"ESTABLISHMENT AND APPLICATION OF A FECAL GLUCOCORTICOID METABOLITE ASSAY FOR 4.1 MAGELLANIC PENGUINS (*SPHENISCUS MAGELLANICUS*) TO BE USED FOR THE ASSESSMENT OF ADRENAL ACTIVITY IN CONJUNCTION WITH BEHAVIORAL OBSERVATIONS TO UNDERSTAND THE POTENTIAL IMPACT ASSOCIATED WITH VARIABLES OF BEHIND THE SCENES TOURS AT A ZOOLOGICAL FACILITY"

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Arts
Interdisciplinary Studies

Committee:

Director

Program Director

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Date: December 4, 2014

Fall Semester 2014
George Mason University
Fairfax, VA

"Establishment and Application of a Fecal Glucocorticoid Metabolite Assay for 4.1 Magellanic Penguins (Spheniscus Magellanicus) for the Assessment of Adrenal Activity in Conjunction with Behavioral Observations to Understand the Potential Impact Associated with Variables of Behind the Scenes Tours at a Zoological Facility"

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DEDICATION

This is dedicated to my loving husband, whose support, encouragement and understanding were essential to my ability to complete this ambition and to continue to follow my passion. Until our retirement days spent in Craftmatic adjustable beds I love every day living alongside you, building our family, working for our individual purpose and becoming better people.

ACKNOWLEDGEMENTS

I would like to thank the many friends, relatives, and supporters who have made this happen. My loving husband, Dr. David DeNardo, whose scientific mind and analytical inspiration assisted me in my research. My son, Glenn Harrison, who brings me the peace, joy, and balance needed to work full-time, complete a master's degree and have a newborn infant. Drs. Dorsey & Kozlowski for their guidance, encouragement, patience and invaluable help. Each of the members of my committee for all they have taught me. Dr. Asa for her interest in and support of this project and for putting me in contact with the right people to make it a successful reality. Eli Basker, Amanda Murti, Karen Bauman, members of the St. Louis Zoo Research Department, who helped me with everything from ordering supplies to scheduling observers to running assays. All of the Research Department volunteers who spent their valuable time collecting behavior data for this project. Mike Macek & Anne Tieber for their knowledge, encouragement, support and skillful management of my enthusiasm. American Association of Zookeepers for financial support awarded by their Research Grant. Finally, thanks go out to the Saint Louis Zoo for providing an amazing work environment full of diverse, skillful, intelligent people who work together towards an ambitious, yet critical, mission pertinent to all the animals that are fortunate enough to call it home.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Adrenocorticotropic Hormone ..... ACTH
Decibels ..... dB
Glucocorticoid Metabolites ..... GCM
F-Ratio ..... F
Human Animal Relationship ..... HAR
Mean ..... $\bar{x}$
Microliters ..... µL
Nanograms ..... ng
Radioimmunoassay ..... RIA
Revolutions per Minute ..... rpm
Significance Value ..... p

ABSTRACT

"ESTABLISHMENT AND APPLICATION OF A FECAL GLUCOCORTICOID METABOLITE ASSAY FOR 4.1 MAGELLANIC PENGUINS (SPHENISCUS MAGELLANICUS) FOR THE ASSESSMENT OF ADRENAL ACTIVITY IN CONJUNCTION WITH BEHAVIORAL OBSERVATIONS TO UNDERSTAND THE POTENTIAL IMPACT ASSOCIATED WITH VARIABLES OF BEHIND THE SCENES TOURS AT A ZOOLOGICAL FACILITY"

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An excrement glucocorticoid metabolite (GCM) assay was established for penguins and was applied as a tool, in conjunction with behavioral observations, to evaluate individual birds responses to participation in a behind the scenes tour program involving potential tactile interactions with zoo guests. An adrenocorticotrophic hormone (ACTH) challenge was used to validate a corticosterone assay for measuring GCM in Magellanic penguin excrement, as well as to develop individual GCM profiles consisting of maximum values, baseline, and percent change for each bird. The GCM assay was used in conjunction with behavioral and environmental data collected during behind the scenes tours as a means to quantify potential stress. Excrement samples and tour observations were collected daily from each bird for one week during which it participated, with a second

bird, in tours with zoo guests twice daily. Excrement samples were also collected from each bird daily during one week in which it participated in no tours. Results indicate that both endocrine and behavioral responses to tours, and associated tour environment components, are variable among the individual birds. While three of the birds did not have significant changes in GCM values ($F_{1,48} = 2.05$, $p = 0.16$) on days they participated in tours, two birds did show increased GCM levels ($F_{1,35} = 4.60$, $p = 0.04$) on days that they participated in tours. These same two birds also showed a lower maximum response to ACTH challenge ($\bar{x}$ = 1,205ng/g compared to $\bar{x}$ =1,750ng/g), lower percent change between baseline GCM and ACTH maximum response ($\bar{x}$ = 1,186% compared to $\bar{x}$ = 5,851%), and had elevated baseline GCM levels ($\bar{x}$ = 72.12ng/g compared to $\bar{x}$ =33.77ng/g) relevant to the other three birds. These results may suggest a down-regulation in the ability of the HPA axis of these individual birds to respond to stress. This could be a result of chronic intermittent stress as part of their subjective experience and resulting affective states when participating in tours. These finding are similar to previous studies with other species that have found associations between chronic stress and compromised adrenal function. Behavioral data analysis showed increases in alternate head turn (AHT) behaviors correlate with lower GCM ($F_{4,41} = 5.53$, $p = 0.02$) and that AHT is positively correlated with reproductive (R), vocalization (V) and preening (P) behaviors. The lower GCM values may signify that higher AHT behavior rates imply a bird is comfortable within the tour environment and the association with R, V & P behaviors indicate the rates of these behavior may also be reflective of some level of comfort within the tour setting. Nip/bite (NB) behavior rates were positively

correlated with all categories involving herding, an involuntary situation where staff manipulates a bird's interaction with guests, and negatively correlated with voluntary approach of guests behaviors. This may indicate the infraction on a bird's opportunity for choice, resulting from herding, may elicit behaviors undesirable in tour scenarios. Other behavioral correlations were specific to individual birds and pairing of birds further implicating the role of individual personality. Study results indicate that some individual animals maybe more suited to the role of ambassador animals within the zoo setting, and individual stress responses should be considered when choosing animals for guest interactions.

INTRODUCTION

An increasingly integral component of zoological mission statements is to connect people to wildlife and animals in a meaningful way, which ideally results in conservation-orientated behaviors by zoo guests. Because positive human-animal contact influences future attitudes towards animals [Kidd and Kidd 1997; Kidd, Kidd Zaslof, 1995], animal encounter experiences and behind-the-scenes tour programs are often utilized as a means of achieving that mission component, as well as generating additional revenue. These programs frequently involve non-domesticated species, which have not undergone selective breeding of temperament traits for accepting human contact or to ease handling [Maciejowski and Zieba, 1982]. While some interactions with familiar humans may have a positive welfare effect, the effects of close interactions with unfamiliar visitors in non-husbandry related scenarios are less understood. Positive interactions with human caretakers have been shown to reduce stress responses during routine husbandry in some species [Baker, 2004; Carlstead et al., 1993; Mellen, 1991; Waitt et al., 2002]. Alternatively, persistent fear of human presence can be a source of psychological stress [Shephardson et al., 2004] as well as physiological stress [Hogan et al., 2011]. It is important for zoological organizations utilizing ambassador animal encounters to understand the impact these interactions have on the individual animals and establish protocols that minimize the opportunity for undesired consequences, such as

additional stress or aberrant behaviors, while also maximizing guest impact. By better understanding an ambassador animals reactions to guest encounters, zoos will be better prepared to handle possible ethical challenges to those programs and to make appropriate adjustments when needed. Zoos accredited by the Association of Zoos and Aquariums (AZA) are subject to the organization's program animal policy as well as several accreditation standards, board-approved policies, and recommendations to assure that the welfare, health and safety needs of the animals, handlers, and public are met and to facilitate the receipt of conservation messages by their audience. The AZA Conservation Education Committee's program animal position statement outlines research supporting the impact of program animals as educational tools to achieve goals conveying important messages about conservation and wildlife issues to zoo visitors.

Objective

The goal of this project was to gain an understanding of how individual birds experience and behave during behind the scenes tour activities, to determine if those observations suggest an increase in stress, and, if so, to use that information to find solutions that mitigate those stressors with the ultimate goal of optimizing individual animal welfare. This goal was accomplished through two primary objectives: (1) develop a protocol that would measure excrement glucocorticoid metabolites (GCM) in order to quantify corticosterone production in 4.1 Magellanic Penguins, and (2) to use that protocol in conjunction with behavioral observations to evaluate the impact of a behind-the-scenes tour program involving tactile interaction with guests.

During the first objective a GCM assay was validated and a range of GCM values including rate of response over 24 hours, maximum level of response, rate to return to baseline and time lag between a stressor and detection of elevated glucocorticoids in excrement material, was established for each individual bird via an adrenocorticotrophic hormone (ACTH) challenge. By inducing the stress response through an ACTH challenge [Norris, 1996; Wasser et al., 2000] and analyzing samples pre- and post-injection, we were be able to confirm the assay detects the appropriate corticosterone metabolites in penguin feces and is sensitive enough to detect biologically significant changes in corticosterone levels. These data also provided a reference for interpreting corticosterone levels during the tour data collection phase of the project.

The second objective of this study was to use the validated glucocorticoid assay in combination with behavior observations and tour surveys to determine whether tour group composition and/or social dynamics among birds contribute to, or mitigate stress during behind-the-scenes tours. Tour variables were evaluated in conjunction with behavioral observations and hormone results to assess whether any of the tour conditions were correlated with increased GCM production in these birds. This information may be applied to animal management practices by amending tour protocols to mitigate any stressors and potentially optimize the welfare of the birds. These data will also allow for the long-term noninvasive evaluation of multiple husbandry parameters, which could be studied to optimize welfare. Having a validated GCM assay will allow this institution, and others, to conduct possible future studies looking at the impact of variables within

behavioral husbandry program protocols, habitation routines, introductions of animals, shipments and reproduction management for this species at zoological institutions.

Hypotheses

Tour conditions involving more experienced birds were hypothesized to result in fewer defensive behaviors and lower levels of glucocorticoid metabolites in the excrement of both of the participating birds. Tours consisting of larger groups of guests, louder guests and more children were hypothesized to correlate with higher levels of glucocorticoid metabolites from the participating birds and more defensive behaviors exhibited by the birds.

To test these hypotheses GCM values were compared between samples collected on tour and non-tour days, between individual bird sample values, between tours associated with different rates of specific behaviors, between tours with specific partner birds, and between tours with different environmental component values including number of guests, volume, and number of children. Rates of behaviors were also used to test the hypotheses by comparing them to differences in aspects of the tour environment, differences in individual bird's rates of behaviors, and comparing them to rates of other behaviors.

BACKGROUND

Stress and Adrenal Activity

While the presence or absence of stress can provide meaningful information regarding the well-being of an animal, it has been scientifically challenging to define a reliable measurement of stress because the concept is often applied to many different phenomena including physical, social or psychological stressors [Moberg, 1985; Terlouw et al., 1997]. In 1936 Hans Selye described the "General Adaptation Syndrome," which has become known as the stress response, and elucidated the role of the hypothalamus, pituitary gland, and adrenocortical tissue (HPA axis) in this response. During the cascade of reactions involved in the stress response the hypothalamus secretes corticotrophin-releasing hormone (CRH), which stimulates the pituitary glad to secrete adrenocorticotrophic hormone (ACTH), which causes the adrenal cortex to release glucocorticoids (such as cortisol in most mammals and corticosterone in birds, reptiles and rodents). Glucocorticoids are important to many normal biological processes essential for survival, normal short-term elevations may occur after a meal or activity and can be affected by an animal's sex, reproductive state, age, or rank [Goymann, 2005; Lane, 2006], as well as seasonal rhythms, temperature, humidity, and other environmental factors [Millspaugh and Washburn, 2004; Mormede et al., 2007]. Additionally acute stress responses can be considered adaptive but chronic stress

responses are associated with long-term health implications [Broom and Johnson, 1993; Sapolsky, 1996; Sapolsky et al. 1990].

