3.9

Behavior	Frequency	Total duration (s)	Duration Mean (s)	IEI Mean (s)	% of total length
Stationary	18	1400.78	77.82 ± 83.87	22.66 ± 26.02	73
Groom Self	7	92.00	13.14 ± 10.92	236.33 ± 284.05	4.8
Investigation - B	1	16.00	16	NA	0.8
Maintenance	1	10.00	10	NA	0.5
Locomotion	21	160.39	7.64 ± 9.96	76.68 ± 89.66	8.4
Climbing	2	5.00	2.5 ± 2.12	1537	0.3
Paw	2	30.00	15 ± 7.07	8	1.6
Vocalization	3	184.00	61.33 ± 16.29	234.5 ± 195.87	9.6
Scent Marking / Rubbing	9	75.00	8.33 ± 3.67	188.75 ± 279.95	3.9
Other	1	10.00	10	NA	0.5

Table 4. Time budget summaries for male *(top)* and female *(bottom)* African lions during trial #3, at Rosamond-Gifford Zoo. Frequency represents the number of times a behavioral event was observed. Total duration represents cumulative time (in seconds) allocated to a specific behavior. Such valuation was utilized to evaluate the percentage of total trial period spent per behavior. Average duration and inter-event intervals (IEI) reported as mean ± SD. There were no inter-event intervals for those behaviors occurring a single time (frequency of 1), and thus IEI means for such were reported as NA. Time trial was conducted on October 1,2017 for a total time of 30 min (1800 sec). Data constructed using BORIS software (Friard *et al.,* 2016). (Sgambelluri, 2018)

Behavior	Frequency	Total duration (s)	Duration Mean (s)	IEI Mean (s)	% of total length
Investigation - O	1	1.10	1.1	NA	0.1
Stationary	28	1410.17	50.36 ± 89.6	13.33 ± 20.86	79.7
Investigation - B	1	8.00	8	NA	0.5
Locomotion	28	164.28	5.87 ± 6.4	36.59 ± 44.9	9.3
Climbing	1	4.00	4	NA	0.2
Hindlimb Scraping	1	6.00	6	NA	0.3
Contact	1	2.00	2	NA	0.1
Vocalization	6	306.00	51 ± 29.79	186.2 ± 118.9	17.3
Scent Marking / Rubbing	3	24.00	8 ± 4.36	2.5 ± 0.71	1.4
Urine Spray	1	12.00	12	NA	0.7

Behavior	Frequency	Total duration (s)	Duration Mean (s)	IEI Mean (s)	% of total length
Investigation - O	1	1.00	1	NA	0.1
Stationary	11	1695.19	154.12 ± 307.72	7.48 ± 6.70	95.8
Yawn	1	2.00	2	NA	0.1
Groom Self	1	32.00	32	NA	1.8
Locomotion	10	46.09	4.61 ± 3.26	83.66 ± 185.93	2.6
Climbing	1	2.00	2	NA	0.1
Contact	1	2.00	2	NA	0.1
Vocalization	4	180.00	45 ± 13.78	318 ± 159.62	10.2
Urine Spray	1	15.00	15	NA	0.8

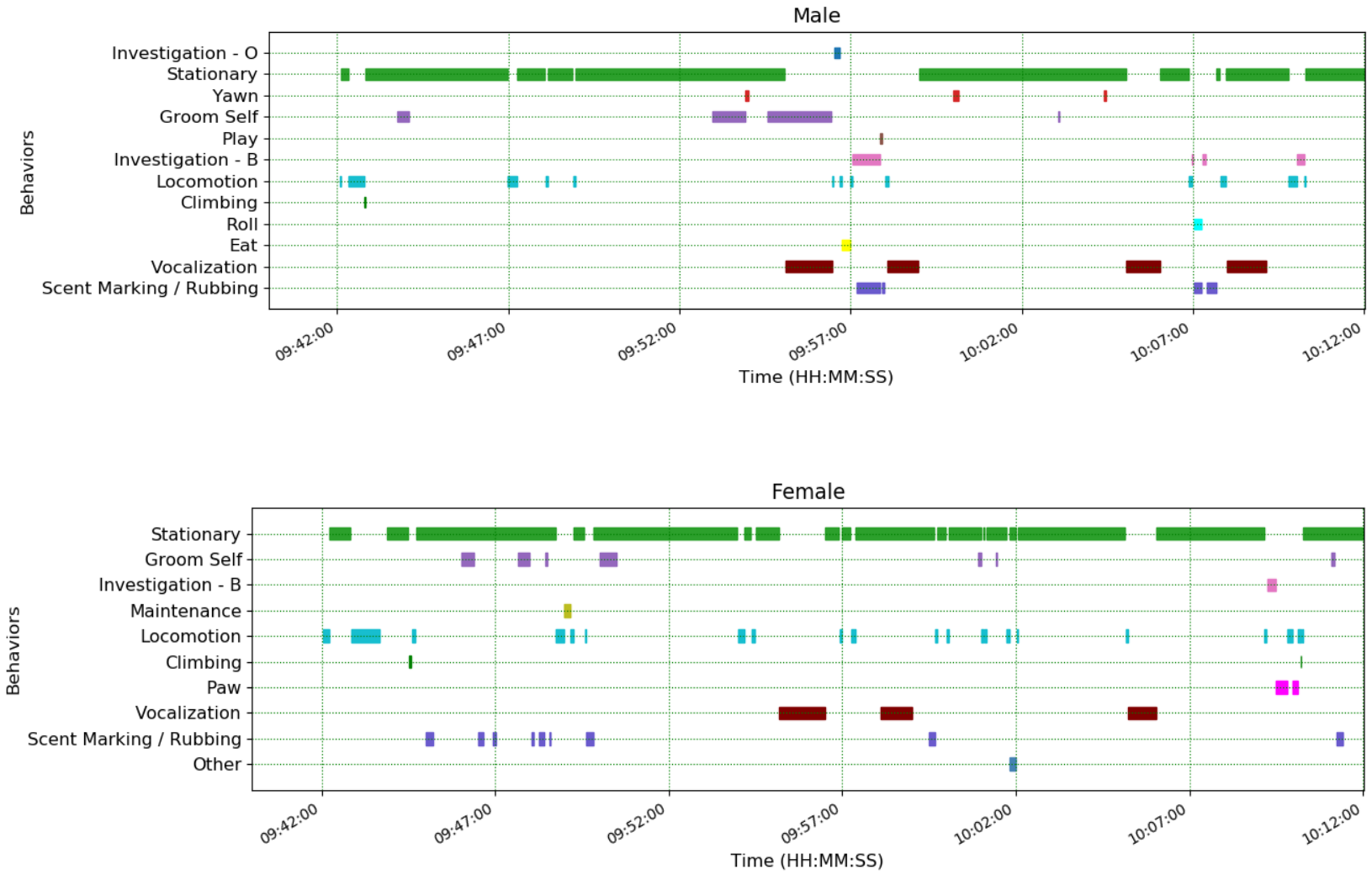


Figure 3. Time budget diagrams for both male *(top)* and female *(bottom)* African lions during trial #1 at Rosamond Gifford Zoo. Behavior parameters include only state events (frequency and duration). Point events (frequency only) were not included. Trial length was 20 minutes, starting at 9:42 AM and ending at 10:12 AM.

