MEASURING FAECAL GLUCOCORTICOID METABOLITES AS A NON-INVASIVE TOOL FOR MONITORING ADRENOCORTICAL ACTIVITY IN SOUTH AMERICAN CAMELIDS

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by

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1. INTRODUCTION

The South American camelids (SACs) include the domestic species alpaca and llama and the wild species vicuña and guanaco, and each of the four species has a diploid number (2n = 74) of chromosomes (MARIN et al., 2007). Alpaca and vicuña are well known to be fine fiber producers, whereas llamas are frequently used for transportation activities. Inbreeding between the four species occurs with fertile hybrid descendants, but this may lead to their genetic degeneration (KADWELL et al., 2001). Genetic evidence suggests that alpacas and llamas were domesticated from their ancestors vicuña and guanaco in South America before the Spanish colonization, and llama with alpaca were hybridized resulting in a reduction of the quality of fine fiber (KADWELL et al., 2001). Nowadays, vicuña and alpaca fiber production industry benefits economically to highland farmers: Furthermore llamas are commonly used for transportation in native communities where car transportation is not present. Since last century, far from South America, alpaca and llama populations have been increasing and colonizing new geographical areas such as North America, Australia, Asia, and Europe with different purposes as companion pets, or economic fiber industry. Therefore, studies on several aspects of these animals in particular on welfare are of interest.

The alpaca is one of the most important camelids with largest population raised in the highlands of Peru. The breeding of this population is halted by high rate of embryo mortality and reproductive disorders of females and males alpacas (FERNANDEZ-BACA et al., 1970). Most of the research conducted so far has addressed the physiology of reproduction, and mainly pathological factors involving bacteria, virus and parasites (LEGUIA 1991; CEBRA et
al., 2000). There is limited number of studies dealing with animal welfare issues. Although alpaca farming systems are distinct in different parts of the world, in all of them, animals will always be exposed to stressful situations. Therefore, changes in farming systems reducing the exposure to stress will ensure optimal impact on animal welfare and a good herd health status and sustainable production.

In farmed and wild animals, the exposures to environmental stimuli that lead to the imbalance of homeostasis are called stressors. Afterwards the animal body produces complex responses leded by the brain (MÖSTL and PALME, 2002). These responses involve changes in behaviour, immune system activity, activation of the hypothalamic-pituitary-adrenal (HPA) axis, and the autonomous nervous system (ANS). Stress is good for the animal since it might help in the adaptation into a new environment, but if the stress responses persist for a long time, they might negatively affect the reproduction, immunity and growth status of the animal (MOBERG, 2000). HPA activity in South American camelids has been studied based on the increase of cortisol concentrations by invasive methods using blood samples which cause additional sampling stress. This limitation can be overcome by evaluating non-invasive methodologies alternative to the invasive sampling collection that do not involve manipulation and capture of the animals. Such methodology was developed to measure cortisol metabolites from faecal samples and it is been extensively used in domestic and wild ruminants (PALME and MÖSTL, 1997; DEHNHARD et al., 2001; MÖSTL et al., 2002; LEXEN et al., 2008; KLEINSASSER et al., 2010; PALME, 2012).
The **aim** of this work was to monitor the adrenocortical activity in alpacas and llamas by using pharmacological stimulation; and in vicuñas by physiological stressors. In this thesis I describe the analysis of faecal cortisol metabolites after pharmacological stimulation of the adrenal cortex in alpacas and llamas, and after a physiological stimulation with anthropogenic stressors such as handling and shearing in vicunas.

The **hypothesis** of this work was that faecal cortisol metabolites represent reliable and sensitive indicators of adrenocortical activity for non-invasive monitoring of stress in South American camelids.
2. LITERATURE REVIEW

The following reviews of literature will summarize the stress definition, the secretion, metabolism and excretion of glucocorticoids, studies related to stress in South American camelids, and fecal cortisol metabolites as a measure of stress.

2.1 Stress

Stress refers to a series of changes in behaviour, neuroendocrine, autonomic and immune systems that are caused by environmental stimuli that lead to the imbalance of homeostasis (MÖSTL and PALME, 2002; McEWEN, 2007). Also, a stress situation can be defined as the response to a noxious stimulus (or stressor) and it is composed of adaptive physiological and behavioural changes (DICKENS et al., 2010). Stress is divided in acute and chronic responses depending on the intensity and duration of the stressor (DALLMAN, 2003) and the difference between these two responses was described by DICKENS et al. (2010).

An acute stress response initially involves a fast “fight-or-flight” response and the slower glucocorticoid production. This response allows for a rapid reaction to the stressor and can be beneficial for the animal to survive. The fast “fight-or-flight” response includes elevation of heart rate, increased blood pressure, and mobilization of energy sources to the central nervous system and muscles. This initial response is followed by the slower hormonal stress response, in which the adrenal gland secretes glucocorticoid hormones. Cortisol or
corticosterone are predominantly secreted (DALLMAN, 2003). A negative feedback suppresses glucocorticoid (GC) release as the stressor is decreased or nullified (DICKENS et al., 2010).