Corticosteroid measurements are valuable in stress research because cortisol shows a graduated response depending on the severity of the stressor, which allows a means to assess events on a scale of averseness [Terlouw et al., 1997]. While elevated corticosterone levels are reflective of HPA axis activity, this alone does not automatically equate to a state of distress [Moberg, 2000; Romero, 2004] and cannot be a conclusive reflection of a possible infringement on animal welfare. For example, a study looking at cortisol secretion in stallions found similar values for horses that were restrained, exercised, or permitted to mate with a mare [Colborn et al., 1991]; however, in regards to the stallions welfare it would be difficult to equate the impacts of being restrained with mating [Moberg, 2000]. Additionally, decreased fecal glucocorticosteriod values are not an automatic indicator of health or lack of stress. A study looking at skin and oral lesions in black rhinoceros, a disease previously thought to be stress induced, found reduced adrenal activity among individuals with lesions [Dorsey et al., 2010]. Although stress has become associated with negative connotations not all stress, and associated increases in corticosteroids, reflect a negative impact on the welfare of an animal. Moberg (1985) states that "stress becomes a threat only when the stress response is of such a magnitude that.... it is sufficient to endanger the general well being of the animal". Taken together these data imply that corticosteroid measurements alone are not sufficient when attempting to quantify animal welfare impacts of stress.

While some studies have demonstrated habituation via decreased responses of animals to frequent human contact, the appearance of external calm may not be reflective of internal physiological changes [Hogan et al., 2011; Wilson et al., 1991]. For example regular handling of wombats lowered reactivity and avoidance of human handlers but did not reduce the increased adrenal activity in response to handling [Hogan et al., 2011]. Additionally stressors that are predictable and uniform are more likely to result in habituation when compared to random and intermittent stressors [Marti and Armario, 1997; Pitman et al., 1988]. Chronic stress can also result in the external appearance of habituation while actually be being associated with elevated levels of corticosterone [Hogan et al., 2011; Romero, 2004], which can have a negative influence on disease resistance & reproduction [Angelier et al., 2010; Wingfiled et al., 1998]. In addition to the HPA axis response to stress, animals may also show changes in other neuroendocrine measurements, reactions by the autonomic nervous system, changes in physiological measures related to reproductive qualities, and behavioral responses. Terlouw et al., (1997), recommends an integrated approach that considers both individual behavioral and physiological measurements for a more accurate interpretation of stress.

Behaviors of specific relevance to animal stress are those that are important to the biological functioning or communication of a species, those that are indicators of disturbance, pain, or illness, and those that are signs of behavioral displacement or suppression [Rushen, 2000]. In an overview of studies looking at behavioral indicators of stress in zoo animals Hosey et al., 2010, noted that abnormal behaviors, increased intra-specific and inter-specific aggression, increased activity, and decreased affiliative

behaviors, including grooming have been found to be associated with the stress response. In situ studies of penguins often look at alert behaviors (such as facing the direction of a perceived threat), posture, activity levels (standing or moving), nest abandonment, vocalizations, aggression/biting, and alternate head turns when evaluating the stress response of human disturbance [Yorio and Boersma, 1992; Fowler, 1999]. Ranges and types of behavioral diversity, relative frequencies of behaviors, temporal patterns and function/purpose of behaviors are all important considerations when studying the welfare impacts of animal behavior. Interpretation of behavioral responses to stress is very complex and requires an understanding of the causal mechanisms underlying a behavior [Rushen, 2000]. Studies using glucocorticoid levels for welfare assessments should include complementary indicators of animal welfare, such as behavioral observations, reproductive measurements, immunological measurements, and other endocrinology parameters [Lane, 2006; Millspaugh and Washburn, 2004].

Magellanic Penguins, Adrenal Activity and Behavior

Studies of the behavioral and hormonal responses of wild populations of Magellanic penguins to human presence via ecotourism and scientific research have revealed various levels of physiological stress responses [Fowler, 1999; Walker, Boersma and Wingfield, 2006]. While penguins may frequently appear to be quite `tame' [Yorio and Boersma, 1992], internal stress responses, such as increased heart rate, can occur at the mere sight of humans [Culik et al., 1990]. Additionally, the type and frequency of human exposure can lead to habituation and influence the adrenal activity of penguins. For example, Fowler [1999] found that free-ranging Magellanic penguins exposed to high

levels of human visitation via tourism (subject to frequent human visits most daylight hours during the nesting season for over 20 years) did not have a significant stress response (as measured via plasma corticosterone analysis and behavioral observations) compared to birds exposed to less, but still moderate levels of human exposure (subject to human visits by researchers for ~1 hour daily). These differences in the adrenal response to human disturbance could be the consequence of habituation [Fowler, 1999] but they could also reflect a decreased capacity of the bird's adrenocortical tissue to secrete corticosterone [Walker, Boersma & Wingfield, 2006]. A 2006 study found differences in the adrenal responses of Magellanic Penguins with different historical exposures to human disturbance correlated with measurements of the physiological functioning of the adrenocortical tissue studied via plasma corticosterone collection following an ACTH challenge [Walker, Boersma & Wingfield, 2006]. Penguins in tourist areas had lower corticosterone responses to capture and restraint than birds in areas without human visitation but this difference was found to correlate with a decreased maximum response to ACTH challenge indicating a decreased capacity of the tourist site birds' adrenocortical tissue to secrete corticosterone [Walker, Boersma & Wingfield, 2006]. The health implications of this reduced adrenal activity response in penguins is not well understood as glucocorticosteriods have wide-ranging physiological impacts; For example, a lower adrenal response is thought to allow animals to avoid negative consequences of repeated elevated glucocorticosteriod levels when responding to stressors [Johnson et al., 1992; Wingfield et al., 1995] while it is also associated with an

inability to adequately access stored energy at times of need [Romero and Wikelski, 2002].

Zoos Visitors, Adrenal Activity and Behavior

There are many factors in a zoo/aquarium environment including loud sounds, aversive noises, various odors, lighting, restricted movement, reduced retreat space, forced proximity to humans and restrictions on behavioral opportunity that could act as potential stressors for animals [Morgan and Tromborg, 2006]. Reponses of zoo animals to visitors are inconsistent both within and between taxa. A number of studies indicate that the presence, and particularly the behavior, of unfamiliar people may be stressful to zoo animals [Hosey, 2008]. For example, three different species of primates increased agonistic behavior and decreased resting and grooming behaviors when zoo visitors were present but that those changes in behavior were reduced when the visitors were asked to crouch instead of stand in front of the habitat [Chamove et al., 1988]. Other studies have found that animals may not be stressed and may even be enriched by the presence of visitors [Hosey, 2008]. For example *Cacatua* species were found to have increased positive social behaviors and vocalizations [Keane, 2005] and one individual appeared to seek out opportunities for interactions with zoo guests [Nimon and Dalziel, 1992]. Much of the research looking at visitor effects on zoo animals have looked at primate behavior and most of those have revealed an increase in stress response associated behaviors [Hosey 2000; Hosey et al., 2010]. Specifically related to penguins, one study found a visitor-related increase in activity, as measured by movement throughout the habitat, with Gentoo (*Pygoscelis papua*) and Black-footed Penguins (*Spheniscus demersus*) [Warren et

al., 2002]. Another study found a decrease in resting in Black-footed Penguins in response to guest presence and density [Brooking & Price, 2004]. Physiological markers of stress have also been correlated with zoo visitors [Shephardson et al., 2004]. For example, a study looking at urinary cortisol in spider monkeys (*Atele geoffroyii rufiventris*) found a significant increase as visitor numbers to the zoo increased [Davis et al., 2005] and fecal corticoid levels in black rhinoceros were higher for animals whose enclosures had a greater degree of public exposure [Carlstead and Brown, 2005].

While these studies address the potential impact of zoo guests outside of an animal's habitat, little research has been done looking at the impact of zoo guests on animals that serve ambassador roles and participate in guest interactions outside of their housing situations or where guests are brought behind the scenes. Two recently published abstracts revealed elevated levels of fecal glucocorticoid metabolites in red-tailed hawks and armadillos that were exposed to increased levels of handling for husbandry and educational programming purposes [Baird et al., 2013; Wilder-Schook et al., 2013].

Excrement Measurements of Adrenal Activity

Corticosterone is heavily metabolized and excreted as species-specific metabolites in excrement (feces and/or urine) material. Fecal glucocorticoid assays can reliably detect endogenous changes in adrenal activity of a diverse array of species [Wasser et al., 2000] and may reflect a wide array of potential stressors [Wingfiled, 1994; Wasser et al., 1997; Wingfiled et al., 1997]. Evaluation of glucocorticoid metabolites (GCM) from excrement material is less invasive than serum [Goyman, 2005; Nilsson et al., 2008], does not typically interfere with behavior, allows multiple samples to be collected from an

individual, is collected with relative ease, and does not conflict with animal welfare [Touma and Palme, 2005]. Excrement samples represent pooled fractions of metabolites thereby providing an integrated measure of adrenal activity [Goymann et al., 1999]. Excrement GCM assays reflect a cumulative secretions and elimination of hormones over several hours [Touma and Palme, 2005], rather than a point sample with serum assays, and therefore may provide a more accurate assessment of long-term glucocorticoid levels [Harper and Austad, 2000].

However, because excrement metabolites vary among species, proper interpretation of glucocorticoid production requires knowledge of the normal range of values for each species. A pharmacological challenge with ACTH can establish whether an assay accurately reflects acute adrenal activation [Wasser et al., 2000]. ACTH challenges have been used to evaluate the adrenal function in several avian species [Goyman, 2005; Nilsson et al., 2008; Touma and Palme, 2005], and is a preferred analytical validation of fecal GCM assays because it stimulates the natural production of steroid in the glands thus generating a physiological increase of hormones to be measured [Goyman, 2005; Nilsson et al., 2008]. In order to establish if a fecal assay can accurately measure acute adrenal activation, pharmacological challenge with ACTH is used to mimic a natural adrenal response to stress causing a rapid rise in circulating glucocorticoids followed by a return to baseline [Norris, 1996; Wasser et al., 2000; Goyman, 2005; Nilsson et al., 2008]. ACTH challenges also allow researchers to use excrement samples to identify the potential magnitude and duration of adrenal response

as well as the specific lag-time between a potential stressor and maximum response [Palme et al., 1998; Nilsson, 2008].

Due to the physiology of avian species, excrement samples collected from birds typically consists of both uric acid and fecal components [Mostl et al., 2005]. Separation of these two fractions can be difficult to impossible depending on the bird species. Separation is not recommended as a more comprehensive estimate of total glucocorticoid metabolites can be obtained by analyzing both fractions together [Millspaugh and Washburn, 2004]. For these reasons samples will be referred to as excrement rather than fecal throughout this paper and GCM will be used rather than fecal GCM [Mostl et al., 2005]. Because fecal steroids have been reported to have unequal distribution within samples and the mixed nature of bird excrement, mixing samples prior to analysis to ensure sample homogeneity is very important [Millspaugh and Washburn, 2004; Mostl et al., 2005].

METHODOLOGY

Prior to starting the project all methods were approved by the Saint Louis Zoo's Institutional Animal Care and Use Committee (IACUC). In compliance with federal laws, regulations, and policy governing the use of non-human, vertebrate species for scientific research and/or instruction, the IACUC is responsible for reviewing research protocols to assure the humane treatment of vertebrate animals. This review is necessary for compliance with provisions of the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act, federal granting agencies of the PHS, and all other applicable research animal welfare laws and regulations.