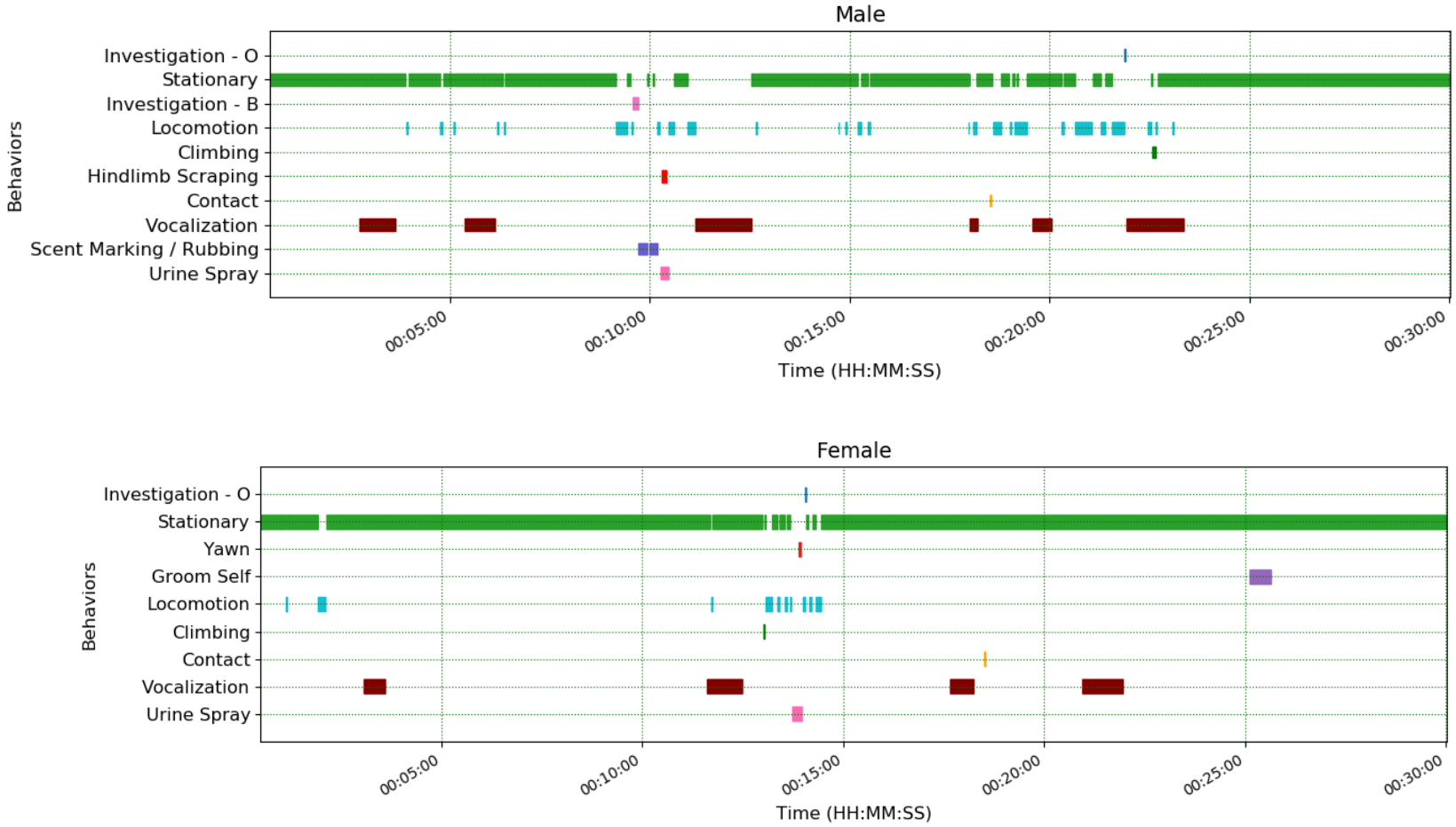


Figure 4. Time budget diagrams for both male *(top)* and female *(bottom)* African lions during trial #3 at Rosamond Gifford Zoo. Behavior parameters include only state events (frequency and duration). Point events (frequency only) were not included. Trial length was 30 minutes, starting at 9:36 AM and ending at 10:06 AM.

Table 5. Observation and documentation of behaviors exhibited by male lion during first trial on September 17, 2017. Total duration of trial was 20 minutes, beginning at 9:42 am and concluding at 10:12 am. Time at which behavior was initiated (*time*) and the total duration of that behavior (in seconds; *duration*) are reported in table. Behaviors recorded using coding provided in table 1. Scent marking predominantly occurred using scent glands in cheeks; this is recorded as SM and described as head rubbing or rubbing of face. As with female, rubbing face against an object was documented and presumed as scent marking (SM), rather than grooming, based on current knowledge of wild felid behavior as described in scientific literature (Gorman *et al.,* 1989; Marnewick *et al.,* 2006; Pageat *et al.,* 2003; Reiger, 1979; Soini *et al.,* 2012). Refer to ethogram (table 2) for behavioral coding.

BEHAVIOR	TIME (h:min:sec)	DURATION (sec)	NOTES
TRIAL STARTED AT 9:42:00			
L	9:42:04	3	male entered enclosure
R-S	9:42:07	12	looking around enclosure and sniffing air
L	9:42:19	17	moved to far side of enclosure by hammock
L	9:42:36	11	ambled around enclosure containing hammock
CL	9:42:47	3	jumped up onto/into hammock
R-L	9:42:50	56	laying in hammock
G-S	9:43:46	20	
R-L	9:44:06	60	
R-L	9:45:06	60	
R-L	9:46:06	53	
L	9:46:59	26	stood up and left hammock. Moved across enclosure, and into adjacent enclosure, which contained saliva device/box
R-S	9:47:15	50	standing in enclosure and sniffing air
L	9:48:05	5	walked to front of enclosure
R-S	9:48:10	43	standing at front of enclosure. Intermittent sniffing
L	9:48:53	5	walked towards back of enclosure, near platform where female was resting
R-L	9:48:58	60	resting on ground
R-L	9:49:58	60	
R-L	9:50:58	60	
R-L	9:51:58	60	
G-S; R-L	9:52:58	57	
Y	9:53:55	5	
R-L	9:54:00	35	
G-S	9:54:35	23	
R-L	9:54:58	7	
V	9:55:05	82	male initiated vocalization. Female joined roughly five seconds in
L	9:56:27	10	Vocalization ended. Walked over to log
I-O	9:56:31	6	investigating log
L	9:56:41	4	walked to food tray
E	9:56:45	15	eating food
L	9:57:00	3	Stopped eating. Walked over to box
I-B	9:57:03	7	sniffing (interacting with) box
I-B; SM	9:57:10	41	Rubbing face and body on box; sniffing box intermittent. Apparent salivation observed, though uncertain if any deposited.
I-B; P	9:57:51	5	trying to play with box. Box held strong
I-B; SM	9:57:56	4	

