

# Ape Conservation Physiology: Fecal Glucocorticoid Responses in Wild *Pongo pygmaeus morio* following Human Visitation

Michael P. Muehlenbein<sup>1,2\*</sup>, Marc Ancrenaz<sup>2,3,4</sup>, Rosman Sakong<sup>5</sup>, Laurentius Ambu<sup>2</sup>, Sean Prall<sup>1</sup>, Grace Fuller<sup>6,7</sup>, Mary Ann Raghanti<sup>6,8</sup>

**1** Department of Anthropology, Indiana University, Bloomington, Indiana, United States of America, **2** Sabah Wildlife Department, Kota Kinabalu, Sabah, Malaysia, **3** Kinabatangan Orangutan Conservation Programme, Sandakan, Sabah, Malaysia, **4** North of England Zoological Society, Chester, United Kingdom, **5** Red Ape Encounters, Sukan, Sabah, Malaysia, **6** Cleveland Metroparks Zoo, Cleveland, Ohio, United States of America, **7** Department of Biology, Case Western Reserve University, Cleveland, Ohio, United States of America, **8** Department of Anthropology, School of Biomedical Sciences, Kent State University, Kent, Ohio, United States of America

## Abstract

Nature-based tourism can generate important revenue to support conservation of biodiversity. However, constant exposure to tourists and subsequent chronic activation of stress responses can produce pathological effects, including impaired cognition, growth, reproduction, and immunity in the same animals we are interested in protecting. Utilizing fecal samples ( $N=53$ ) from 2 wild habituated orangutans (*Pongo pygmaeus morio*) (in addition to 26 fecal samples from 4 wild unhabituated orangutans) in the Lower Kinabatangan Wildlife Sanctuary of Sabah, Malaysian Borneo, we predicted that i) fecal glucocorticoid metabolite concentrations would be elevated on the day after tourist visitation (indicative of normal stress response to exposure to tourists on the previous day) compared to samples taken before or during tourist visitation in wild, habituated orangutans, and ii) that samples collected from habituated animals would have lower fecal glucocorticoid metabolites than unhabituated animals not used for tourism. Among the habituated animals used for tourism, fecal glucocorticoid metabolite levels were significantly elevated in samples collected the day after tourist visitation (indicative of elevated cortisol production on the previous day during tourist visitation). Fecal glucocorticoid metabolite levels were also lower in the habituated animals compared to their age-matched unhabituated counterparts. We conclude that the habituated animals used for this singular ecotourism project are *not* chronically stressed, unlike other species/populations with documented permanent alterations in stress responses. Animal temperament, species, the presence of coping/escape mechanisms, social confounders, and variation in amount of tourism may explain differences among previous experiments. Acute alterations in glucocorticoid measures in wildlife exposed to tourism must be interpreted conservatively. While permanently altered stress responses can be detrimental, preliminary results in these wild habituated orangutans suggest that low levels of predictable disturbance can likely result in low physiological impact on these animals.

**Citation:** Muehlenbein MP, Ancrenaz M, Sakong R, Ambu L, Prall S, et al. (2012) Ape Conservation Physiology: Fecal Glucocorticoid Responses in Wild *Pongo pygmaeus morio* following Human Visitation. PLoS ONE 7(3): e33357. doi:10.1371/journal.pone.0033357

**Editor:** Sharon Gursky-Doyen, Texas A&M University, United States of America

**Received** August 30, 2011; **Accepted** February 8, 2012; **Published** March 15, 2012

**Copyright:** © 2012 Muehlenbein et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** Funding was provided by the Great Ape Conservation Fund of the United States Fish and Wildlife Service, the Disney Wildlife Conservation Fund, and Indiana University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have read the journal's policy and have the following conflicts: At the time of sample collection, Dr. Sakong was an employee of Red Ape Encounters (but is no longer). Dr. Ancrenaz was also a founding member of Red Ape Encounters. These previous affiliations have not influenced data analyses, interpretation of results, manuscript preparation or decision to publish. These previous affiliations do not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

\* E-mail: mpm1@indiana.edu

## Introduction

Tourism generates more than 9% of the global gross domestic product [1], and may account for almost half of the GDP in developing countries with biodiversity-rich ecotourism draws [2]. Whale- and dolphin-watching activities in 1998 alone generated more than US\$1 billion [3]. Ecotourism represents “environmentally responsible travel and visitation to relatively undisturbed natural areas, in order to enjoy and appreciate nature (and any accompanying cultural features — both past and present) that promotes conservation, has low visitor impact, and provides for beneficially active socio-economic involvement of local populations”, p. 20 [4]. Ecotourism accounts for a significant proportion of all international tourism and contributes billions of dollars to the

national income of various countries [5]. Such revenue could enhance economic opportunities for local residents, support environmental education, and protect the natural and cultural heritage of the area, including the conservation of biodiversity and improvement of local facilities [6–8].

Rapid, unmonitored development of nature-based tourism projects can also lead to degradation of habitats and deleterious effects on animal well-being, negatively impacting the very species we wish to conserve [9]. Animals can become crowded in restricted habitats, pollution may increase in otherwise pristine areas, and invasive species could be introduced accidentally. Animals can become displaced, altering their activity budgets and preventing them from feeding properly [10–15]. Animals used for tourism may also have reduced opportunities to interact with



unhabituated animals not used for tourism, prohibiting social interactions. Habituation of animals to human presence can increase the likelihood that animals will actively seek out contact with humans, particularly in the form of crop raiding and invasion of garbage pits and latrines. Habituation may make animals more vulnerable to poaching because of their loss of fear of humans.

Tourists also likely serve as a source of infection, particularly to nonhuman primates who are genetically closely related to humans and share a number of common infections. A significant proportion of visitors to primate rehabilitation centers are not adequately vaccinated and are likely infectious, creating unnecessary risk of pathogen transmission to wildlife because they are largely unaware of the impacts they may directly have on animal health, despite their interests in environmental protection [16–18].

Anthropogenic disturbances may also affect adversely animal physiology. Stressors are noxious stimuli that trigger responses to maintain homeostasis. Normal activation of stress responses (both sympathetic nervous system and hypothalamic-pituitary adrenal axis) is important for survival via alteration of metabolism and behaviors. Chronic exposure to stressors can lead to ‘allostatic overload’ [19]. However, constant exposure to stressors and chronic activation of stress responses can produce pathological effects, including impaired cognition, growth, reproduction, and immunity [20–25]. The chronic stressor of tourism exposure to wildlife could theoretically cause immunosuppression, increasing susceptibility to infectious diseases, and decreasing reproductive success.

Field endocrinology has recently been used to assess alterations of the hypothalamic-pituitary-adrenal axis in endangered and economically valuable wildlife species [26–32]. Fecal samples collected noninvasively under natural conditions are the ideal media for assessing extended secretion of glucocorticoid metabolites in response to anthropogenic stressors in wildlife [33]. Fecal extracts represent the accumulation of glucocorticoids and their metabolites over a period of time approximately equal to gut retention time of the animal. Because of this, and because concentrations of glucocorticoid metabolites in feces parallel concentrations of glucocorticoids in plasma [34], fecal glucocorticoid metabolites (fGM) have been used to assess stress in a variety of animal species [35–37]. For example, in areas with elevated recreational intensity (compared with animals in other areas), higher fGM levels were identified in wolves (*Canis lupus*), pine martens (*Martes martes*), elk (*Cervus elaphus*), and wood grouse (*Capercaillie Tetrao urogallus*) [35,38–40].

The present study proposed to evaluate the effects of tourist exposure on fGM levels of wild habituated orangutans. Fecal steroids [41–46] have been used previously to describe endocrine correlates of behavior [47,48], reproduction [49,50], and immune function [51], among other things, in populations of wild nonhuman primates. Here we aimed to test the null hypothesis that wild orangutans exposed to tourists as part of the Red Ape Encounters (RAE) program in Sabah, Malaysian Borneo, exhibit normal fGM responses, indicative of a lack of chronic stress in these animals. According to the Red List of Threatened Species, Bornean orangutans (*Pongo pygmaeus* ssp.) are endangered [52]. Orangutan tourism has focused mainly around rehabilitation centers that use tourism for public education and to generate resources for conservation activities [53]. Red Ape Encounters (see below) is the only ecotourism program that facilitates trekking for wild orangutans. Given the increasing demand of tourists to encounter wild orangutans, it is critical to evaluate any potential physiological effects this and future programs may have on this charismatic and endangered species. We predicted that: 1) fGM concentrations would be elevated on the day after tourist visitation

(indicative of normal stress response to exposure to tourists on the previous day) compared to samples taken before or during tourist visitation; 2) samples collected from unhabituated animals not used for tourism would have higher fGM concentrations than from those habituated animals utilized for tourism.

