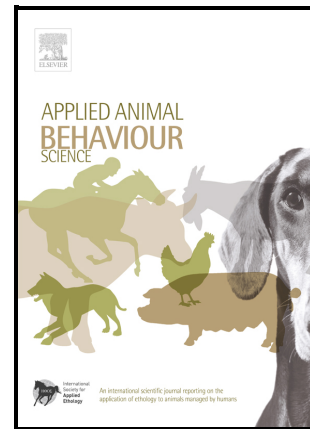


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PII: S0168-1591(23)00283-6

DOI: <https://doi.org/10.1016/j.applanim.2023.106111>

Reference: APPLAN106111

To appear in: *Applied Animal Behaviour Science*

Received date: 21 August 2023

Revised date: 30 October 2023

Accepted date: 3 November 2023

Please cite this article as: Mishaal Akbar and Neil Price Evans, Elevated hair cortisol and decreased hair testosterone indicates chronic disruption of the HPA/HPG axis and is reflective of poor welfare in Rhesus Macaques used as performing (dancing) monkeys in Pakistan., *Applied Animal Behaviour Science*, (2023) doi:<https://doi.org/10.1016/j.applanim.2023.106111>

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Elevated hair cortisol and decreased hair testosterone indicates chronic disruption of the HPA/HPG axis and is reflective of poor welfare in Rhesus Macaques used as performing (dancing) monkeys in Pakistan.

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Declarations of interest: none

Abstract

Throughout South and South East Asia it is common to see rhesus monkeys being used in public entertainment as Dancing Monkeys (DM). In Pakistan, native rhesus macaques are captured from the wild during infancy and trained using negative reinforcement methods to perform for public entertainment. This study aimed to quantify and compare physiological stress in 50 DM with 77 controls from an outdoor harem- housed colony of captive rhesus

macaques, and to assess whether certain behavioural indices observed in DM were reflective of physiological state. DM had significantly higher hair cortisol concentrations ($t(77.358)=-2.8099$ CI(-0.519,-0.088), $p<0.01$, $d=0.531$) and lower hair testosterone concentrations ($t(66.6)=4.917$, 95CI(0.474, 1.1108), $p<0.0001$, $d=1.16$) compared to the controls. Linear modelling indicated that hair cortisol was positively associated with fear and ectoparasite load, and negatively associated with ectoparasite presence. Hair testosterone was negatively associated with aggression and body welfare and positively associated with abnormal behaviour. The results were assessed relative to semi-structured trainer interviews to better understand observed negative physiological and behavioural markers indicating stress and hence poor welfare. Overall, the results exemplify the merit of using a two-fold biobehavioural approach for assessing welfare and indicate that DM are under chronic physiological stress which is associated with negative behavioural outcomes.

Keywords

Rhesus macaque, Hair, Cortisol, Testosterone, Welfare, Behaviour, ELISA

1. Introduction

Animals have been used in and as forms of entertainment, across the world and throughout human history. With increased animal rights advocacy and a better understanding of the implications of such practices for animal welfare, however, the use of animals as performers for entertainment purposes is decreasing (Waller and Iluzada, 2020). Synonymous with the more globally well-known and now phased-out practice of Dancing Bears in Europe and

Asia, Dancing Monkeys (DM) remain common throughout South and South East Asia (Agoramoorthy and Hsu, 2005).

In Pakistan, native rhesus macaques are typically captured from the wild during infancy and trained via negative enforcement and fear based methods to perform actions such as somersaulting, jumping and saluting (appendix 1), in public areas, as a means of income generation, particularly within underprivileged communities. This practice is generally accepted by local communities with limited legal protections for the DMs.

Trainers belong to specific '*bradaris*' or groups which are hierarchical. These groups may have different practices, teach different performances to their animals and be of varying sizes specifically operating in different geographical regions. DM trainers prefer working with males as they are larger and attract more public attention, however high levels of conspecific aggression are observed between animals so despite being naturally gregarious, rhesus macaques kept as DM are kept separated and when not used in performance remain collared and leashed either in solitary conditions or close to DM owned by nearby trainers. Many DM undergo body modifications such as ear piercing and dyed or cut fur as this is believed to increase public engagement and the entertainment value of the animals leading to greater monetary gain. Access to veterinary care and the provision of adequate nutrition can be limited by trainer income. The training, performance and husbandry conditions of DM may therefore violate fundamental established principles of animal welfare and thus DM are likely to be subject to a reduced quality of life compared to other wild rhesus Macaques or captive primate populations which are subject to stricter animal welfare regulations (Fooden, 2000; Mellor, 2016)

The physiological stress response is a homeostatic mechanism that functions to protect an organism from harm or disturbance (i.e. a stressor). The main mediators of the response are increased activity within the sympatho-adreno-medullary (SAM) and hypothalamic-pituitary-adrenal (HPA) axes. Cortisol is the end product of the HPA axis in primates, it is quantifiable in biological media and is therefore a widely used measure of physiological stress (Kopin et al., 1988; Maestriperi and Hoffman, 2011; Smith and Vale, 2006).

Cumulative repeated or chronic elevations in plasma cortisol can result in increased allostatic load and has been linked with behavioural disturbances, immune system suppression, disease, accelerated aging and permanent physiological dysregulation (Maestriperi and Hoffman, 2011; Novak et al., 2013). As hair cortisol concentrations (HCC) in rhesus macaques have been shown to be related to salivary cortisol and have stable inter-individual variability in the absence of any identifiable major stressor (Meyer and Novak, 2012; Stalder et al., 2012) it is considered a highly useful, non-invasive and valid measure of chronic HPA activity and, by extension, stress (Novak et al., 2013).

Although significant intraindividual variability exists in HCC, HCC can be negatively related with rank (Qin et al., 2013; Wooddell et al., 2016) and age (Dettmer et al., 2012; Heimbürge et al., 2019). Basal HCC have been shown to be influenced by the maternally environment experienced by an individual before birth (Kapoor et al., 2014; Kapoor et al., 2016), and permanently altered by adverse early childhood experiences (Feng et al., 2011).

Functionality within physiological systems are normally interlinked and the operation of the hypothalamus-pituitary-gonadal (HPG) axis although capable of being regulated independently by environmental factors may be disturbed secondary to chronic stress. In males, the HPG axis is depressed under conditions of chronic stress, as cortisol can act directly on the testes to limit testosterone production (Bingaman et al., 1994; Cameron and

Nosbisch, 1991; Hayashi and Moberg, 1987; Pickering et al., 1987; Toufexis et al., 2014). In conditions of social stress, however, it has been reported that testosterone may be increased, particularly where the behavioural effects of testosterone (increased dominance and aggression) serve to benefit the individual e.g. maintaining social status (Higham et al., 2013; Mann et al., 1998; Rose et al., 1971; Stanton et al., 2009). Assessing the changes in circulating cortisol and gonadal steroids can therefore be useful when assessing physiological stress (Stalder and Kirschbaum, 2012). Steroids are secreted into the peripheral circulation and can be measured in serum, plasma, saliva, urine and faeces (Maestriperi and Hoffman, 2011), the different media having different steroid profiles relative to the time of secretion but covering the range of seconds to hours. Steroids are also stably deposited into the medullary region of the hair shaft during the anagen phase of the hair growth cycle via diffusion from the blood stream (Heimbürge et al., 2019; Kapoor et al., 2018). As hair grows and steroids can be extracted from the hair and then measured hair steroid concentrations can provide a measure of peripheral concentrations over much longer time periods of weeks to months. Similar to humans, rhesus macaques undergo a 3-month hair cycle with three phases (anagen (growth), telogen (resting), catagen (degradation) with a hair grows that is similar to scalp hair (~1cm per month); thus the quantification of steroid hormones from hair can provide a 3-month historical picture of HPA and HPG activity(Lutz et al., 2019) (Meyer and Novak, 2012)and a means to assess chronic stress and its secondary effects on the HPG axis in DM (Meyer and Novak, 2012). In this regard, HCC has been shown to be a useful tool for assessing depressive and anxiety-like behaviours in rhesus macaques (Adams et al., 2017; Dettmer et al., 2012) endocrine disturbances in polar bears (Bechshøft et al., 2012; Bechshøft et al., 2011) and how endocrinological and neuropsychiatric disorders response to treatment (Meyer and Novak,

2012). Given these features, HCC measurements have gained credibility as a valid, objective and standardised tool with which to assess welfare (Heimbürge et al., 2019; Yamanashi et al., 2016). Although salivary testosterone has been previously used as an acute stress marker in pigs (Escribano et al., 2014) and hair testosterone has been quantified in rhesus macaques (Kapoor et al., 2014), the methods in this study are novel as they incorporate the quantification of both hair cortisol and testosterone for welfare applications in primates.