In a chronic stress situation, the animal is exposed to a persistent stressor or to a series of acute stressors that initiates multiple consecutive stress responses. The initial physiological and behavioural changes that facilitate immediate survival are not longer helpful and may be detrimental for the animal. Dysregulation of mediators such as the chronic release of CG and catecholamines can lead to changes in the body and brain that can be deleterious for the animal (DALLMAN, 2003; DICKENS et al., 2010). Changes in the physiological system during acute and chronic response are presented in Table 1.

Table 1. Responses of the behavioural and physiological system during acute and chronic stress. Modified from DICKENS et al. (2010)

<table>
<thead>
<tr>
<th>Behavioural system - Physiological system</th>
<th>ACUTE Stress</th>
<th>CHRONIC Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune system</td>
<td>Mobilization of immune system</td>
<td>Immunosuppression Autoimmune reactions</td>
</tr>
<tr>
<td>Glucocorticoids – HPA axis</td>
<td>Energy mobilization Inhibit reproduction</td>
<td>Immunosuppression Increased energy requirement Reproductive suppression</td>
</tr>
<tr>
<td>Catecholamines – cardiovascular</td>
<td>Fight of flight response Energy mobilization</td>
<td>Attenuated fight or flight response Dysregulated metabolism</td>
</tr>
<tr>
<td>Coping strategy – behavioural</td>
<td>Fleeing or freezing Increased food intake Increased vigilance</td>
<td>Anxiety Attempts to return home to decrease stress exposure</td>
</tr>
</tbody>
</table>
2.2 Secretion, metabolism and excretion of glucocorticoids

The glucocorticoid secretion is controlled by the hypothalamic-pituitary-adrenal (HPA) axis and is regulated by diurnal signals and activated by stress (DALLMAN et al., 2002). HPA activity is controlled by the corticotropin releasing hormone (CRH) and arginine vasopresin (AVP). After exposure to a stressor, the nervous system stimulates a group of hypophysiotropic neurons located in the hypothalamus, then CRH/AVP is liberated in the pituitary-portal circulation which stimulate the release of adrenocorticotropic hormone (ACTH) into the general circulation that stimulates the production of adrenal glucocorticoids in the systemic circulation. Circulating cortisol is metabolized in the liver and excreted via urine and faeces (PALME et al., 1997; MÖSTL and PALME, 2002). In faecal samples of ruminants there is a large number of metabolites, but cortisol itself was not found in the faeces of sheep (PALME and MÖSTL, 1997).

In summary, the increases of glucocorticoids after stress affect various systems allowing the animal to face the challenge of the stressor and survive. The responses include temporary increases in cardiovascular and cognitive activity and glucose mobilization, while the immune, digestive and reproductive function is suppressed (DALLMAN 2003).
2.3 Stress measurement in SACs

An abstract from a conference in Peru by GUERRA-GARCIA et al. (1975) published results of adrenocortical stimulation in 28 alpaca by injection of 0.25 mg of ACTH. There was a great individual variation in responses, and after chromatographic studies corticosterone seemed to be the most important glucocorticoid in alpacas (GUERRA-GARCIA et al., 1975). Following, the next early efforts to evaluate stress in alpacas using heart rate and behaviour as stress indicators, demonstrated that social companion of familiar alpacas helped to reduce stress during confinement, but if the animal was restrained there was no reduction of heart rate and behaviour (POLLARD and LITTLEJOHN, 1995). There was a significant increase of cortisol in serum in alpacas after transportation, however behavioural characteristics and heart rate were not found to be useful indicators of stress (ANDERSON et al., 1999b). In another study, it was found that the concentrations of cortisol in saliva did not increase after transportation as compare to cortisol in serum, therefore cortisol in saliva was not a sensitive indicator of stress after transportation (ANDERSON et al., 1999a). HEATH et al. (2001) demonstrated that whole-body shearing of alpacas could have a beneficial effect on thermoregulation (heat disipation) when used as a preventative measure against heat stress.

In alpacas, increased concentrations of cortisol in serum were found after birth and weaning (stressors) which decreased to baseline levels during time (BRAVO et al., 2001). In guanacos, it was found high concentrations of cortisol in plasma and increased neutrophil-lymphocyte ratios after transportation. However, in this study, blood glucose concentration,
heart rate and body weight values were not associated with the stress response (ZAPATA et al., 2004). In the course of the “Chacu”, the ancient tradition of capture and handling for shearing of vicuñas, plasma cortisol levels were significantly increased after capture, transport, and captivity of this species (BONACIC et al., 2003b). Additionally, challenge of the adrenal cortex by injection of ACTH in vicuñas resulted in a 4.5-fold increase of cortisol concentration in plasma within the first hour, decrease of lymphocytes and increase of neutrophils (BONACIC et al.2003a). Undesirable consequences of the Chacu are injuries, body trauma, death, separation of mothers and their newborns, abortion of pregnant females (BONACIC et al., 2006)