L	9:58:00	4	Left box and moved to center of enclosure
V	9:58:04	54	In center of pen. Initiated vocalization and joined by female.
R-L	9:59:00	26	Stopped vocalizing. Laid down in middle of enclosure.
G-S	9:59:26	32	Lots of grooming
R-L	9:59:58	2	
Y	10:00:00	9	
R-L	10:00:09	51	laying in center of enclosure
R-L	10:01:00	60	appears to be napping
R-L	10:02:00	3	joined by female. Lifted head briefly when female approached, then lowered back to floor
R-L	10:02:03	60	
G-S	10:03:03	6	Lifted head and repositioned body (shifted from laying on side to sternal recumbant)
R-L	10:03:06	60	
R-L	10:04:06	17	
Y	10:04:23	5	
R-L	10:04:28	35	
V	10:05:03	60	male started vocal at 10:05:03. Still laying down
R-L	10:06:03	50	Male stopped vocalizing. Remained laying down, and soon joined by female
L	10:06:53	5	Stood up and moved back over to box
I-B	10:06:58	3	
SM; RL	10:07:01	15	Rubbed head against box. Then started rolling on his back against box, continuing to rub cheeks (where scent glands are located) against box.
I-B	10:07:16	8	Stopped rolling and stood up. Sniffed box. Seems to have saliva pooling at corners of mouth. Licked left side mouth. Did not lick grate on top of box.
SM	10:07:24	17	Rubbing head against top of box
R-S	10:07:41	7	Stopped rubbing. Standing at box. Looking to right side
L	10:07:48	10	moved to far side of enclosure by hammock
R-S	10:07:58	2	standing by hammock, facing front of enclosure
V	10:08:00	60	male vocalized. Female did not join vocalization
V	10:09:00	8	Still vocalizing
R-S	10:09:08	14	stopped vocalizing. Standing in same spot. Intermittently moving head to shift gaze around enclosure.
R-S	10:09:22	6	Still standing, but now watching female investigate box
R-S	10:09:28	20	Stopped focus on female. Returned to standing with intermittent shift in gaze around enclosure.
L	10:09:48	15	moved back across enclosure and approached saliva device
I-B	10:10:03	12	sniffing box
L	10:10:15	3	moved to center of enclosure
R-S	10:10:18	60	laying down in center of enclosure
R-S	10:11:08	52	
TRIAL ENDED AT 10:12:00			

Trial #2 Results and Observations

Both lions failed to approach and/or interact with the device. In general, they seemed undisturbed and uninterested in the box. Bulk of 20 minute trial period was allocated between grooming, resting, and moving between areas of the enclosure for both individuals. No vocalizations or scent marking activities were observed. Additionally, no level one or two behaviors were observed or recorded. In accordance, the trial was deemed as unsuccessful for both individuals, yielding an overall success value of zero.

Trial #3 Results and Observations

While the device was seen by the female, she failed to pursue any further interaction with such. After approximately 13 minutes, this individual approached within one foot of the box. A total of nine seconds were spent at that location, with three instances where the female looked directly at the device; device was approached and observed from a distance. No level one behaviors occurred at that time, nor during time following (Figure 4). However, a level two behavior did occur. Within 10 seconds of leaving the spot near the device, the female sprayed along the back wall of the enclosure (Figure 4). Specific motivation behind such behavior was ambigous; such may have been in direct response to the device or in response to human presence previously in the enclosure. Remainder of trial period was allocated between resting and vocalization (Figure 4). Frequent head rubbing and sneezing episodes that had been observed during trial one were absent in this trial. Three mililiters (3000 uL) of urine – from urine spray – was collected upon trial termination (Table 6). Since the specific motivation behind the urine spray was not clearly defined, the behavior was not recorded as a device-specific response in an attempt to avoid potential bias. Consequently, a success value of one was assigned for this individual in this trial.

The male, conversely, exhibited a strong, favorable response to the device. Approach occurred approximately nine minutes after entering the enclosure (Figure 4), with time spent at the device exceeding one minute. Scent marking via facial rubbing against the device occured, as well as scent marking via urine spray (Table 4). Duration of such behaviors totaled to 50 seconds, representing 3% of trial (Table 4). The urine spray occurred immediately following cessation of head rubbing, and was projected towards the front of the box, alluding territorial motivation. Unlike that of the female, the male's urine spray was directly associated with the device, in that it was in response to and directed at the device specifically. Approximately 2 mL (2000 uL) of urine was collected amongst three collection syringes, along with retrieval of mane hair fibers from the wooden crate (Table 6). While this trial had a success value of three due to lack of saliva deposition and collection, it should be noted that the device did succeed in eliciting deposition of body fluid – the urine spray – from at least one individual.

Table 6. Complete table of time trial data, as well as documentation of information relative to success ranking. Level 1 and level 2 behaviors observed for each individual per trial are described. Whether body fluid was deposited and collected during each trial is illustrated, as well as the amount that was collected if deposition occurred (1 cc = 1 mL). Damage occurrence refers to whether or not the device sustained any damage requiring repair during/after a trial period. This factor served as a metric in success evaluation as a primary determinant of durability.

Approach refers simply to the lion walking up to the device and remaining within a 1 ft radius of device. Such behavior does not deal with a direct interaction with the device; it does not involve investigation of device or any other behaviors elicited therein. Investigation differs from approach in that lion made direct and explicit effort to interact with the box, typically through sniffing, and involved the lion standing at the device for more than five seconds. Scent marking predominantly occurred using scent glands in cheeks; this is described as head rubbing (level 2 behavior); described as scent marking based on current knowledge of wild felid behavior as described in scientific literature (Gorman *et al.,*1989; Marnewick *et al.,* 2006; Pageat *et al.,* 2003; Reiger, 1979; Soini *et al.,* 2012). A urine spray is different from normal urination. Urination usually involves squatting and typically no stimulus involved. Urine spray done while standing, with a jet of urine being released straight behind the animal. Tail is usually erect in air, and hindlimb scraping may be involved.

*Female head rubbing during first trial was against enclosure walls, not the device itself. Animal curator indicated that the frequency at which the female exhibited this behavior was far greater than normally observed.