Abnormal behaviour is often used as an outward manifestation of stress or deviation from standard wellbeing (Animals, 2011) and it is accepted that primates display a variety of behavioural stress indicators. Abnormal stereotypies such as self-injurious, hyper-aggressive, hyper-fearful and stereotypical behaviour (Fittinghoff et al., 1974; Novak et al., 2013) are thought to be maladapted analogues of normal behaviour and are often observed in primates that have been separated from their mother before their natural age of independence (1 year for rhesus macaques) and those in social isolation, legitimizing its use as a welfare indicator (Novak and Suomi, 2008; Philbin, 1998). However, there is some evidence that the expression of these 'maladaptive' behaviours arise from an inability to display natural behaviours, and in so doing certain stereotypies may have a self-soothing or self-stimulating purposes and hence may not be a consistent valid indicator of physiological stress (Jacobson et al., 2016; Poirier and Bateson, 2017).

The aim of this study was to quantify welfare in DM using a two-fold biobehavioural approach.. We tested the hypotheses that relative to controls that were subject to more stringent welfare and husbandry oversight and were socially-housed (Mannheimer

Foundation (MHF), FL, USA), DMs which are subject to reduced standards of care and are mainly housed individually would have higher chronic cortisol concentrations and lower chronic testosterone concentrations. Furthermore, DMs will display specific negative behaviours (abnormal behaviour, fear, aggression) compared to control macaques. Finally, that these physiological and behavioural indicators of stress will both be associated with poor body condition and mistreatment by trainers. The combination of both physiological and behavioural findings will provide a holistic picture of possible welfare standards of rhesus macaques used for entertainment in South Asia.

2. Materials and methods

2.1 Ethics: Both animal and human components of the study were approved by the University of Glasgow Animal Ethics committee (Ethical approval(s) 31a/18; 200170136) and all hair sampling was MHF IACUC-approved. Sample importation was approved in accordance with Regulation 26 and Schedule 3, paragraph 3 of the Trade in Animals and Related Products (Scotland) Regulations 2012 (TARP(S)2018/15). No export licences were required from Pakistan. Explicit prior verbal consent was obtained from trainers for the collection of hair samples and video footage.

2.2 Dancing Monkeys: 50 DM (48 males, 2 females, aged 0.5 - 12 years) were sampled from three '*bradari*' groups (A=10,B=10,C=30) of trainers around Islamabad, Pakistan (July-August 2018). All sampled DM had been used as DM for at least 3 months prior to data collection. The age of the animal was given by the trainer who, under direction, provided the hair

samples. Hair samples from the anterior lateral chest and anterior shoulder were first tied using small elastic bands and then cut using precleaned (ethanol) scissors. The anterior lateral chest and anterior shoulder were chosen as collection sites as hair could be cut inconspicuously from these locations and thus was acceptable for the trainers. These areas should have the highest HCC due to blood flow (Carlitz et al., 2015). Hair samples were packed in individual airtight Ziploc bags (protected from heat and light) before shipment to the University of Glasgow for analysis. Each animal's performance was video recorded by MA for later characterisation of body modifications, animal-trainer interactions and a systematic behavioural analysis. Trainers were interviewed in a semi structured manner during the sample collection to understand standard trade practices and conditions.

2.3 Controls: As it is not possible to collect standardised control samples from wild populations of macaques, samples were obtained from a captive outdoor breeding colony of Indian-origin rhesus macaques at The Mannheimer Foundation, an AAALAC-accredited facility in LaBelle, FL, USA. Within this facility macaques are housed in groups of one adult male, 5-8 adult females and their offspring (up to 12 months old). Field cages are constructed from galvanised 4.45cm² mesh chain-link fencing and fully roofed by raised sheet metal panels on silica sand substrate. Enclosures are furnished with species appropriate environmental enrichment including manipulanda, swings, perches and barrels. Animals are fed a commercial nonhuman primate diet (5LB2, Lab Diet, St. Louis, MO) and automatically watered ad libitum (lixit, Napa, CA). Animals are given a foraging feed mix (sunflower seeds, dried corn and peanuts) and observed at least once daily by trained staff for illness, injury and abnormal behaviours and had average lifespan seen in captivity (median 27 years). The preventative medicine program at the centre includes semi-annual

physical examination, intradermal tuberculin skin testing, deworming and routine vaccination against *Clostridium tetani*, *Measles morbillivirus*, and *Rabies lyssavirus*. Animals are specific-pathogen-free (serologically negative) for *Macacine alphaherpes virus 1*, *Simian retrovirus serotype 1*, *Simian T-lymphotropic virus 1*, and *Simian immunodeficiency virus*. No individual showed any sign of illness, alopecia or abnormal behaviour at the time of sample collection and all subjects were of healthy BCS. No sampled control animal had any history of abnormal behaviours. Hair samples were clipped from the anterior chest of 77 individuals (66 male, 11 female; 0.7–18 years) during routine semi-annual physical examinations, stored at room temperature in individually labelled, airtight plastic zip-top bags, protected from light and heat, and mailed to the University of Glasgow for analysis (appendix).

2.4 Body modifications, animal trainer interactions: DM were scored relative to body alterations from 0 (none) to 4 (piercings, scars, fur dyed and cut); observed mistreatment by trainer from 1 (none) to 5 (reprimanded with training stick eliciting audible and visible pain response) (tables 4, 5). These were not scored in the control macaques as they had no body modifications. Controls also had no close human interactions such as training or hand-feeding; staff only entered once a day to clean the cages and the animals were given the option to move to another section of the cage, plus all other observations and feeding was conducted from a distance or outside from the cage.

2.5 Behavioural Measures: DM behaviour was scored (1=no presence to 4=high exhibition) for fear, aggression towards both humans and conspecifics, and abnormal behaviour including stereotypies and self-injury (Barik et al., 2013; Pomerantz et al., 2012) (table 1-3) from their recorded performances. As the DM performances were of different lengths,

ranging for 30 seconds to 4 minutes and were made up of different tricks, it was difficult to obtain standardised rates or proportions of behaviour. Hence, behaviour was standardised by making ethograms of severity of behaviours previously established as 'negative' welfare indicators and whether or not they were displayed during the DM performance (appendix). These behavioural measures were not scored in the control macaques as no sampled individual displayed stereotypic or abnormal behaviour in their lifetime.