2.4 Faecal cortisol metabolites as a measure of stress

Initial studies using the infusion of radio-labeled cortisol came up to understand the metabolism and excretion of steroids in urine and faeces in sheep, ponies, and pigs (PALME et al., 1996). High amounts of radioactivity were found in urine and lower levels in faeces. Then, more studies of faecal glucocorticoid metabolites were performed and two enzyme immunoassays (EIA) validated. These EIAs cross-react with a group of metabolites sharing the same functional group. For instance, an 11-oxoaetiocholanolone EIA, first described by PALME and MÖSTL (1997) measures 11,17-dioxoandrostanes. This was worldwide the first EIA to measure cortisol metabolites. In the meantime, several antibodies have been designed to measure groups of metabolites present in faeces and have been validated for different
species (PALME, 2012). These EIAs provided the basis for a non-invasive evaluation of adrenocortical activity. The biological relevance of this non-invasive method has been proven in ruminants following stimulation (ACTH) or suppression (dexamethasone) of cortisol release by the adrenal cortex and transportation stress (MÖSTL et al., 2002; PALME et al., 2005). This validated method is able to monitor stress hormone metabolites in faecal samples with several applications in different species.

Measurement of glucocorticoid metabolites in faecal samples is feasible and less complicated than measurement of cortisol from blood or saliva samples in particular from wild animals such as vicuñas. Using this approach the capture of these animals is not necessary and therefore the read out are not affected by the capture itself. Another advantage of this methodology is the application for long-term studies (MÖSTL and PALME, 2002).

There are considerations to be taken into account during sample collection for the measurement of glucocorticoid metabolites (reviewed by PALME, 2012): Samples should be collected at known time shortly after defecation and stored immediately at -20 °C to avoid degradation of steroids due to bacterial enzymatic activity (BOKKENHEUSER et al., 1978; MÖSTL et al., 1999). Samples should be homogenised before proceeding to extraction as within-sample variation might exist (PALME et al., 2005). Individual variability among animals might exist when measuring stress responses that can interfere with differences between experimental groups. In this case, large number of animals should be sampled or the response expressed as percentage of increase above baseline values and in this way each animal acts as its own control (PALME et al., 2005). An antibody that detects the majority of
the excreted metabolites and the time of excretion should also be determined for each animal species. Other factors that might affect fecal metabolite measurements are the reproductive cycle, pregnancy, sex and season of the year (PALME, 2012).
3. Manuscript

Measuring faecal glucocorticoid metabolites as a non-invasive tool for monitoring adrenocortical activity in South American camelids

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Measuring faecal glucocorticoid metabolites as a non-invasive tool for monitoring adrenocortical activity in South American camelids

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Abstract
The welfare and productivity of South American camelids may be affected by stressful events.
The purpose of this study was to validate a non-invasive method for stress monitoring using faecal samples and to apply it to evaluate a stressful event, such as confinement. For physiological validation, nine alpacas (Vicugna pacos) and six llamas (Lama glama) were subjected to pharmacological stimulation of their adrenal cortex. Serial faecal samples were collected during 48 h before and after stimulation. During confinement, faecal samples from six llamas were collected twice per day during six consecutive days. Faeces belonging to 18 vicuñas (Vicugna vicugna) were collected before and one day after their capture for confinement (Chacu). Faecal cortisol metabolites (FCM) were extracted from each sample
and quantified by an 11-oxoaetiocholanolone enzyme immunoassay. Thirty-three and 28 h (median) after ACTH stimulation, FCM concentrations peaked with a ten- and eight-fold increase (median) above baseline in alpacas and llamas, respectively. There were no significant differences in FCM concentrations between sexes. In llamas, FCM concentrations peaked (4.7 times higher than baseline) after five days of confinement in females and after three days (2.7 times) in males. In vicuñas, three times higher FCM levels were observed the day after the start of confinement (in comparison to the starting values). Based on our findings, this non-invasive method is well suited to measure adrenocortical activity in alpacas, llamas and vicuñas. Thus, this method could help to improve management, handling and welfare in wild and domesticated South American camelids.