## Materials and Methods

### Ethics statement

Ethical approvals were obtained from the Institutional Animal Care and Use Committee of Indiana University (protocol #08-047) and by the Scientific Research Review Committee of Cleveland Metroparks Zoo (project #CS2005-030). Permission to conduct this research was granted by the Sabah Wildlife Department. As fecal samples were collected noninvasively from the captive and wild orangutan populations, all research complied with all recommendations of the 2006 Weatherall report for the use of non-human primates in research.

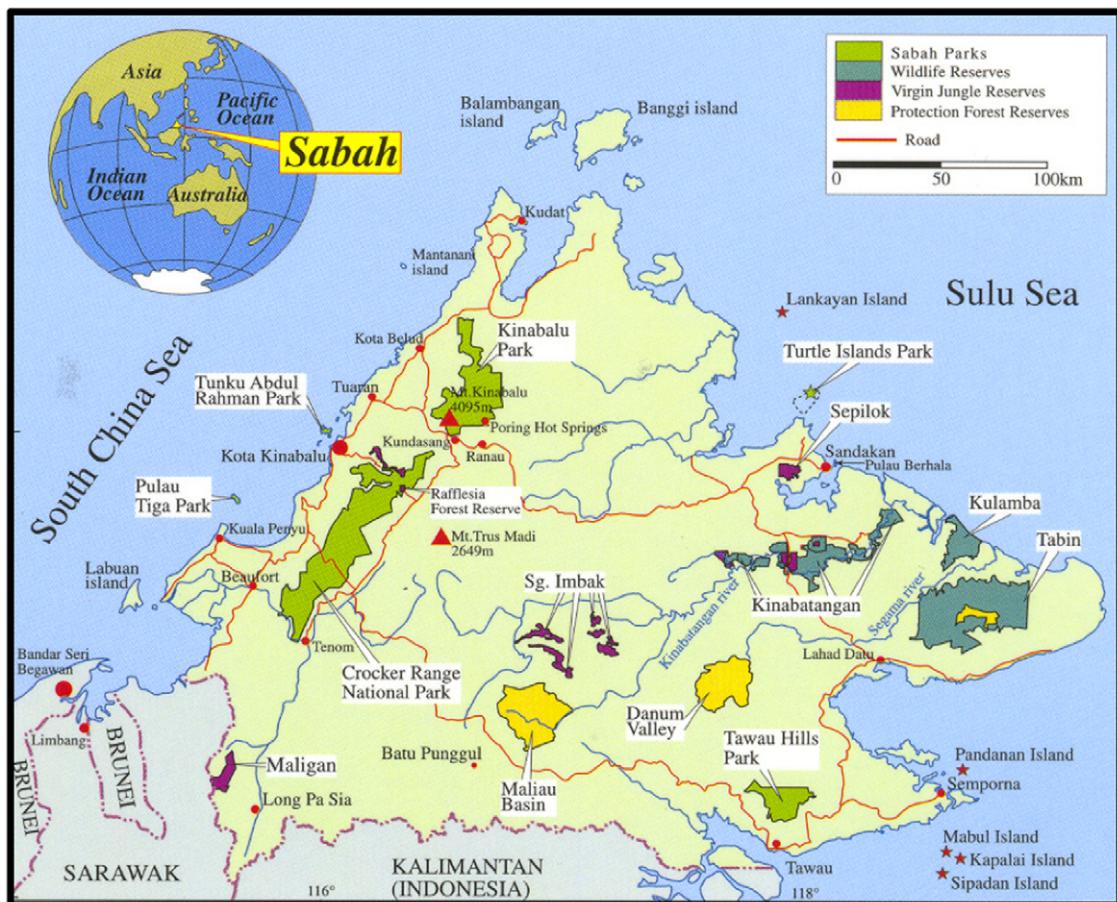
### Samples for fecal degradation test

Under field conditions, it is not usually possible to process fecal samples immediately following defecation or collection. Therefore, we determined the effects of time on hormone degradation using samples from one male and one female adult orangutan (*Pongo pygmaeus pygmaeus*) at Cleveland Metroparks Zoo. Using a wooden applicator stick, first-morning samples were collected between 7 and 8 am (immediately following defecation) off of a clean enclosure floor, uncontaminated with water or urine. Three samples were collected from each animal on non-consecutive days. Immediately following defecation, a portion of the fecal mass was stored in a closed centrifuge tube at enclosure temperature. A portion of each sample was removed from the tube and processed (see below) precisely every 30 minutes for up to 6 hours (for a total of 78 measurements {39 measurements per animal}; 6 fGM values for each of 13 different 30-minute time points). Solid-phase extraction for this portion of the project took place in a laboratory at Cleveland Metroparks Zoo; sample solvolysis and enzyme immunoassay took place in the Evolutionary Physiology and Ecology Laboratory at Indiana University.

Because gut biota, diet and environmental conditions differ between captive animals at Cleveland and wild animals in Borneo, we also collected a single fecal sample from each of two previously unknown, unhabituated wild orangutans (one adult male and one adult female) when they were encountered during area surveys in the Kinabatangan (see below). Like the Cleveland samples, these samples (presumably first morning voids before 8 am) were collected immediately following defecation and processed in a similar fashion described above (a solid-phase extraction every 30 minutes for up to 6 hours) so as to determine the effects of time on hormone degradation.

### Samples for tourist visitation test

Between 2008 and 2009, 36 fecal samples were collected and processed from Jenny, an adult (at the time, approximately 32 years of age) female orangutan (*Pongo pygmaeus morio*), and 17 samples from her 11-year old sub-adult male offspring, Etin. These wild animals have been monitored semi-continuously since 1998 as part of the Kinabatangan Orangutan Conservation Programme (KOCP) near the village of Sukau, in the Malaysian state of Sabah (north Borneo) (Figures 1 and 2). These animals inhabit the 26,000 hectare Lower Kinabatangan Wildlife Sanctuary, an area of refuge surrounded by encroaching agricultural productions. The lowland dipterocarp forest is home to approximately 1,100 orangutans (of the approximately 55,000 left in Borneo), 8 other



**Figure 1. Location of Sabah, Malaysian Borneo.** Illustration provided by the Sabah Wildlife Department.  
doi:10.1371/journal.pone.0033357.g001

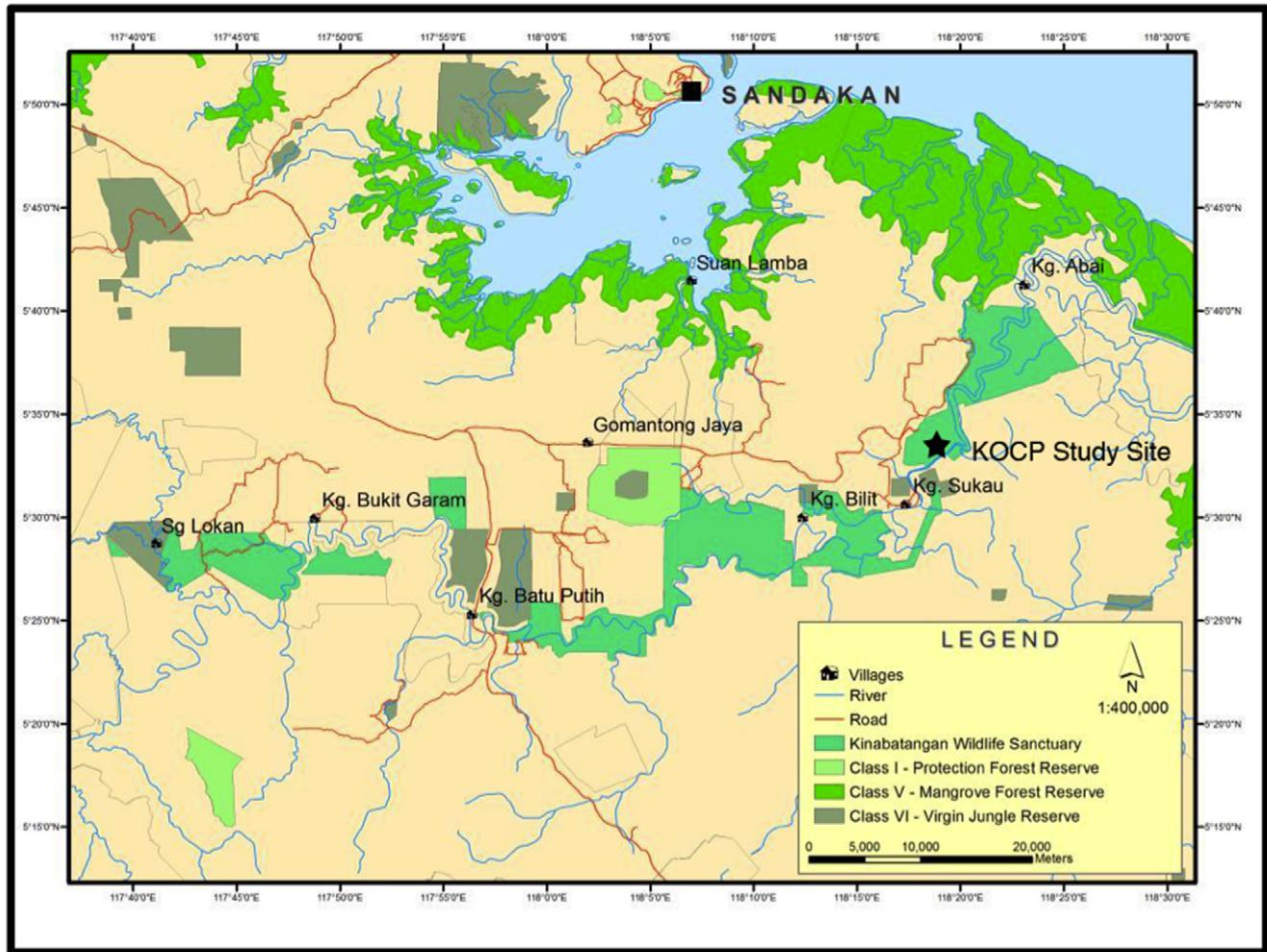
species of primates, Bornean elephants, estuarine crocodiles, more than 300 species of birds, and other wildlife [54]. KOCP has been involved in general bio-monitoring, particularly of orangutan population trends, analysis of orangutan behavioral ecology, and community-oriented initiatives in Sabah.