Table 1: Ethogram used to assess severity of fearfulness behaviours exhibited during DM performance

Fear Score	Behaviours Included
1	No fearful behaviour observed
2	Ears pulled back, lip smacking, brow pulled up, freezing
3	2 + tense mouth and body, cringing and crouching, alarm chirps
4	3 + O shaped mouth, grimacing, screaming, attempting to flee

Table 2: Ethogram used to assess severity of aggressive behaviours during DM performance

Aggression Score	Behaviours Included
1	No aggression observed
2	Open mouth stare, grunting
3	2 + teeth barring, pseudo-charge, piloerection
4	3 + patting ground, charging and eliciting response from trainer

Table 3: Ethogram used to assess severity of abnormal behaviours displayed during DM performance

Abnormal Behaviour Score	Behaviours Included
1	No abnormal behaviour observed
2	Stereotypic behaviours e.g. digit sucking
3	2 + some self-directed behaviour (e.g. self-biting, hair-pulling) with no wounds
4	3 + self-injury with visible wounds

Table 4: Number of body modifications assessed as body welfare visible during the DM performances

Body Welfare Score	Alterations: Scars/Sores, Piercings, Fur Dyed/Cut/Shaves, Parasite Presence
0	No alterations
1	1 alteration e.g. scars OR piercing
2	2 alterations e.g. scars AND piercings
3	3 alterations
4	All body alterations present

Table 5: Trainer interaction and force observed during the DM performances

Ethical Treatment Score	Behaviours Included
1	No visible mistreatment
2	Trainer using leash and training stick to direct behaviour, no contact between stick and animal

3	Trainer pulling leash (animal may hold leash), training stick in contact with animals body
4	Trainer jerking leash, training stick making contact with animal body with moderate force or for reprimand
5	Trainer harshly pulling leash, training stick used with severe force and with visible pain response from animal

2.6 Hair Sample Preparation: As all of the samples were collected as a convenience sample, each animal was only sampled once. The incidental finding of louse eggs (appendix figure 3) was observed in DM hair samples only. Hatched and unhatched lice eggs have been shown to be a valid measure of ectoparasite load in Japanese macaques (Ishii et al., 2017) and so DM hair samples were classified by visual external evidence of ectoparasites and eggs as high (>100 eggs visible), medium (30-100 eggs visible), low (0-20 eggs visible) or none. Each sample was washed with distilled water and ethanol, twice, 30 seconds each time. Samples were then air dried at room temperature before being cut with scissors, flash frozen in liquid nitrogen and powdered in a Retsch Ball mill (Retsch, Haan, Germany). Powdered hair was stored in air-tight Eppendorf tubes at room temperature and protected from light until ELISA. Initial analysis based on a pooled sample revealed the required weight needed for extraction of cortisol and testosterone for accurate quantification in the assays.

2.7 Cortisol: Samples were processed and analysed as in (Maxwell et al., 2019; Meyer et al., 2014) using a commercially available Cortisol Elisa Kit (Caymen Chemicals, Ann Arbor, Michigan, USA). Briefly, 12 mg of powdered hair was incubated with 2mL HPLC-grade

methanol, in an orbital shaker oven, for 17 hours at 51°C. A 750 µL aliquot was dried in a vacuum centrifuge, covered and stored at -20°C. Samples were thawed and reconstituted in 300 µL of ELISA buffer, vortexed for 30 min and assayed following the manufacturer's instructions (Caymen Chemicals, 2018). Plates were read using an automated microplate reader (LT-450, Labtech, Sussex, UK) and results interpreted using AssayZap software (Biosoft, Cambridge, UK). Assay sensitivity over 4 assays averaged 0.039pg/ml, and intra- and inter-assay coefficients of variation averaged 6.8% and 6.2% respectively.

2.8 Testosterone: All DM males (n = 31) that were older than 6 months were selected for testosterone analysis. These were compared to 44 male controls (aged 3–12 years). Testosterone was extracted from 24 mg of powdered hair as above. Samples were reconstituted with 450 µL of diluent, vortexed for 30 minutes and assayed following the manufacturer's instructions (R&D Systems, 2017). AssayZap was used for result interpretation. Assay sensitivity was 0.056ng/ml, and the intra and inter assay coefficient of variation over 2 assays averaged 11 % and 5.09% respectively.

2.9 Statistical analysis: Behavioural and welfare scores from the videos were coded by MA, 3 times each, in random order. The average score was then used for final analysis within DM. The sex distribution of the DM was heavily biased towards males and as no sex differences are observed in HCC in the rhesus macaques, data from both sexes were combined for cortisol analysis (Dettmer et al., 2012; Yamanashi et al., 2016). All analysis was conducted in R using the RStudio interface (ver. 3.5.3, <http://www.R-project.org>). Log transformed values were used for both hair cortisol concentration (HCC) and hair testosterone concentration (HTC) to meet normality assumptions, based on the Schapiro-

Wilk Normality test ($p > 0.05$). A Welch T Test was used to identify the difference in HCC and HTC in the controls and DM. HCC was seen to vary between the three sampled trainer groups ($ICC=0.27$). This variation was not associated with any other measured variables in this study and may reflect different practices between the groups or '*bradaris*'. As such, the lme4 package was used to fit mixed effect linear models to characterise the relationship between HCC and behavioural and welfare indicators within the DM group. Group was added as a random effect (3 level factor), all behaviour scores (aggression, fear, abnormal behaviour), welfare scores (body welfare and observed mistreatment) and parasite density were added as integer fixed effects. BCS was included as a numeric fixed effect. The presence or absence of each ethogram score (ethical treatment, aggression, abnormal behaviour, and parasites) were also included as 2 level factors (yes/no) variables to see whether the presence of the behaviour was statistically related to hormone concentration or to the severity of the behaviour exhibited during the performance. All sampled DM displayed at least one indicator of fear so presence of fear was not included. As age is known to alter behaviour, an interaction between age and fear, aggression and abnormal behaviour score was also added. A stepwise model simplification was then conducted, using a nested modelling approach by removing one variable at a time. Models were compared by ANOVA and the minimum AIC value model (with $p > 0.05$) was accepted as the best fit model.

HTC did not vary between groups ($ICC=0$), possibly due to a smaller sample size and misrepresentative proportion of individuals in each group (appendix table 1). Thus, OLS linear regression models were used to characterise the relationship between HTC and behaviour and welfare scores as above, removing group as an explanatory variable. The best

model was selected by a stepwise removal of the highest p value variable in the model output.

In both HCC and HTC models, along with adding age as a numeric variable, age groups were assigned to all animals based on previous reports which were added as a categorical variable (infant >0.5 years; juvenile 0.6 - 1.5 years; adolescent 1.6 - 3 years; adult 4 - 7 years; older adult 8-11 years, senior adult 12 - 18 years) (Fooden, 2000).

OLS linear regression models were also fitted to analyse the relationship between age and HCC and HTC in the DM and controls, and to test whether age had a significant effect of any behavioural or welfare scores in the DM.

3. Results

3.1 Trainer interview results

DMs were sampled from 3 groups of trainers (*bradaris*), though many exist based in different geographical regions. The trainers are often from the same background and tend to be insular towards outsiders. Each trainer had been inducted into the DM trade by a male family member at a young age and had no formal education. Trainers reported that they were well below the poverty line and only fed their animals human leftovers, which mainly consisted of '*rotis*' (flatbread). There was some local knowledge about a pro-bono human doctor who sometimes treated DM if they become unwell, however, trainers reported that they are then generally unable to afford any medicines required and so are unable to provide medical care to their animals. There was limited knowledge about animal-human interaction, or health and safety and trainers and their animals maintained very close contact with no personal protection. There was therefore a high risk of both zoonotic and reverse-zoonotic disease. There is a defined hierarchy within the DM trade, with trainers

earning the least, as well as having to give a percentage of their earnings to their local leader to be allowed to operate in their area. Local leaders also train and sell or rent DM out to trainers and gain a significant portion of their income from the sale of animals. In addition to sales to trainers local leaders sell monkeys to local biomedical facilities and universities for animal testing. One local leader indicated that they were also active in the Dancing Bear trade and had procured animals for local individuals and zoos for additional income. Many of the trainers specifically reported that they worked with DMs to provide for their children and put them through school so that their children did not have to carry on within this field due to the personal difficulties that arise within it.

3.2 Behaviour and welfare score summary

During the DM performances, every sampled DM had the presence of one or more behavioural indicators of fear (score 2 and above, mean 2.7 ± 0.122). 40% and 54% of DM had a score of 2 or higher for aggression (mean 1.78 ± 0.157) and abnormal behaviour (mean 1.76 ± 0.123), respectively. Every sampled DM had at least one body modification with a mean body welfare score of $2.22 \pm \text{SEM } 0.15$ and every sampled DM had an ethical treatment score of 2 or higher with a mean of $3.5 \pm \text{SEM } 0.13$. No sampled DM was of a healthy BCS (mean $1.6 \pm \text{SEM } 0.05$). The mean age of sampled DM was 3.5 (0.5 – 12 years). The trainers reported an average lifespan of 12 years for their animals.