**Keywords:** animal welfare, faeces, glucocorticoids, New World camelids, plasma, stress

**Introduction**

South American camelids (SAC) include the domesticated alpacas (*Vicugna pacos*) and llamas (*Lama glama*) along with the wild species, vicuña (*Vicugna vicugna*) and guanaco (*Lama guanicoe*). There is a growing population of alpacas and llamas in the USA, Europe and Australia, but more than 95% of them are located in the Andean region of Perú and Bolivia where they provide fibre, meat, pelts and faeces, the latter being used as fuel and fertiliser. Worldwide, alpacas and llamas are used for breeding, fibre production, trekking, and as companion animals. Moreover, llamas are utilised as pack animals in some isolated regions (Fernández-Baca 1993).
Reproductive disorders in SAC (especially in females) are one of the main problems that make their reproductive management complicated for breeders and veterinarians (Vaughan & Tibary 2006). Stressful situations during common management activities, such as transportation, handling, and restraint for medication or venipuncture are considered to contribute to this problem. For example, stress from transportation has been associated with foetal losses in alpacas (Knight et al 1995) and embryo mortality seems to be the main cause of low reproductive efficiency in alpacas (Fernández-Baca 1993). Moreover, it is hypothesised that stressors such as long walks, antiparasitic shower-dip and shearing may result in a fulminant systemic infection, known as ‘Alpaca fever’ (Hewson & Cebra 2001). Stress triggers a physiological response that involves a cascade of events ending with the release of glucocorticoids (cortisol, corticosterone) by the adrenal cortex (Möstl & Palme 2002; Palme 2012). Initial attempts to evaluate stress in SAC began with studies measuring cortisol in plasma samples. Anderson et al (1999a) found a significant increase in serum cortisol concentration in alpacas following transportation, while behavioural characteristics and heart rate were not found to be useful indicators of stress. In a later study, concentration of salivary cortisol did not rise after transportation while serum cortisol did, suggesting that cortisol in saliva is not a sensitive indicator of transport stress (Anderson et al 1999b). High concentrations of serum cortisol were found in alpacas after birth and weaning (stressors) and decreased to baseline values with time (Bravo et al 2001).

Similarly, in wild SAC species, Zapata et al (2004) found significant increases in plasma cortisol concentrations and increased neutrophil:lymphocyte ratios in guanacos after transportation. However, in their study, blood glucose concentration, heart rate and bodyweight values were not associated with the stress response. During the Chacu (the
ancient tradition of capture and handling events for shearing of vicuñas) plasma cortisol levels were greatly increased after capture, transport, and captivity of this species (Bonacic et al 2003b). Furthermore, stimulation of the adrenal cortex by injection of ACTH in a group of vicuñas resulted in a 4.5-fold increase of plasma cortisol concentrations (Bonacic et al 2003a). Blood sample collection has the limitation of being stressful itself, especially in wild animals. Therefore, a non-invasive method such as faecal cortisol metabolites (FCM) monitoring may be more appropriate. Faecal samples can be collected without causing stress to the animal and FCM values are not affected by hormonal pulsatile secretion (burst-like or episodic; Palme 2012). Glucocorticoids are metabolised by the liver, excreted via faeces and the metabolites can be detected in faecal methanol extracts by a group-specific enzyme immunoassay (EIA; Palme & Möstl 1997). This method has proven useful to evaluate adrenocortical activity in wild and domestic ruminants such as deer, goat, cattle and sheep (Palme et al 1999; Möstl et al 2002; Huber et al 2003; Pesenhofer et al 2006; Lexen et al 2008; Kleinsasser et al 2010; Rouha-Mülleder et al 2010; Konjević et al 2011). However, there is no information about such a measurement in SAC. Due to expressed species differences regarding metabolism and excretion of glucocorticoids (Palme et al 2005), analogous conclusions cannot be drawn from other species (Palme 2005).

The aim of this study was to select and validate an EIA for measuring faecal cortisol metabolites (FCM) to evaluate adrenocortical activity in SAC. Therefore, stimulation of the adrenal cortex (ACTH challenge test) and confinement in alpacas, llamas and vicuñas were performed.
Material and Methods

All experimental procedures were approved by the Cayetano Heredia University, Lima, Perú, Ethical committee for animal use (SIDISI 57461).

**Study animals and housing**

Clinically healthy adults, between two to four years of age, that had not participated previously in any experimental studies were randomly selected from the Santa-Ana INIA Research Station Farm in Huancayo, Perú (latitude 12°0’50” S, longitude 75°13’9” W; altitude 3,300 m) where animals live under free-range conditions grouped only during the night in an open-air facility. In total, nine alpacas of the Huacaya breed (three non-pregnant females, three males and three castrated males) and two groups, each of six llamas of the Ccara breed (three non-pregnant females and three males), were utilised. In order to collect individual samples, each animal was housed indoors in a room of 9 m², with access to natural light and with an average temperature of 15°C. All animals were able to interact visually and aurally through open windows. Animals were exposed to daily management activities such as cleaning, supply of alfalfa hay and water ad libitum.

The vicunas participating in this study belonged to the National Reserve Pampa Galeras, Ayacucho, Perú (latitude 14°39’ S, longitude 74°20’ W). These wild animals live without human intervention, in the semi-arid highlands (more than 3,800 m above sea level) with the vegetation available for food being determined by season.
**Stimulation of adrenal cortex in alpacas and llamas**

In order to validate physiologically whether the pattern of excreted FCM in SAC reflects the predicted cortisol profile following ACTH challenge test (Touma & Palme 2005), nine alpacas and six llamas received a single jugular intravenous injection of 0.25 mg synthetic ACTH (Synacthen®, Defiante Farmaceutica, Portugal). All voided faeces were collected during 48 h post-injection in alpacas and llamas and, in the latter, samples had also been collected for an extra 48 h before injection to establish baseline FCM levels. After defaecation, faeces not contaminated with urine were collected into plastic bags, homogenised within the bag and immediately stored at –20°C for further analysis. Additionally, blood samples were collected from the jugular vein in EDTA tubes from the six llamas 15, 30, 60, 120 and 180 min after ACTH administration. After centrifugation (2500 × g; 30 min), plasma samples were transferred to eppendorf tubes and stored at –20°C prior to analysis.

