Long-term quantification of faecal glucocorticoid metabolite concentrations reveals that Mexican grey wolves may habituate to captivity

I. ESCOBAR-IBARRA1,2, L. MAYAGOITIA-NOVALES3, A. ALCÁNTARA-BARRERA2, A. L. CERDA-MOLINA1, R. MONDRAGÓN-CEBALLOS3, R. RAMÍREZ-NECOECHEA2, & M. ALONSO-SPILSBURY2*

1Programa de Doctorado en Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana Unidad Xochimilco, México, DF, Mexico, 2Depto. de Producción Agrícola y Animal, Área de Investigación: Ecodesarrollo de la Producción Animal, Universidad Autónoma Metropolitana Unidad Xochimilco, México, DF, Mexico, and 3Depto. de Etología, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Tlalpan, México, DF, Mexico

(Received 14 October 2016; accepted 6 May 2017)

Abstract
Mexican grey wolves are considered the most endangered wolf in the world. The aim of this longitudinal study was to assess the physiological stress level of 24 captive-born wolves confined at four zoos and two parks, by measuring faecal glucocorticoid (fGC) metabolite concentrations in 1005 samples, for 1 week in every season of the year over a 2-year period. Total overall mean fGC concentration of individual scats was 153.83 ± 8.16 standard error mean (SEM) ng/g dry matter (DM). Paired samples from the same individuals showed a decline in fGC levels between years (173.09 ± 12.15 and 135.94 ± 10.93 for 2010 and 2011, respectively; \( P = 0.02 \)). Significant differences were also observed between reproductive and non-reproductive seasons (116.78 ± 12.48 vs. 173.20 ± 10.50, respectively; \( P < 0.0001 \)), and between genders (males: 214.53 ± 13.43 vs. females: 121.51 ± 10.03; \( P < 0.0001 \)). Tukey’s post hoc comparisons showed that elder wolves excreted higher levels than young adults and reproductive adults (\( P = 0.016 \)). High-social-ranked wolves showed higher (\( P < 0.0001 \)) fGC levels (202.03 ± 19.94; \( n = 229 \)) than medium- (175.33 ± 19.94 ng/g DM; \( n = 214 \)) and low- (162.06 ± 16.15; \( n = 377 \)) ranked wolves did; however, no correlation was found between social rank and fGC levels. Anthropogenic acute stressors during husbandry procedures a day before sampling resulted in considerable elevations of fGC concentrations above 1270.34 ng/g DM, returning to baseline levels after 2 days. Our results suggest that wolves are becoming habituated to confinement at the zoos; these findings may contribute to a more comprehensive definition of confinement, which has traditionally been perceived as a stressful habitat for wild animals. Using fGC concentrations as an overall physiological state in long-term studies may provide crucial information on the resilience of captive animal populations.

Keywords: Canis lupus baileyi, wolf, faecal glucocorticoid metabolites, physiological stress

Introduction
Mexican grey wolves (Canis lupus baileyi Nelson and Goldman, 1929) are considered the smallest subspecies of the grey wolf and the most endangered wolf in the world (Endangered Wolf Center 2016). Moreover, the Mexican government has determined that free-ranging Mexican grey wolves are probably extinct (DOF 2015; SEMARNAT 2015: NOM-059-SEMARNAT-2010). Currently, there are only 248 Mexican wolves total in captivity (FWS 2014).

Large packs of European free-ranging wolves (Canis lupus Linnaeus, 1758) show higher levels of stress than confined ones, and intra-specific competition during breeding is likely to cause elevated levels of glucocorticoids (Barja et al. 2008; Eggermann et al. 2013). Long-term production of elevated glucocorticoids has serious negative effects on health and reproduction (Sapolsky 2002), with long-lasting stress adversely affecting the fitness of wolves (Creel et al. 2002; Sands &
Creel 2004). Thus, the evaluation of stress is important for conservation of threatened species.

Wolves are highly social, and captive wolves typically maintain a stricter hierarchy within the pack than wild wolves do (Frank & Frank 1982), which is enforced with more frequent intraspecific aggression and dominance displays (Zimen 1976). Spercoski et al. (2012) discussed the possibility that stress and aggression levels are higher in captivity than in the wild.

In addition to confinement, there are temporal pattern-, breeding season-, social rank-, sex- and age-mediated cortisol differences. Romero (2002) explains the seasonal modulations of glucocorticoid concentrations in many species; in maned wolf Chrysocyon brachyurus (Illeger, 1811), Spercoski et al. (2012) observed two-fold higher levels during spring compared to the other seasons; however, in timber wolves no seasonal variation has been found in glucocorticoid concentrations (McLeod et al. 1996). In wild populations of wolves, Eggermann et al. (2013) and Sands and Creel (2004) reported an increase of faecal glucocorticoid (fGC) during the annual mating season. Sands and Creel (2004) found also that fGC levels were significantly higher in dominant wolves than in subordinates, for both sexes, in three packs for 2 years, although they were not associated with high rates of aggression or agonistic interaction.