3.3 Cortisol

Mean HCC was significantly higher in DM ($44.70 \pm 4.75\text{pg/mg}$) compared to the controls ($28.77 \pm 1.67\text{pg/mg}$) ($t(77.358)=-2.8099\text{CI}(-0.519,-0.088)$, $p<0.01$) (Figure 1). The Cohens d was calculated as 0.531 showing a moderate effect size.

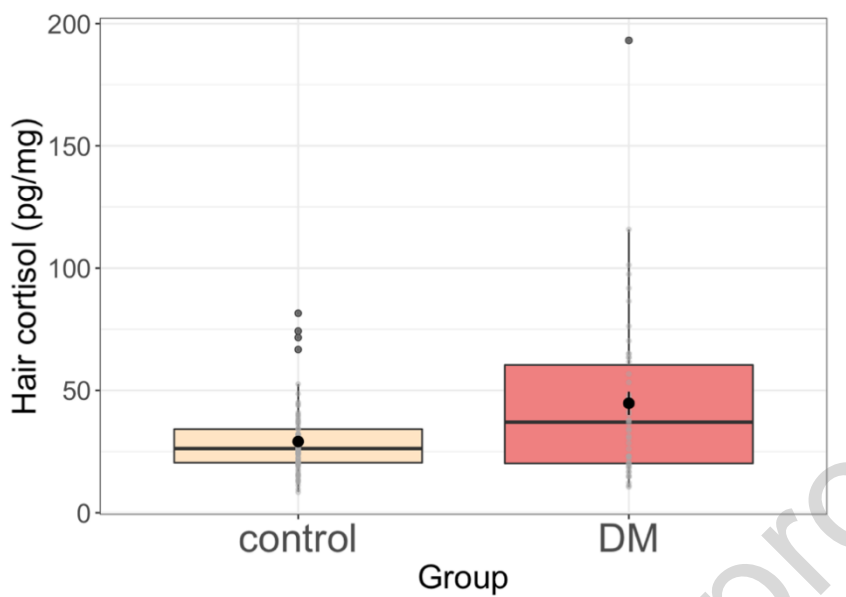


Figure 1: Hair cortisol concentrations sampled from dancing monkeys (DM) and the control group (outdoor colony housed rhesus macaques). DM had significantly higher mean hair cortisol compared to the controls ($p < 0.01$), where the centre dot in the rectangular region indicates the mean, the top and bottom of the boxplot are defined quartiles, the vertical line extends to the full range of the data with outliers in denoted in smaller grey dots, and the horizontal line represents the median.

Within the DM group, the statistical model that fitted the cortisol data best (cortisol \sim fear score + parasite presence + parasite density) explained 47.4% of the observed variation (AIC=103.74, BIC=115.21, Pseudo R^2 (fixed effects) = 0.28, Pseudo R^2 (total)=0.47).

<i>Predictors</i>	cort				
	<i>Estimates std. Error</i>		<i>CI</i>	<i>p</i>	<i>df</i>
(Intercept)	2.77	0.33	2.03 – 3.52	<0.001	10.11
fearscore	0.37	0.10	0.17 – 0.58	0.001	45.79
parasitepresence [yes]	-0.87	0.31	-1.50 – -0.25	0.007	44.77
parasitedensity	0.35	0.15	0.05 – 0.64	0.023	44.76
Random Effects					
σ^2	0.30				
τ_{00} group	0.11				
ICC	0.27				
N_{group}	3				
Observations	50				
Marginal R^2 / Conditional R^2	0.279 / 0.474				

Figure 2: The best fit mixed effect model for hair cortisol in Dancing Monkeys output summary

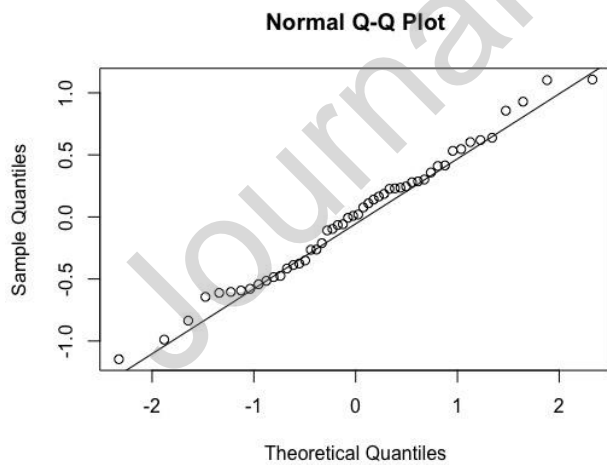


Figure 3: Q-Q Plot diagnostic plot for best fit mixed effect model for hair cortisol in Dancing Monkeys ($\text{cortisol} \sim \text{fear score} + \text{parasite presence} + \text{parasite density}$).

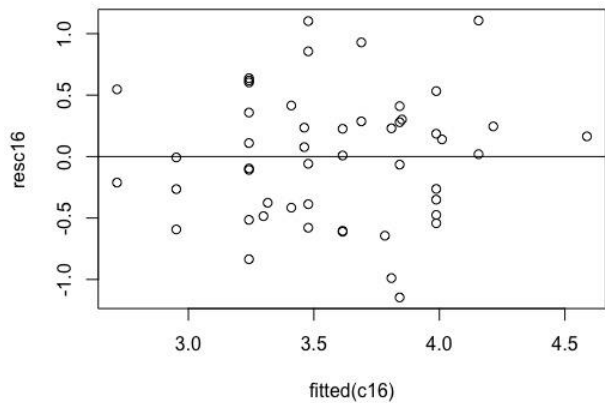


Figure 4: Residual vs. fitted values plotted for the best fit mixed effect model for hair cortisol in dancing monkeys ($\text{cortisol} \sim \text{fear score} + \text{parasite presence} + \text{parasite density}$).

Fear score was positively associated with HCC ($p < 0.001$, SE 0.10, 95CI(0.17,-0.58)). HCC increased with ectoparasite density ($p < 0.05$, SE 0.15, 95CI(0.05,0.64), but was negatively associated with the presence of ectoparasites ($p < 0.01$, SE 0.31, 95CI(-1.59,-0.25)).

3.4 Testosterone

Mean HTC was significantly lower in DM (195 ± 26.66 ng/mg) compared to the controls (429.41 ± 42.41 ng/ml) ($t(66.6) = 4.917$, $p < 0.0001$, 95CI(0.474, 1.1108) with a large effect size ($d = 1.16$).

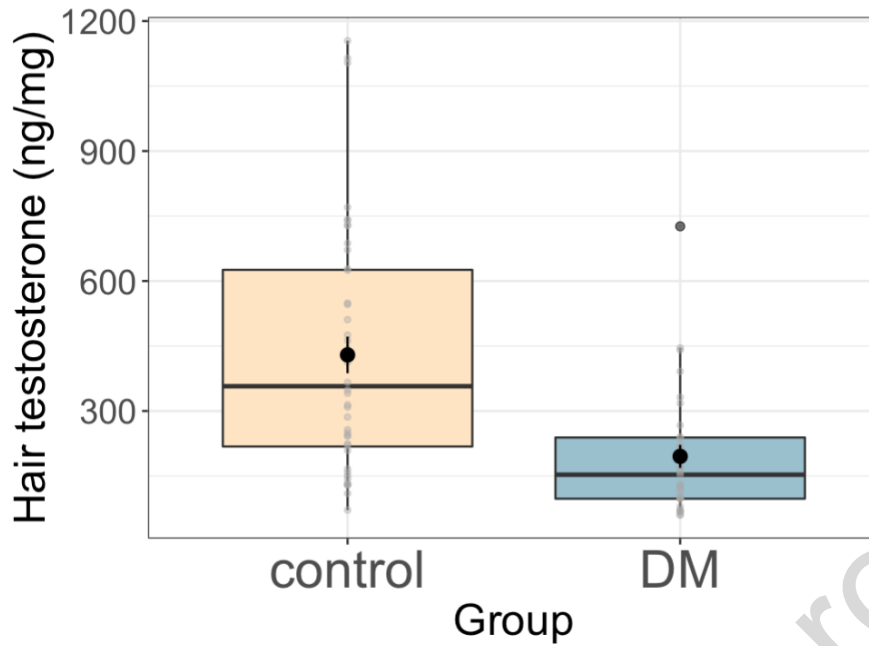


Figure 5: Hair testosterone concentration sampled from Dancing Monkeys compared to the control group (outdoor harem-housed colony). Dancing Monkeys had significantly lower mean hair testosterone compared to the controls ($p > 0.0001$), where the centre dot in the rectangular region indicates the mean, the top and bottom of the boxplot are defined quartiles, the vertical line extends to the full range of the data and the horizontal line represents the median

Within the DM group, the best fit fixed effect linear model (testosterone \sim age + body welfare score + aggression score + abnormal behaviour score + age*fear score) explained 56% of the observed variation ($F(6,24) = 7.30$, $p = 0.00$, $\text{cond.}R^2 0.558$).