Study Population

The Saint Louis Zoo houses five (4 male & 1 female) Magellanic Penguins (*Spheniscus magellanicus*) who act as ambassador birds and participate in behind the scenes tours with zoo guests on a routine basis, (see Table 1). Two of the birds (1 male & 1 female) are full siblings who hatched in May 2002 and have been participating in tours since 2004 (~10 years). The other three male birds (two of which are also full siblings) hatched in June of 2007 and have been participating in tours since 2009 (~5 years). All birds are housed together in an off-exhibit area that is made up of two joined rooms (total of 364ft^2), each containing a fresh water pool that is 79ft^3. The lighting is on a southern hemisphere schedule and during the course of the study it was set at 14.5 hours on and

9.5 hours off. Birds are hand-fed all of their daily diet, which is made up primarily of capelin. To ensure adequate nutrition while on a diet of previously frozen fish each bird is supplemented with ½ tab of Mazuri Marine Mammal Vitamin daily.

Currently tours are conducted between zero and three times daily and consist of two birds, with each bird participating in a maximum of two tours daily. Tours typically last between 10-20 minutes and guest group size can range from 2-10 people. During tours the birds walk from their off exhibit housing room to a neighboring open space while guests, typically sitting on the floor or in a chair, are allowed to gently touch the birds along their back. The tour space has a center area, which the guests are seated around, where guests are asked not to enter, allowing the birds to have a retreat space while still fully visible to guests. Often birds are guided, by zoo staff, to walk near or past the tour guests and are occasionally gently restrained to allow a guest to have a "touching opportunity". In 2013 over 205 tours were conducted, reaching over 690 guests

Table 1: Study Individuals
Individual penguin's number within the study, hatch date, age and years participating in tours

Bird	Sex	Date of Hatch	Age at Time of Study	Number of Years as Ambassador Bird
1	Male	5/18/2002	12	10
2	Female	5/16/2002	12	10
3	Male	6/8/2007	7	5
4	Male	6/14/2007	7	5
5	Male	6/1/2007	7	5

ACTH Challenge

The study investigated each of the five Magellanic Penguins that participate in behind the scenes tours with zoo guest interactions on a routine basis. Excrement samples were collected opportunistically from each bird on baseline days and for 24 hours after an ACTH challenge injection. This approach was chosen because studies have shown significant individual variation in baseline corticosterone levels [Cockren and Silverine, 2002; Vleck et al., 2000] as well as the magnitude and pattern of corticosterone response, as demonstrated by differences the shape & magnitude of adrenal response curves [Cockren & Silverine, 2002; Nakagawa, 2003; Nilsson et al., 2008; Touma and Palme, 2005; Vleck et al., 2000]. During the ACTH challenge each bird was briefly physically restrained to receive an intramuscular injection of 200 IU of adrenocorticotrophic hormone (Sigma-Aldrich product #A6303) [Legagneux et al., 2011] into the breast muscle, administered by a veterinarian using a 25-gauge needle on a 1ml syringe [Nakagawa, 2003]. To minimize restraint time and any potential stress associated with the presence of vet staff all injection preparation was completed prior to entering the room with the bird. The keeper gently manually restrained the bird, holding its wings against its body and supporting its feet, while vet staff administered the injection.

For 24 hours following the injection any naturally voided excrement samples were collected every hour and pooled into 0.5g samples. To ensure the samples collected during the ACTH challenge were assigned to the correct individual each bird was separated into a clean, dry room. To facilitate the social needs of this species and minimize stress during separation, a mesh gate was used to

allow visual and auditory access to the other birds in this group. Baseline excrement sample collection occurred one week before ACTH challenge when possible, otherwise a minimum of one week after the ACTH challenge injection to ensure sufficient time for GCM levels to return to baseline. Baseline, non-ACTH challenge, excrement collection occurred opportunistically over the course of multiple days while birds were kept in social groupings to reduce the potential influence of stress from social separation.

Excrement Hormone Analysis

Fecal samples were collected from a clean, dry floor and frozen at -20 °C in insulated boxes prior to extraction using the established St. Louis Zoo Endocrine Lab protocol [Kozlowski et al., 2013]. During extraction process excrement samples were mixed well and distributed into 0.5 g aliquots of wet feces to which 2.5 mL of phosphate-buffered saline, pH 7.0, and 25 µL of β-Glucuronidase /Arylsulfatase (Roche Diagnostics 10-127-698001) was added and incubated overnight at 37 °C. The next day 2.5 mL methanol was added to each aliquot and the samples were shaken at 200 rpm at room temperature overnight. Liquid was decanted and centrifuged at 4000g at 4 °C for 1 hour. The supernatant containing the hormone extract was then frozen at -80 °C until assay. The solid fecal material was placed in a drying oven overnight at 80 ºC and weighed the next day for conversion of final results from ng/ml to ng/g by dividing the concentrations from the liquid extract by the amount of dried fecal material. Samples were assayed in duplicate, according the manufacture's instructions, using a double-antibody ^{125}I-corticosterone radioimmunoassay (ImmuChem DA Corticosterone ^{125}I, MP Biomedicals;

catalog #07-120-102) and counted on a Perkin Elmer Wizard2 Automatic Gamma Counter (model #2470).

Tour Data and Sample Collection

Two conditions were evaluated for each bird during the study. The "tour-condition" was one full week during which a focal bird participated in two tours each day with a randomly assigned and rotating "partner-bird", reflecting the current tour protocol of two tours maximum for any individual and each tour involving two birds. The "non-tour condition" was one full week during which a focal bird participated in no tours. Over the course of the five weeks of the study each bird was assigned one week as the focal "tour-condition" study bird, one week assigned as the focal "non-tour condition" control bird, and three weeks of random daily assignment as the partner bird for the focal bird participating in tours that week. See Appendix I for detailed schedule. This was done to reflect the current protocol for penguin tours at this institution, which calls for two birds to participate in each tour and allows each bird to participate in a maximum of two tours daily. Tour data collection consisted of behavioral observations of both birds participating in the two tours that day and excrement sample collection of the "tour-condition" study bird and the "non-tour condition" control bird for that week.

Each bird had one week of non-invasive excrement samples collected under the "tour condition" and one week of sample collection done under the "non-tour condition." Each week excrement samples were collected from two birds: the focal "tour condition" bird and the control "non-tour condition" bird. The tour and collection schedule can be found in Appendix I. Sample collection was conducted as described in the ACTH section

above. Most samples were collected within 30minutes of separation with a few taking up to 1 hour. If birds did not produce any excrement after 1 hour they were returned to the rest of the group under routine housing and husbandry parameters. Separation for sample collection occurred 15-30 minutes after the second tour for the day, which was typically 2-4 hours after the first tour of the day. This timing was chosen based on the results of the ACTH challenge which showed GCM values elevated for all birds within 1 hour after HPA axis activation and values reaching peak levels between 1-4 hours after injection depending on individual birds response rates.

To assess whether specific aspects of the tours impact GCM levels and/or behaviors of birds, surveys that documented variables present in tour conditions were used in conjunction with behavioral observations. See Appendix III for data collection form. The form includes quantitative observations of relevant behaviors and measures potentially influential tour variables. Behavior observers standing outside of the primary tour area collected tour data and completed the tour survey. Behavior observers were trained using an ethogram (see Appendix II) and a video catalog of behaviors demonstrating each behavior of interest from multiple angles. Inter-observer reliability was checked using a recorded tour session to ensure behaviors were identified consistently between individual observers. Analysis of data also looked for potential observer effect. Tour variables believed to be potentially influential on bird behavior were tracked and included: number of people, number of children, duration of tour, maximum tour volume level as measured using a decibel meter, staff member guiding the tour, and combination of individual birds. Observations of relevant behaviors including

the presence of participating behavior indicators such as approaching guests without guidance, standing for tactile interaction with a guest, or interacting with a novel object, as well as other behaviors, such as vocalizations, nipping/biting, or alternate head turns (a defensive behavior) [Eggelton and Siegfried, 1977; Ellenberg et al., 2006; Yorio and Boersma, 1992]. Tour conductor was also noted. All tours were conducted by one of five staff members, with assignment to tour responsibilities based on availability.

Data Analysis

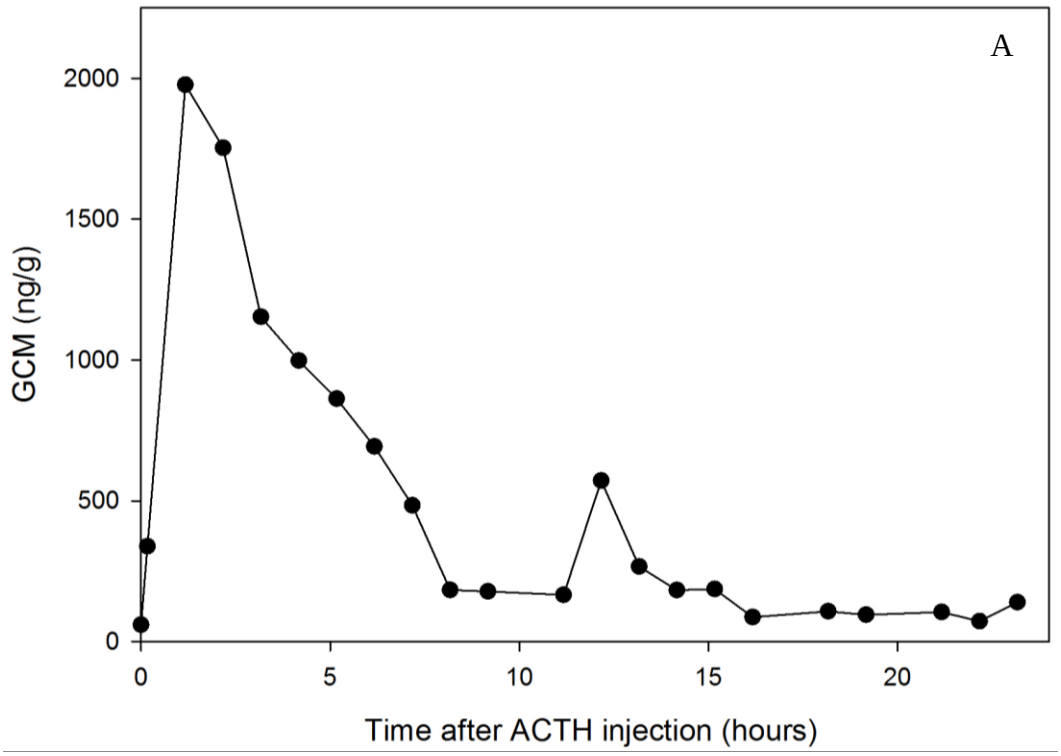
Percent change for GMC peaks during the ACTH challenge were calculated for each individual by dividing the difference between the baseline average and the maximum peak ACTH value by the baseline average value and multiplying by 100. Because corticosterone concentrations are not normally distributed GCM values from the tour study were log transformed to establish normality. Behaviors were converted into rates per minute of tour duration (tours ranged between 7 and 34 minutes) prior to data analysis. Guest minutes were determined by multiplying the number of guests on a given tour by the duration of that tour. Total rate (TRate) of behavior was calculated for each tour by adding the rates of all behaviors that occurred during that specific tour together. Total herding (TotalH) was calculated for each tour by adding the rates of herding (H) and tactile with herding (TH) for that tour together. Total tactile (TotalT) was calculated for each tour by adding the rates of tactile (T) and tactile with herding (TH) together for each tour. For GCM data analysis the behavior rates were averaged between the two tours conducted that day, since the GCM reflect excretion during the window of time in which both tours occurred. Behavior correlations were run without GCM data and the behavior