**Confinement of llamas and vicuñas**

Complementary to the physiological validation of the EIA, this method was applied to evaluate a stressful event such as a confinement. A different group of six llamas (three males and three females) were confined individually in rooms (3×3×2.5 m; length × width × height) for six days and fresh faecal samples were collected twice daily (0800 and 1600h). These animals had never previously been confined.

Faecal samples from nine vicuñas were randomly obtained during the period from chasing until the beginning of confinement (Chacu) carried out between 1000 and 1400h. These were considered to contain baseline FCM concentrations. The following day, nine faecal samples were collected after confinement and just before animals were individually sheared around
Samples were immediately stored on ice until freezer facilities were available within the next 12 h.

Steroid analysis

Extraction of faecal samples was performed as described previously (Palme & Möstl 1997). Briefly, 0.5 g of each thawed sample was mixed with 5 ml of 80% methanol, shaken on a hand vortex for 5 min and centrifuged (2,500 × g; 30 min). Subsequently, 0.5 ml of the supernatant was transferred into eppendorf microtubes and dried down overnight at 50°C. For plasma cortisol extraction, 0.5 ml of plasma was thawed, mixed with 5 ml diethyl ether by vortexing for 2 min and then stored in a freezer at –20°C for 3 h. Supernatants (ether phase) were quickly transferred to new eppendorf tubes and evaporated at 50°C in a ventilated oven. Dried samples were transported to the University of Veterinary Medicine, Vienna, Austria, where they were rehydrated with 80% methanol (0.5 ml) overnight, vortexed for 10 min (for faeces) or with 0.5 ml EIA buffer (for plasma) and all stored at –20°C until EIA analysis.

To select the best-suited EIA to measure FCM in SAC, the following EIAs were tested in a subset of llama faecal samples from the ACTH test: two different 11-oxoaetiocholanolone EIA (Palme & Möstl 1997; Möstl et al 2002), an 11ß-hydroxyaetiocholanolone EIA (Frigerio et al 2004) and a 5α-pregnane-3ß,11ß,21-triol20-one EIA (Touma et al 2003). Only the 11-oxoaetiocholanolone EIA measuring 11,17-dioxoandrostanes (11,17-DOA) was found to be suitable. All other EIAs showed unexpected profiles of increase and decrease in FCM after ACTH injection (data not shown). Subsequently, all samples were analysed with this 11-oxoaetiocholanolone EIA. Plasma cortisol concentrations were determined using a cortisol EIA described previously (Palme & Möstl 1997; Palme et al 1999).
**Stability of 11,17-DOA**

To evaluate the influence of the time interval between defaecation and freezing on FCM concentrations, a fresh faecal sample was collected immediately after defaecation from one alpaca. The sample was homogenised and a total of 16 subsamples (0.5 g each) weighed. A group of four subsamples was frozen (–20°C) immediately (hour 0) and the others (n = 4, each) after storing at room temperature for 2, 4, and 8 h, respectively. Samples were processed and analysed as described above.

**Statistical analysis**

For each alpaca, the baseline FCM level was established as the median concentration of all samples collected during the first 6 h after the ACTH challenge. In every llama, the baseline was the median value of all samples voided during the first 24 h (from hours –48 to –24) before ACTH injection (hour 0). In each confined llama, baseline FCM concentration was calculated as the mean of two samples collected the first day of confinement at 0800 and 1600h and individual increases above the baseline are then supplied. In confined vicuñas, starting (reflecting pre-stress) values were compared (t-test) with post-Chacu values. For graphical presentation and statistical analysis all values of the stimulation experiment were grouped into 6-h intervals. Statistical analysis was performed by repeated measures of ANOVA using Prism for Windows version 5.01 (GraphPad Software Inc, USA). The threshold for statistically significant differences was defined as $P < 0.05$. Results are given as the mean ($\pm$ SD) or as a range (from minimum to maximum) plus median, if the data were not normally distributed.
Results

Stimulation of the adrenal cortex in alpacas and llamas

Plasma cortisol levels (mean [± SD]; ng ml−1) in llamas were 36 (± 24), 56 (± 35), 94 (± 68), 58 (± 12) and 60 (± 42) after 15, 30, 60, 120 and 180 min following ACTH challenge, respectively. Peak concentrations were observed 60 min after ACTH injection. They were significantly (P < 0.05) higher than the starting values (after 15 min).