On the other hand, Molnar et al. (2015) found no relationship between fGC levels and age, sex or social status in free-ranging wolves. To our knowledge, social stability of packs has not been investigated as a potential stressor in confined wolves.

Measurement of glucocorticoid metabolites in scats has become a widely employed and accepted method for the non-invasive evaluation of adrenocortical response to stressors in carnivores (Goymann et al. 1999; von der Ohe & Servheen 2002; Sands & Creel 2004; Young et al. 2004; Paz et al. 2015). As part of a more comprehensive study on physiological stress and reproductive behaviour in captive Mexican grey wolves, we sought to analyse the effects of breeding season, social rank, pack social grouping, gender and age on the quantification of faecal concentrations of glucocorticoid metabolites within six wolf facilities in a longitudinal study. We hypothesise that social stable wolf packs in captivity are likely to show low fGC metabolite concentrations. In addition, higher hormone levels would be obtained during the breeding season, in dominant animals, in males, in elder wolves and in zoo-housed wolves compared to those living in parks.

### Materials and methods

#### Facilities and wolves

Twenty-four Mexican grey captive-born wolves (nine males and 15 females) from a 5.8-year generation time from four zoos, one centre for wildlife conservation and one park in Mexico were monitored from 16 January 2010 to 2 December 2011. The locations were: (1) Africam Safari in Puebla, (2) León Zoo in Guanajuato, (3) Parque del Pueblo Nezahualcoyotl (Neza), (4) Zacango Zoo, (5) San Cayetano Centre for Wildlife Conservation and Research (3–5 in the State of Mexico), and (6) El Tecuán Park, located in Durango. Table I provides information on the facilities’ characteristics and the number of animals, their studbook number, group social composition, and feeding schedule at each zoo.

Wolves are part of the Mexico–US Binational Conservation Program, so zookeepers are specially trained and management is similar across the zoos, although feed provision differs in the two parks, where wolves are fed once a day with dry dog food. San Cayetano Centre and El Tecuán Park do not give access to the public, whereas the four zoos are open to visitors throughout the year.

#### Packs’ composition and wolves’ ages

New wolf packs were formed at Neza Zoo, Zacango Zoo and Tecuán Park in January 2010, when our study started. The other packs, with the exception of the breeding pair at San Cayetano, were already established when we started our study. The San Cayetano breeding couple was formed in 2011,

| Facility          | Groups | Pairs | Solitary | Total |
|-------------------|--------|-------|----------|-------|
| Africam Zoo*      | ♀ 3    | ♂ 1   | ♀♀ 1     | 4     |
| León Zoo          | ♂ 1    |       | ♂♂ 2     | 4     |
| Zacango Zoo       | 1      |       | 2        | 4     |
| Neza Zoo          |       | ♂ 2   | ♂♂ 2     | 4     |
| Tecuán Park       | 1      |       | 1        | 4     |
| San Cayetano**    | 1      | 1     | 6        | 8     |
| Total             | 4      | 4     | 2        | 24    |

* Africam Zoo housed their four wolves with a schedule of solitaire during the breeding season in winter, and rotation for the rest of the year, leaving the resident male with a different female every day.
** San Cayetano Centre housed one mixed group of one neutered male commingling with three females, and a reproductive couple (1♂ : 2♀); the two packs were divided by a mesh-wired fence.
whereas the related group of sisters and the neutered male were formed in 2004; León Zoo groups were already established between 2003 and 2007, and the Africam Zoo pack was established during 2003. Wolves were classified into three groups according to their age (Hayes & Harestad 2000): (a) young adult: age between 1.5 and 3.4 years; (b) reproductive adults: age 3.5–6 years; and (c) elders: age > 6 to 11 years old. It is worth mentioning that the two wolves from Neza Zoo died in the summer of 2011.

**Rank order**

Behavioural observations were carried out during seven consecutive days for every season and every year. Wolves were individually identified based on physical characteristics. Seasons were defined as follows: winter from 21 December to 20 March, spring from 21 March to 20 June, summer from 21 June to 20 September, and autumn from 21 September to 20 December. It is worth mentioning that the breeding season for wolves in Mexico is during winter. Data collection was carried out following Altmann (1974) on focal animal sampling; also, spontaneous social interactions for every wolf pack in the study were video-recorded during continuous monitoring from 10:00 to 17:00 (time when open to the public in most zoos) and from 7:00 to 20:00 at San Cayetano and El Tecuán Park. Patterns to discriminate between dominant and submissive behaviours were according to Schenkel (1948, 1967) and Fox (1971), and agonistic behaviours were according to the ethogram of Escobar et al. (2005). Records of agonistic interactions were used to create win–loss input matrices (Martin & Bateson 1988). Thus, a dominance index metric as described by Zumpe and Michael (1986) was calculated for each confined group; solitary housed wolves were not considered in this analysis. No software was used to extract the behavioural data; an observer (the same person) watched all recorded videos.

**Faecal samples**

Faecal samples were collected from January to December for two consecutive years: 2010 and 2011. In accordance with Mexican laws (LGVS 2015) and NOM-126-SEMARNAT-2000 (SEMARNAT 2001), a permit was required for the collection of faecal samples from all wolves, and from the zoos involved in the study (SGPA/DGVS/0384/10).