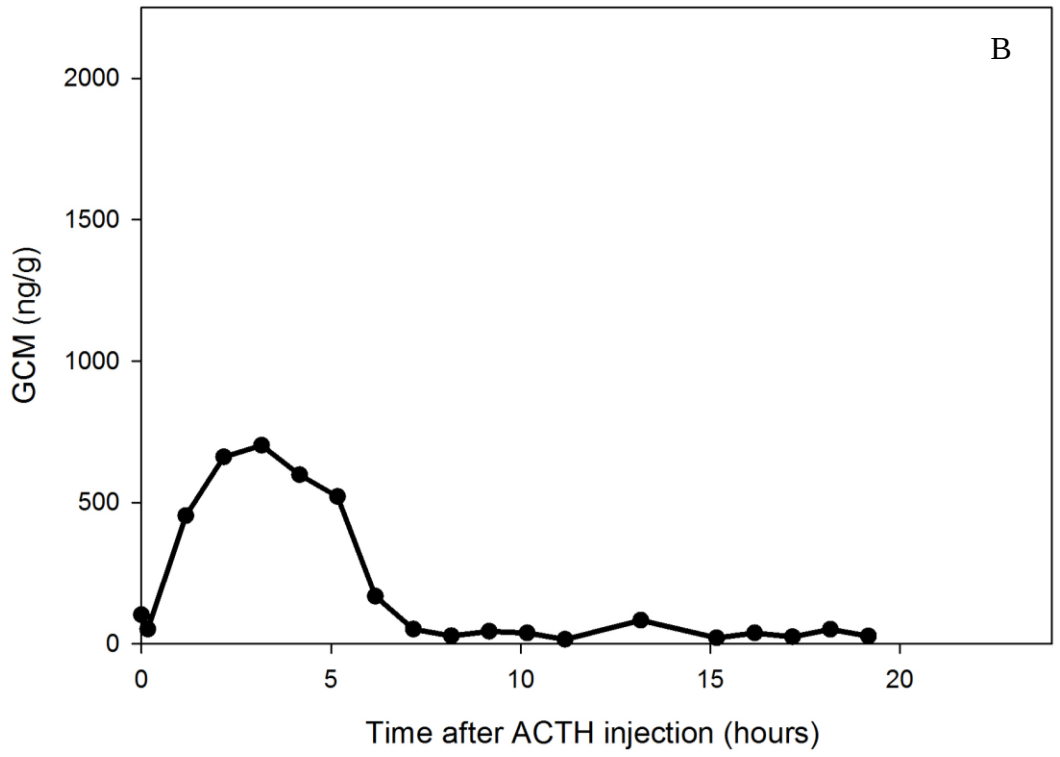
rates from each tour were included as individual data points. Data analysis was conducted using the statistical program NCSS9 © (Kaysville, UT). Running a general linear model (GLM) ANOVA analysis of GCM values for samples collected on the day's birds participated in tours and day's birds did not participate in tours revealed differences in individual responses. A Pearson's correlation matrix analysis was conducted using all behaviors rates to reveal which specific behaviors were found to correlate with each other. To evaluate environmental parameters against behavior rates a linear regression correlation test was used. Statistical results are presented using APA style, as detailed in the Publication Manual of the American Psychological Association. ANOVA results are expressed using F-value, which is the ratio of the variance between groups to the variance within groups, with the two degrees-of-freedom numbers in parentheses (separated by a comma). The first number reports the between-groups degrees of freedom; the second number reports the within-groups degrees of freedom. After each (F) statistic (rounded off to two decimal places) is the significance level (p).

RESULTS

ACTH Challenge

The magnitude and timing of glucocorticoid peaks during the ACTH challenge were characterized for each individual bird over the course of 24 hours post injection (see Figures #1 A-E). Peak GCM values were found to occur at one-hour post injection for birds 1 & 5, three hours post injection for birds 2 & 4, and four hours post injection for bird 3 (see Table #2). This time at which a substantial peak in fecal glucocorticoid levels was measured following injection was used to estimate lag-time between when a potential stressor is experienced and when the associated glucocorticoid metabolites are detectable in excrement. This lag-time estimate was used to determine fecal collection schedules during the tour data collection portion of the study. A second, significantly smaller peak was also noted between 12-16 hours for all birds. This second peak was 71-88% smaller than first peak GCM value.


A
GCM (ng/g)
2000
1500
1000
500
0
0
5
10
15
20
Time after ACTH injection (hours)


B
GCM (ng/g)
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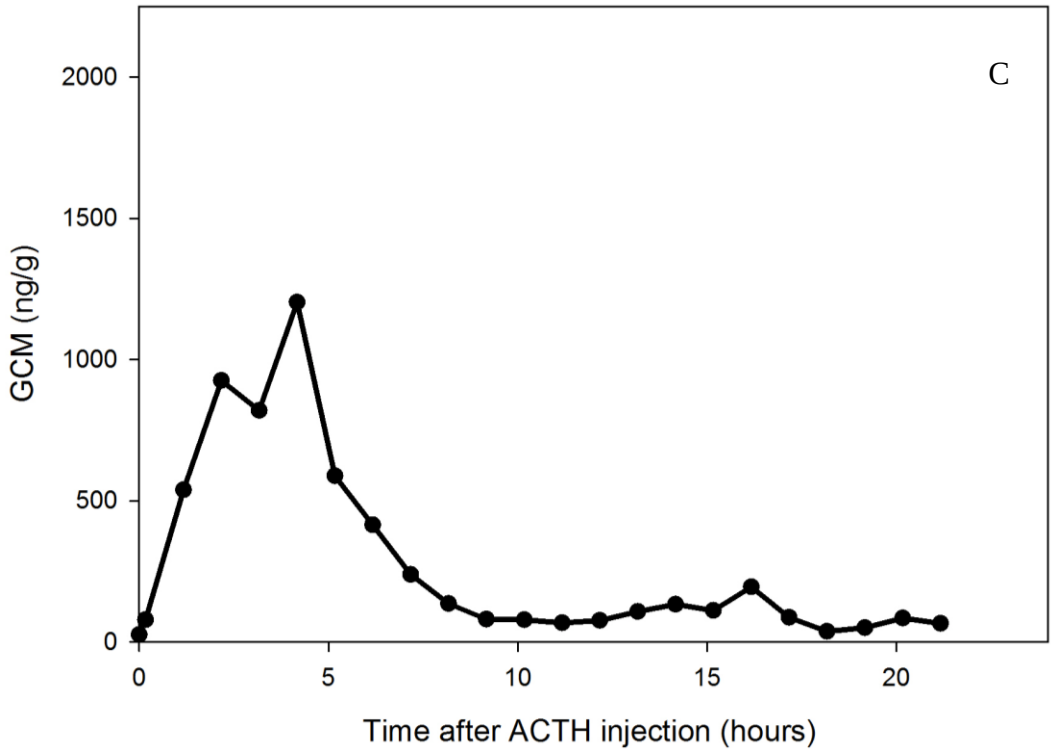
Time after ACTH injection (hours)


C
2000
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GCM (ng/g)
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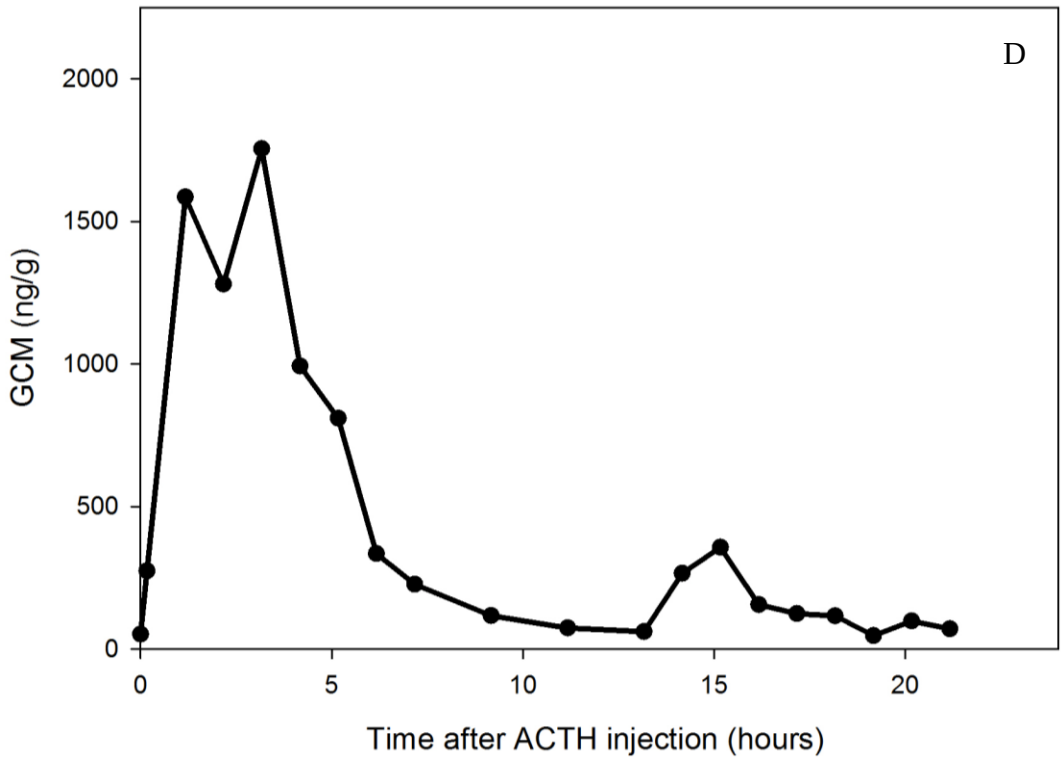
Time after ACTH injection (hours)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GCM (ng/g)
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Time after ACTH injection (hours)

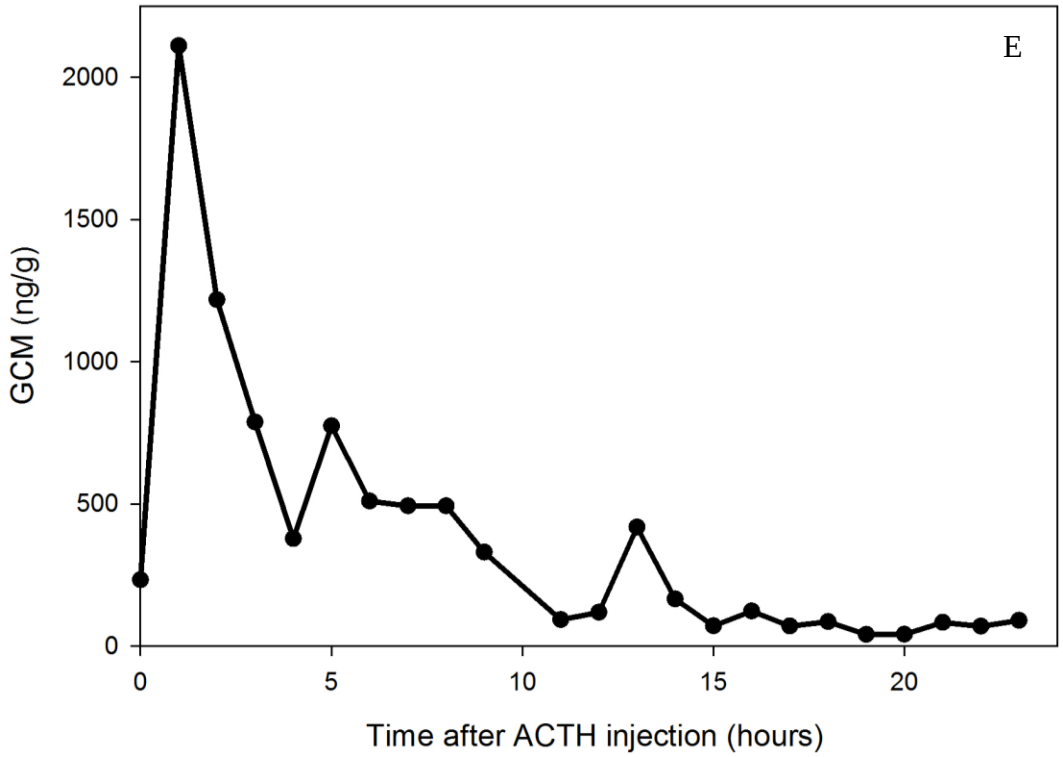

Figure 1A, B, C, D, E: ACTH Challenge
Glucocorticoid metabolite concentrations from Magellanic penguin excrement samples collected over a 24-hour time course after administration of ACTH challenge for bird 1, **B** bird 2, **C** bird 3, **D** bird 4, and **E** bird 5.