In 2001, KOCP, the Sabah Wildlife Department and the Danish International Development Agency formed the Red Ape Encounters (RAE) community-based ecotourism program in the village of Sukau in the Lower Kinabatangan Wildlife Sanctuary, an area acknowledged as a prominent tourism destination in Sabah's National Ecotourism Plan [55]. Of the tourism operations in the Kinabatangan, RAE is the only community-owned and operated program (with significant socioeconomic return to indigenous people of Sukau and for conservation purposes), and the only agency that facilitates trekking for wild orangutans [54,56]. RAE is therefore one of few true ecotourism programs in Borneo. Their program is designed to enhance knowledge about the local wildlife and environment, clearly communicating conservation messages. Visitor guidelines are similar to those described in the Best Practice Guidelines for Wild Great Ape Tourism by the International Union for Conservation of Nature (IUCN) [57]. For example, sick individuals are not allowed to participate, a maximum of 7 tourists are allowed with the animals at any given time, length of visitation with the animals is limited to 1 hour (with animals rarely exposed to tourists for more than 3 hours per week), distance between visitors and orangutans is limited to 10 meters minimum, and visitors must adopt appropri-

ate behaviors at all times [58]. Currently, RAE hosts approximately 250 tourists per year, with most of the activity centered around two habituated orangutans: Jenny and Etin. This is the only commercial operation that regularly brings tourists to view wild orangutans.

To evaluate changes in fecal cortisol levels associated with tourist visitations, our goal was to obtain fecal samples from both Jenny and Etin i) 24 hours before tourist visitation (to act as a baseline, with no human exposure within the preceding 48 hours), ii) during tourist visitation (to measure the effects of the previous day's exposure to our two field assistants) and iii) 24 hours after tourist visitation (to measure the effects of the previous day's exposure to the tourists). But obtaining samples for each animal and time point is difficult because of the relatively few tourists that presently utilize RAE, and because these animals are completely wild and utilize a very large territory, coming and going as they please. It is very difficult to stay with a single animal for more than a few days at a time, leading to a compromised sampling schedule.

Samples were collected in association with 25 tourist visitation events. Of the 53 samples (36 from Jenny and 17 from Etin), 17 were obtained on the day before tourist visitation, 24 were obtained during tourist visitation, and 12 were obtained the day after tourist visitation. Samples were collected by 8 am each day (which may represent first morning void when the animals first awoke from their night nest) to minimize any diurnal effects. Animal identification and behaviors (including potential behavioral indicators of stress like hair pulling, self-scratching,



**Figure 2. Location of Kinabatangan Orangutan Conservation Programme (KOCP) study site where Red Ape Encounters bring tourists to visit wild orangutans.**

doi:10.1371/journal.pone.0033357.g002

vocalizations, hiding, fleeing, teeth baring, and defensive head turns), animal physical condition, height of animal in canopy, tourist behaviors (including grouping, volume of conversation, camera flashes, etc.), number of tourists present, distance between animal and tourists, date, time of animal defecation and sample extraction were recorded. Given the nature of fecal hormone secretion, elevated fGM levels detected in the samples obtained the day after tourist visitation would be indicative of elevated stress response on the previous day (during the tourist visitation), assuming a gut retention time of approximately 24 hours for a large-bodied primate with a high fibrous diet [48,59,60]. The average gut retention time of wild orangutans is unknown.

Fecal samples were also obtained from 4 previously unidentified (and unhabituated) orangutans (2 sub-adult males and 2 adult females) when they were encountered during area surveys, expeditions and line transects in the Kinabatangan. Once these animals were located, they were followed every day that was possible. Ten fecal samples were obtained from the 2 unknown females (9 samples collected upon initial encounter, or an encounter with no previous contact for at least 2 days; only 1 sample was collected within a day after previous encounter), and 16 samples from the 2 unknown males (10 samples collected upon first encounter, or an encounter with no previous contact for at

least 2 days; and 6 samples collected within a day after previous encounter).

Solid-phase extraction for this portion of the project took place in the KOCP laboratory in Sukau; sample solvolysis and enzyme immunoassay took place in the Evolutionary Physiology and Ecology Laboratory at Indiana University.

#### Solid-phase extraction

Each sample was thoroughly mixed within its collection tube. 0.2 grams (g) of feces (no fibrous plant or other undigested material) was weighed and mixed with 2.5 milliliters (ml) of distilled water and 2.5 ml of 95% ethanol in a new tube. The tube was then vortexed for exactly 5 minutes (min) (it was critical that each sample be processed exactly the same way to ensure equal extraction). The tube was then centrifuged at 3300 revolutions per minute (rpm) for 10 min, and the fecal extract (supernatant) decanted into a clean tube. An Alltech Prevail C18 Maxi-Clean reversed-phase non-polar silica solid-phase extraction cartridge (500 milligrams [mg] bed weight) was primed with 2 ml of distilled water. Using a syringe, 2 ml of the fecal extract was slowly loaded into the cartridge, followed by another 2 ml of distilled water. Both ends of the cartridge were capped, the cartridge was labeled and placed into a zip-lock bag containing a silica gel packet. Cartridges

were stored at ambient temperature in the dark for two weeks before being shipped to the laboratory and frozen at -20 degrees Celsius (°C). This method of adhering the fecal analytes onto a sorbet matrix in the field requires the availability of organic solvents and electricity, but also allows for shipping diagnostic, non-infectious samples at ambient temperature, eliminating the need for shipping alcohol, dry ice or liquid nitrogen. This method was also preferred in the present study because of unpredictable freezer/electricity availability in the forest.

Using a vacuum manifold, the cartridges were washed using 1 ml of 20% methanol and eluted using 2 ml of 100% methanol. The eluted samples were evaporated to dryness using an Organomation Multi-Vap nitrogen evaporator (70°C, 15 pounds per square inch [PSI]). Samples were rehydrated with 1 ml of 100% ethanol and vortexed. Samples underwent solvolysis (50 microliters [ $\mu$ l] of a sulfuric acid solution [6.94 ml H<sub>2</sub>SO<sub>4</sub> with 43.06 ml water], 100  $\mu$ l of a saturated sodium chloride solution [31.13 g made up to 100 ml water], and 4.5 ml of ethyl acetate, mixed with sample, incubated overnight in a water bath [40°C]) to hydrolyze conjugated steroids. The next morning, 2.5 ml of water were added, the tubes vortexed for 5 min, and then centrifuged for 3 min at 1000 rpm. The ethyl acetate fraction was transferred into clean tubes, evaporated until dryness and rehydrated with 1 ml of 100% ethanol.