Trial	Date	Time (24 hr; hr:min:sec)	Duration (min.)	Gender	Approach (Y/N)	Time to Approach (min.)	Level 1 Behaviors	Level 2 Behaviors	Elicitation of Licking (Y/N)	Obvious Salivation (Y/N)	Body Fluid Deposited and Collected	Amount deposited (cc ; uL)	# of Samples Collected	Damage Occurrence (Y/N)	Success Value
1	9/17/2017	9:42:00	20	Male	Y	9	Investigation Sniffing Pawing Play	Salivation/Drooling Head Rubbing	N	Y	Saliva	0.1 cc ; 100 uL	1	N	4
1	9/17/2017	9:42:00	20	Female	Y	10	Investigation Sniffing Pawing	Head Rubbing*	N	N	None	-	0	N	3
2	9/17/2017	17:10:00	30	Male	N	X	X	X	N	N	None	-	0	N	0
2	9/17/2017	17:10:00	30	Female	N	X	X	X	N	N	None	-	0	N	0
3	10/1/2017	9:28:00	30	Male	Y	9	Investigation Sniffing	Urine Spray Head Rubbing	N	Y	Urine	2 cc ; 2000 uL	3 (+ hair)	N	3
3	10/1/2017	9:28:00	30	Female	Y	13	X	Urine Spray	N	N	Urine	3 cc ; 3000 uL	1	N	1

DISCUSSION

Overall, the device was successful, with a success rating of greater than or equal to three in two of the three trials conducted. In general, there was a higher success in elicitation of behavioral responses and actual collection of saliva with the male.

The female exhibited some interesting behaviors that may have been correlated with the presence of the device, and/or a reaction towards one of the attractants. During the first trial, the female exhibited excessive head rubbing against grating of enclosure, as well as frequent sneezing fits – both of which were deemed as unusual for this individual by the animal curator. Furthermore, after approaching the device, the female went alongside the device and began pawing at the ground. This may have been a type of behavior called scratching, a variation of scent marking wherein the individual marks territorial areas by scratching or pawing at the ground (Ghoddousi *et al.,* 2008). This typically is followed by spraying, which was not observed in this event. It is unclear whether one of the attrancts was percieved positively by the male, but negatively by the female, and if such was illiciting such behavior in the female. It is possible that such behavior was conducted more as an act of submission, since the male seemed to claim the device during the first trial. It would be interesting to know whether the female was indeed reacting negatively to the device, or if one of the attractants used was perceived differently by the genders.

Both individuals were not nearly as interested in the device when they were reintroduced to it on the evening of the first trial. It is difficult to determine causation for failed success in this second trial – whether such was a function of time of day or lack of interest, and there was not enough data to make such determination. However, based on the data obtained, it appears that the device yielded most success when removed for a period of time and reintroduced for each trial, rather than remaining in the enclosure. It is possible that the individuals habituated to the stimulus

when it was left in the enclosure between trials. For the third trial, it is possible that a decreased response from the lions occurred because the attractants were less concentrated (only one sponge soaked with each attractant was used, rather than two), relative to the first trial. The less concentrated the attractant(s), the less noxoious the stimuli, and – potentially – the lower the degree of response. Furthermore, installation of the device occurred while the individuals were already inside the enclosure; per request of the zookeepers. Though they could not access the device during installation, the lions were able to observe it being installed and were also exposed to the scents at that time. It is possible that direct observation of such, rather than finding of device upon entry into enclosure, detered or influenced the behavior of the lions towards the device; for instance, such may explain why the female lion would only approach the device at a distance, and why less time was spent at the device for both lions in general. It could be argued that both urine sprays observed during the trial were, rather, territorial responses to an unknown human being having been in the enclosure previously. Howerver, if such were the case, there likely would have been a greater amount of strong scent marking behavior made throughout the entire enclosure by both individuals. Furthermore, the general relaxed attitude of the lions belies such arguement. Throughout the entire installation and time trial, both individuals appeared undisturbed and unagitated. There were no aggressive behaviors observed or displayed towards myself or the animal curator during device installation. Rather, the female was resting on the horizontal platform and the male was sleeping in the hammock. Pacing, another behavior that would be expected in such scenario, was not exhibited by either individual.

Based on the obsersvations and results of these trials, the device was indeed successful, with the potential to not only elicit and collect saliva, but also collect other body fluids as well. In this case, urine spray. The combination of attractants employed succeeded in eliciting approach by

the individuals, as well as a number of interesting behaviors. In this particular case, the almost allergic like response of the female versus the territorial response by the male. The device itself carries great potential for how it may be used in the future, particularly in the realm of wildlife health assessment. However, before such can be enacted, followup studies must be conducted. The replicative power, or its ability to reproduce saliva collection in other individuals, needs to be evaluated for this device. It is not to say that, while it indeed worked with the lions used at the Rosamond-Gifford Zoo, it could yield greatly different responses in lions at different zoos.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

The goal of this thesis was to devise a novel technique for saliva collection in African lions (*Panthera leo*), while providing a new way to noninvasively assess health in wildlife populations. The fact that this device was able to elicit responses from the study individuals, and successfully collected an adequate sample of saliva, indicates that this method was indeed successful for this study. In one of the trials, not only were level 2 behaviors elicited (e.g. scent marking and head rubbing), but the device succeeded in eliciting the deposition of body fluid – the urine spray. Furthermore, this fluid was collectable. This finding is significant, in that it indicates (1) the device has the potential to trigger a variety of responses, and (2) it can collect several different types of samples. In the three trials performed in this study to test this device, the device was able to collect saliva, urine, and hair samples, all of which can be utilized to gain information relative to individual health. This highlights the potential for this device to be used beyond only saliva collection, and thus broadens scope of how it may be used in the future.

Future Research and Considerations

Obviously, further research needs to be conducted to supplement the findings obtained here. The device needs to be tested on different individuals in different locations. This can be taken a step further, and tested on different felid species to compare responses. Next steps for the device would also include the addition of a self-closing lid system, to ensure collection of individual samples and avoid cross contamination between individuals.

Saliva can be used to provide a wealth of knowledge ranging from immunologic to reproductive health. Based on this understanding, it could be used as a valuable medium for

assessing health across various spectra. Efforts should be, and need to be, made to begin incorporating the use of saliva and employing it to regularly monitor health of captive species; particularly if health is impaired by stress events.

Regarding the utilization of saliva, steps also need to be made to biologically validate feline salivary health measures. This requires the acquisition and/or production of ELISA assays that are validated for feline saliva. An original objective in this study was the biological validation of salivary assays for feline secretory immunoglobin A (IgA) and c-reactive protein (CRP), and using such for evaluation of stress-induced immunosuppression. During the progression of this study, it became clear that such objective was better suited as a separate, ongoing project in and of itself. At this current time, IgA and CRP ELISAs operative on domestic feline saliva are rare; for lions they are nonexistent. Most of the ELISAs for evaluations of such levels in felines only detect – and thus function – with serum or plasma samples. Attempts to contact individuals who analyzed immunoglobin levels in saliva of domestic felines for evaluation of feline leukemia were made, although such attempts were unsuccessful. Furthermore, valid and thorough completion of the biological validations requires careful collaboration of several entities – diagnostic laboratories and companies with appropriate ELISAs in particular.