In both alpacas and llamas there were no significant differences in FCM values between females, males and castrated males. Therefore, data from all the individuals were grouped. Baseline FCM concentrations varied among individuals; they were 12 to 47 (median: 24) ng g−1 faeces in alpacas and 36 to 226 (median: 83) ng g−1 faeces in llamas. Following the ACTH challenge in alpacas, FCM concentrations increased after 12 h and peaked after 33 h (median) with concentrations ranging between 196 and 601 (median: 343) ng g−1 faeces. After the ACTH injection in llamas, FCM concentrations peaked at 28 h (median) with values ranging from 422 to 1,272 (median: 760) ng g−1 faeces. Expressed as a percentage (above individual baseline levels), peak concentrations represented a 963% (ten-fold) increase in alpacas and an 805% (eight-fold) increase in llamas. Subsequently, concentrations decreased over time. However, in alpacas and llamas, even 48 h after the injection of ACTH, levels had not returned to baseline in all individuals, although FCM concentrations at the later time points were not significantly different from those of the baseline intervals in llamas (see Figure 1).
**Confinement of llamas and vicuñas**

Again, there were no significant sex differences in absolute FCM levels. Baseline concentrations were comparable to those of the ACTH test (ranging from 10 to 161; median: 56 ng g–1 faeces). In female llamas, individual 11,17-DOA concentrations started to increase on the second day, peaked on day 5 (4.7 times higher than baseline levels; \( P < 0.001 \)) and decreased thereafter. In contrast, in male llamas, there was an increase in relative FCM levels during the first two days, peaking at day 3 (2.7 times higher than baseline values; \( P < 0.001 \)) followed by a decrease to approximately baseline levels from the fourth day until the end of the experiment (Figure 2). FCM levels (% increase) were significantly different (\( P < 0.0001 \)) from baseline (day 1) on days 3, 4, 5, 6 in females and on days 3, 4, 5 for males (Figure 2). In the group of wild vicuñas, mean (± SD) 11,17-DOA concentrations were 110 (± 47) ng g–1 faeces at the beginning of confinement (starting value) and 327 (± 64) ng g–1 faeces one day later, which was significantly higher (\( P < 0.0001 \)).

**Stability of 11,17-DOA**

After defaecation (0 h), mean (± SD) concentrations of 11,17DOA were 79 (± 13) ng g–1 faeces. Two hours later, concentrations remained unchanged 86 (± 6) ng g–1 faeces. However, there was a significant (\( P < 0.001 \)) increase after 4 h (137 [± 15] ng g–1 faeces; see Figure 3) and after 8 h (158 [± 16] ng g–1 faeces) resulting in approximately two times higher FCM concentrations compared to the initial hour 0.
Figure 1 Boxplot of grouped concentrations (intervals of 6 h) of cortisol metabolites (11,17-dioxoandrostanes) in faeces (ng g⁻¹) after administration of ACTH (Synacthen® 0.25 mg) in (a) alpacas (n = 9) and (b) llamas (n = 6). *** P < 0.001, ** P < 0.01, * P < 0.05 compared to baseline as analysed by repeated measures analysis of variance (with Tukey correction).
Figure 2

Mean (± SD) changes in relation to the baseline (day 1) of faecal cortisol metabolites (11,17-dioxoandrostanes) concentrations (in three male [○] and three female [●] llamas) during six days of confinement (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to baseline as analysed by one-way repeated measures analysis of variance [with Dunnett’s correction]). Crosses denote a significant difference (+++ $P < 0.001$ and + $P < 0.05$) between females and males at indicated time points as analysed by two-way repeated measures analysis of variance (with Bonferroni correction).
Figure 3 Mean (± SD) concentrations of faecal cortisol metabolites (11,17-dioxoandrostanes) from one homogenised alpaca faecal sample. Subsamples were divided into four groups (n = 4, each) and stored at room temperature for 0, 2, 4 and 8 h. *** Differ significantly at \( P < 0.001 \) compared to initial values (0) as analysed by one-way analysis of variance (with Bonferroni correction)
Discussion

To our knowledge this is the first study describing a measurement of faecal cortisol metabolites (FCM) in South American camelids. Based on our successful physiological and biological validation in alpacas, llamas and vicuñas such a method could be used as a potential tool to measure stressful events in these species.

In all animals, expected patterns of FCM concentrations after ACTH were found. Plasma cortisol after ACTH injection was also measured in the llamas. Although variability among animals concerning both baseline and peak values was observed, concentrations of 11,17-DOA in faeces paralleled those of cortisol in plasma as shown in other ruminant species (Palme et al 1999).

Peak FCM concentrations were found after 33 h in alpacas and after 28 h in llamas. These values were longer than those seen in other ruminants, such as cattle, sheep, goats and red deer, where peak values were detected between 10 and 18 h (Palme et al 1999; Huber et al 2003; Kleinsasser et al 2010). The delay time of FCM excretion reflects the intestinal transit time from duodenum to rectum which is species specific (Palme et al 1996, 2005). In the literature a much longer intestinal gut passage time for SAC, when compared to other ruminants, is reported (Sponheimer et al 2003). This corresponds very well with the much longer times until peak FCM concentrations that we observed in SAC. This also underlines the importance of running validation experiments in every species of interest (Palme 2005).