### Enzyme immunoassay

Cortisol antibody and cortisol-horseradish peroxidase (HRP) were obtained from Coralie Munro at the University of California-Davis. 200  $\mu$ l of antibody were mixed with 1.8 ml carbonate buffer (2 Sigma carbonate-bicarbonate capsules with 200 ml water). 124  $\mu$ l of this solution were mixed with 198 ml of carbonate buffer to yield a 1:16,000 antibody dilution. 100  $\mu$ l of this was pipetted into each well of a Nunc Maxisorb plate. The plates were incubated inside a humidity chamber in a refrigerator overnight, after which they were washed (4 cycles with 10 second soaks on a Bio-Tek ELx-405 plate washer) with buffer (1800 ml water with 200 ml concentrated buffer [87.66 g NaCl, 5 ml Tween 20, made up to 1000 ml water]), blotted, 150  $\mu$ l buffer (10.842 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 17.324 g Na<sub>2</sub>HPO<sub>4</sub>, 17.4 g NaCl, 2 g bovine serum albumin, made up to 2000 ml water; pH adjusted to 7) added to each well, sealed and frozen until use.

Eight standard concentrations (5–1000 picograms [pg] per well) of cortisol were created by dilution of a stock solution (10 mg of cortisol in 100 ml 100% ethanol). 900  $\mu$ l of unknowns were transferred into clean tubes, and these along with standard and controls (62.5 pg and 17 pg) were evaporated to dryness. 300  $\mu$ l of cortisol-HRP conjugate solution (17 ml buffer with 11.3  $\mu$ l HRP solution [5  $\mu$ l HRP with 495  $\mu$ l buffer]) were added to each tube, vortexed, and then transferred to a deep-well plate. 200  $\mu$ l of the cortisol-HRP conjugate solution were added to the zero concentration well, and 300  $\mu$ l buffer to the non-specific binding well.

Antibody-coated plates were removed from the freezer and allowed to reach room temperature within a humidity chamber, after which the buffer was dumped and the plates blotted. 100  $\mu$ l of from each well in the deep-well plate were then pipetted in duplicate to the corresponding wells on the antibody-coated plate. The plate was sealed and incubated for 2 hours (5 min on a plate rotator and then 155 min in the humidity chapter at room temperature). The plate was washed with buffer as before, and 100  $\mu$ l of substrate-citrate buffer solution (250  $\mu$ l of 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] [ABTS]-solution [0.439 g ABTS made up to 20 ml water] with 80  $\mu$ l H<sub>2</sub>O<sub>2</sub> solution [0.5 ml H<sub>2</sub>O<sub>2</sub> with 7.5 ml water]), and 24.67 ml citrate

buffer [19.21 g citric acid with 1966 ml water, pH adjusted to 4]) were added to each well. The plate was resealed and rotated for 5 min, and incubated in the humidity chamber for 25 min at room temperature. 100  $\mu$ l stop solution (10 ml of water with 20  $\mu$ l ethylenediaminetetraacetic acid (EDTA) solution [41.623 mg EDTA made up to 100 ml water] and 10 ml hydrofluoric acid solution [5.048 ml of 49% hydrofluoric acid with 1.2 NaOH [5 normal]]) were added to each well. Absorbance (optical density) of each well was read at 415 nanometers (nm) (reference wavelength of 570 nm) using a Bio-Tek Synergy 2 plate reader. The average optical density of the non-specific binding wells was subtracted from the average optical density of all other wells. Percent binding was plotted against the standard concentrations using a log-logit transformation. Based on the dilutions used above, the value of each unknown was transformed from pg per well to nanograms (ng) of glucocorticoid metabolite per gram of feces by multiplying by 0.042, with separate dilution factors for standards and controls. A detailed protocol can be made available upon request. This assay was adapted from previous methods used by Dr. Toni Ziegler and Dan Wittwer at the Wisconsin National Primate Research Center for other nonhuman primate species [61,62].

### Statistical analyses

Data were entered into an Excel database that was then imported into SAS version 9.1 (SAS Institute, Cary, NC) for statistical analysis. Intra-assay coefficients of variation were assessed using the mean coefficients of variation of control duplicates. Intra-assay coefficients of variation were less than 8%. Interassay coefficients of variation were assessed using the mean coefficients of variation of control (high and low) duplicates in five separate assays. Interassay coefficients of variation were less than 10%.

Continuous measures were summarized by mean, median, and standard deviation. For the fecal degradation samples (N=2 Cleveland {3 samples per Cleveland animal} and 2 wild animals), average cortisol values for each time point (every 30 minutes between 0 and 6 hours) were plotted, and a first-order auto correlation was used to evaluate change over time. For the Cleveland samples, a repeated measures analysis of variance was run on their sequential samples (3 samples collected on non-consecutive days) to evaluate stability in fGM across days.

Samples from the wild habituated animals (Jenny and Etin) collected before (N = 17), during (N = 24) and after (N = 12) tourist visitation were compared using analysis of co-variance (to control for amount of time between defecation and extraction, although this window was always less than 3 hours). Samples from the 4 unhabituated animals collected at initial encounter (N = 19) and after exposure to researchers (N = 7) were compared using analysis of co-variance. Samples from the wild, habituated animals were compared with those of the unhabituated animals (at corresponding time point: before visitation for Jenny and Etin versus initial encounters for the unhabituated animals, and after visitation for Jenny and Etin versus secondary encounters for the unhabituated animals) as well as the captive Cleveland samples using non-parametric Mann-Whitney U tests. Level of significance (alpha) was set at 0.05. Because of low sample size (and subsequent power less than 80%), borderline significant results are interpreted conservatively to avoid conclusions based on false negatives.

## Results

### Fecal degradation

Under field conditions, it is not usually possible to process fecal samples immediately following defecation or collection. Therefore,

we determined the effects of time on hormone degradation using samples from one male and one female adult orangutan (*Pongo pygmaeus pygmaeus*) at Cleveland Metroparks Zoo as well as two wild orangutans (*Pongo pygmaeus morio*) in Borneo. Results suggest that, if samples cannot be extracted immediately following defecation, then they should be extracted within 3 hours following defecation. At that point, variation in fGM increases substantially (Figure 3). Results of the repeated measures analysis on the Cleveland samples indicate stability in fGM within individuals across days (within-subjects effects,  $F = 1.657$ ,  $p = 0.141$ ), but with increased variability within days if samples are processed after more than three hours.

Increased variation in fGM over time likely reflects bacterial metabolism, conjugate cleavage and other physiological processes [63–65]. With this knowledge, we always processed fecal samples from the wild orangutans within three hours of defecation (mean time between defecation and extraction of 2 hours). We contend that results from other studies dependent upon samples collected randomly without witnessing defecation, and those not extracted (or frozen) within 3 hours of defecation, are limited. The time between defecation and extraction should be standardized for a given research population, ideally with all samples and cohorts extracted at approximately the same time since defecation. Samples extracted immediately can yield very different results from those extracted after 3 hours.

#### Effects of tourist visitation and researcher contact

Of the 25 different tourist visitations that were monitored, mean number of tourists at each visitation was 6.2 (SD 5.1) with a mean

