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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.

Materials 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 m^2, with access to natural light and with an average temperature of 15ºC. 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 vicuñas 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 –20ºC 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 –20ºC 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 1000h. 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 centrifugated (2,500 × g; 30 min). Subsequently, 0.5 ml of the supernatant was transferred into eppendorf microtubes and dried down overnight at 50ºC. 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 –20ºC for 3 h. Supernatants (ether phase) were

quickly transferred to new eppendorf tubes and evaporated at 50ºC 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 –20ºC 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 11β-hydroxyaetiocholanolone EIA (Frigerio *et al* 2004) and a 5α-pregnane-3β,11β,21-triol-20-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 (± 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

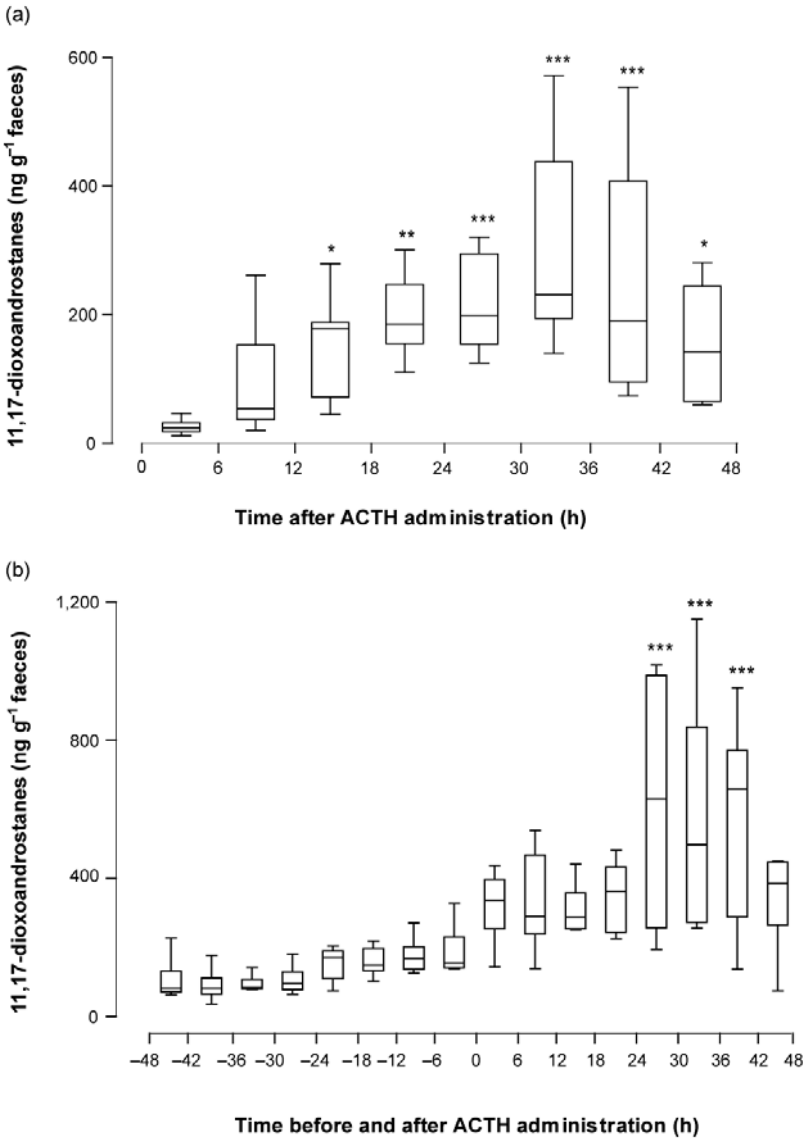
Plasma cortisol levels (mean [± SD]; ng ml^{-1}) in llamas were 36 (± 24), 56 (± 35), 94 (± 68), 58 (± 12) and 60 (± 42) after 15, 30, 60, 120 and 180 min following ACTH challenge, respectively. Peak concentrations were observed 60 min after ACTH injection. They were significantly ($P < 0.05$) higher 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 ($P < 0.0001$).

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).