Once the ability of the EIA method to measure the increase of FCM after adrenocortical stimulation in SAC was proven, it was important to examine whether the adrenocortical response induced by a biological stressor can be quantified by means of FCM concentrations.
Both male and female llamas excreted higher FCM concentrations after physical confinement, but females had a more expressed increase than males. This difference is in agreement with studies of cortisol concentrations in other ruminant species. In guanacos, female cortisol levels were higher than in males when captured for shearing and this difference was shown to be due to pregnancy (Carmanchahi et al 2011). However, female llamas studied here were not pregnant at the time of the experiment. Also, in sheep, females had a greater cortisol response to the physical/psychological stressor of isolation/restraint compared to males (Turner et al 2002). These sex differences appear to occur at the brain level, as the cortisol response to an ACTH challenge did not differ between the sexes (Turner et al 2002). However, further studies with a greater number of animals are needed to evaluate the significance of this interesting finding and to elucidate possible causes.

We also used 11,17-DOA concentrations as a tool to monitor stress in vicuñas during Chacu management in Perú. This study confirmed that chasing and confinement are stressors for vicuñas as indicated by high FCM concentrations ($P < 0.0001$). It was demonstrated in vicuñas that captivity induced physiological changes including elevated cortisol that peaked around 120 min after capture, increased levels of creatin kinase during the course of captivity and higher respiratory and heart frequency (Arzamendia et al 2010). Although the Chacu carried out only by humans might cause less stress compared to other methods of chasing such as the use of motorised vehicles (Arzamendia et al 2010), other factors such as waiting in the stockyard, handling, presence of humans, lack of access to their natural environment, loss of fibre and attempts to escape (Bonacic et al 2003a; Sahley et al 2006) might also add to the elevated FCM found in this study. In wild guanacos, elevated serum cortisol concentrations were observed after 80 min of restraint (Carmanchahi et al 2011). The vicuña
is a notable species that offers, among other features, the production of valuable fine fibre with economic benefits for communities (Sahley et al 2006) and therefore monitoring of stress using non-invasive methods as described here has great implications for conservation and management of this species.

In an effort to minimise changes in FCM concentrations after defaecation, it is critical for samples to be frozen immediately or collected onto ice, otherwise 11,17-DOA concentrations may increase if maintained at room temperature (Möstl et al 1999). Results of the stability experiment presented here demonstrate that it is possible to maintain samples up to 2 h at room temperature until freezing without altering final results in alpacas. In cattle, whose faecal sample consistency differs from camelids, there was a significant increase (136%) in metabolite concentration maintained at room temperature for the first hour and then rising until 24 h (Möstl et al 1999). The same was observed in sheep, where there was a significant increase when samples were stored at room temperature for 1 h (Lexen et al 2008). We also observed a similar increase of FCM concentrations at later time points (4 and 8 h), which might be due to a further conversion of glucocorticoid metabolites into other metabolites by bacterial enzymes (Winter et al 1979; Möstl et al 1999; Lexen et al 2008).

Although the stability of FCM from wild SAC needs to be tested, the fact that in alpaca faeces there is at least a 2-h interval between sample collection and freezing without a significant change of FCM values is of great importance, since it facilitates studies in the field where freezing equipment is not immediately available.
Animal welfare implications

For the first time, measurement of faecal glucocorticoid metabolites was performed and successfully validated in SACs. This method can be applied as a non-invasive tool for evaluating disturbances in these species, especially in wild camelids where it is difficult to obtain blood samples and with which it’s hard to monitor within their natural environment. Such a method could help to improve management, handling and welfare in both domesticated and wild SAC.

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4. Discussion

This work presents robust data on the measurement of faecal cortisol metabolites and its successful validation as a non-invasive sampling methodology for monitoring adrenocortical activity in South American camelids. Groups of Alpacas and Llamas were subjected to the stimulation of the adrenal cortex by an analogue of ACTH and by an environmental stressor of confinement. There were significant increases of glucocorticoid metabolites in their faeces. In the same way, in the group of vicuñas significant increases were observed after their chaising and captivity.

To establish the dose of Synacthen® (ACTH) to be injected in every animal, I followed the protocol described by PALME et al. (1999) in which one ampoule was injected to each animal. However, it has never been considered that camelids show differences for the metabolism of several drugs as compared with other ruminants (NAVARRE et al., 2001; CAVILLA et al., 2010): Thus it is important to study the appropriate dose of ACTH for South American camelids.

The selected 11-oxoaetiocholanolone enzyme immunoassay measuring a group of faecal cortisol metabolites (11,17-dioxoandrostanes) in South American camelids had been proven to work in other ruminant species, too (PALME and MÖSTL, 1997).

Due to individual variations in the FCM concentrations after confinement and ACTH challenge, there was not a significant difference between sexes. Further analysis with
individual increases in values revealed sex differences over the days only in the confined group of llamas (see Figure 2 of the manuscript). Females and males could not be differentiated only by absolute values (ng/g). In this respect, a large number of animals might help to elucidate whether there difference between sexes exist.

Samples for this study were collected for 48 consecutives hours; however, besides environmental stressors, daily rhythms may influence the HPA axis induce the release of cortisol. In this respect, it was found that melatonin concentrations in two alpacas measured every 2 hours reveals high values between 10 P.M. and 2 A.M. (MORGANTE et al., 2007) but there is no information whether melatonin can affect cortisol production in camelids.