<i>Predictors</i>	<i>Estimates</i>		test		
			<i>std. Error</i>	<i>CI</i>	<i>p</i>
(Intercept)	5.18	0.64	3.85 – 6.50	<0.001	24.00
age	0.32	0.10	0.11 – 0.54	0.005	24.00
bodywelfare score	-0.18	0.08	-0.34 – -0.02	0.029	24.00
aggression score	-0.24	0.08	-0.41 – -0.07	0.007	24.00
abnormal behaviour score	0.20	0.09	0.01 – 0.38	0.038	24.00
fear score	0.06	0.19	-0.34 – 0.46	0.751	24.00
age * fear score	-0.12	0.04	-0.21 – -0.03	0.012	24.00
Observations	31				
R ² / R ² adjusted	0.646 / 0.558				

Figure 6: The best fit fixed effect model for hair testosterone in Dancing Monkeys output summary

Aggression score was negatively associated with HTC ($p=0.007$, SE 0.08, 95CI(-0.41, -0.07)) as was body welfare score ($p=0.029$, SE 0.08, 95CI(-0.34, -0.02)) whereas abnormal behaviour score was positively associated with HTC ($p=0.038$, SE 0.09, 95CI(0.01, 0.38)). HTC was also seen to increase with age in DM ($p=0.005$, SE 0.10, 95CI(0.11, 0.54) and decrease in older DM with a lower fear score ($p=0.012$, SE 0.04, 95CI(-0.21, - 0.03)).

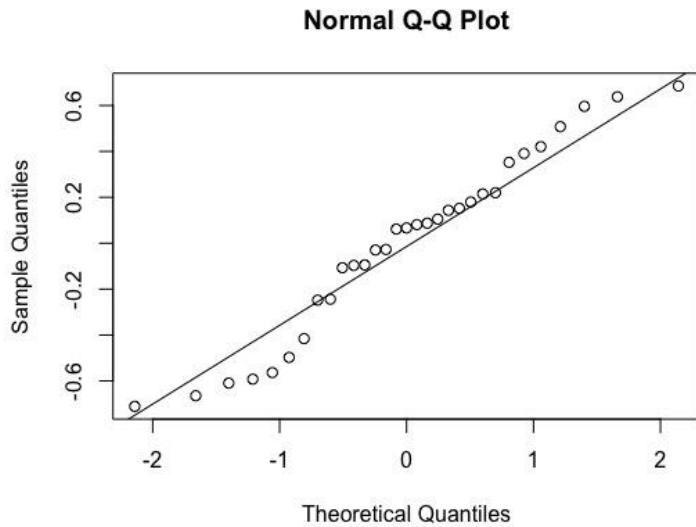


Figure 7: Q-Q Plot diagnostic plot for best fit fixed effect model for hair testosterone in Dancing Monkeys ($\text{testosterone} \sim \text{age} + \text{body welfare score} + \text{aggression score} + \text{abnormal behaviour score} + \text{age} * \text{fear score}$)

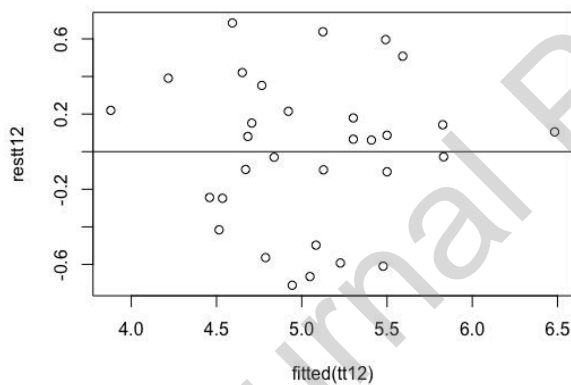


Figure 8: Residual vs. fitted values plotted for the best fit fixed effect model for hair testosterone in dancing monkeys ($\text{testosterone} \sim \text{age} + \text{body welfare score} + \text{aggression score} + \text{abnormal behaviour score} + \text{age} * \text{fear score}$)

3.5 Hormonal profiles associated with age in DM and Controls

In the controls, HCC had a significant negative association with age ($p=0.002$, SE 1.05, 95CI(-5.41,-1.22) model fit ($F(1,75) = 9.95$, $p = 0.00$) but no relationship was observed with HTC ($p=0.78$, SE 0.62, 95CI(-1.07, -1.43) model fit ($F(1,42) = 0.08$, $p = 0.77$))

age					
<i>Predictors</i>	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>p</i>	<i>df</i>
(Intercept)	16.54	3.47	9.63 – 23.45	<0.001	75.00
cort	-3.31	1.05	-5.41 – -1.22	0.002	75.00
Observations	77				
R ² / R ² adjusted	0.117 / 0.105				

age					
<i>Predictors</i>	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>p</i>	<i>df</i>
(Intercept)	4.04	3.64	-3.31 – 11.40	0.273	42.00
test	0.18	0.62	-1.07 – 1.43	0.774	42.00
Observations	44				
R ² / R ² adjusted	0.002 / -0.022				

Figure 9: Model for hair cortisol and testosterone in the Controls output summary

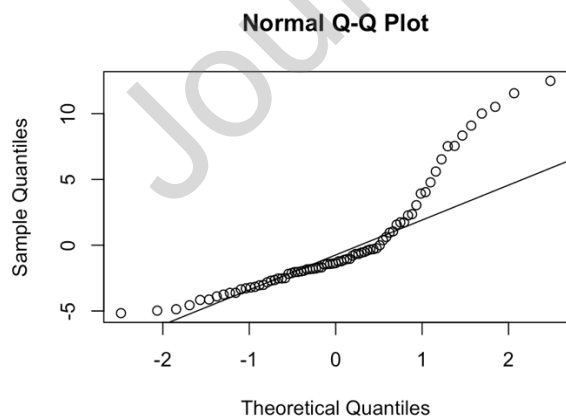


Figure 10 Q-Q Plot diagnostic plot for best fit fixed effect model for age in the controls ($age \sim cortisol$)

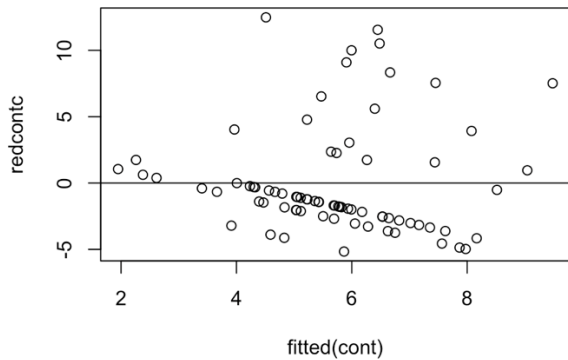


Figure 11: Residual vs. fitted values plotted for the best fit fixed effect model for age in the controls ($age \sim cortisol$)

In DM, HTC had a positive association with age ($p=0.037$, SE 4.19, 95CI(-12.65, 4.12)) model fit $F(1,29) = 4.77$, $p = 0.04$ but no relationship was observed with HCC ($p=0.109$, SE 0.60, 95CI(-2.20, 0.23)) model fit ($F(1,48) = 2.66$, $p = 0.11$)