Table 2: Summary of ACTH Results
Summary of ACTH results for each bird including average baseline values, maximum peak values of GCM in ng/g, time of maximum peak in hours, percent change from baseline to maximum peak, value and time of second peak, and percent change of second peak from baseline and from maximum peak.

Bird #	GCM Baseline Average	ACTH Max Peak GCM Value	ACTH Time to Max Peak	Percent Change from Baseline to Max ACTH	ACTH 2nd Peak GCM Value	ACTH Time to 2nd Peak	% change from Max to 2nd peak	% change from baseline to 2nd peak
1	42.5 ng/g	1977 ng/g	1 hour	4,546	572 ng/g	12 hours	71	1,246
2	62.06 ng/g	702 ng/g	3 hours	1,031	83 ng/g	13 hours	88	34
3	82.18 ng/g	1203 ng/g	4 hours	1,364	195 ng/g	16 hours	84	137
4	27.96 ng/g	1754 ng/g	3 hours	6,175	357 ng/g	15 hours	80	1,177
5	30.87 ng/g	2110 ng/g	1 hour	6,832	418 ng/g	13 hours	80	1,254

Individual corticosterone baseline values were calculated by averaging the values of samples collected opportunistically at various time points throughout a week following ACTH challenge. Of note is the range in the magnitude of change over baseline values. Birds 1, 4 and 5 had peak values of over 1750ng/g and a percent of change of 4,546-6,832% over their baseline values while birds 2 & 3 had peak values under 1205ng/g and a percent of change of only 1,031-1,341% over their baseline values (see Figures #2 & 3). This reduced percent change for birds 2 & 3 is also seen with the second peak ranging from 34-137% compared to 1,177-1,254% for birds 1, 4, & 5 (see Table #2 & Figure #3). This is also reflective of the fact that birds 2 & 3 had baseline levels around 90ng/g, while birds 1, 4, & 5 had baseline values ranging from 27.96ng/g to 42.56ng/g (see Table #2 and Figure #2).

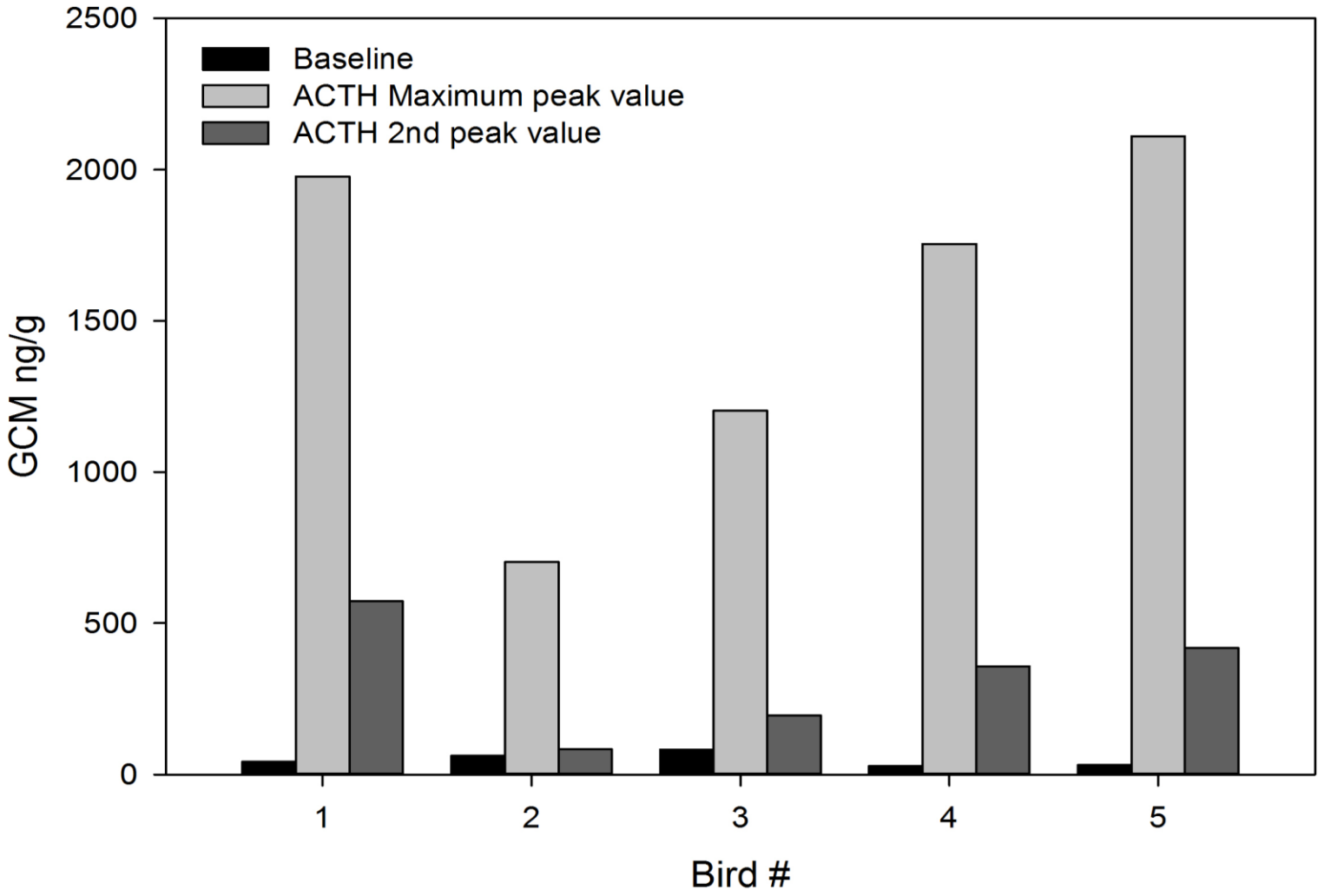

Figure 2: ACTH Challenge Peak & Baseline Results
Baseline values, first ACTH challenge peak, and second ACTH challenge peak values of glucocorticoid metabolite concentrations (ng/g) in Magellanic penguin excrement samples for each bird studied.

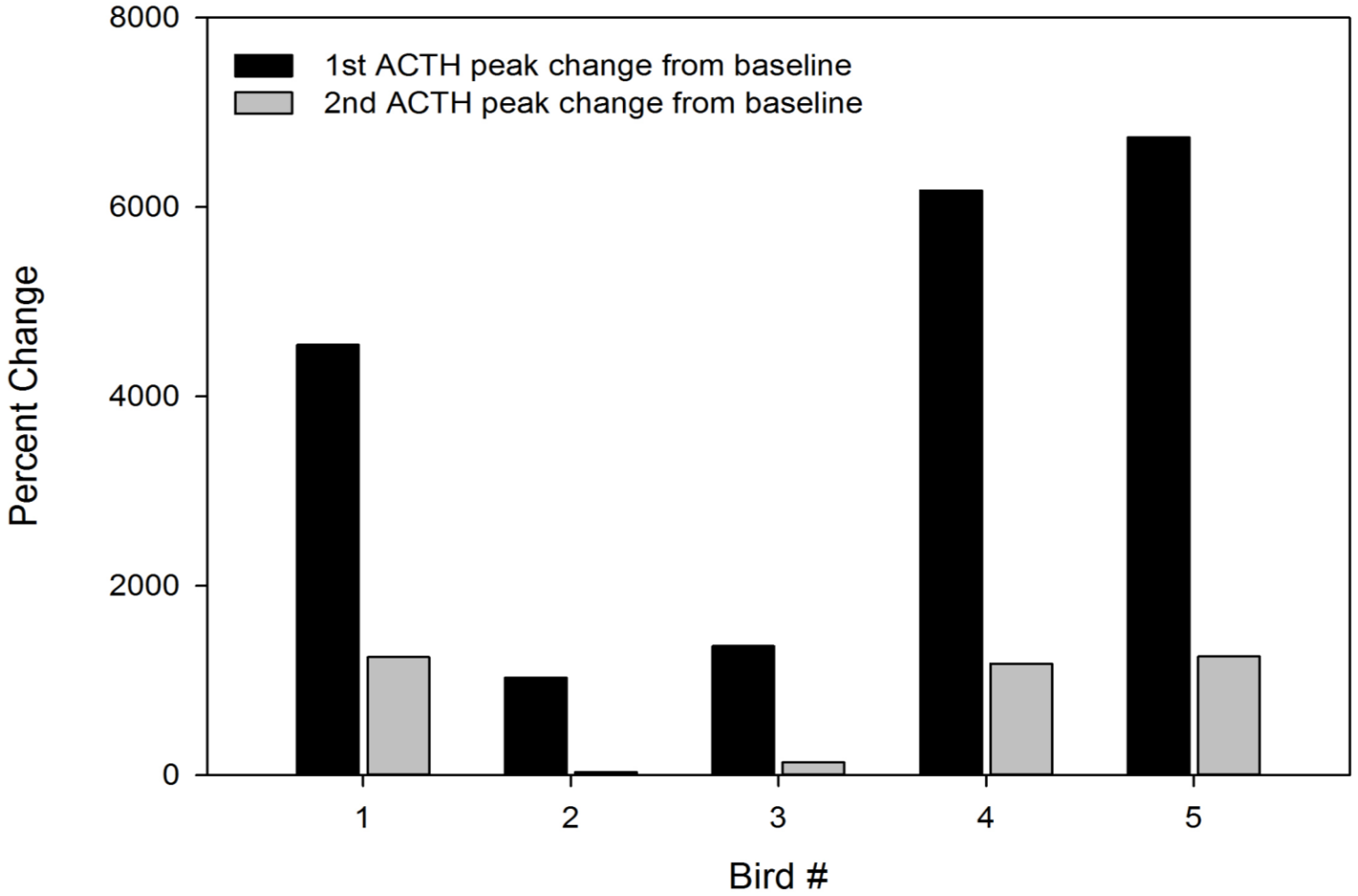

Figure 3: Percent Change ACTH Challenge Results
Percent change of glucocorticoid metabolite concentrations (ng/g) in Magellanic penguin excrement samples from baseline to peak values during ACTH challenge

Data from Tour Condition

Birds 2 & 3 had an increase in GCM ($F_{1,35} = 4.60$, $p = 0.04$) on days they participated in tours, while birds 1, 4 & 5 did not have significant changes in GCM values relevant to tour participation ($F_{1,48} = 2.05$, $p = 0.16$), see figure #4. All birds showed a decrease in GCM values with an increase in the rate of alternate head turn behavior (AHT) ($F_{4,41} = 5.53$, $p = 0.02$). Further analysis of AHT behavior revealed a significant difference ($F_{4,140} = 18.55$, $p < .001$) in individual rates of performing this behavior. Birds 1 & 2 performed the behavior at rates 6.7 times higher than birds 3, 4, &5 (see Figure #5). See table #3 for summary of individual behavior rate differences.