Fresh faecal samples were collected for seven consecutive days every season for every wolf of the six facilities; collections were done in the morning from 09:00 to 10:00, from the dorm or by the exhibitors, during their daily cleaning routine. Samples were collected 1 day after the behavioural observations.

Based on their sampling dates, samples were also classified as collected during the breeding period (January to March) or the non-breeding period (April to December). On the day prior to collection, wolves were given artificial bakery colouring (red, blue, green, purple, yellow and orange) in their food for individual identification of their faeces. These colourants have not been shown to be toxic for non-human animals, and had been used previously in non-human primates (Cerda-Molina et al. 2006). Samples were kept in hermetically sealed plastic bags, correctly identified and frozen unpreserved within a few hours after collection, and stored at −20°C until analysis, as recommended by Millspaugh and Washburn (2004).

**Hormonal extraction and quantification**

Faecal samples were dried in a solar oven at 60–70°C for 6 hours; they were cleaned of soil, and residual feed, leaves and hairs, and were pulverised. We used the methanol extraction method suggested by Wasser et al. (2000), with some modifications. Briefly, 4 mL of 100% methanol was added to 1 g of dried faeces, thoroughly mixed in a vortex for 3 min and left for 24 hours in a vertical shaker. The next day, samples were centrifuged at 3000 g for 30 min at 4°C, and the supernatants were recovered on glass tubes. Faecal pellets were washed by adding 1 mL of methanol, mixed in a vortex for 3 min, and left in the vertical shaker for 10 hours. Samples were centrifuged again at 3000 g for 30 min at 4°C and the supernatant was added to the previous one; afterwards they were dried in a water bath. Samples were reconstituted with 2000 µL of phosphate buffer (0.01 M), pH 7, mixed with ethanol 100% (2:1) and tween 20 (0.02%). Finally, concentrations of faecal glucocorticoid metabolites (cortisol) were measured with a commercially supplied radioimmunoassay kit (Immunotech s.r.o. Prague, Czech Republic, Cat: IM1841™).

**Cortisol quantification and validation**

The fGC methanol recovery was assessed by adding, on average, 2244 ± 152.21 cpm 125I-labeled cortisol to 1 g of dry faeces (n = 10) and stored overnight at room temperature before methanol extraction. Mean (± standard deviation, SD) extraction recovery and variation coefficient (VC) from a faecal pool were: 61.00% ± 3.7, VC = 6.07%. The commercial kit used has an analytical sensibility of 5 nM, and the antibody used in the immunoassay is highly specific.
for cortisol with extremely low cross reactivity against other steroids, such as aldosterone, corticosterone, cortisone, 11-desoxycortisol and progesterone. The intra-assay and inter-assay coefficients of variation were 9.3% and 13.4%, respectively. All samples were assayed in duplicate, and we corrected hormone concentrations for this extraction to express concentrations as nanograms per gram of faecal dry matter (DM).

Since in endangered species a rigorous physiological validation might not be possible, to further establish the biological validation of the faecal cortisol assay, each animal can be used as its own control before and after a known stressful event (Touma & Palme 2005). For routine procedures, five wolves from the study were subjected to capture, immobilisation and confinement, and were found to produce up to a 92-fold rise in fGC (Table II), returning to basal levels 2 days after, demonstrating that the technique could detect biologically meaningful changes in circulating glucocorticoid levels. Wolves’ physical restraint was used to validate fGC determination in another study (Pifarré et al. 2012).

Statistical analyses

For statistical analysis the SPSS statistics package (version 19.0; IBM SPSS, Armonk, NY, USA) was used. The distribution of our data indicated that fGC data were normally distributed after Kolmogorov–Smirnov; therefore, we used untransformed fGC values for graphical representation of the data. In addition, means were tested for significant differences with the t-test for repeated and independent samples, and comparisons between groups for zoo location, seasons, social group and age class were analysed by one-way analysis of variance (ANOVA). In the case of overall significant effects, when appropriate, post hoc comparisons were conducted by Tukey, Wilcoxon signed-rank, and Kruskall–Wallis tests. For all statistical tests, the alpha value was set at \( P = 0.05 \). Assays results are expressed as mean ± standard error of mean ng metabolite concentrations of fGC per dry gram of faeces. To determine correlations between fGC levels and social rank, Spearman correlation analysis was used.

Ethical note and underlying open-access data

We adhered to the “Guidelines for the use of animals in research” as published in *Animal Behaviour* (1991, 41, 183–186). The Academic Committee of the Programa de Doctorado en Ciencias Biológicas y de la Salud, UAM-X (Universidad Autónoma Metropolitana-Xochimilco), approved the experimental protocols and the handling of non-human animals. Maximal care was taken to avoid animal disturbance during faecal sampling and behaviour video-recording. All data in this study are available from the corresponding author.