<i>Predictors</i>	<i>Estimates std. Error</i>		age		
			<i>CI</i>	<i>p</i>	<i>df</i>
(Intercept)	-4.26	4.10	-12.65 – 4.12	0.307	29.00
test	1.76	0.81	0.11 – 3.41	0.037	29.00
Observations	31				
R^2 / R^2 adjusted	0.141 / 0.112				

<i>Predictors</i>	<i>Estimates std. Error</i>		age		
			<i>CI</i>	<i>p</i>	<i>df</i>
(Intercept)	7.04	2.20	2.62 – 11.46	0.002	48.00
cort	-0.99	0.60	-2.20 – 0.23	0.109	48.00
Observations	50				
R^2 / R^2 adjusted	0.053 / 0.033				

Figure 12: Model for hair cortisol and testosterone in the Dancing Monkeys output summary

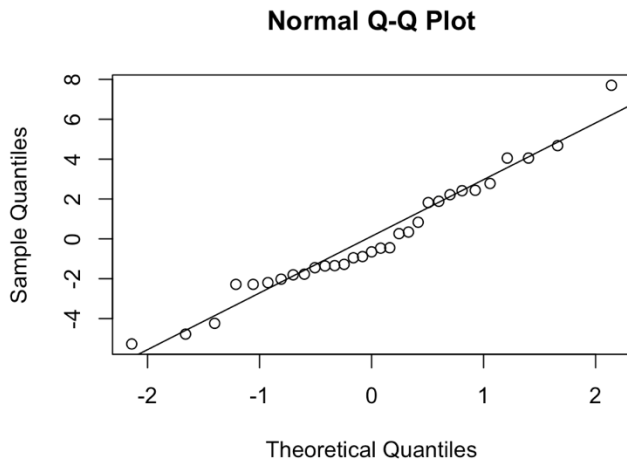


Figure 13: Q-Q Plot diagnostic plot for best fit fixed effect model for age in the dancing monkeys ($age \sim testosterone$)

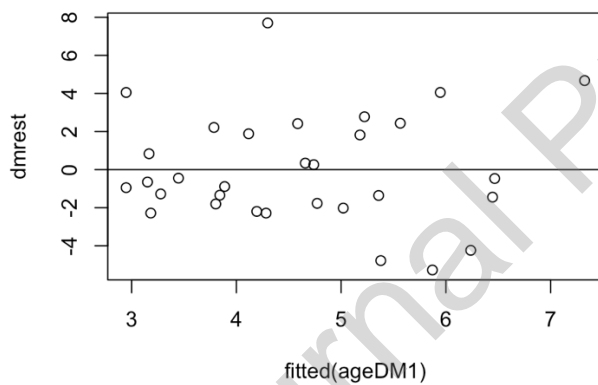


Figure 14: Residual vs. fitted values plotted for the best fit fixed effect model for age in dancing monkeys ($age \sim testosterone$)

Though statistically significant, both these models weakly explained observed variation as seen by low R^2 s.

3.6 Age and behavioural indices observed within DM

Within DM, only ethical treatment score ($p=0.006$, SE 0.39, 95CI(-1.91, -0.34)) and parasite density had a significant negative relationship ($p=0.028$, SE 0.36, 95CI(-1.52, -0.09)) with age

($F(2,47)=7.63$, $p=0.00$, $R^2=0.25$, Adj. $R^2= 0.21$)

<i>Predictors</i>			age		
	<i>Estimates</i>	<i>std. Error</i>	<i>CI</i>	<i>p</i>	<i>df</i>
(Intercept)	8.05	1.41	5.20 – 10.89	<0.001	47.00
ethicaltreatmentscore	-1.13	0.39	-1.91 – -0.34	0.006	47.00
parasitedensity	-0.81	0.36	-1.52 – -0.09	0.028	47.00
Observations	50				
R^2 / R^2 adjusted	0.245 / 0.213				

Figure 15: Best fit fixed effect model for age and measured behavioural indices in the Dancing Monkeys output summary

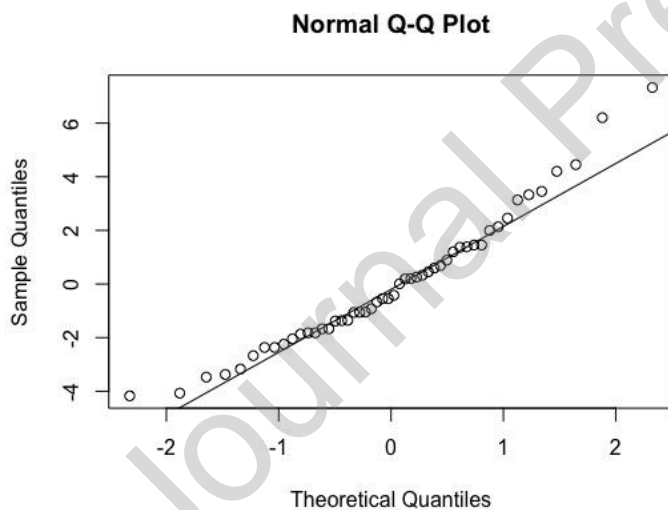


Figure 16: Q-Q Plot diagnostic plot for best fit fixed effect model for behaviour and age in Dancing Monkeys ($age \sim$ ethical treatment score + parasite density)

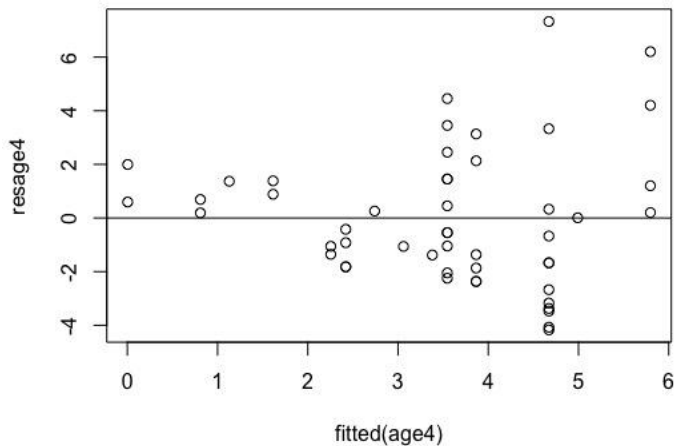


Figure 17: Residual vs. fitted values plotted for the best fit fixed effect model for behaviour and age in Dancing Monkeys ($age \sim ethical\ treatment\ score + parasite\ density$)

3.7 General Model Diagnostics

Best fit models were further accepted by checking diagnostic plots (figure 3-17).

Goodness of fit of the models is indicated by the Q-Q plots (figure 3, 7, 10, 13, 16) where the vast majority of data points lie along or close to the linear line, some showing small deviations only in the extreme ends of the data set showing normality of the dataset and valuable goodness of fit of the chosen models.

Generally, residuals (figures 4, 8, 11, 14, 17) are symmetric with point agreement ranges sitting at about zero showing minimal offset bias further showing goodness of fit of selected models and significant variables quantified by p values.

4. Discussion

The results of this study demonstrate that rhesus macaques used and trained for street entertainment in Pakistan have chronically higher HPA activity and depressed HPG activity relative to outdoor harem-housed rhesus macaques maintained in more stable captive conditions which allow them to express more natural behaviours. These physiological measures provide quantitative evidence of chronic physiological stress and poor welfare. The higher HCC in DM was positively associated with increased expression of fear responses during performances, and ectoparasite density but was negatively associated with the presence of parasites. Lower HTC in DM was positively associated with severity of abnormal behaviour expressed during performance, but negatively associated with observed aggression during performance and amount of body modifications. These physiological results in DM compared to the controls, combined with the association of our measured behavioural indices within DM provide quantitative evidence of chronic stress. Compared to the controls, DM also had limited provision to adequate housing, nutrition, veterinary access, and an inability to express natural behaviours which along with the evidence of chronic stress point towards an overall negative welfare state experienced by DM.