Broader Impacts

The importance of this research is (1) the creation of a novel technique that provides a noninvasive alternative to current health sampling methods, which can then (2) be employed in investigating how incessant exposure to stress stimuli may impact population health in the future. Understanding occurrence of physiologic issues augmenting immunosuppression could be crucial in constructing successful and effective conservation strategies. If we are to obtain accurate data

relative to stress-induced immunosuppression, it is imperative that noninvasive techniques be created and employed.

REFERENCES

Adamo, Shelley A., Ilya Kovalko, and Brianna Mosher. (2013). The behavioural effects of predator-induced stress responses in the cricket (*Gryllus texensis*): the upside of the stress response. *The Journal of experimental biology* 216.24: 4608-4614.

Agarwal, Ashok, *et al.,* "Effect of oxidative stress on male reproduction." *The world journal of men's health* 32.1 (2014): 1-17.

Aguirre, A. A., Ostfeld, R., & Daszak, P. (2012). *New directions in conservation medicine: applied cases of ecological health*. OUP USA.

Aich, P., Potter, A. A., & Griebel, P. J. (2009). Modern approaches to understanding stress and disease susceptibility: A review with special emphasis on respiratory disease. *International journal of general medicine*, 2, 19.

Buchanan, K. L. (2000). Stress and the evolution of condition-dependent signals. *Trends in Ecology & Evolution*, 15(4), 156-160.

Busch, D. Shallin, and Lisa S. Hayward. (2009) Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables. *Biological Conservation* 142.12: 2844-2853.

Caro, T. M., and C. J. Stoner. (2003). The potential for interspecific competition among African carnivores. *Biological Conservation* 110.1: 67-75.

Caro, T. M., *et al.,* (1987). Health of adult free-living cheetahs. *Journal of Zoology* 212.4: 573-584.

Chiappin, S., Antonelli, G., Gatti, R., & Elio, F. (2007). Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clinica Chimica Acta*, *383*(1), 30-40.

Chrousos, George, M.D. (2013). *The Stress Response and its Implications for Pituitary Tumors*. Pituitary Network Association. Pituitary Network Association. Web.
<https://pituitary.org/medical-resources/pavilions/women-s-health/women-s-health-archive/the-stress-response-and-its-implications-for-pituitary-tumors>.

Creel, Scott, and Nancy Marusha Creel. (1996). Limitation of African wild dogs by competition with larger carnivores. *Conservation Biology* 10.2 (1996): 526-538.

Creel, S., Christianson, D., & Schuette, P. (2013). Glucocorticoid stress responses of lions in relationship to group composition, human land use, and proximity to people. *Conservation Physiology*, *1*(1), cot021.

Darnell, A. M., Graf, J. A., Somers, M. J., Slotow, R., & Gunther, M. S. (2014). Space use of African wild dogs in relation to other large carnivores. *PloS one*, 9(6), e98846.

Dohms, J. E., & Metz, A. (1991). Stress—mechanisms of immunosuppression. *Veterinary Immunology and Immunopathology*, 30(1), 89-109.

Drewe, J. A., *et al.,* (2009). Pathology of *Mycobacterium bovis* infection in wild meerkats (*Suricata suricatta*). *Journal of comparative pathology* 140.1: 12-24.

Drewe, Julian A. (2009). Who infects whom? Social networks and tuberculosis transmission in wild meerkats. *Proceedings of the Royal Society of London B: Biological Sciences*: rspb20091775.

East, Marion L., *et al.* (2013). Factors influencing *Dipylidium* sp. infection in a free-ranging social carnivore, the spotted hyaena (*Crocuta crocuta*). *International Journal for Parasitology: Parasites and Wildlife* 2 (2013): 257-265.

eBioscience. (2015). *Introduction to Immunoglobulins*. Retrieved March 17, 2016, from affymetrix website:
http://www.ebioscience.com/knowledge-center/antigen/immunoglobulin.htm

French, M. (Ed.). (2012). *Immunoglobulins in health and disease* (Vol. 1). Springer Science & Business Media.

Friard, O. and Gamba, M. (2016), BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol Evol, 7: 1325–1330.

Ghoddousi, A., Hamidi, A. K., Ghadirian, T., Ashayeri, D., Hamzehpour, M., Moshiri, H., ... & Julayi, L. (2008). Territorial marking by the Persian Leopard (*Panthera pardus saxicolor*) in Bamu National Park, Iran. *Zoology in the Middle East*, 44(1), 101-103.

Glaser, R., Rice, J., Sheridan, J., Fertel, R., Stout, J., Speicher, C., & Kiecolt-Glaser, J. (1987). Stress-related immune suppression: Health implications. *Brain, behavior, and immunity*, 1(1), 7-20.

Goller, Katja Verena. *Pathogens in free-ranging African carnivores*. Diss. Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I, 2011.

Gorman, M. L., & Trowbridge, B. J. (1989). *The role of odor in the social lives of carnivores*. In Carnivore behavior, ecology, and evolution (pp. 57-88). Springer, Boston, MA.

Hansen, Fawne. (2014) *How Does Stress Affect Your Immune System?* The Adrenal Fatigue Solution. PERFECT HEALTH, 20 July 2014. Web. http://adrenalfatiguesolution.com/stress-immune-system/.

Hayward, Matt W., and Gina J. Hayward. (2007). Activity patterns of reintroduced lion (*Panthera leo*) and spotted hyaena (*Crocuta crocuta*) in the Addo Elephant National Park, South Africa." *African journal of ecology* 45.2: 135-141.

Hill, J. O., Pavlik, E. J., Smith III, G. L., Burghardt, G. M., & Coulson, P. B. (1976). Species-characteristic responses to catnip by undomesticated felids. *Journal of Chemical Ecology*, *2*(2), 239-253.

Hoover, E. A., and James I. Mullins. (1991). Feline leukemia virus infection and diseases. *Journal of the American Veterinary Medical Association* 199.10: 1287-1297.

Joubert, Dereck (1992). *Eternal Enemies: Lions and Hyenas*. Screenplay by Dereck Joubert. National Geographic Society. Film.

Laudenslager, M. L., Bettinger, T., & Sackett, G. P. (2006). Saliva as a medium for assessing cortisol and other compounds in nonhuman primates: collection, assay, and examples. In *Nursery rearing of nonhuman primates in the 21st century* (pp. 403-427). *Springer US*

Laurenson, M. Karen. (1995). *Implications of high offspring mortality for cheetah population dynamics*. Serengeti II: dynamics, management and conservation of an ecosystem: 385-399.