Results

Overall data

We were able to collect a total of 1005 faecal samples since not all wolves defecated on every scheduled sample day. The total overall mean of fGC metabolite concentration was 153.83 ± 8.16 ng per gram of DM. The mean fGC metabolite concentration of individual scats was 173.09 ± 12.15 and 135.94 ± 10.93 ng per gram of DM for years 2010 (n = 481) and 2011 (n = 524), respectively (paired t-test: \( t = 1.537, P = 0.02 \)).

Hormone concentrations varied among wolf packs from 1.47 to 2147.70 for 2010, and 2.23 to 3380 for year 2011 (Table III). Only in one park, wolves showed higher mean fGC levels as compared with the other park, the four zoos and the conservation centre (post hoc Tukey test, \( P = 0.02 \)).

After husbandry procedures, five samples contained considerably higher concentrations (Table II) than all other samples (maximum 1270.34 ng/g DM).

| Facility          | n    | GCC (ng/g DM) mean ± SEM | Range        |
|-------------------|------|--------------------------|--------------|
| Africam Zoo       | 135  | 120.97 ± 22.95\(^a\)     | 8.51–2147.70 |
| León Zoo          | 149  | 147.13 ± 18.81\(^a\)     | 8.51–1654.10 |
| Zacango Zoo       | 188  | 111.90 ± 15.10\(^a\)     | 1.74–1433.26 |
| Neza Zoo          | 45   | 133.54 ± 27.03\(^a\)     | 1.47–913.84  |
| Tecuan Park       | 148  | 311.00 ± 22.21\(^b\)     | 32.09–1260.56|
| San Cayetano      | 340  | 126.94 ± 14.79\(^a\)     | 2.23–3380.64 |

One-way repeated measures analysis of variance (ANOVA).

\(^a\)Different letters indicate significant differences (post hoc Tukey test, \( P = 0.02 \)). DM: dry matter. SEM: standard error mean.
However, for these specific wolves, a day later cortisol was lower than baseline levels.

Seasonal and reproductive season variations
Mean ± standard error mean (SEM) overall fGC concentration varied across seasons (ANOVA, $F_{4,519} = 8.85$, $P < 0.05$) with lower levels during winter vs. spring and winter vs. autumn (post hoc Tukey test, $P < 0.01$; Table IV).

Seasonal fluctuations were demonstrated for 2010, whereas in 2011 no seasonal variations were observed (Table V). However, between years there was a difference in autumn (Wilcoxon signed-rank test, $z = -2.43$, $P < 0.05$, $n = 24$ wolves).

An increase of fGC metabolites was observed during non-reproductive vs. reproductive seasons (paired $t$-test: $t = -9.14$, $P < 0.0001$; Figure 1(a)). However, when comparing only the breeding couple, there were no differences between breeding seasons (paired $t$-test: $t = -0.082$, $P = 0.76$).

Social rank
In general, we obtained a total of 14 hours per wolf per behavioural sampling, with a total of 2688 video hours. A total of 2294 agonistic encounters were registered: 265 for wolves from El Tecuán, 1404 from Zacango, 96 from León and 1228 from San Cayetano. Average aggression encounters per year were greater in 2011 compared to 2010 (8.97 ± 2.27 vs. 54.01 ± 10.50; Wilcoxon signed-rank test $z = -4.890$, $n = 70$, $P < 0.0001$). Zoo location indicated a significant difference between years for aggression events at San Cayetano (15.06 ± 5.39 in 2010, and 108.38 ± 21.45 in 2011, Wilcoxon signed-rank test, $P < 0.0001$, $n = 29$).

After analysing social ranks, Zacango’s female wolves showed a rank from 38 to 51. Therefore, individuals were classified into three dominance rank classes: low (0–39%), medium (40–50%) and high (51–100%). High-social-ranked wolves showed higher ($P < 0.0001$; Kruskal-Wallis) fGC levels (202.03 ± 19.94; $n = 229$) than medium- (175.33 ± 19.94 ng/g DM; $n = 214$) and low- (162.06 ± 16.15; $n = 377$) ranked wolves did. Spearman’s $r$ analysis showed no correlation ($r = -0.062$, $P = 0.135$) between fGC and social rank.

Pack social grouping
No significant differences were observed for group composition (one-way ANOVA, $F_{2,867} = 1.19$, $P = 0.36$). Solitary wolves had 149.87 ± 22.94 ng/g, both types of pairs from the same or different gender had 137.09 ± 26.69 ng/g, and groups from the same gender or mixed showed 168.51 ± 10.39 ng/g.

Gender and age class
Significant differences for fGC concentrations were obtained between genders (independent $t$-test, $t = 5.50$, $P < 0.0001$): males (214.53 ± 13.43, $n = 348$) showed higher levels compared to females (121.51 ± 10.03, $n = 657$; Figure 1(b)).