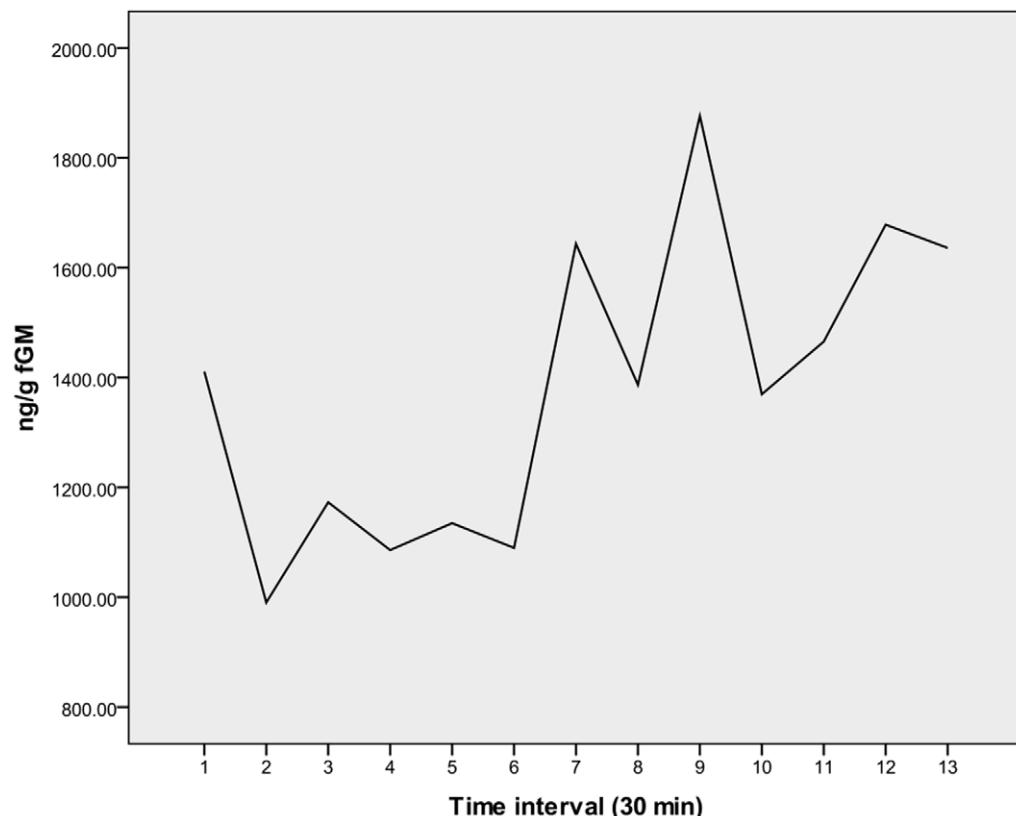
length of visit of 143 min (SD 111). These variables were unassociated with fGM levels in either animal (results not shown). The two habituated animals exhibited no behavioral indicators of stress (i.e., no kiss-squeak or agonistic vocalizations, hiding or fleeing, or branch breaking) and animal physical condition appeared excellent. Height of animal in canopy, tourist behaviors (including grouping, volume of conversation, camera flashes, etc.), number of tourists present, and distance between animals and tourists did not vary significantly to allow further analysis.

In comparing Jenny and Etin's (the habituated animals') samples obtained the day before, the day of and the day after tourist visitation (ANCOVA controlling for time between defecation and sample extraction), fGM levels were significantly elevated in samples collected the day after tourist visitation (indicative of elevated cortisol production on the previous day: during tourist visitation) ( $F = 3.140$ ,  $p = 0.052$ ).

For the unknown animals, fGM levels in samples obtained after previous contact with researchers were numerically higher but not statistically significantly different from their initial samples ( $F = 0.070$ ,  $p = 0.794$ ; ANCOVA again controlling for time between defecation and sample extraction) (see Table 1).

#### Comparisons between habituated and unhabituated animals

In comparing the habituated animals' (Jenny and Etin's) samples obtained the day before tourist visitation ( $N = 17$ ; their baseline samples reflective of no contact with humans the day prior) with the samples obtained from the 2 unhabituated animals



**Figure 3. Analysis of fecal glucocorticoid metabolite degradation over time in captive and wild animals (4 animals total, 8 measurements at each time point) indicates that, if samples cannot be extracted immediately (time point 1), they should be extracted between 1 and 3 hours of defecation (between time points 2 and 6 on figure). The time between defecation and sample extraction should also be recorded.**  
doi:10.1371/journal.pone.0033357.g003

**Table 1.**

Orangutans	Sample paradigm <sup>§</sup>	Mean ng/g fGM (SD)
Habituated (N = 2)	Before tourists (N = 17)	1272 (526)
	During tourists (N = 24)	1367 (704)
	After tourists (N = 12)	1933 (1336)
Unhabituated (N = 4)	First contacts (N = 19) <sup>†</sup>	1821 (812)
	Secondary contacts (N = 7) <sup>‡</sup>	2188 (1630)

<sup>§</sup>Indicates number of samples collected for each time period.

<sup>†</sup>Samples collected upon initial contact with unhabituated animals as well as samples collected from these same animals after no previous contact with researchers for at least 2 days.

<sup>‡</sup>Samples collected within 24 hours of previous contact with researchers.

doi:10.1371/journal.pone.0033357.t001

upon initial contact or no contact with humans in the 2 preceding days (N = 19), fGM levels were lower in the habituated animals ( $z = -2.234$ ,  $p = 0.025$ ). However, fGM levels in the habituated animals' samples the day after tourist visitation (N = 12; when fGM levels are elevated in response to tourist exposure on the previous day) were not statistically different ( $z = -0.169$ ,  $p = 0.902$ ) from samples obtained from the 2 unhabituated animals the day after exposure to the researchers (N = 7).

## Discussion

fGM levels following an anthropogenic stressor provide valuable biometric data that complement previous work illustrating behavioral alterations in wild populations of nonhuman primates in response to habituation and tourism [66–68]. For example, mountain gorillas used in tourism spend more time moving and monitoring and exhibit more self-directed indicators of stress (e.g., self-grooming and scratching) compared to other gorillas [69]. Other studies indicate costs of primate-based tourism, like increased food-associated aggression in adult Tibetan macaques with subsequent major increases in infant mortality [70], increased frequency of threat displays in Tibetan macaques in response to tourist noises [71], and worse coat condition in ring-tailed lemur groups [72]. Populations of black howlers (*Alouatta pigra*) in Belize regularly visited by tourists have significantly higher fGM values than other study groups not visited by tourists [73]. fGM levels were elevated in Barbary macaques (*Macaca sylvanus*) in Morocco only following ‘aggressive’ human behavior towards the animals (and not other tourist behaviors), although self-scratching in males (used here as a proxy for anxiety) was related directly to rates of all tourist-macaque interactions [74].

The present study, although not without limitations, utilizes a more powerful study design (sampling the same animals before, during and after tourist exposure, across several tourist visitation events, in the absence of potential social or nutritional stressors) to identify apparently normal fGM responses to tourist exposure in wild orangutans, with fGM levels increasing in response to tourist visitation, and then returning to a lower level. fGM levels were also lower in habituated than in nonhabituated animals. We therefore conclude that these habituated animals utilized for tourism in Sabah are not chronically stressed, unlike other animals with documented permanent alterations in stress responses. For example, habituated wild adult Magellanic penguins (*Spheniscus magellanicus*) in Argentina exposed to tourism exhibit blunted corticosterone responses following capture and restraint as well as blunted responses following exogenous treatment with adrenocorticotropin hormone [75,76]. They also exhibit lowered baseline

glucocorticoid concentrations compared to non-tourist-visited animals [77]. In contrast, Australian Little penguins (*Eudyptula minor*) at tourist sites exhibit higher glucocorticoid concentrations than animals at non-tourist sites [78], and tourist-exposed Yellow-eyed penguins (*Megadyptes antipodes*) exhibit significantly higher corticosterone responses to capture and restraint [79]. Tourist-exposed marine iguanas (*Amblyrhynchus cristatus*) in the Galápagos islands exhibit reduced stress responses to capture and restraint compared to more isolated animals [80]. Others have identified elevated corticosterone concentrations in iguanas at different tourist sites, with elevated stress responses to restraint and handling compared to animals at undisturbed sites [81]. Others have identified no effects of tourism on glucocorticoid responses in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) [82] or spotted hyenas (*Crocuta crocuta*) [83]. It would appear that different species and populations of animals react differently to human exposure. Differences may also be due, in large part, to variation in tourism intensity (including distance between animals and visitors) and stage of habituation.

It is difficult to interpret the differential causes and consequences of hypo- versus hyper-secretion of glucocorticoids. Insight might be found in cases the etiology of post-traumatic stress disorder (PTSD). Those with PTSD (including civilian trauma like loss of loved one, disaster, sexual abuse, etc.) typically display lower baseline (tonic) cortisol levels compared to healthy controls [84–88]. In contrast, PTSD patients show elevated reactive (phasic) cortisol levels when asked to review personalized trauma scripts [89]. The cause of lowered baseline levels of cortisol in PTSD may be a combination of altered function of the adrenal cortex [90] and enhanced negative feedback on the hypothalamus and anterior pituitary [91].

Other studies have paradoxically observed increased baseline cortisol levels in those with PTSD [92,93], suggesting that hypo- and hyper-secretion are both evident in PTSD. This might be explained by variation in stressor type and personality factors. For example, “uncontrollable” stressors (like abuse, disaster or loss) are frequently associated with chronically elevated cortisol levels [94]. Furthermore, while humans habituating to stress (e.g., Trier Social Stress Test) sometimes exhibit lower salivary and plasma cortisol over time [95], this habituation likely happens most readily in only sub-groups of participants ('low responders') with specific personality traits (those with more self-esteem, less physical health problems and less depressed mood) [96].