Stability of 11,17-DOA

After defaecation (0 h), mean (± SD) concentrations of 11,17-DOA 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.

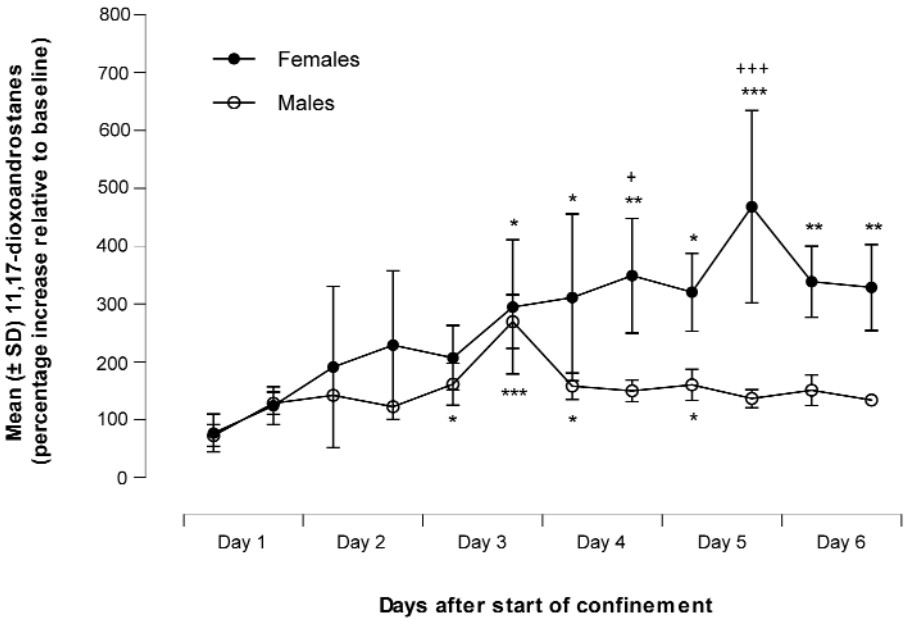
Discussion

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).

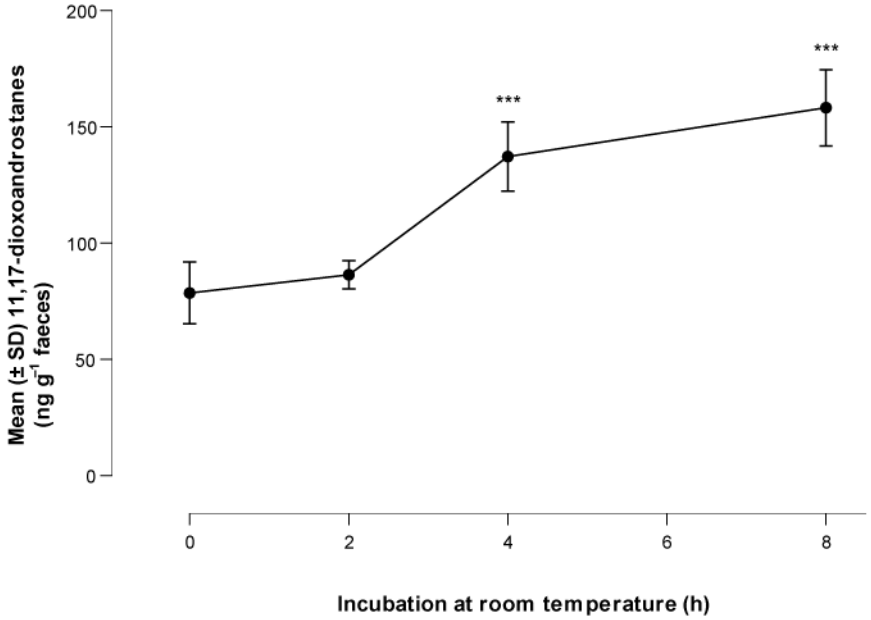
Peak FCM concentrations were found after 33 h in alpacas and after 28 h in llamas. These values were longer than those seen in other ruminants, such as cattle, sheep, goats and red deer, where peak values were detected between 10 and 18 h

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).

(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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