### Table IV. Comparison of overall mean faecal glucocorticoid metabolite concentrations (GCC, expressed in ng/g dry matter) over four seasons in six wolf packs.

| Season | n  | GCC (ng/g DM) Mean ± SEM | Range          |
|--------|----|--------------------------|----------------|
| Winter | 347| 116.78 ± 12.48$^{b}$     | 2.23–3378.40   |
| Spring | 219| 192.58 ± 18.76$^{c}$     | 1.47–1433.26   |
| Summer | 267| 145.21 ± 12.95$^{ab}$    | 4.57–1654.10   |
| Autumn | 172| 191.96 ± 25.18$^{a}$     | 1.74–2147.70   |

One-way repeated measures analysis of variance (ANOVA). $ab$Different letters indicate significant differences ($P < 0.01$).

### Table V. Comparison of means for faecal glucocorticoid metabolite concentrations (GCC, expressed in ng/g dry matter) over two successive years and seasons in six wolf packs.

| Season | 2010 | 2011 |
|--------|------|------|
|        | GCC (ng/g DM) mean ± SEM | n | Range | GCC (ng/g DM) mean ± SEM | n | Range |
| Winter | 104.20 ± 13.44$^{ac}$   | 102 | 5.20–1074.59 | 122.02 ± 16.77$^{ac}$ | 245 | 2.23–3380.64 |
| Spring | 227.73 ± 28.46$^{ac}$  | 126 | 1.48–1433.27 | 144.96 ± 20.75$^{ac}$ | 93 | 14.18–1116.73 |
| Summer | 194.55 ± 30.01$^{ac}$ | 140 | 6.68–1654.10 | 132.46 ± 17.28$^{ac}$ | 127 | 4.57–1242.49 |
| Autumn | 156.79 ± 19.10$^{ac}$ | 113 | 1.74–2147.70 | 155.70 ± 38.96$^{ad}$ | 59 | 6.95–1720.59 |

One-way repeated measures analysis of variance (ANOVA). $abc$Different letters within columns indicate significant differences between seasons ($P < 0.0001$). $ab$Different letters within rows indicate significant differences between years ($P < 0.05$).
Older wolves excreted higher levels of fGC than young adults and reproductive adults did (one-way ANOVA, $F_{2,997} = 4.33$, $P = 0.016$; Figure 1(c)). The youngest individuals in the study were 3 years old, whereas the oldest ones were 11 years old.

**Discussion**

**General data**

Cortisol concentrations in free-ranging wolves ranged from 872 to 1468 ng/g dry faeces according to the results of Creel et al. (2002) and Sands and Creel (2004). In the present study we found 1.47–3380.64 ng of fGC/g DM, extremes which are incomparably low and high levels. The range differences among studies may be explained by different factors. Field samples seem to have higher concentrations (4–7 times) than confinement ones do. Bryan et al. (2015) recently showed that the disrupted social structure caused by hunting causes physiological stress with elevated levels of cortisol in free-ranging wolves, although some studies have discussed the possibility that stress and aggression levels are higher in captivity than in the wild (e.g. Spercoski et al. 2012). In addition, the extracted hormones from faeces sampled by Creel et al. (2002) and Sands and Creel (2004) were different from the one used in this study.

Sustained elevation of cortisol level generally indicates increased adrenal function, as was the case in five females with extremely high levels. These were coincidental with previous stressful husbandry events such as capture of group mates, fighting with neighbour wolves through the fence, cleaning of the exhibit, and wolf off-exhibitor remaining in social isolation on the day before samples were taken. These wolves’ fGC glucocorticoid concentrations returned to basal levels 2 days after the husbandry procedures. Likewise, the translocation of a male to a female enclosure resulted in a 3.5-fold increase compared to baseline concentrations in foxes (*Cerdocyon thous* Linnaeus, 1766) (Paz et al. 2015), and two-fold elevated fGC concentrations were observed 1 day after a veterinary procedure in zoo-housed armadillos (*Tolypeutes matacus* Desmarest, 1804) (Howell-Stephens et al. 2012). Our results also agree with Creel et al. (2002), Van Meter et al. (2009) and Eggermann et al. (2013), who state that anthropogenic disturbance may also influence stress hormones. Thus, an acute stressor resulted in the short-term elevation of glucocorticoid concentrations (Sheriff et al. 2011). It is worth mentioning that in the case of Africam Zoo, where three females were rotated every day during the non-breeding season, allowing one of them to stay with the resident male, this social disruption did not seem to affect their adrenal activity, or the male’s (data not shown).

Of the six facilities studied, only in one were fGC levels significantly higher and, contrary to expectations, corresponded to one of the parks without visitors, but also to the one feeding with dog pellets as the only feed source. It is important to note that glucocorticoids increase in circulation in response to energetic needs, and their levels are generally interpreted as indicators of allostatic load (Bonier et al. 2009). Furthermore, a diet type or lack of proper diet can influence measurements of glucocorticoid metabolites in faeces, and may be a stressing...
factor to non-human animals (Keay et al. 2006; Goymann 2012).