Similarly, animal temperament may influence the outcome of anthropogenic disturbance, with some animals responding more negatively to human disturbance [97,98]. In fact, animals likely disperse based partly on individual temperaments as well as the distribution of food, mates and predators. Therefore “endocrinal differences between animals occupying disturbed and undisturbed areas may not be solely a direct effect of stress response to disturbance by humans, but may also reflect the non-random spatial distribution of individuals of different temperaments”, p. 66–67 [98]. In short, elevated fGM levels in tourist-exposed versus unexposed groups [73], as well as elevated fGM in animals interacting (like aggressively, or during feeding) versus not interacting (or neutral interactions) with tourists [74] must be interpreted more conservatively. The effects of stress on wildlife populations are likely context dependent. Elevated fGM may simply reflect normal response to stimuli, with little effects on fitness in the presence of coping/escape mechanisms [99]. Furthermore, it is possible that previous studies are confounded by social interactions among animals (i.e., dominance and sexual interactions among animals resulting in elevated fGM) as well as variations in diet between animals/groups.

Tourism seems to negatively affect fitness of some wildlife populations. Survival is lower in tourist-exposed groups of Amazonian hoatzin juveniles (*Opisthocomus hoatzin*) than undisturbed groups [100]. Similarly, Yellow-eyed penguins (*Megadyptes antipodes*) exposed to tourism exhibit significantly lower breeding success than those in other populations [79]. Permanently altered acute stress responses in habituated animals could be detrimental because normally-functioning acute responses are necessary for ‘fight-or-flight’ reactions [23,25]. Such costs would be higher in pregnant females for which elevated glucocorticoids would be problematic for both the female and her fetus [101–103].

Whatever the case may be, nature-based tourism programs that result in permanent alterations of stress physiology in their animals should not be viewed as ‘sustainable,’ since they violate the basic ‘do no harm’ principle of ecotourism. With increasing demand from tourists to experience direct encounters with wildlife, it is important to produce definitive guidelines that will protect wildlife. Funds must be redirected directly from these programs into species conservation, enabling the employment of more local community members to supervise tourist-animal interactions. Third-party monitoring programs (formed from organizations such as the International Union for the Conservation of Nature, International Ecotourism Society, Convention on Biological Diversity, Conservation International, the Cooperative Research Centre for Sustainable Tourism, the World Tourism Organization and the United Nations Environment Programme) could be utilized to ensure the balance between animal well-being and economic opportunities. Furthermore, animal usage must be kept minimal (at least for pregnant females and ill animals, and during times of resource restriction), and the habituation processes gradual. The costs associated with tourism development must be carefully considered before new groups of animals are habituated. And most importantly, consumer behavior and visitor management must change. The public must be informed better about the risks they may pose to wildlife.

Low sample sizes (for reasons explained above) prevent us from concluding if acute variation in cortisol in these orangutans correlates with survival or reproduction probabilities. It is unknown if there are changes in neurotransmitter or endogenous

opioid levels or receptor functions in these animals. An adrenocortotropic hormone (ACTH)-challenge as well as a ‘capture-and-restraint’ test would certainly yield useful information [104], but are not feasible with wild orangutans. The present sample also cannot assess diurnal cortisol patterns or the cortisol awakening response, both of which can be informative for chronic stress [105–108]. However, the orangutans at this site are generally in excellent health (no visible external signs of illness) with no predation, few social stressors (these animals are largely solitary) and little seasonal fluctuation in food resources. Low levels of predictable disturbance likely result in low physiological impact on these animals. This may be particularly the case with tempered (“low responding”) animals with adequate coping or escape mechanisms. Wildlife at this and other sites should be continually monitored for potential adverse reactions, and the results of continued monitoring of fGM must be interpreted conservatively (i.e., that acute elevation in fGM represents normal response to stimuli, particularly in social species) in the absence of fitness data or physiological measures indicative of chronic stress. In the interim, we continue to recommend relatively low levels of tourism (i.e., predictable, heavily regulated disturbance) of wild apes, although best practices must be location/population/species-specific [57].

## Acknowledgments

Kristen Lukas, Kym Gopp, Joanna Malukiewicz, members of the Cleveland Metroparks Zoo, members of the Kinabatangan Orangutan Conservation Programme, and members of Red Ape Encounters all provided valuable logistical support. Toni Ziegler and Dan Wittwer provided critical assistance with the cortisol assays.

## Author Contributions

Conceived and designed the experiments: MPM MA. Performed the experiments: MPM. Analyzed the data: MPM SP. Contributed reagents/materials/analysis tools: RS LA GF MAR. Wrote the paper: MPM. Collected and extracted the wild samples: RS. Collected and extracted the captive samples: GF MAR. Edited the manuscript: LA SP GF MAR.

## References

- World Travel and Tourism Council (1999) Global importance of tourism. Background paper no. 1, prepared for the Commission on Sustainable Development, seventh session, April 19–30.
- World Tourism Organization (2004) Tourism 2020 Vision. Madrid, Spain: World Tourism Organization.
- Hoyt E (2000) Whale watching 2000: Worldwide tourism numbers, expenditures and expanding socioeconomic benefits. Report to the International Fund for Animal Welfare.
- Ceballos-Lascuráin H (1996) Tourism, Ecotourism, and Protected Areas: The State of Nature-Based Tourism Around the World and Guidelines For Its Development. Gland, Switzerland: World Conservation Union.
- Filion FL, Foley JP, Jacqemot JA (1994) The economics of global ecotourism. In: Munasinghe M, McNealy J, eds. Protected Area Economics and Policy: Linking Conservation and Sustainable Development. Washington, DC: The World Bank. pp 235–252.
- Balmford A, Beresford J, Green J, Naidoo R, Walpole M, et al. (2009) A global perspective on trends in nature-based tourism. PLoS Biol 7: 1–6.
- Walpole MJ, Thouless CR (2005) Increasing the value of wildlife through non-consumptive use? Deconstructing the myths of ecotourism and community-based tourism in the tropics. In: Woodroffe R, S. T, Rabinowitz A, eds. People and Wildlife: Conflict or Coexistence?. Cambridge: Cambridge University Press. pp 122–139.
- Naidoo R, Adamowicz WL (2005) Economic benefits of biodiversity exceed costs of conservation at an African rainforest reserve. Proc Natl Acad Sci USA 102: 16712–16716.
- Kuss FR, Graef AR, Vaske JJ (1990) Recreation Impacts and Carrying Capacity. Washington, DC: National Parks and Conservation Association.
- Amo L, Lopez P, Martin J (2006) Nature-based tourism as a form of predation risk affects body condition and health state of *Podarcis muralis* lizards. Biol Conserv 131: 402–409.
- Beale CM, Monaghan P (2004) Human disturbance: people as predation-free predators? J Appl Ecol 41: 335–343.
- de la Torre S, Snowdon CT, Bejarano M (2000) Effects of human activities on wild pygmy marmosets in Ecuadorian Amazonia. Biol Conserv 94: 153–163.
- Fernandez-Juricic E, Telleria JL (2000) Effects of human disturbance on spatial and temporal feeding patterns of Blackbird *Turdus merula* in urban parks in Madrid, Spain. Bird Study 47: 13–21.
- Griffiths M, van Schaik CP (1993) The Impact of Human Traffic on the Abundance and Activity Periods of Sumatran Rain-Forest Wildlife. Conserv Biol 7: 623–626.
- Mann J, Smuts B (1999) Behavioral development in wild bottlenose dolphin newborns (*Tursiops* sp.). Behaviour 136: 529–566.
- Muehlenbein MP, Martinez LA, Lemke AA, Ambu L, Nathan S, et al. (2008) Perceived vaccination status in ecotourists and risks of anthroponozoonoses. Ecohealth 5: 371–378.
- Muehlenbein MP, Ancrenaz M (2009) Minimizing pathogen transmission at primate ecotourism destinations: the need for input from travel medicine. J Travel Med 16: 229–232.
- Muehlenbein MP, Martinez LA, Lemke AA, Ambu L, Nathan S, et al. (2010) Unhealthy travelers present challenges to sustainable primate ecotourism. Travel Med Infect Dis 8: 169–175.
- McEwen BS, Wingfield JC (2003) The concept of allostasis in biology and biomedicine. Horm Behav 43: 2–15.
- Abbott DH (1987) Behaviourally mediated suppression of reproduction in female primates. J Zool 213: 455–470.
- Avitsur R, Stark JL, Sheridan JF (2001) Social stress induces glucocorticoid resistance in subordinate animals. Horm Behav 39: 247–257.
- Moberg GP, Mench JA, eds. The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. Wallingford, CT: CABI Press.