Marnewick, K. A., Bothma, J. D. P., & Verdoorn, G. H. (2006). Using camera-trapping to investigate the use of a tree as a scent-marking post by cheetahs in the Thabazimbi district. *South African Journal of Wildlife Research*-24-month delayed open access, 36(2), 139-145.

Martin, L. B., Andreassi, E., Watson, W. & Coon, C. (2011) Stress and Animal Health: Physiological Mechanisms and Ecological Consequences. *Nature Education Knowledge* 3(6):11

Mills MGL. (1990). *Kalahari Hyaenas: The Behavioural Ecology of Two Species*. Unwin Hyman, London.

Mills, M. G. L. (2005). Large carnivores and biodiversity in African savanna ecosystems. *Large carnivores and the conservation of biodiversity*, 208-229.

Munson, Linda, *et al.,* (2004). Serosurvey of viral infections in free-ranging Namibian cheetahs (*Acinonyx jubatus*). *Journal of Wildlife Diseases* 40.1: 23-31.

Munson, L., Terio, K. A., Worley, M., Jago, M., Bagot-Smith, A., & Marker, L. (2005). Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. *Journal of wildlife diseases*, 41(3), 542-548.

M. W. Hayward, and G. Kerley. (2005). Prey preferences of the lion *(Panthera leo)*, *Journal of Zoology* 267 (issue 3): 309–322

Nat Geo Wild (2015) *Hyenas and Lions Face-Off*. Nat Geo Wild. National Geographic Channel, 1996-2015. Web. 8 Dec. 2015.
<http://channel.nationalgeographic.com/wild/built-for-the-kill/videos/hyenas-and-lions-face-off/>.

Pageat, P., & Gaultier, E. (2003). Current research in canine and feline pheromones. *Veterinary Clinics: Small Animal Practice*, 33(2), 187-211.

Palomares, Francisco, and Tim M. Caro. (1999). Interspecific killing among mammalian carnivores. *The American Naturalist* 153.5: 492-508.

Råberg, L., Grahn, M., Hasselquist, D., & Svensson, E. (1998). On the adaptive significance of stress-induced immunosuppression. *Proceedings of the Royal Society of London B: Biological Sciences,* 265(1406), 1637-1641.

Rabinowitz, Alan. (2010). A New Strategy for Saving the World's Wild Big Cats. Interview by E360. *Yale Environment 360* 17 Feb. 2010: Print.

Randall, M. (2012, February 3). The Physiology of Stress: Cortisol and the Hypothalamic-Pituitary-Adrenal Axis. Retrieved from *Dartmouth Undergraduate Journal of Science* website: http://dujs.dartmouth.edu/2011/02/the-physiology-of-stress-cortisol-and-the-hypothalamic-pituitary-adrenal-axis/#.WAWNbOgrKhc

Reiger, I. (1979). Scent rubbing in carnivores. *Carnivore*, 2(1/2), 17-25.

Ritchie, Euan G., and Christopher N. Johnson. (2009). Predator interactions, mesopredator release and biodiversity conservation. *Ecology letters* 12.9: 982-998.

Roelke, M. E., Brown, M. A., Troyer, J. L., Winterbach, H., Winterbach, C., Hemson, G., & Alexander, K. A. (2009). Pathological manifestations of feline immunodeficiency virus (FIV) infection in wild African lions. *Virology*, *390* (1), 1-12.

Roth, J. A. (1985). Cortisol as mediator of stress-associated immunosuppression in cattle. In *Animal stress* (pp. 225-243). Springer New York.

Salimetrics, L. L. C., & SalivaBio, L. L. C. (2011). *Saliva collection and handling advice*. Available at www. salimetrics.com.

Salimetrics, L. L. C., & SalivaBio, L. L. C. (2015). *SALIVARY SECETORY IgA INDIRECT ENZYME IMMUNOASSAY KIT*. Available at www. salimetrics.com. https://www.salimetrics.com/assets/-documents/1-1602n.pdf

Sapolsky, Robert M. (2002). Endocrinology of the Stress Response. *Behavioral Endocrinology*. By Jill B. Becker. MIT Press. 409-49. Print.

Sapolsky, Robert M., L. Michael Romero, and Allan U. Munck. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine reviews* 21.1: 55-89.

Soini, H. A., Linville, S. U., Wiesler, D., Posto, A. L., Williams, D. R., & Novotny, M. V. (2012). Investigation of scents on cheeks and foreheads of large felines in connection to the facial marking behavior. *Journal of chemical ecology*, 38(2), 145-156.

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. *Journal of Wildlife Diseases*, 40(2), 259-266.

Trinkel, Martina, and Gerald Kastberger. (2005). Competitive interactions between spotted hyenas and lions in the Etosha National Park, Namibia. *African Journal of Ecology* 43.3: 220-224.

Valeix, M., Hemson, G., Loveridge, A. J., Mills, G., & Macdonald, D. W. (2012). Behavioural adjustments of a large carnivore to access secondary prey in a human-dominated landscape. *Journal of Applied Ecology*, 49(1), 73-81.

Van Meter, P. E., French, J. A., Dloniak, S. M., & Watts, H. E. (2009). Fecal glucocorticoids reflect socio-ecological and anthropogenic stressors in the lives of wild spotted hyenas. *Hormones and Behavior*, *55*, 329-337.

Vaz, J., Narayan, E. J., Kumar, R. D., Thenmozhi, K., Thiyagesan, K., & Baskaran, N. (2017). Prevalence and determinants of stereotypic behaviours and physiological stress among tigers and leopards in Indian zoos. *PloS one*, 12(4), e0174711.

Wells, A., Terio, K. A., Ziccardi, M. H., & Munson, L. (2004). The stress response to environmental change in captive cheetahs (Acinonyx jubatus). *Journal of Zoo and Wildlife Medicine*, 35(1), 8-14.

Williams, Brianna M., *et al.,* (2014). Prevalence and diversity of Babesia, Hepatozoon, Ehrlichia, and Bartonella in wild and domestic carnivores from Zambia, Africa. *Parasitology research* 113.3: 911-918.

Woodroffe R, Ginsberg JR. (2005). King of the beasts? Evidence for guild redundancy among large mammalian carnivores. See Ray *et al.,* 2005, pp. 154-175

Young, K. M., *et al.,* (2004). Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. *General and comparative endocrinology* 137.2: 148-165.