The total overall mean fGC concentrations in this study were lower than the results of Pifarre (2004: 153.83 ± 8.16 vs. 203.74 ng/g DM), who did not find differences in faecal cortisol concentrations among 14 Mexican grey wolves from three zoos. Low adrenal activity was also observed in maned wolves in a protected area compared to those in farmlands and park boundaries (Spercoski et al. 2012). According to Keay et al. (2006), once a non-human animal becomes habituated to a stressor, there may be a reduction in the extent of elevation of faecal cortisol levels in response to that stressor. Furthermore, cortisol concentrations could also be used as indicators of overall physiological state and provide vital information on the health and resilience of a population as a whole (Kershaw & Hall 2016); thus, our findings suggest that wolves are becoming habituated to confinement at the zoos.

Seasonal and breeding season variations

Overall, data showed that paired samples of fGC levels significantly declined during the second year of monitoring. However, when we considered only the two wolf groups formed in 2010 (Zacango Zoo and El Tecuán Park), there were no significant differences between years. Seasonal fGC concentrations were lower during winter – the breeding season – and peaked in spring, indicating modest seasonal variability; there was a difference between years in autumn, with higher levels in 2010. No seasonal or circadian variation in glucocorticoid concentrations has been reported in the species (Seal et al. 1987; McLeod et al. 1996) under free-ranging conditions. However, Romero (2002) explained that there is a normal seasonal variation in glucocorticoid concentrations in vertebrates.

According to Zimen (1976), breeding is the period when more aggression is observed in free-ranging wolves, in response to sexual competition. Similarly, Sands and Creel (2004) observed higher glucocorticoid levels during the breeding season, and so did Teodoro et al. (2012) in captive male maned wolves. Nevertheless, in the present study, wolves in the non-reproductive season showed two-fold higher levels of fGC compared to the reproductive season. These results are consistent with the work of Spercoski et al. (2012) on maned wolves living on farmlands, where home range varied between the two reproductive periods. In our opinion, these conflicting results may be explained at least partially by the composition of the wolf packs at the different zoos and parks in the present study including singletons, iso-pairs and just one breeding couple, which may have biased the effect of the reproductive season on fGC levels. Another explanation of the lower, clearly temporal fGC levels found during the breeding season – in winter – is that it coincided with shortening day length and decreasing ambient temperatures (ranging from 13.9 to 22°C); and, according to Smith et al. (2004), wolves are well adapted to winter conditions.

Social rank

In previous studies, higher glucocorticoid levels were reported in identified (Sands & Creel 2004) or suspected (Barja et al. 2008) dominant compared to subordinate free-ranging wolves, and no correlation between social status and glucocorticoid was found in a captive pack, similar to our results. Moreover, Creel et al. (2002) showed that high-ranking wolves were more stressed (about 50% more) than low-ranking subordinates. Similarly, van Kesteren et al. (2012) found that dominant males had higher average fGC than did subordinates. Our results are consistent with these findings, demonstrating that dominant individuals in societies where dominance is maintained through frequent physical aggression exhibit higher physiological stress indices due to the physical demands of fighting and the challenge of maintaining social rank.

Higher levels of aggression were observed in the resident wolves reacting to a newly introduced breeding female at San Cayetano. At this park, the two packs (see Table I) were unable to move out of sight of each other because their enclosures were divided by a mesh-wired fence.

Pack size

We found no differences in fGC levels from wolves housed in groups, in pairs or in solitaire. A similar result was found in another study in captive wolves (Post 2007). Similarly, Aurich et al. (2015) observed that individual stabling in horses did not affect salivary cortisol.

Gender and age

Data analysed by gender showed that wolf males’ fGC concentrations were almost 2 times higher than females’. These findings are in keeping with a previous study by Touma et al. (2003), who observed that male mice (Mus musculus Linnaeus, 1758) excreted higher amounts of faecal corticosteroid metabolites than their female counterparts did. However, most studies have not found gender differences in stress levels, including
Pifarre (2004), Creel et al. (2002), Sands and Creel (2004), Pifarré et al. (2012), and Molnar et al. (2015) in wolves, and Vynne et al. (2014) and Spercoski et al. (2012) in the free-ranging managed wolves. Palme et al. (2005), on the other hand, reported that sex plays an important role in the metabolism and excretion of faecal glucocorticoids, and this might be the reason why we observed gender differences regardless of the breeding season.

Tukey’s post hoc comparisons showed that fGC varied between elderly wolves and adults. This finding contrasts those of Nogueira et al. (2002), who observed that cortisol decreased during horse (Equis caballus Linnaeus, 1758) aging and physical conditioning, and those of Aurich et al. (2015) who failed to find age differences in cortisol levels.

Conclusions

In conclusion, our data shows that the availability of food and stability of the environment, both constant in confinement enclosures such as zoos, may contribute to the low and constant fGC metabolite levels observed in this study: Mexican grey wolves showed significantly lower differences after a year, when most groups were established, and – contrary to expectations – during the reproductive season. These results suggest wolves’ good adjustment or habituation to captivity; however, acute stress of anthropogenic husbandry increased fGC levels up to 90-fold. Our results show that evaluating variation in faecal glucocorticoid excretion over time might be informative for assessing both acute and chronic physiological stress in long-term studies, providing crucial information on the resilience of captive animal populations. Our findings may contribute to a more comprehensive definition of confinement, which has traditionally been perceived as a stressful habitat for wild animals.