The comparator for DM used in the current study was a colony of captive, outdoor, socially housed macaques i.e. a group of animals maintained in controlled conditions and subject to strict welfare requirements and laws. This approach was taken as it is not feasible to obtain standardised hair samples from wild rhesus macaques in Pakistan. Furthermore, in a wild populations there may be additional external stressors which cannot be accounted for e.g. competition, rank, territory and health (Meyer and Novak, 2012; Novak et al., 2013). Wild primates are generally used as a comparator for 'good welfare' for captive primates but

considering differences in social and environmental factors in the wild and in captivity this may also provide imperfect controls, hence the importance of using multiple indices to infer affective or welfare states (Howell and Cheyne, 2019). Thus, the specific questions answered by this study are whether 1) two captive rhesus macaque populations maintained in very distinct conditions have differences in historic hormone profiles commonly associated with stress; and 2) within DM are these physiological parameters associated with expression and severity of behaviours generally used as negative welfare indices. It is important to note, however, that behavioural and welfare scores were only measured during DM performances, and so may show an incomplete picture of the animal's overall welfare state. However, using this two-fold biobehavioural approach allows for us to make stronger inferences about the current conditions faced by DM.

The higher mean HCC in DMs compared to the high welfare controls is indicative of higher activity within the HPA axis in the DM in the three-month period prior to hair collection. Segmental analysis of HCC has also been shown to be possible in longer haired primates such as orangutans whereby it can provide a historic record of HPA activity (Carlitz et al., 2014). In rhesus macaques, which have shorter hair, HCC has been shown to be consistent among proximal and distal ends of hair samples, possibly due to diffusion of cortisol across the hair shaft (Davenport et al., 2006). In this study an integrative measure of HPA activity was assessed as the full length of hair was used. Previous work has shown that a variety of stressors can result in elevated cortisol in nonhuman primates (Novak et al., 2013) and that chronic stress can result in elevated HCC in a number of species (Bechshøft et al., 2011; Carlitz et al., 2015; Davenport et al., 2008; Dettmer et al., 2012; Gow et al., 2010; Maxwell et al., 2019). The high levels of HCC seen in the DM monkeys indicates that, as reported previously, early and repeated exposure to stressors may not result in physiological

adaptation of the stress response (Dettmer et al., 2012; Heimbürge et al., 2019; Ruys et al., 2004) or it could reflect repeated activation of the HPA axis in response to the varied and unpredictable nature of the stressors to which DM are exposed during training and performance throughout their life. Although the exact causes of stress for the DMs which has led to the chronic activation of the HPA axis cannot be defined, based on observation and the trainer interviews, a series of stressors that have been shown to result in elevated cortisol in dogs could be implicated including spatial and social restriction (Beerda et al., 1996; Grigg et al., 2017), conspecific aggression (Kelly and Vitousek, 2017), starvation (Fujiwara et al., 1996), extreme temperatures (Assia et al., 1989) and dehydration (Slater et al., 1963) fear (Hydbring-Sandberg et al., 2004), pain (Kang et al., 2022) and anxiety (Shin and Shin, 2016). The presence of visitors, irrespective of numbers, has also been shown to increase stress in zoo felids (Suárez et al., 2017). As the nature of DM is public facing, this may be a continuous stressor leading to repeated HPA activation. In the DM, unlike in previous studies (Lutz et al., 2016) and in our control samples, a decrease in HCC was not seen with age. This suggests that the physiological stress associated with being a DM does not decrease i.e. there is no habituation of the DM to their lifestyle/performance. Such repeated stress would add to the allostatic load and could be a contributory factor to the relatively short lifespan of DM (max 12 years) compared to rhesus monkeys kept in captivity where the median lifespan is 27 and the maximum 40 years (Fooden, 2000; Robertson et al., 2017).

The responsiveness and activity of the HPA axis may also have been influenced by longer-term life history, as it has been reported that early maternal separation in rhesus macaques can result in increased cortisol secretion in infancy and anxious behaviours (Dettmer et al., 2012; Dettmer et al., 2009) and decreased cortisol secretion with abnormal stress responses

and decreased social behaviour in adulthood (Barik et al., 2013; Feng et al., 2011). Based on trainer testimony, DM in this study would have been removed from their natal troop approximately ten months before their natural age of weaning (Fooden, 2000) and so the elevated HCC observed in DM could be a reflection of a higher baseline as a result of early maternal separation.

Fearfulness was exhibited during each observed DM performance, the severity of which was positively associated with HCC. This may be due to a learned fear (Nelson et al., 2003) of the training stick which was used as a negative enforcement reprimand during training and performances by all trainers (appendix fig. 1, 2). This constant fear of reprimand and the observer/visitor effect (Suárez et al., 2017) may have led to the high exhibition of fearful behaviours during filmed performances, repeated activation of the HPA axis and the high HCC in DM.

Within this study, an incidental finding was the presence of hatched and unhatched louse eggs on DM hair samples, which is a valid indicator for ectoparasite burden (Ishii et al., 2017). Though difficult solely by eggs, it is thought that the lice observed are from the *Anoplura* order which are endemic to the region, easily transferable between mammalian species including between humans and DM; especially the *pediculosis* species which is particularly common in low-income communities in Pakistan and may be an important zoonotic vector (Durden et al., 1990; Tufail et al., 2017). Grooming is an important social activity in rhesus macaques as it serves to remove ectoparasites and skin debris (Tanaka and Takefushi, 1993) and it has been reported that 98.9% of the objects removed during social grooming in Japanese Macaques are lice and louse eggs (Ishii et al., 2017). DM, however, were generally kept in solitary conditions or with limited social interactions and thus deprived of this natural social behaviour. As lice are primarily spread through social contact,

the presence of these ectoparasites may act as a proxy for social contact. Ectoparasite presence was associated with decreased HCC, reflecting a possible social buffering effect on stress (Culbert et al., 2019; Novak et al., 2013). Increased ectoparasite density was associated with increase HCC which may reflect a heavy parasite burden and a lack of social grooming that would normally serve to build social bonds and release endorphins that reduce physiological stress (Akefe et al., 2017; Aureli et al., 1999; Duboscq et al., 2016; Hopla et al., 1994; Keverne et al., 1989; Toft, 1986; Wooddell et al., 2017). The results also indicated a negative relationship between ectoparasite density and age, as older DM were no more likely to be allowed social contact compared to younger DM, the reduced density of ectoparasites in older DM may be due to increased self-grooming with age, as reported in wild rhesus macaques (Liao et al., 2018).

As control samples were harem-housed with one male alpha adult present and stable hierarchal structures, social stress is not thought to be the reason for the observed higher HTC in controls (Beisner et al., 2012). The low HTC observed in DMs could be a direct consequence of allostatic adaptations to chronic activation of the HPA axis but could also be associated with perceived low dominance (Mazur and Booth, 1998) of the DM relative to its trainers. This is further supported by the fact that DM with increased body welfare scores (i.e. more body modifications leading to more handling by trainer) had lower HTC. In primates, high testosterone may manifest as aggression and associated behaviours in response to competitive need where such behaviours are beneficial, e.g. fighting off competitors or up the dominance hierarchy (Higham et al., 2013; Wingfield et al., 1990). In DMs, increased dominance, competitive and aggressive behaviours are repressed by the trainer, potentially through painful treatments which can further activate the HPA axis and

further suppress HPG activity promoting non-dominant behaviours (Viau, 2002). Based on the positive relationship described above between testosterone and aggression, the negative correlated observed in DM between HTC and aggression score during performance suggests that in this population, observed aggression may be reactive towards negative stimuli such as the training stick or public crowds rather than dominance based. Interestingly, fear score was not related to HTC but a positive interaction between fear score and age was observed. Therefore, as DMs age they appear to express less fear which may suggest that they become more compliant and less likely to display behaviours that elicit a negative response from the trainer leading to a lower ethical treatment score (less negative enforcement used by trainer). Age was shown to be positively associated with HTC in DM but this was not observed in the controls. Circulating testosterone is known to decrease with age (Sorwell and Urbanski, 2013); with prepubescent changes detected in males starting from 2.5 years to 5.5 years (Bernstein et al., 1991). A sub-selection of controls and DM were chosen for testosterone analysis to account for skewed ages in our study. The average age of DM sampled for HTC was 4.6 years and for controls it was 5 years, however, the overall DM HTC sample contained younger animals (0.6 – 12 years, 4 individuals > 2) compared to the control HTC sample (3 – 12 years) due to the nature of the trade and convenience sampling method. Thus, the observed positive associations between age and age and fear score with higher HTC in DM may be reflective of a more stable physiological state due to a more compliant and stable relationship with the trainer and possible habituation but may also be linked to the fact that our DM sample contained a large amount of sexually immature individuals.