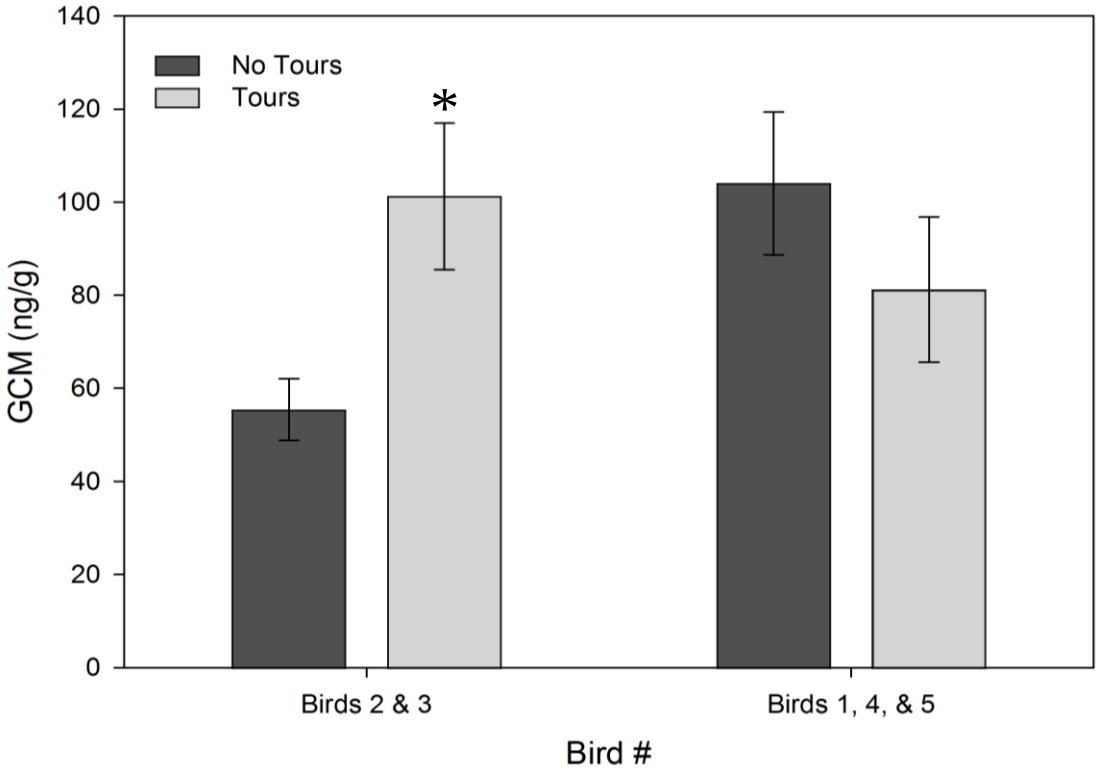

Figure 4: GCM for Tour Days and Non-Tour Days
The mean (+/- SE) concentrations of glucocorticoid metabolite values in excrement samples on days each bird participate in tours compared to days those individuals did not participate in any tours. Birds grouped according to ACTH results and similar response values. Asterisk indicates $p < 0.001$.

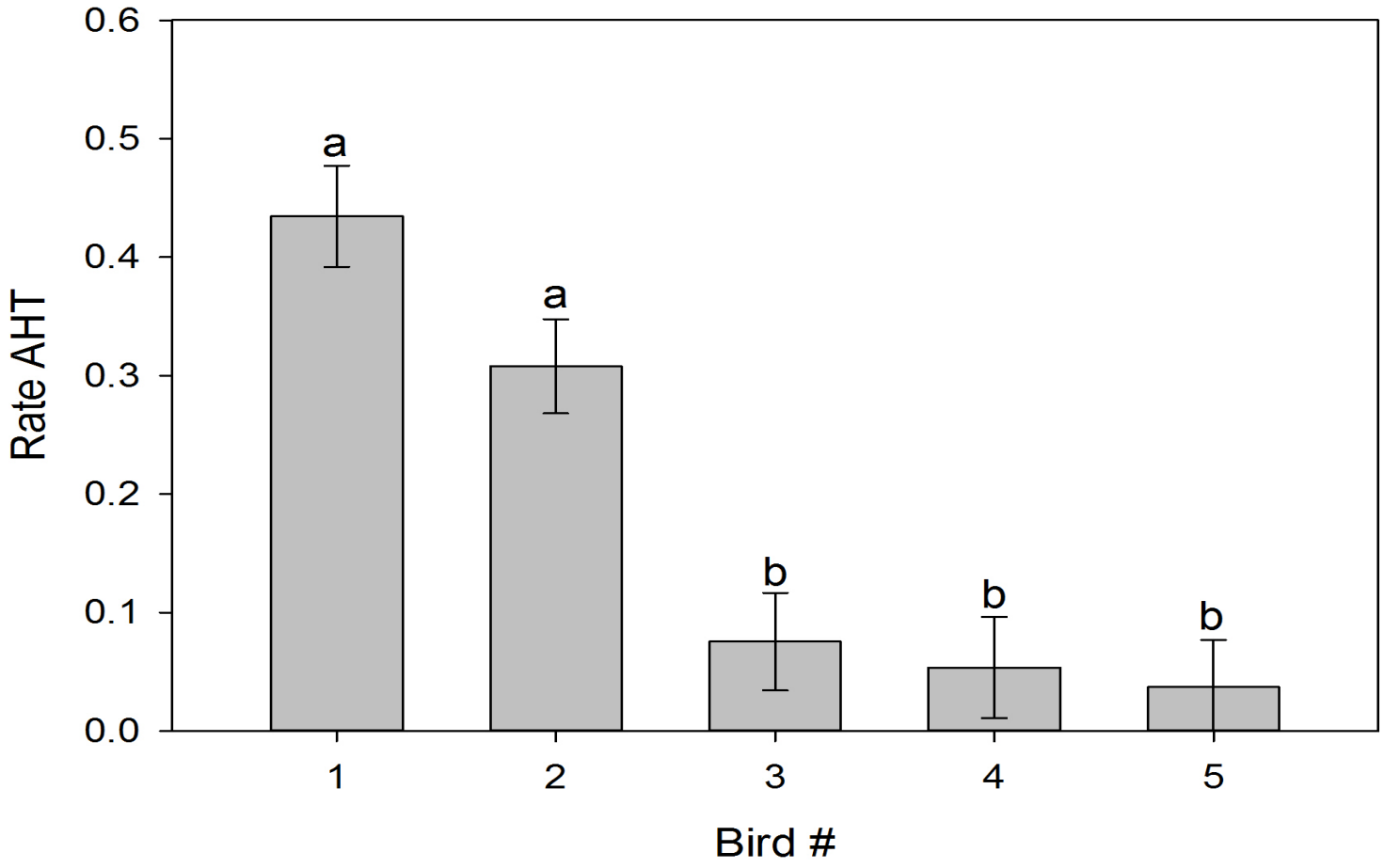

Figure 5: Rate of AHT by Bird
The mean (+/- SE) rate, per minute of tour duration, of alternate head turn behavior (AHT) for each individual bird. Shared letters indicate values which do not differ ($p>.05$) from each other.

Other individual behavior rate differences found between the birds include engage in novel object (ENO) ($F_{4,140} = 11.69$, $p < .001$), approach group (AG) ($F_{4,140} = 4.518$, $p = 0.003$), voluntary tactile interaction with guests (T) ($F_{4,140} = 4.63$, $p = 0.002$), reproductive behavior (R) ($F_{4,140} = 8.13$, $p < .001$), preening behavior (P) ($F_{4,140} = 12.93$, $p < .001$), vocalization (V) ($F_{4,140} = 14.48$, $p < .001$), and total rate of behavior (TRate) ($F_{4,140} = 6.64$, $p < .001$). Other than vocalization behavior, which was exhibited most frequently by individual 1, individual 2 consistently had the higher rates of all behaviors, while individuals 3 & 5 consistently exhibited lower rates of behavior.

The bird with which an individual penguin was paired for a tour also impacted behaviors. AHT rates were increased when paired with bird 5 and decreased when paired with birds 1 & 2 ($F_{4,140} = 4.22$, $p = 0.003$). The partner bird on tour also impacted the rates at which birds were herded towards guests for interactions and tactile experiences. Bird 2 as a partner bird decreased and bird 3 as a partner bird increased approach group with herding (AGH) ($F_{4,140} = 3.23$, $p = 0.014$), tactile with herding (TH) ($F_{4,140} = 2.66$, $p = 0.036$), and total herding ($F_{4,140} = 3.23$, $p = 0.014$) for all birds.

Table 3: Behavior Correlations with Individuals
Behaviors that differed significantly among birds as individuals (blue) and when bird was partner bird during tours (red). Behaviors that occurred significantly more frequently are indicated by + and behaviors that occurred less frequently are indicated by -. Behavior differences greater than 3 fold change are noted by double ++ or --.

Behavior	Bird 1	Bird 2	Bird 3	Bird 4	Bird 5
Alternate Head Turn	+/-	+/-	-	-	-/+
Nip/Bite					
Engage in Novel Object	-	++		+	-
Approach Group Voluntary		+	-		
Approach Group with Herding		-	+		
Tactile Voluntary		+	-		-
Tactile with Herding		--	++		
Reproductive	++		-	+	--
Preening		++			
Vocalization	++				
Total Tactile Interaction		+	-		-
Total Herding		--	++		
Total Rate of Behavior		+	-		-

Specific combinations of individual birds as pairs also impacted tour behaviors. When bird 1 was paired with bird 4 it had increased rates of tactile with herding and total herding while the same rates were decreased when paired with bird 2 ($F_{3,22} = 4.412$, $p = 0.012$). Bird 1 also had increased rates of vocalization when paired with bird 2 ($F_{3,22} = 8.14$, $p = 0.001$). Bird 2 had increased rates of tactile interaction with bird 3 as a partner and decreased rates with bird 1 ($F_{3,26} = 3.05$, $p = 0.046$). Bird 3 had increased rates of approaching group with herding when paired with bird 5 and decreased rates when paired with bird 2 ($F_{3,24} = 3.81$, $p = 0.023$). Bird 4 had no significant changes in behavior rates when paired with any of the other birds. Bird 5 approached guests voluntarily more often when paired with bird 2 ($F_{3,26} = 3.42$, $p = 0.032$) and vocalized more often when paired with bird 1 ($F_{3,26} = 3.77$, $p = 0.023$).

Table 4: Behavior Correlations with Pairing of Birds
Behaviors that differed significantly when paired with specific other partner birds for tours. Upward pointing arrows (↑) indicate an increase in behavior of bird list on left column when paired with bird listed on upper row. Downward pointing arrows (↓) in indicate a decrease in behavior of bird list on left column when paired with bird listed on upper row. Behavior abbreviations can be found in Table #3.

		Partner Bird				
		Bird 1	Bird 2	Bird 3	Bird 4	Bird 5
Focal Bird	Bird 1		↓ TH ↓ TotalH ↑ V		↑ TH ↑ TotalH	
	Bird 2	↓ T		↑ T		
	Bird 3		↓ AGH			↑ AGH
	Bird 4					
	Bird 5	↑ V	↑ T			

Specific behaviors were found to correlate with each other (see Table 5). Negative correlations were found between nip/bite and voluntary approach of group as well as between engagement with novel object and vocalization. Nip/bite was positively correlated with all herding behavior categories. Engagement with novel object was positively correlated with voluntary approach of group, voluntary tactile, and preening

behaviors. Voluntary tactile interactions were positively correlated with voluntary approach of group while tactile interactions with herding were positively correlated with approaches that involved herding. Also, of note is the finding that herding behaviors (both tactile and approach) were negatively correlated with voluntary approach, voluntary tactile and reproductive behaviors.

Table 5 Behavioral Correlations

Behaviors that correlate significantly with each other are noted inn the boxes. Positive correlations greater than 0.175 are blue. Negative correlations greater than 0.175 are red. Weaker correlations, <0.11 & >0.175 are not underlined and fainter in color. Correlations less than 0.11 are not noted.