23. Sapolsky RM (1992) Neuroendocrinology of the stress response. In: Becker JB, Breedlove SM, Crews D, eds. *Behavioural Endocrinology*. Cambridge, MA: MIT Press. pp 287–324.
24. Wingfield JC, Romero LM (1999) Adrenocortical responses to stress and their modulation in free-living vertebrates. In: McEwan B, ed. *Handbook of Physiology – Coping with the Environment*. New York: Oxford University Press. pp 211–234.
25. Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21: 55–89.
26. Busch DS, Hayward LS (2009) Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables. *Biol Conserv* 142: 2844–2853.
27. Keay JM, Singh J, Gaunt MC, Kaur T (2006) Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. *J Zoo Wildl Med* 37: 234–244.
28. Millspaugh JJ, Washburn BE (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *Gen Comp Endocrinol* 138: 189–199.
29. Schoech SJ, Lipar JL (1998) Conservation endocrinology: Field endocrinology meets conservation biology. In: Fielder PL, Kareiva PM, eds. *Conservation Biology for the Coming Decade*. New York: Chapman & Hall. pp 461–477.
30. Walker BG, Boersma PD, Wingfield JC (2005) Field endocrinology and conservation biology. *Integr Comp Biol* 45: 12–18.
31. Wikelski M, Cooke SJ (2006) Conservation physiology. *Trends Ecol Evol* 21: 38–46.
32. Wingfield JC, Hunt K, Breuner C, Dunlap K, Fowler GS, et al. (1997) Environmental stress, field endocrinology, and conservation biology. In: Clemons JR, Buchholz R, eds. *Behavioral Approaches to Conservation in the Wild*. Cambridge: Cambridge University Press.
33. Tarlow EM, Blumstein DT (2007) Evaluating methods to quantify anthropogenic stressors on wild animals. *Appl Anim Behav Sci* 102: 429–451.
34. Sheriff MJ, Krebs CJ, Boonstra R (2010) Assessing stress in animal populations: Do fecal and plasma glucocorticoids tell the same story? *Gen Comp Endocrinol* 166: 614–619.
35. Millspaugh JJ, Woods RJ, Hunt KE, Raedeke KJ, Brundige GC, et al. (2001) Fecal glucocorticoid assays and the physiological stress response in elk. *Wildl Soc Bull* 29: 899–907.
36. Smith J, Wingfield JC, Veit R (1994) Adrenocortical response to stress in the common diving petrel, *Pelecanoides urinatrix*. *Physiol Zool* 67: 526–537.
37. von der Ohe CG, Wasser SK, Hunt KE, Servheen C (2004) Factors associated with fecal glucocorticoids in Alaskan brown bears (*Ursus arctos horribilis*). *Physiol Biochem Zool* 77: 313–320.
38. Creel S, Fox JE, Hardy A, Sands J, Garrott B, et al. (2002) Snowmobile activity and glucocorticoid stress responses in wolves and elk. *Conserv Biol* 16: 809–814.
39. Barja I, Silvan G, Rosellini S, Pinciro A, Gonzalez-Gil A, et al. (2007) Stress physiological responses to tourist pressure in a wild population of European pine marten. *J Steroid Biochem* 104: 136–142.
40. Thiel D, Jenni-Ernemann S, Braunisch V, Palme R, Jenni L (2008) Ski tourism affects habitat use and evokes a physiological stress response in capercaille *Tetrao urogallus*: A new methodological approach. *J Appl Ecol* 45: 845–853.
41. Bechner JC, Whitten PL (2004) Modifications of a field method for fecal steroid analysis in baboons. *Physiol Behav* 82: 269–277.
42. Heistermann M, Palme R, Ganswindt A (2006) Comparison of different enzyme-immunoassays for assessment of adrenocortical activity in primates based on fecal analysis. *Am J Primatol* 68: 257–273.
43. Hodges JK, Heistermann M (2003) Field endocrinology: monitoring hormonal changes in free-ranging primate. In: Setchell JM, Curtis DJ, eds. *Field and Laboratory Methods in Primatology: A Practical Guide*. New York: Cambridge University Press. pp 282–294.
44. Mohle U, Heistermann M, Palme R, Hodges JK (2002) Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. *Gen Comp Endocrinol* 129: 135–145.
45. Whitten PL, Brockman DK, Stavisky RC (1998) Recent advances in noninvasive techniques to monitor hormone-behavior interactions. *Am J Phys Anthropol* S27: 1–23.
46. Ziegler TE, Witwer DJ (2005) Fecal steroid research in the field and laboratory: improved methods for storage, transport, processing, and analysis. *Am J Primatol* 67: 159–174.
47. Bechner JC, Phillips-Conroy JE, Whitten PL (2005) Female testosterone, dominance rank, and aggression in an Ethiopian population of hybrid baboons. *Am J Primatol* 67: 101–119.
48. Muehlenbein MP, Watts DP, Whitten PL (2004) Dominance rank and fecal testosterone levels in adult male chimpanzees (*Pan troglodytes schweinfurthii*) at Ngogo, Kibale National Park, Uganda. *Am J Primatol* 64: 71–82.
49. Strier KB, Ziegler TE (2005) Variation in the resumption of cycling and conception by fecal androgen and estradiol levels in female Northern Muriquis (*Brachyteles hypoxanthus*). *Am J Primatol* 67: 69–81.
50. Shimizu K (2005) Studies on reproductive endocrinology in non-human primates: application of non-invasive methods. *J Reprod Develop* 51: 1–13.
51. Muehlenbein MP (2006) Intestinal parasite infections and fecal steroid levels in wild chimpanzees. *Am J Phys Anthropol* 130: 546–550.
52. IUCN (2010) IUCN Red List of Threatened Species. Version 2010. 4 ed. Gland, Switzerland: International Union for Conservation of Nature, Species Survival Commission.
53. Aveling RJ, Mitchell A (1982) Is rehabilitating orangutans worthwhile? *Oryx* 16: 263–271.
54. Rajaratnam P, Pang C, Lackman-Ancrenaz I (2008) Ecotourism and indigenous communities: The Lower Kinabatangan experience. In: Connell J, Rugendyke B, eds. *Tourism at the Grassroots: Villagers and Visitors in the Asia Pacific*. London: Routledge. pp 236–255.
55. Ministry of Culture, Arts and Tourism (1996) *National Ecotourism Plan*. Kuala Lumpur, .
56. Ancrenaz M, Dabek L, O'Neil S (2007) The costs of exclusion: recognizing a role for local communities in biodiversity conservation. *PLoS Biol* 5: e289.
57. Macfie EJ, Williamson EA (2010) Best Practice Guidelines for Wild Great Ape Tourism. Gland, Switzerland: IUCN/SSC Primate Specialist Group.
58. Ancrenaz M (2006) Guidelines for Tourists Visiting the Red Ape Encounter Habituated Orang-Utans. Sabah, Malaysia: Kinabatangan Orang-Utan Conservation Project.
59. Milton K, Demment MW (1988) Digestion and passage kinetics of chimpanzees fed high and low fiber diets and comparison with human data. *J Nutr* 118: 1082–1088.
60. Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, et al. (2000) A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *Gen Comp Endocrinol* 120: 260–275.
61. Van Belle S, Estrada A, Ziegler TE, Strier KB (2009) Sexual behavior across ovarian cycles in wild black howler monkeys (*Alouatta pigra*): male mate guarding and female mate choice. *Am J Primatol* 71: 153–164.
62. Van Belle S, Estrada A, Ziegler TE, Strier KB (2009) Social and hormonal mechanisms underlying male reproductive strategies in black howler monkeys (*Alouatta pigra*). *Horm Behav* 56: 355–363.
63. Adlercreutz H, Martin F, Pulkkinen M, Dencker H, Rimer U, et al. (1976) Intestinal metabolism of estrogens. *J Clin Endocrinol Metab* 43: 497–505.
64. Bokkenheuser VD, Winter J (1980) Biotransformation of steroid hormones by gut bacteria. *Am J Clin Nutr* 33: 2502–2506.
65. Washburn BE, Millspaugh JJ (2002) Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. *Gen Comp Endocrinol* 127: 217–222.
66. Blom A, Cipollatta C, Brunsting AM, Prins HH (2004) Behavioral responses of gorillas to habituation in the Dzanga-Ndoki National Park, Central African Republic. *Int J Primatol* 25: 179–196.
67. Doran-Sheehy DM, Derby AM, Greer D, Mongo P (2007) Habituation of western gorillas: the process and factors that influence it. *Am J Primatol* 69: 1354–1369.
68. Johns BG (1996) Responses of chimpanzees to habituation and tourism in the Kibale Forest, Uganda. *Biol Conserv* 78: 257–262.
69. Muyambi F (2005) The impact of tourism on the behavior of mountain gorillas. *Gorilla J* 30: 14–15.
70. Berman CM, Li J, Ogawa H, Ionica C, Yin H (2007) Primate Tourism, Range Restriction, and Infant Risk Among *Macaca thibetana* at Mt. Huangshan, China. *Int J Primatol* 28: 1123–1141.
71. Ruesto LA, Sheeran LK, Matheson MD, Li J-H, Wagner RS (2010) Tourist behavior and decibel levels correlate with threat frequency in Tibetan macaques (*Macaca thibetana*) at Mt. Huangshan, China. *Prim Conserv* 25: 99–104.
72. Jolly A (2009) Coat condition of ringtailed lemurs, *Lemur catta* at Berenty Reserve, Madagascar: I. Differences by age, sex, density and tourism, 1996–2006. *Am J Primatol* 71: 191–198.
73. Behie AM, Pavelka MS, Chapman CA (2010) Sources of variation in fecal cortisol levels in howler monkeys in Belize. *Am J Primatol* 72: 600–606.
74. Maréchal L, Semple S, Majolo B, Qarro M, Hiestermann M, et al. (In press) Impacts of tourism on anxiety and physiological stress levels in wild male Barbary macaques. *Biol Conserv*.
75. Walker BG, Boersma PD, Wingfield JC (2005) Physiological and Behavioral Differences in Magellanic Penguin Chicks in Undisturbed and Tourist-Visited Locations of a Colony. *Conserv Biol* 19: 1571–1577.
76. Walker BG, Dee Boersma P, Wingfield JC (2006) Habituation of Adult Magellanic Penguins to Human Visitation as Expressed through Behavior and Corticosterone Secretion. *Conserv Biol* 20: 146–154.
77. Fowler G (1999) Behavioral and hormonal responses of Magellanic penguins (*Spheniscus magellanicus*) to tourism and nest site visitation. *Biol Conserv* 90: 143–149.
78. Turner E (2001) The effects of human activity on the behavioural and hormonal stress response of Little Penguins (*Eudyptula minor*) on Phillip Island. Sydney: Macquarie University.
79. Ellenberg U, Setiawan AN, Cree A, Houston DM, Seddon PJ (2007) Elevated hormonal stress response and reduced reproductive output in Yellow-eyed penguins exposed to unregulated tourism. *Gen Comp Endocrinol* 152: 54–63.
80. Romero L, Wikelski M (2002) Exposure to tourism reduces stress-induced corticosterone levels in Galápagos marine iguanas. *Biol Conserv* 108: 371–374.
81. French SS, DeNardo DF, Greives TJ, Strand CR, Demas GE (2010) Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (*Amblyrhynchus cristatus*). *Horm Behav* 58: 792–799.
82. Dalmau A, Ferret A, Chacon G, Manteca X (2007) Seasonal Changes in Fecal Cortisol Metabolites in Pyrenean Chamois. *J Wildlife Manage* 71: 190–194.