Acknowledgements

I. Escobar-Ibarra was supported by a scholarship from CONACyT (179126). ALCM, RMC and MAS are members of the National System of Researchers (SNI) of Mexico. The authors are grateful to authorities from the different zoos and parks – Parque el Tecuán, CIVS San Cayetano, Zacango Zoo (DVMs Guillermo Díaz & Jesús Frieventh), León Zoo (DVM Ivonne Ruiz), Parque del Pueblo Nezahualcóyotl (DVM Magda García Trinidad), Africam Safari (Mr Juan Govea, mammal curator) – and to Dirección General de Vida Silvestre and SEMARNAT (DVM Fernando Cortes and Eduardo Martínez), for all their support and cooperation. The authors are also indebted to DVMs Omar García and Lilia Meza, and veterinary field assistants Ricardo Trejo, Nadyeli Nava, Victor Yamasaki, Fernanda Nuñez, Aldo Michel, Samantha Romo, Miguel Orozco, Isy Aguilar, Teresa Villanueva, Evelia Padilla and Fabiola Gómez, who kindly helped with faeces collection and video recording of behavioural observations. We also acknowledge Dr Milagros Méndez-Ubach for lending equipment from her laboratory (Depto. de Neuroquímica, Subdirección de Investigaciones Clínicas, IMPRFM). Special thanks to the anonymous referee whose suggestions improved our manuscript.

References

Altmann J. 1974. Observational study of behavior: Sampling methods. Behaviour 49:227–266. DOI:10.1163/156853974X00534.

Aurich J, Wulf M, Ill N, Erber R, Von Lewinski M, Palme R, Aurich C. 2015. Effects of season, age, sex, and housing on salivary cortisol concentrations in horses. Domestic Animal Endocrinology 52:11–16. DOI:10.1016/j.domaniend.2015.01.003.

Barja I, Silván G, Illera JC. 2008. Relationships between sex and stress hormone levels in feces and marking behavior in a wild population of Iberian wolves (Canis lupus signatus). Journal of Chemical Ecology 34:697–701. DOI:10.1007/s10886-008-9460-0.

Bonier F, Martin PR, Moore IT, Wingfield JC. 2009. Do baseline glucocorticoids predict fitness? Trends in Ecology & Evolution 24:634–642. DOI:10.1016/j.tree.2009.04.013.

Bryan HM, Smits JEG, Kore L, Paquet PC, Wynne-Edwards KE, Musiani M. 2015. Heavily hunted wolves have higher stress and reproductive steroids than wolves with lower hunting pressure. Functional Ecology 29:347–356. DOI:10.1111/1365-2435.12354.

Césped-Molina AL, Hernández-López L, Páez-Ponce D, Rojas-Maya S, Mondragón-Ceballos R. 2006. Seasonal variations of fecal progesterone and 17α-estradiol in captive female black-handed spider monkeys (Ateles geoffroyi). Theriogenology 66:1985–1993. DOI:10.1016/j.theriogenology.2006.03.038.

Creel S, Fox JE, Hardy A, Sands J, Garrott B, Peterson RO. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology 16:809–814. DOI:10.1046/j.1523-1739.2002.00554.x.

Diario Oficial de la Federación. 2015. Proyecto de Modificación del Anexo Normativo III, Lista de especies en riesgo de la Norma Oficial Mexicana NOM 059SEMARNAT 2010, Protección ambiental Especies nativas de México de flora y fauna silvestres Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio Lista de especies en riesgo. Available: http://www.dof.gob.mx/nota_detalle.php?codigo=5420810&fecha=21/12/2015&print=true. Accessed May 2017 11.

Eggermann J, Theuerkauf J, Pirgá B, Milanowski A, Gula R. 2013. Stress-hormone levels of wolves in relation to breeding season, pack size, human activity, and prey density. Annales Zoologici Fennici 50:170–175. DOI:10.5735/086.050.0304.

Endangered Wolf Center. 2016. Mexican wolf. Available: http://www.endangeredwolffcenter.org/educational-resources/mexican-gray-wolf/. Accessed Jan 2016 24.

Escobar I, Alonso SML, Mayagoitia NL, Ramírez NR, Mota RD. 2005. Elaboración de un etograma empático del lobo gris
mexicano (Canis lupus baileyi). Cuadernos de Etnología Fauna Silvestre No. 1. México: Universidad Juárez del Estado de Durango. Universidad Autónoma Metropolitana. 89 pp. (In Spanish).

Fox MW. 1971. Behaviour of wolves, dogs, and related canids. New York: Harper & Row. 220 pp.

Frank H, Frank MG. 1982. On the effects of domestication on canine social development and behavior. Applied Animal Ethology 8:507–525. DOI:10.1016/0303-7462(82)90025-2.

FWS. 2014. Mexican wolf recovery program. 2014 Progress Report. US Fish and Wildlife Service. 70 pp.

Goymann W. 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: The problem with sex, diet, metabolic rate and the individual. Methods in Ecology and Evolution 3:757–765. DOI:10.1111/j.2041-210X.2012.00203.x.