	Alternate Head Turn	Nip/Bite	Engage Novel Object	Approach Group	Approach Group With Herding	Tactile	Tactile with Herding	Reproductive	Preening	Vocalization	Total Tactile	Total Herding
Alternate Head Turn								0.514	0.182	0.337		
Nip/Bite				-0.179	0.277		0.240				0.121	0.270
Engage Novel Object				0.385		0.179			0.314	-0.234	0.128	
Approach Group					-0.122	0.389	-0.128				0.290	-0.134
Approach Group With Herding							0.741	-0.102	0.148		0.321	0.892
Tactile								0.148			0.885	
Tactile with Herding								-0.111			0.437	0.965
Reproductive										0.252		-0.115
Preening												
Vocalization												0.420
Total Tactile												

Approach group with herding (AGH) was impacted by duration of tour, decreasing rates with longer tour durations ($t(140) = -2.78$, $p = 0.006$). Rates of AGH increased with louder maximum volume of guests ($t(140) = 3.16$, $p = 0.006$), and increased number of kids ($t(140) = 2.54$, $p = 0.0124$). Total amounts of herding (TotalH) increased with increases in maximum volume of guests ($t(140) = 2.12$, $p = 0.036$), and TotalH decreased as duration of tour increased ($t(140) = -2.22$, $p = 0.028$). Voluntary approach of guests (AG) increased as total number of guests ($t(140) = 3.48$, $p = 0.001$), guest minutes ($t(140) = 2.84$, $p = 0.005$), and number of kids ($t(140) = 2.04$, $p = 0.043$) each increased. Voluntary tactile interactions (T) increased when total number of guests ($t(140) = 3.49$, $p = 0.0001$), guest minutes ($t(140) = 2.68$, $p = 0.008$), and number of kids ($t(140) = 2.75$, $p = 0.007$) increased. Total tactile interaction increased when total number of guests ($t(140) = 3.37$, $p = 0.001$), number of kids ($t(140) = 2.80$, $p = 0.050$) and maximum volume of guests ($t(140) = 2.08$, $p = 0.040$) increased. Preening decreased as duration of tour ($t(140) = -2.43$, $p = 0.017$), and number of kids ($t(140) = -2.30$, $p = 0.023$) increased. Rates of reproductive behaviors increased when total number of guests ($t(140) = 2.26$, $p = 0.025$) and guest minutes ($t(140) = 2.02$, $p = 0.045$) increased. Total rate of all behavior increased when total number of guests ($t(140) = 3.29$, $p = 0.001$), number of kids ($t(140) = 3.48$, $p = 0.001$), and maximum volume of guests ($t(140) = 2.53$, $p = 0.013$) increased.

ANOVA analysis revealed no significant difference in tour observer ($F_{7,82} = 0.65$, $p = 0.72$), or enrichment provided ($F_{3,82} = 1.42$, $p = 0.24$) on GMC values on

any of the thirteen behavior rates observed. ANOVA and linear regression analysis revealed no significant difference in tour observer, time of day, or enrichment provided on any of the thirteen behavior rates observed. Focal bird did not have any correlation with duration ($F_{4,140} = 0.48$, $p = 0.75$), guest minutes ($F_{4,140} = 0.27$, $p = 0.89$), maximum volume ($F_{4,140} = 0.23$, $p = 0.92$), and number of guests ($F_{4,140} = 0.49$, $p = 0.74$). Partner bird also did not have any correlation with duration ($F_{4,140} = 0.50$, $p = 0.73$), guest minutes ($F_{4,140} = 0.34$, $p = 0.85$), maximum volume ($F_{4,140} = 0.24$, $p = 0.91$), and number of guests ($F_{4,140} = 0.64$, $p = 0.63$).

Staff who provided tours was found to have overall correlations with three specific behaviors: engagement with novel object ($F_{4,130} = 3.40$, $p = 0.001$) increased with J and decreased with S, reproductive behaviors ($F_{4,135} = 2.74$, $p = 0.031$) increased with J and decreased with C, and vocalizations ($F_{4,135} = 2.46$, $p = 0.048$), decreased with S and C. Much of these differences were noted within specific combinations of individual birds and tour providers but because of unequal assignment of staff to tour roles, due to scheduling limitations, these differences are not reported.

DISCUSSION

This study used an ACTH challenge with five individuals to validate a radioimmunoassay used to quantify glucocorticoid metabolites in penguin excrement samples as an indicator of adrenal response. The results from the ACTH challenge verify that the RIA, used in conjunction with the extraction protocol as described, is able to detect and evaluate levels of GCM from excrement samples of Magellanic penguins. The study demonstrated that maximum GCM levels from stimulation of the HPA axis are found between 1-4 hours after ACTH injection for these individuals. A second, significantly smaller peak was also noted between 12-16 hours for all birds. The timing of both of these peaks is similar to findings in a study of three species of owls, with their first peak at 2hours and second around 12hours [Wasser et al., 2000]. Because bird excrement samples contain both fecal and urate material this second peak is reflective of the fecal metabolites while the earlier and more notable peak results from the more quickly processed urate metabolites [Mostl et al., 2005]. The implications of mixed excrement samples with bird's highlights the importance for consistent sample collection techniques, thorough mixing of samples prior to starting any assay, and properly designating samples as excrement rather than feces [Goymann, 2005; Mostl, Rettenbacher and Palme, 2005].

Patterns in elevations of GCM in excrement can be regarded as indicative of the physiological adrenal response [Wasser et al., 2000] and assays evaluating GCM have been found to have predictive and explanatory value in avian species at times of physiological or psychosocial stress [Wasser et al.,1997; Kotrschal et al., 1998]. While all five birds in this study were reared under similar conditions, experience the same daily husbandry parameters and participate in the same tour program with the same guest interaction protocols, it is important to interpret the results in the context of each individual. Specific variables may affect animals differently and the influence of a potential stressor depends on an individual's subjective experience [Ladewig, 2000].

The lower peak values in response to ACTH challenge and higher baselines for birds 2 & 3 could imply these two individuals have decreased capacity of adrenal tissues or receptor down-regulation in the adrenal gland due to repeated stressor exposure. This concept is also supported by the increase in GCM values on tour days compared to non-tour days for these two individuals. Organisms that experience long-term intermittent stress have been shown to change their responses to stressors over time [Ladewig, 2000]. These changes can reflect return to basal levels, adaptation at the cognitive level (behavioral responses), desensitization (flooding or habituation) or sensitization and the type of change depends on how a specific stressor affects a specific animal [Ladewig, 2000]. Chronic adrenal activity can change the responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to other stressors [Ladewig and Smidt, 1989], including blunted activation of the HPA response to acute stress [Goliszek et al., 1996]. For example, dairy cows exposed to heat stress responded to an ACTH challenge with

reduced cortisol peaks [Gwazdauskas et al., 1975; Roman-Ponce et al., 1981]. Tethered cattle housed on concreted and partially slated floors react with reduced cortisol responses to ACTH injection when compared to cattle that are not tethered and kept on deep straw [Ladewig and Smidt, 1989], which is likely reflective of chronic intermittent stress [Ladewig, 2000].

Different stress response systems (behavioral, HPA, autonomic nervous system, immune system, opioid system and various neurotransmitters) may become sensitized concurrently while others becomes desensitized and the interaction between different systems may change with repeated stressor exposure [Ladewig, 2000]. Repeated social isolation of swine resulted in gradually diminished ACTH and cortisol responses while adrenaline, noradrenaline, and heart rate responses remained elevated [Schrader and Ladewig, 1999]. Naive wombats reduced behavioral reactivity and flight response distance in response to regular handling but did not have reduced fecal cortisol metabolites secretion [Hogan, 2011]. It is possible for a stress response system to stop responding due to some physiological mechanism that suppresses the response rather than actual adaptation at the cognitive level impacting the subjective experience [Ladewig, 2000]. Mechanisms that dampen excess corticosteroid release may have evolved to protect the individual from the dramatic effects these hormones have on many body functions [Ladewig, 2000]. While behaviorally these two individuals do not have profound external negative stress responses "it would be a grave fault to claim an animal had adapted to a specific housing system if its lack of a stress response was, in fact, due

to receptor down-regulation in the adrenal glad and not due to changes (adaptation) at the cognitive level" [Ladewig, 2000].

The GCM values for birds 1,4 & 5 during the ACTH challenge and tour condition suggest the subjective experience by birds 2 & 3 is not a universal species response to the tour program and environment but rather an individual bird phenomenon. Individual differences in temperament and personality can affect human animal relationship (HAR) [Hosey, 2008], which can impact an animal's subjective experience of human interactions and associated physiological responses. Some of the factors that may determine the quality of HAR in a zoo setting include exhibit design, types of interactions (negative, neutral or positive), extent of handling experienced early during life, and individual temperament/personality [Hosey, 2008]. The potential of this individuality component may be reflected when noting that all five birds in this study hatched at the same institution, experienced similar rearing environments, were transferred to the current institution at similar ages, have been housed together, participate in tours together, and each of the birds with lower GCM peak response values to ACTH challenge and higher GCM values on days participating in tours is a full-sibling and clutch mate of a bird which did not show these same responses.

Behavioral data showed a decrease in GCM values as alternate head turn (AHT) behavior rates increase. This species-typical behavior is usually reported as a defensive behavior, but its association with low GCM values in this study may reflect a bird that is confident and comfortable within the tour environment. Further support for this is that AHT was not associated with other defensive behaviors, such as nip/bite responses, and

the bird who showed the highest rates of AHT, bird 1, also had the lowest GCM values during tour conditions. AHT was positively correlated with reproductive, vocalization and preening behaviors, which are also species-typical behaviors and could reflect a bird's comfort level within the tour environment. Other behavioral correlations were specific to individual birds and pairing of birds during tours, further suggesting a component of individual personality and indicating that some individual animals maybe more suited to the role of ambassador animals within the zoo setting.

Nip/bite behavior rates were positively correlated with all categories involving herding, an involuntary situation where staff manipulates a bird's interaction with guests, and negatively correlated with voluntary approach of guests behaviors. These results indicate that staff manipulation of retreat opportunity and the resulting reduction in a bird's opportunity for choice may elicit behaviors that are undesirable in tour scenarios. Studies of exhibit design have shown that, when available, animals will make use of opportunities to retreat from public interactions [Carlstead et al., 1993; Anderson et al., 2002; Mallapur et al., 2005]. Studies on human animal relationships (HAR) predict that animals are less likely to show fear of humans when enclosures give them some control over whether or not they interact with unfamiliar humans [Hosey, 2008], while opportunities for choice and control may prevent the development of behavioral problems [Bloomsmith et al., 2000], and some researchers view giving animals control as a critical factor in promoting psychological well-being [Bloomsmith et al., 2000; Markowitz 1979; Snowden and Savage 1989]. A review of potential sources of stress in captivity by Morgan and Tromborg in 2007 found restricted choice brings a host of other potential

stressors and the perceived or actual inability to control most aspects of their surroundings is perhaps the greatest stressor in populations of captive animals [Morgan & Tromborg, 2007; Sambrook and Buchanan-Smith, 1997]. Allowing petting zoo animals' access to a full-retreat space from human victors was shown to reduce behaviors undesired in human-animal interactions [Anderson et al., 2002]. Lack of sufficient retreat space is potentially a significant stressor for animals and adding retreat spaces improved many indicators of animal welfare [Morgan and Tromborg, 2007]. Therefore, the reduction or elimination of herding by staff during tours in conjunction with strict adherence to retreat space boundaries could benefit both tour guests by soliciting fewer incidents of nip/bite behaviors, and birds by offering more control and choice in guest interactions which may also increase positive welfare.