Among the DMs, abnormal behaviour was associated with high HTC but no relationship was observed with HCC. Abnormal behaviours, like stereotypies and self-harm are generally

accepted as measures of psychological state (Bloomsmith et al., 2019) but it has also been suggested that their expression maybe a coping mechanism (Bernstein and Mason, 1970; Pomerantz et al., 2012). In this regard, our results suggest that abnormal behaviour in the DMs may not be directly associated with physiological stress (i.e. cortisol) but rather a self-soothing coping mechanism or displacement behaviour that may in turn function to maintain long-term physiological balance.

Individuals in the DM trade often inherited the profession from their forefathers with little formal education or alternative means for income generation. Interviews with leaders of the DM trade in 2018 revealed that a significant portion of income comes from capturing infants from the wild and selling them to medical and veterinary universities, however, all individuals in the trade were still facing daily poverty as income from DM performances is highly unpredictable. Testing on live animals was banned in industrial and university complexes in the federal capital territory in 2020, where the interviewees resided and worked, and the impact of this on members in the DM community remains unknown. In April 2020, The Islamabad High Court recognised that animals have legal rights and protection under the constitution. While there are no specific legal protections for DMs, under the Pakistan Animal Welfare Act (1890, section 3a/3b and 6a/6b) it is illegal to bind, beat or treat any animal to unnecessary pain or suffering and to employ animals unfit for labour. However, these are rarely enforced and with no specific protections, the DM practice is effectively legal in Pakistan. A model solution to the issue would be similar to that deployed by Wildlife SOS and other groundworkers in India to tackle the synonymous issue of dancing bears where trainers and their families were provided alternate sources of livelihood before surrendering the bears to reputable purpose-built sanctuaries. Over 600

bears and 3000 families have directly benefited from the scheme, reducing the need for individuals to depend on wildlife trade to support themselves. There are currently no projects working to tackle the DM trade in Pakistan and the practice is still widely accepted by the public, showcasing a great need for local groundwork on every level.

5. Conclusions

Rhesus macaques used as Dancing Monkeys (DM) in Pakistan have higher chronic HPA activity (as measured by hair cortisol) and depressed HPG activity (as measured by hair testosterone) compared to rhesus macaques kept at a high standard of welfare. The increased HPA activity in DMs was correlated to increase fear responses and ectoparasite burden. Depressed HPG activity in DMs was correlated with increased aggressive behaviours and body alterations (e.g. fur dyed and cut), whereas increased abnormal behaviour, age and ectoparasite presence was associated with increased HPG activity. All sampled DMs displayed at least one behavioural indicator of fear and 56% exhibited stereotypical or self-harm behaviours during their performances. The results suggest that animals kept at a lower standard of welfare, such as those used in this unique cultural practice are subject to long-term physiological and behavioural disruptions and are chronically stressed.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Acknowledgements

The authors wish to thank Dr Wesley Burnside and the staff at the Mannheimer Foundation for provision of the control hair samples and their collaboration with this project.

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Appendix



Figure 1: Image still from Dancing Monkey performance showing one of the most common tricks, a salute (Islamabad, 2018)



Figure 2: Image still from Dancing Monkey performance showcasing the training stick and rope used by the trainer. (Islamabad, 2018)

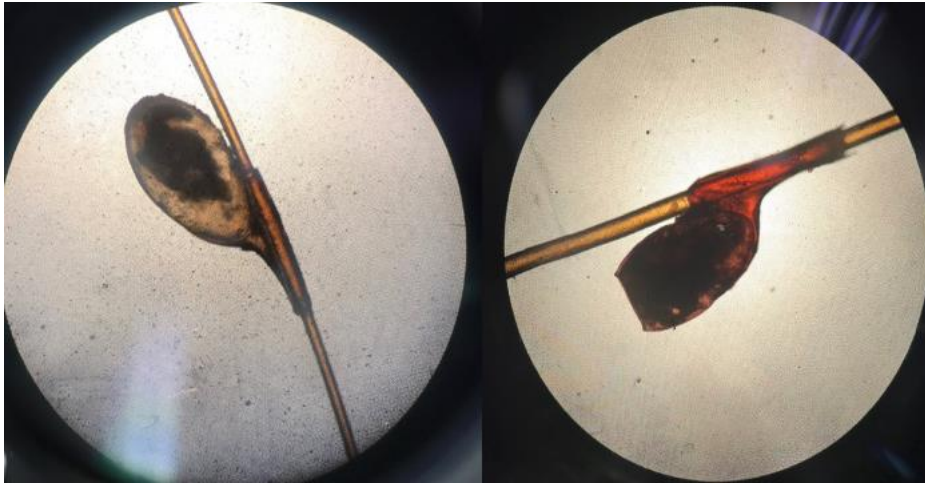


Figure 3: Louse eggs observed in Dancing Monkey hair samples under a light microscope

Age(years)	Number of Samples
Controls	77
F	11
4	11
M	66
0.7	4
3	20
4	22
8	5
9	2
10	2
12	3
15	3
16	1
17	3
18	1
DM	50
F	2
2.5	1
3	1
M	48
0.5	1
0.6	4
0.9	1
1	1
1.2	2
1.3	2
1.5	6
2	6
2.5	3

3	5
4	2
5	4
6	3
7	3
8	2
10	1
12	2
Grand Total	127

Table 1 Demographics of Dancing Monkey and Control population sampled

Journal Pre-proof

Fear Score	Behaviours Included
1	No fearful behaviour observed
2	Ears pulled back, lip smacking, brow pulled up, freezing
3	2 + tense mouth and body, cringing and crouching, alarm chirps
4	3 + O shaped mouth, grimacing, screaming, attempting to flee

Figure 6: Ethogram used to assess fear score for filmed dancing monkey performances

Aggression Score	Behaviours Included
1	No aggression observed
2	Open mouth stare, grunting
3	2 + teeth barring, pseudo-charge, piloerection
4	3 + patting ground, charging eliciting response from trainer

Figure 7: Ethogram used to assess aggression score for filmed dancing monkey performances

Abnormal Behaviour Score	Behaviours Included
1	No abnormal behaviour observed
2	Stereotypic behaviours e.g. digit sucking
3	2 + some self-directed behaviour (e.g. self-biting, hair-pulling) with no wounds
4	3 + self-injury with visible wounds

Figure 8: Ethogram used to assess abnormal behaviour in filmed dancing monkey performances, including stereotypic and self-harm behaviours.

Body Welfare Score	Alterations: Scars/Sores, Piercings, Fur Dyed/Cut/Shaves, Parasite Presence
0	No alterations
1	1 alteration e.g. scars OR piercing
2	2 alterations e.g. scars AND piercings
3	3 alterations
4	All four body alterations

Figure 9: Ethogram used to establish body condition and number of alterations for each individual dancing monkey

Observed Mistreatment Score	Behaviours Included
1	No visible mistreatment
2	Trainer using leash and training stick to direct behaviour, no contact between stick and animal
3	Trainer pulling leash (animal may hold), training stick in contact with animals body
4	Trainer jerking leash, training stick making contact with animal body with moderate force or for reprimand
5	Trainer harshly pulling leash, training stick used with severe force and with visible pain response from animal

Figure 10: Ethogram used to assess human animal interaction and observed mistreatment of animal by trainer during filmed dancing monkey performances

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Dancing Monkeys have chronically high hair cortisol and low hair testosterone
- Hair cortisol was positively associated with fear
- Hair testosterone was negatively associated with aggression
- Dancing Monkeys have lower welfare compared to outdoor colony rhesus macaques