Hays RD, Harestad AS. 2000. Demography of a recovering wolf population in the Yukon. Canadian Journal of Zoology 78:36–48. DOI:10.1139/cjz-199-186.

Howell-Stephens JA, Brown JS, Bernier D, Mulkerin D, LGVS. 2015. Ley General de Vida Silvestre. Última reforma DOF 26-01-2015. Available: http://www.diputados.gob.mx/LeyesBiblio/pdf/146_260115.pdf. Accessed Jan 2016 24.

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. Journal of Zoo and Evolution 3:757–765. DOI:10.1111/j.1568-5396.2006.00065.x.

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. Journal of Zoo and Evolution 3:757–765. DOI:10.1111/j.1568-5396.2006.00065.x.

Molnar B, Detante B, Delahanty B, Palme R, Boonstra R. 2011. Measuring stress in wildlife: Techniques for quantifying glucocorticoids. Oecologia 166:889–897. DOI:10.1007/s00442-011-1943-y.

Sheriff MJ, Dantzer B, Delahanty B, Palme R, Boonstra R. 2011. Measuring stress in wildlife: Techniques for quantifying glucocorticoids. Oecologia 166:889–897. DOI:10.1007/s00442-011-1943-y.

Smith SW, Drummer TD, Murphy KM, Gueneyre DS, Evans SB. 2004. Winter prey selection and estimation of wolf kill rates in Yellowstone National Park, 1995–2000. Journal of Wildlife Management 68:153–166. DOI:10.2193/0022-541X(2004)068[0153:WPSEAO]2.0.CO;2.

Spercoski KM, Moraes RN, Morato RG, De Paula RC, Azevedo FC, May-Junior JA, Santos JP, Reghelin AL, Wildt DE, Songsasen N. 2012. Adrenal activity in maned wolves is higher than in domestic dogs. General Comparative Endocrinology 189:148–155. DOI:10.1016/j.ygcen.2012.04.003.
on farmlands and park boundaries than within protected areas.
General Comparative Endocrinology 179:232–240. 
DOI:10.1016/j.ygcen.2012.08.002.
Teodoro LO, Melo-Jr AA, Spercoski KM, Morais RN, Souza FF. 
2012. Seasonal aspects of reproductive physiology in captive 
maned wolves (Chrysocyon brachyurus, Illiger 1815). 
Reproduction in Domestic Animals 47:250–255. 
DOI:10.1111/rda.12071.
Touma C, Palme R. 2005. Measuring fecal glucocorticoid meta-
bolites in mammals and birds: The importance of validation. 
Annals of the New York Academy of Sciences 1046:54–74. 
DOI:10.1196/annals.1343.006.
Touma C, Sachser N, Möstl E, Palme R. 2003. Effects of sex 
and time of day on metabolism and excretion of corticoster-
one in urine and feces of mice. General Comparative 
Endocrinology 130:267–278. DOI:10.1016/s0016-6480(02) 
00620-2.
van Kesteren F, Sillero-Zubiri C, Millar R, Argaw K, Macdonald 
DW, Paris M. 2012. Sex stress and social status: Patterns in 
fecal testosterone and glucocorticoid metabolites in male 
Ethiopian wolves. General Comparative Endocrinology 
179:30–37. DOI:10.1016/j.ygcen.2012.07.016.
Van Meter P, French JA, Dloniak SM, Watts HE, Kolowski JM, 
Holekamp KE. 2009. Fecal glucocorticoids reflect socio-eco-
logical and anthropogenic stressors in the lives of wild spotted 
hyenas. Hormones and Behavior 55:329–337. DOI:10.1016/j. 
yhbeh.2008.11.001.
von der Ohe CG, Servheen C. 2002. Stress and fecal glucocorti-
roids. Wildlife Society Bulletin 30:1215–1225.
Vynne C, Booth RK, Wasser SK. 2014. Physiological implications 
of landscape use by free-ranging maned wolves (Chrysocyon 
brachyurus) in Brazil. Journal of Mammalogy 95:696–706. 
DOI:10.1644/12-MAMM-A-247.
Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, 
Bechert U, Millspaugh JJ, Larson S, Monfort SL. 2000. A 
generalized fecal glucocorticoid assay for use in a diverse 
array of nondomestic mammalian and avian species. General 
Comparative Endocrinology 120:260–275. DOI:10.1006/ 
gcen.2000.7557.
Young KM, Walker SL, Lanthier C, Waddell WJ, Monfort SL, 
Brown JL. 2004. Noninvasive monitoring of adrenocortical 
activity in carnivores by fecal glucocorticoid analyses. General 
Comparative Endocrinology 137:148–165. DOI:10.1016/j. 
ygcen.2004.02.016.
Zimen E. 1976. On the regulation of pack size in wolves. Ethology 
40:300–341. DOI:10.1111/j.1439-0310.1976.tb00939.x.
Zumpe D, Michael RP. 1986. Dominance index: A simple mea-
sure of relative dominance status in primates. American 
Journal of Primatology 10:291–300. DOI:10.1002/ 
ajp.1350100402.