The experiments were performed in Peru at 3300 meters above sea level, where the high altitude sickness is a rare pathology in animals and humans living at these altitudes (CUEVA et al., 1974; SILLAU et al., 1980). However, studies in humans suggest that steroid hormones production are decreased at higher altitude (BRAUN et al., 2000): Thus it is hypothetized that hypoxia may affect the cortisol production (PANESSAR 2004). This assumption can be tested in camelids by evaluating the responsiveness of adrenal cortex to pharmacological (ACTH) and physiological stimulation both at sea level and a high altitude (PANESSAR 2004).

Studies on reproductive physiology in female South American camelids are limited compared to other farm ruminants. Interestingly, camelids are induced ovulators as cats or rabbits (SAN MARTIN et al., 1968). High embryo mortality has been reported in the first
month of pregnancy in alpacas (FERNANDEZ-BACA et al., 1970). The causes might be some management practices involving stressful situations to the animals during reproduction. For example it is a common practice to breed the female when adopting sternal recumbency to the male, but this is not a reliable indicator of plasma oestradiol concentration (BRAVO et al., 1991; POLLARD et al., 1994; BRAVO et al., 2010) therefore breeding can fail.

Finally, faecal samples were previously used to measure other steroid hormone (progestagens) metabolites in female vicuñas and evaluate the corpus luteum function (SCHWARZENBERGER et al., 1995). However the work presented in this thesis is the first that measured faecal cortisol metabolites in South American camelids and monitor the adrenocortical activity in the context of stress.

In this work, I validated physiologically and biologically an enzyme immunoassay for measuring adrenocortical activity in South American camelids.
4.1 Conclusions

- Alpacas and llamas increase cortisol levels after adrenocortical stimulation with intravenous injection of Synacthen® (synthetic analogue of adrenocorticotropic hormone).
- Faecal cortisol metabolites (FCM; 11,17-dioxoandrostanes) were measured in the South American camelids alpaca, llama and vicuña.
- 11,17-dioxoandrostanes proved suited for non-invasive measurement of adrenocortical activity in alpacas, llamas and vicuñas.
- In both alpacas and llamas, after 2 hours plasma cortisol levels return to baseline and after 2 days FCM concentrations start dropping.
- There is variability in FCM concentrations between individuals, but with the same trend after adrenocortical stimulation.
- Vicuñas are stressed after the chasing and capture.
- This is the first work using non-invasive methods to measure adrenocortical activity in South American camelids.
4.2 Further Research

Non-invasive monitoring of stress in South American camelids by measuring faecal cortisol metabolites (11,17-dioxoandrostanes) can be applied in the following topics:

1. Immunosuppressive effects of stress predispose animals to the “alpaca fever” (*Streptococcus zooepidemicus* infection), whose pathogenesis is not well understood but it is presumable attributed to stress. The same may be the cause with a predisposition to respiratory infections in newborns.

2. Influence of stress on the embryo mortality in alpaca and vicuña (KNIGHT et al., 1995; ALLER et al., 2003).

3. Increase alpaca and llama fertility through better management practices (SUMAR, 1996).

4. Improve management practices in the wild environment for conservation of vicuña and guanaco; moreover prevent undesirable consequences of chacu (BONACIC et al., 2006).

5. Finally, there is an open question that needs to be addressed: if stress may lead to dandruff in humans (SCHWARTZ et al., 2006). Would stress be the cause of dandruff in the vicuña fiber?
5. **Summary**

The welfare and productivity of South American camelids may be affected by stressful events. The purpose of this thesis was to validate a non-invasive method for stress monitoring using faecal samples and to apply it to evaluate a stressful event, such as confinement.

For physiological validation, nine alpacas (*Vicugna pacos*) and six llamas (*Lama glama*) were subjected to pharmacological stimulation of their adrenal cortex. Serial faecal samples were collected during 48 h before and after stimulation. During confinement, faecal samples from six llamas were collected twice per day during six consecutive days. Faeces belonging to 18 vicuñas (*Vicugna vicugna*) were collected before and one day after their capture for confinement (Chacu). Faecal cortisol metabolites (FCM) were extracted from each sample and quantified by an 11-oxoaetiocholanolone enzyme immunoassay.

Thirty-three and 28 h (median) after ACTH stimulation, FCM concentrations peaked with a ten-and eight-fold increase (median) above baseline in alpacas and llamas, respectively. There were no significant differences in FCM concentrations between sexes. In llamas, FCM concentrations peaked (4.7 times higher than baseline) after five days of confinement in females and after three days (2.7 times) in males. In vicuñas, three times higher FCM levels were observed the day after the start of confinement (in comparison to the starting values). Together this thesis has proven that the non-invasive method is well suited to measure adrenocortical activity in alpacas, llamas and vicuñas. Thus, this method could help to improve management, handling and welfare in wild and domesticated South American camelids.
6. Zusammenfassung


7. References


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