APPENDICES

APPENDIX 1. COLLECTION PROTOCOLS

Collecting saliva

1. **Remove** grate from top of device. This can be done either by 1) unfastening one of the rounded bolts and pivoting the grate to reveal the bucket opening, or 2) unfastening both bolts and removing the grate.
2. **Collect** deposited saliva from basin using a 1 to 5 mL syringe, depending on the amount of saliva visible. Cap with black cap to prevent loss of contents.
3. **Label** each syringe using a cryolabel, recoding the following information using a sharpie:
    - Sample number (refer to number 5)
    - Date of Collection (mnth/day/yr)
    - Gender (M / F)
4. **Record**, on the Excel sheet provided, the following information for each sample:
    - Sample number (matching that on syringe)
    - Date of Collection
    - Collection time
    - Gender of lion depositing sample (M / F)
    - Approximate amnt of saliva collected (mL)
    - Internal basin temperature @ time of collection (according to reading on digital thermometer)
    - Ambient temperature of enclosure
    - Zoo activity – in general (busy, moderate, quiet)
    - Outdoor weather
5. Sample numbering system: label for each sample will begin with SPZ. This provides information on the location of the sample (Seneca Park Zoo). This will then be followed with either M or F, depending on which lion deposited the sample. If there are only two lions at this zoo, just M or F is sufficient. If there are several individuals of the same gender, then a number would follow assigned to each individual. Following this code (either SPZM or SPZF), put 00 followed by whatever sample number this is collected for that individual. If this is the sixth sample deposited by the male lion, then the sample number/label would look as follows: SPZM006 if only one male, or SPZM2_006 if it was male number two of several males.
6. **Wipe** base and walls of basin dry with paper towel between each collection to prevent mixing of saliva or contamination. Do this for the grate as well, making sure to wipe each rod individually and thoroughly.

Sample Storage

1. Immediately following collection, samples/syringes should be placed in zoo freezer until they can be transported either by zoo veterinarian or by myself to Cornell University.

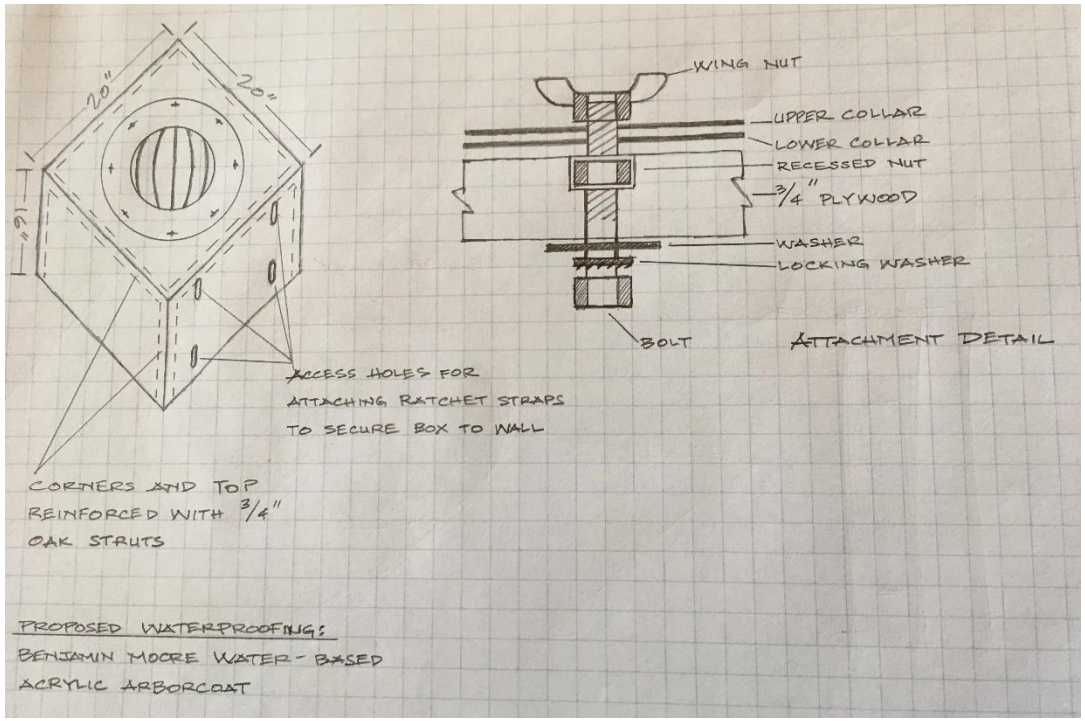
Device Care

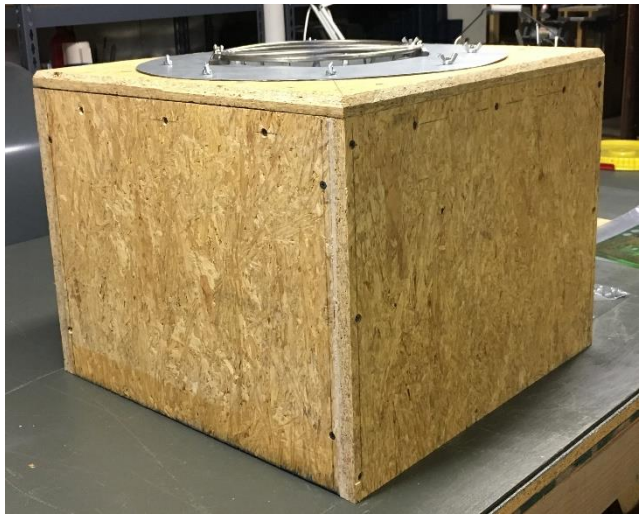
1. **Replace** ice every six to eight hours during testing periods (times the device is out for saliva collection). When saliva is not being collected, or lions are absent from enclosure, check ice and replace if necessary. Old ice that has melted can be discarded (remove both buckets to access the outer bucket housing the ice) and replaced with new ice. Scent wicks should be replaced biweekly.
2. **Wipe** base and walls of basin dry with paper towel between each collection to prevent mixing of saliva or contamination. Do this for the grate as well, making sure to wipe each rod individually and thoroughly.

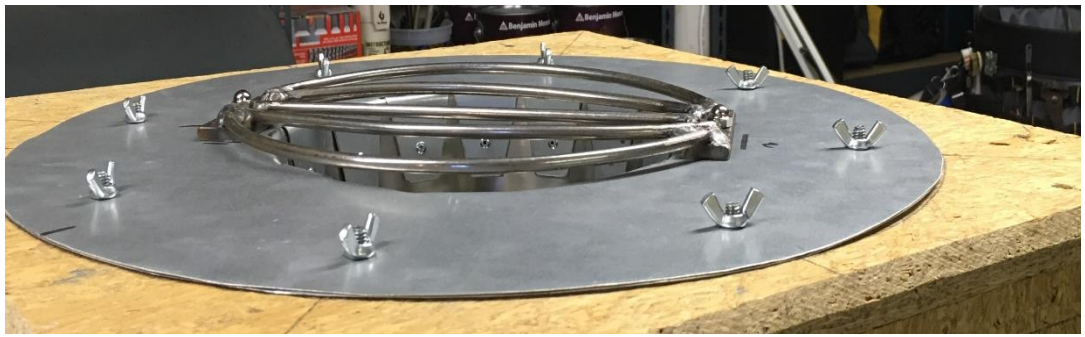
Camera Care

1. Batteries and SD cards should be changed on a biweekly basis. At each exchange, please verify that cameras are set to the following settings: 30 second video; 30 seconds between each recording.