83. Van Meter PE, French JA, Dloniak SM, Watts HE, Kolowski JM, et al. (2009) Fecal glucocorticoids reflect socio-ecological and anthropogenic stressors in the lives of wild spotted hyenas. *Horm Behav* 55: 329–337.
84. Mason JW, Giller EL, Kosten TR, Ostroff RB, Podd L (1986) Urinary free-cortisol levels in posttraumatic stress disorder patients. *J Nerv Ment Dis* 174: 145–149.
85. Oliff M, Guzelcan Y, de Vries GJ, Assies J, Gersons BP (2006) HPA- and HPT-axis alterations in chronic posttraumatic stress disorder. *Psychoneuroendocrinology* 31: 1220–1230.
86. Pervanidou P, Chrousos GP (2010) Neuroendocrinology of post-traumatic stress disorder. *Prog Brain Res* 182: 149–160.
87. Yehuda R, Kahana B, Binder-Brynes K, Southwick SM, Mason JW, et al. (1995) Low urinary cortisol excretion in Holocaust survivors with posttraumatic stress disorder. *Am J Psychiatry* 152: 982–986.
88. Yehuda R, Boisneau D, Lowy MT, Giller EL, Jr. (1995) Dose-response changes in plasma cortisol and lymphocyte glucocorticoid receptors following dexamethasone administration in combat veterans with and without posttraumatic stress disorder. *Arch Gen Psychiatry* 52: 583–593.
89. Elzinga BM, Schmahl CG, Vermetten E, van Dyck R, Bremner JD (2003) Higher cortisol levels following exposure to traumatic reminders in abuse-related PTSD. *Neuropsychopharmacology* 28: 1656–1665.
90. Kanter ED, Wilkinson CW, Radant AD, Petrie EC, Dobie DJ, et al. (2001) Glucocorticoid feedback sensitivity and adrenocortical responsiveness in posttraumatic stress disorder. *Biol Psychiatry* 50: 238–245.
91. Yehuda R, Southwick SM, Krystal JH, Bremner D, Charney DS, et al. (1993) Enhanced suppression of cortisol following dexamethasone administration in posttraumatic stress disorder. *Am J Psychiatry* 150: 83–86.
92. Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, et al. (1997) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am J Psychiatry* 154: 624–629.
93. Pitman RK, Orr SP (1990) Twenty-four hour urinary cortisol and catecholamine excretion in combat-related posttraumatic stress disorder. *Biol Psychiatry* 27: 245–247.
94. Miller KK, Wexler TL, Zha AM, Lawson EA, Meenaghan EM, et al. (2007) Androgen deficiency: association with increased anxiety and depression symptom severity in anorexia nervosa. *J Clin Psychiatry* 68: 959–965.
95. Schommer NC, Hellhammer DH, Kirschbaum C (2003) Dissociation between reactivity of the hypothalamus-pituitary-adrenal axis and the sympathetic-adrenal-medullary system to repeated psychosocial stress. *Psychosomatic Medicine* 65: 450–460.
96. Kirschbaum C, Prussner JC, Stone AA, Federenko I, Gaab J, et al. (1995) Persistent high cortisol responses to repeated psychological stress in a subpopulation of healthy men. *Psychosomatic Medicine* 57: 468–474.
97. Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, et al. (1999) Coping styles in animals: current status in behavior and stress-physiology. *Neurosci Biobehav Rev* 23: 925–935.
98. Martin JG, Reale D (2008) Animal temperament and human disturbance: implications for the response of wildlife to tourism. *Behav Process* 77: 66–72.
99. Treves A, Brandon K (2005) Tourism impacts on the behaviour of black howler monkeys (*Alouatta pigra*) at Lamanai, Belize. In: Paterson JD, Wallis J, eds. *Commensalism and Conflict: The Human–Primate Interface*. Norman, OK: American Society of Primatologists Publication. pp 146–167.
100. Müllner A (2004) Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (*Opsithocomus hoazin*). *Biol Conserv* 118: 549–558.
101. Coe CL, Lubach GR (2005) Developmental consequences of antenatal dexamethasone treatment in nonhuman primates. *Neurosci Biobehav Rev* 29: 227–235.
102. Obel C, Hedegaard M, Henriksen TB, Secher NJ, Olsen J, et al. (2005) Stress and salivary cortisol during pregnancy. *Psychoneuroendocrinology* 30: 647–656.
103. Ranjit N, Young EA, Kaplan GA (2005) Material hardship alters the diurnal rhythm of salivary cortisol. *Int J Epidemiol* 34: 1138–1143.
104. Baltic M, Jenni-Eiermann S, Arlettaz R, Palme R (2005) A noninvasive technique to evaluate human-generated stress in the black grouse. *Ann N Y Acad Sci* 1046: 81–95.
105. Clow A, Thorn L, Evans P, Hucklebridge F (2004) The awakening cortisol response: methodological issues and significance. *Stress* 7: 29–37.
106. Dowd JB, Simaneck AM, Aiello AE (2009) Socio-economic status, cortisol and allostatic load: a review of the literature. *Int J Epidemiol* 38: 1297–1309.
107. Rystedt LW, Cropley M, Devereux JJ, Michalianou G (2008) The relationship between long-term job strain and morning and evening saliva cortisol secretion among white-collar workers. *J Occup Health Psych* 13: 105–113.
108. Wust S, Wolf J, Hellhammer DH, Federenko I, Schommer N, et al. (2000) The cortisol awakening response - normal values and confounds. *Noise Health* 2: 79–88.