Sound was measured and evaluated as an environmental factor during tours to determine its impact on the birds in this study. Many studies have found elevated noise levels to cause stress in animals [Morgan and Tromborg, 2007] and that as zoo visitor numbers increase the noise volume also increases which has been correlated with increased vigilance behaviors in multiple species [Morgan and Tromborg, 2007]. Others have found animals habituated to visitor sounds and did not show changes in behaviors base on guest volume changes up to 32% [Sherwen et al., 2014]. The birds in this study did not seem to be impacted by volume levels during tours, which ranged between 66.5 to 105.9 decibels (average 84.5 dB), with no changes found in rates of behavior or GCM values associated with volume.

Tour group composition, number of total guests and number of children, also had no significant impact on birds in this study. While studies of behaviors of petting zoo animals found higher human density levels resulted in increased rates of undesirable behaviors [Anderson et al., 2002], we did not see any correlation with group size, or number of children, and behavior rates or GCM values. This possibly reflects parameters in the tour protocol, which limit guest number during interactions to ten people total and encourage most guests to sit or be stationary, thereby creating a more predicable environment.

Research has shown that many different species can distinguish between individual humans [Davis, 2002; Morgan and Tromborg, 2007; Hosey, 2008], including penguins [Davis, 2002] and it is predicted that the HAR will differ between individual animals and different keepers [Hosey, 2008]. This individuality component of the HAR is possibly reflected in the correlations with specific behaviors and the staff member facilitating the tour. It is important to recognize that none of the behaviors noted to correlate with individual keepers (engagement with novel object, reproductive behaviors, and vocalizations) were correlated with GCM values or tour participation levels. Therefore they may reflect the individual nature of the HAR rather than a quality of HAR.

Conclusions

The extraction technique and radioimmunoassay as described are valid tools in detecting and quantifying GCM metabolite levels from excrement samples of Magellanic Penguins (*Spheniscus magellanicus)* relative to changes associated with acute adrenal

responses, as simulated using an ACTH challenge, and more subtle changes like those noted during the tour study. The ACTH challenge is useful in both validation of the assay as well as in developing individual HPA response profiles that define maximum GCM response and lag-time from stressor to the presence of correlating measurable metabolite of GCM in excrement. Even when genetics and rearing are very similar individuals can show unique responses to potentially stressful stimuli. Evaluating ambassador or program animals that participate in guest interactions may be beneficial to help establish their suitability for roles within a zoo environment and adjustments could be made to accommodate individual differences in subjective experiences and resulting affective states. Allowing animals choice within the context of program interactions with guests, and avoiding directing their behaviors via herding or other manipulative techniques, might improve both the guests experience and the animal's perception of the interactions. More research is needed on the impact of ambassador animal interactions on individual animals, the determination of animal personalities better suited for these roles within zoological institutions, techniques for selection of animals with temperaments best suited to the context of program animals, and rearing techniques that can maximize the impact of careful selection of program animals as well as facilitate the development of positive HAR in the zoo environment.

APPENDICES

Appendix I: Tour Study Schedule

	Sunday 7/13		**Monday 7/14**		**Tuesday 7/15**		**Wednesday 7/16**		**Thursday 7/17**		**Friday 7/18**		**Saturday 7/19**	
Times:	11:00 (ErinH)	2:00(jhd)	10:30 (gs)	1:30 (sam)	10:30 (dev)	1:00 (NateA)	10:30(gs)	1:15(dev)	10:00 (TBD)	11:00 (Sam)	10:15(dev)	1:30 (gs)	11:00 (Sam)	1:30(Mac)
TOURS:	Alejandro	Frejoles	Alejandro	Shadow	Alejandro	Fidgit	Alejandro	Frejoles	Alejandro	Shadow	Alejandro	Fidgit	Alejandro	Fidgit
NO TOURS:	Cantante		Cantante		Cantante		Cantante		Cantante		Cantante		Cantante	
COLLECTION:	Alejandro	Cantante	Alejandro	Cantante	Alejandro	Cantante	Alejandro	Cantante	Alejandro	Cantante	Alejandro	Cantante	Alejandro	Cantante
	Sunday 7/20		**Monday 7/21**		**Tuesday 7/22**		**Wednesday 7/23**		**Thursday 7/24**		**Friday 7/25**		**Saturday 7/26**	
Times	11:00 (Sam)	1:30 (Lily)	10:30 (gs)	11:45 (dev)	10:00 (Sam)	1:30 (TBD)	10:30 (gs)	1:30 (gs)	11:35(dev)	2:30 (Chawna)	10:30 (gs)	1:00(gs)	11:00(dev)	1:15 (dev)
TOURS:	Shadow	Alejandro	Shadow	Cantante	Shadow	Fidgit	Shadow	Cantante	Shadow	Fidgit	Shadow	Alejandro	Shadow	Alejandro
NO TOURS:	Frejoles		Frejoles		Frejoles		Frejoles		Frejoles		Frejoles		Frejoles	
COLLECTION:	Shadow	Frejoles	Shadow	Frejoles	Shadow	Frejoles	Shadow	Frejoles	Shadow	Frejoles	Shadow	Frejoles	Shadow	Frejoles
	Sunday 7/27		**Monday 7/28**		**Tuesday 7/29**		**Wednesday 7/30**		**Thursday 7/31**		**Friday 8/1**		**Saturday 8/2**	
Times	12:30(Sara)	1:30 (Lily)	10:30 (gs)	1:30(Sam)	10:30 (GS)	1:30(Caity)	11:00 (GS)	1:30(Chawna)	10:15 (Maddie)	1:30(TBD)	10:15 (dev)	1:30(dev)	10:30 (GS)	3:00 (GS)
TOURS:	Cantante	Alejandro	Cantante	Shadow	Cantante	Alejandro	Cantante	Frejoles	Cantante	Alejandro	Cantante	Frejoles	Cantante	Shadow
NO TOURS:	Fidgit		Fidgit		Fidgit		Fidgit		Fidgit		Fidgit		Fidgit	
COLLECTION:	Cantante	Fidgit	Cantante	Fidgit	Cantante	Fidgit	Cantante	Fidgit	Cantante	Fidgit	Cantante	Fidgit	Cantante	Fidgit
	Sunday 8/3		**Monday 8/4**		**Tuesday 8/5**		**Wednesday 8/6**		**Thursday 8/7**		**Friday 8/8**		**Saturday 8/9**	
Times	11:00 (Julie)	1:30 (Julie)	10:30 GS	1:15 Dev	10:30 GS	1:00 (TBD)	11:00 (Samantha)	1:30 (TBD)	10:15 Development	1:30 GS	10:30 GS	1:30 (Christy Poelker)	10:30 (Kim Downey	1:30 (TBD)
TOURS:	Frejoles	Fidgit	Frejoles	Shadow	Frejoles	Fidgit	Frejoles	Cantante	Frejoles	Shadow	Frejoles	Cantante	Frejoles	Shadow
NO TOURS:	Alejandro		Alejandro		Alejandro		Alejandro		Alejandro		Alejandro		Alejandro	
COLLECTION:	Frejoles	Alejandro	Frejoles	Alejandro	Frejoles	Alejandro	Frejoles	Alejandro	Frejoles	Alejandro	Frejoles	Alejandro	Frejoles	Alejandro
	Sunday 8/10		**Monday 8/11**		**Tuesday 8/12**		**Wednesday 8/13**		**Thursday 8/14**		**Friday 8/15**		**Saturday 8/16**	
Times	11:00(JHD)	1:30 (JHD)	10:30 (GS)	1:15 (Erin H)	10:30 (GS)	1:30 (GS)	11:00 (Sam)	1:00 (GS)	10:15 (MaddieS)	3:00 (Sam)	10:05 (dev)	1:40 (dev)	10:30 (KimD)	1:30 (ChristyP)
TOURS:	Fidgit	Frejoles	Fidgit	Cantante	Fidgit	Alejandro	Fidgit	Cantante	Fidgit	Alejandro	Fidgit	Frejoles	Fidgit	Cantante
NO TOURS:	Shadow		Shadow		Shadow		Shadow		Shadow		Shadow		Shadow	
COLLECTION:	Fidgit	Shadow	Fidgit	Shadow	Fidgit	Shadow	Fidgit	Shadow	Fidgit	Shadow	Fidgit	Shadow	Fidgit	Shadow

Appendix II: Magellanic Penguin Ethogram

Alternate Head Turn: Bird looks at person or other bird using only one eye while head is cocked sideways (hard look). Bird swivels head back & forth 180 degrees to look at person, or other bird, first with one eye then the other eye while rest of body is relatively still. Does not involve movement of feet or change in location.

Nip/Bite: Bird uses beak to make aggressive or forceful contact with any part of a person or other bird – not in the context of using beak for gentle exploring or accessing a novel object within the tour environment. The penguin may turn their heads sideways to bite (for example, towards tour guides hand during herding) or pull their head back before striking. Nip/Bite is NOT any beak contact with an arm or leg—if the contact with a bare arm or leg is gentle/exploratory, it can be counted as Engage with Novel Object (in these cases, the novel object is the unfamiliar leg/arm/etc). Always record the recipient of a Nip/Bite in Notes.

Engage with Novel Object: Bird interacts with any novel object in tour area, including, but not limited to, guests' personal items such as purses, camera bags, coats, shoe laces, etc. Bird may use bill/beak to interact with object but if bird bites or nips at a person note behavior under the bite/nip category. Make a note of what object the bird interacts with.

Approach Group: Bird walks to within 2 feet (~arm's length or 2 floor tiles) of the 180 degree range of the **front** of tour guests. Counts both if the penguin walks up directly face-to-face with a guest, as well as if the penguin approaches a guest from the front/side and walks past the front of the guest. Approach Group would be scored for EACH person the penguin walks by (as long as the penguin is within two feet of the 180 degrees forward scope of reach of the person). Will fall under one of the following two categories:

> **Without Herding:** Without the direction the bird is walking being directed or influenced by person walking behind it.
>
> **With Herding:** Person guiding the tour uses their body position and/or walking and/or hands to guide bird to walk within 2 feet of the front facing body of any person participating in tour.

Tactile: Bird is touched by tour guests. Tactile is scored for EACH individual pet/touch a guest gives the penguin. Be sure to include the approach score prior to the tactile. Will fall under one of the following two categories:

> **Without Herding:** Bird stands still and allows a person to touch it without being restrained or guided.
>
> **With Herding:** Person guiding the tour uses their body position and/or walking and/or hands to restrain and/or guide bird into position for tactile interaction. Make a note if bird actually held.

Reproductive/Broody/Nesting: Bird hunkers down in lower body posture, typically in a corner or under an object, bird may bill at ground and/or a wall, digging on floor is also a broody behavior. May also involve bill rubbing with another bird (or keeper) and mounting behaviors.

Preening: Bird uses bill or foot to preen feathers. Also includes a feather shake off.

Vocalize: Bird makes brief trumpet or full out call. Note one of the following subcategories of vocalization only if it is explicitly clear – if unable to determine what bird is vocalizing at just note the vocalization without a subcategory.

> **At Person:** bird is within 3 feet of a person, facing them and vocalizing directly at them.
>
> **At Bird:** bird is within 3 feet of the other tour bird, facing them and vocalizing directly at them.
>
> **At Birds in Holding:** bird is vocalizing while facing the holding rooms AND the birds in holding return the call.
>
> **Undetermined:** bird is vocalizing but no clear intended receiver of vocalization

Appendix III: Tour Data Collection Sheet

1st or 2nd Tour	Observer Name:									
Tour Details			Tour Parameters							
Bird	Date	Time Start	Time End	Tour Provider	Duration	Total # of Guests	# children present	Max Volume	Entrance/Exit	Notes
1										
2										
Behaviors (tick mark for each occurance)										
Alternate Head Turn	Nip or Bite	Engage With Novel Object	Approach Group		Tactile		Reproductive	Preening	Vocalize	Notes
			w/OUT herding	w/herding	w/OUT herding	w/herding			When possible note who @	
1										
2										
Both										
Notes:										

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BIOGRAPHY

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