Appendix 2. Device Design and Outer Shell







Upper left: building designs for wooden holding container with attachment detail for bolt and wing nut or acorn nut. Upper right: rectangular wooden box with collection device attached internally. Both buckets can be removed and replaced from the outside. Lower left: top of collection device bolted into wooden box. Locking washer holds bolts in place to prevent slipping/dislodging when either bucket (inner or outer) are removed. Lower right: back wall of wooden box with four slits for threading industrial grade ratchet strap. Strap will secure this back-panel flush to grated wall of enclosure, while keeping strap outside of enclosure out of reach of lions. (Sgambelluri, 2017)

Appendix 3. Metrics of Success

Lists of *(left)* measures to determine success of device and *(right)* possible factors that may influence success. (Sgambelluri, 2017)

Metrics	Influential Factors
Approach (Y / N)	Time of day
Time to approach (sec.)	Time since last ate
Amnt. of saliva deposited (mL or µL)	Fluid intake / hydration
Elicitation of licking (Y / N)	Gender
Time before ice melt (sec.)	Age
Sample temperature (@ collection) (°F)	Activity level
Ambient temperature (°F)	Tourist activity
# samples collected per trial	Attractant used and concentration
Damage occurrence / durability	Individual behavior

Antioch University New England

Environmental Studies Department

Library Permission Slip

I give my permission for one copy of this thesis to be permanently housed in the Antioch New England Graduate School Library and to circulate to patrons as a part of the library collection. I understand that the thesis circulation process will be subject to all the rules and regulations of the Antioch New England Graduate School Library.

Title of Thesis:

Noninvasive Collection of Saliva in *Panthera leo*

Creation and Validation of a Novel Technique for Health Assessment in Captive African Lions (*Panthera leo*)

Student Signature: (on file)

Student Name (Print): Elizabeth Sgambelluri

Date: 5/8/18

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/720,961	08/22/2018		140	Sgambelluri 0100		

CONFIRMATION NO. 2978

27981
WENDY W. KOBA
PO BOX 556
SPRINGTOWN, PA 18081

FILING RECEIPT

OC000000101907265

Date Mailed: 08/29/2018

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Elizabeth Sgambelluri, Kintnersville, PA;

Applicant(s)

Elizabeth Sgambelluri, Kintnersville, PA;

Power of Attorney:
Wendy Koba--30509

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/28/2018
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/720,961**
Projected Publication Date: None, application is not eligible for pre-grant publication
Non-Publication Request: No
Early Publication Request: No
**** SMALL ENTITY ****
Title

Non-Invasive Device For Collecting Wildlife Saliva

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

NON-INVASIVE DEVICE FOR COLLECTING WILDLIFE SALIVA

Technical Field

The present invention relates to a device for collecting saliva samples from wildlife and, more particularly, to a non-invasive device that allows for the passive collection of the sample without the need for an individual to directly engage with the animal.

Background of the Invention

Faunal species are exposed to various sources of stress, whether from environmental change, competition, or habitat degradation. These stressors influence physiological systems largely in terms of behavioral modifications. Various personnel working in the field are interested in evaluating the immunological impacts of stress on these species, particularly if stressors are burdened on a chronic basis. However, there is known to be a deficit in regard to non-invasive techniques for performing wildlife health assessment. Currently, methods for collecting bodily fluids to be used in health assessment are nonexistent. The absence of non-invasive techniques for saliva collection (as well as urine collection) is likely attributed to the fact that it is difficult, if not impossible, to predict where these bodily fluids will be secreted by the individual animal of interest. Furthermore, there are currently no methods existent in the literature that enable a controlled collection of bodily fluids for evaluation, and none that do not require the presence of a researcher, nor physical interaction with the animal being studied.

Detailed Description of the Invention

The present invention addresses the needs remaining in the prior art and relates to a device for collecting saliva samples from wildlife and, more particularly, to a non-invasive device that allows for the passive collection of the sample without the need for an individual to directly engage with the animal.

In exemplary embodiments, the inventive device takes the form of a "bucket-in-a-bucket" (BiaB) that is placed in the habitat of the animal under evaluation. The BiaB is configured as discussed below to encourage the animal to investigate and eventually lick the BiaB, allowing the saliva to be captured in the smaller bucket nested in the larger bucket. The smaller bucket is configured to be removable, allowing a researcher to come at a time when the animal is not in the immediate vicinity and remove the smaller bucket, transfer the collected saliva to another container, and replace the smaller bucket in place for its next use.

FIG. 1 illustrates an exemplary pair of buckets that may be used to form a non-invasive saliva collection device 10 configured in accordance with the present invention. FIG. 2 is an isometric view of device 10 as placed within a larger container 30. FIG.3 is a photograph of an exemplary configuration as constructed the diagram of FIG. 2. Referring back to FIG. 1, device 10 comprises a larger, outer bucket 12. In use, particular "attractants" (e.g., sponges soaked in an attractant liquid) are preferably placed within larger, outer bucket 12 to encourage an animal to investigate the configuration and trigger the licking reaction. The soaked sponges may be disposed on a separate, rimmed support element (not shown) that keeps the attractant from touching the bottom surface of larger, outer bucket 12. Additionally, it is preferably to add ice (or another type of coolant) to large, outer bucket 12 to keep the temperature of the collected saliva below that where bacteria may grow.

Smaller, inner bucket 14 serves as the collection chamber for drooled saliva. In use, smaller, inner bucket 14 is disposed within a central opening 16 of outer, larger bucket 12. Smaller, inner bucket 14 includes an extended flange 18 around its top perimeter, where extended flange 18 mates with a lower flange 20 formed around the perimeter of outer, larger bucket 12. In this manner, smaller, inner bucket 14 is held suspended above outer, larger bucket 12. Various types of connection arrangements (such as screws, locking mechanisms, and the like) may be used to provide the removable attachment between extended flange 18 and lower flange 20.

As shown in both FIGs. 1 and 2, smaller, inner bucket 14 further comprises a plurality of rods 22 that extend across its central opening 24. Rods 22 encourage the licking reaction of the animal, thereby facilitating the production of saliva that is captured within smaller bucket 14. Rods 22 also function to prevent the animal from attempting to insert its head into smaller bucket 14 and becoming injured (or breaking the device).

In preferred embodiments of the present invention, sidewalls 26 of smaller bucket 14 are formed to include several holes (or slots) that enable the scent from the attractant material (if used) to efficiently waft through and out of device 10, encouraging the animal's interaction. When device is disposed within outer box 30, additional holes/slots 32 may be formed around the perimeter of box 30 to further encourage the movement of the scent into the environment.

Buckets 12 and 14 may be formed of any appropriate material, stainless steel being one choice. Outer box 30 may be formed as a reinforced wooden housing, which itself may be attached to an enclosure wall in the animal's habitat.