Accepted refereed manuscript of:

Ash H, Smith TE, Knight S & Buchanan-Smith HM (2018) Measuring physiological stress in the common marmoset (Callithrix jacchus): Validation of a salivary cortisol collection and assay technique, *Physiology and Behavior*, 185, pp. 14-22..

DOI: 10.1016/j.physbeh.2017.12.018

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Measuring physiological stress in the common marmoset (*Callithrix jacchus*): Validation of a salivary cortisol collection and assay technique

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Abstract

Cortisol levels are often used as a physiological measure of the stress response in captive primates, with non-invasive measures of this being an important step in welfare assessment. We report a method of collecting saliva samples voluntarily from unrestrained captive common marmosets (*Callithrix jacchus*), and validate an enzyme-linked immunosorbent assay (ELISA) technique previously unused in this species. Saliva samples were collected from marmosets housed in pairs in a UK laboratory. The assay showed parallelism, precision, accuracy and sensitivity, meeting the criteria typically used to investigate the effectiveness of new analytical techniques. Use of Salimetrics® Oral Swabs considerably increased the amount of cortisol recovered in comparison with previous studies using cotton buds. However, while use of banana on the swabs can encourage chewing, it may influence results. Although increases in cortisol levels have traditionally been interpreted as an indicator of stress in primates, there are many factors that affect the hypothalamic-pituitary-adrenal axis, with some studies showing decreases in cortisol levels post-stressor. Following a likely stressful event (capture for weighing), we also found cortisol levels significantly decreased, possibly due to social buffering or 'blunting' of the HPA axis. Order of weighing also had an effect. The method therefore provided an effective non-invasive means of assessing acute changes in cortisol level that may be more useful than previous methods, improving our ability to study physiological aspects of welfare in primates. We discuss methodological considerations, as well as implications of using cortisol as a measure of stress.

Key words: common marmoset; HPA axis; salivary cortisol; ELISA; swabs; validation

1 Introduction

Cortisol as a measure of stress

When aroused, the body undergoes a set of characteristic changes, including activation of the hypothalamic-pituitary-adrenal (HPA) axis. During activation, the hypothalamus releases CRH (corticotropin releasing hormone), causing the pituitary gland to release ACTH (adrenocorticotropic hormone) into the blood, which in turn causes the adrenal gland to increase the output of glucocorticoids (Sapolsky, 1992), making more energy available for immediate use and preparing the body for increased demands. While HPA axis activation is an adaptive response, very strong or prolonged periods of activation can lead to failure to reproduce (Rivier and Rivest, 1991); abnormal behaviour (Fraser, 2008); impaired cognitive function (Teixeira et al, 2015); immunosuppression (Martin, 2009), which could increase severity of infections (reviewed in McEwen, 1998); or heightened risk of cardiovascular and metabolic syndromes (reviewed in Walker, 2007), all of which can have substantial implications for the wellbeing of animals.

Cortisol is the main glucocorticoid in many mammals. Numerous studies have therefore used it as an indicator of stress (Mason and Mendl, 1993, eg. *Equus caballus*: Pawluski et al, 2017; *Canis familiaris*: Hennessy, 2013; *Macaca mulatta*: Clarke, 1993; Reinhardt, 2003; *Callithrix sp*.: Smith & French, 1997; Norcross & Newman, 1999; Cross et al, 2004). Baseline samples can be taken, to look at relative stressfulness of certain situations, or a stressor can be imposed to examine HPA axis activation (Novak et al, 2013). In this case, the intensity of the response from baseline to post-exposure is thought to reflect the degree of averseness, with large changes in cortisol indicating unusually high activation of the stress response, and so greater psychological and physiological stress (Fraser, 2008). Primates face a number of potentially stressful experiences when kept in laboratories, resulting from the captive environment and routine husbandry procedures, as well as experimental manipulations (Bassett et al, 2003). Increased cortisol levels have been well documented in primates following stressors such as loud unfamiliar noise and human activity (*Callithrix jacchus:* Cross et al, 2004; Kaplan et al, 2012),

restraint (*M. mulatta*: Reinhardt et al, 1995), human handling (*Saimiri sciureus*: Hennessy et al, 1982) and maternal separation (reviewed in Hennessy, 1997). Relocation (reviewed in Novak et al, 2013), watching other animals undergo procedures (*M. fascicularis:* Flow and Jaques, 1997), isolation (*C. jacchus:* Cross et al, 2004), and death of a social group member (*C. jacchus*: Kaplan et al, 2012) have also been shown to be physiologically stressful.

However, the use of cortisol does have its difficulties. Levels vary across the day and season, depend on the history of the individual, the type of stressor, the presence of social companions and the collection method used (Reinhardt, 1990, 2003; Smith et al, 1998; Cross et al, 2004; de Kloet et al, 2005). For example, Johnson et al (1996) provided comprehensive data on blood cortisol levels in *C. jacchus*, measuring differences depending on sex, social status, housing and time of day, with concentrations ranging more than ten-fold from 31.2+/-2.8μg/dl to 317.5+/-82.2 μg/dl. In the same species, Dettling et al (2002) found that brief separations from the family in the first month of life led to lower basal cortisol levels in 28 day old infants, compared to controls. However, there are no established normal adaptive fluctuations in levels of cortisol (Fraser, 2008).

As well as this, there are a number of studies showing decreases in cortisol concentration following potential stressors in common marmosets. For example, Bowell (2010) found that salivary cortisol level decreased significantly from baseline levels by 30 minutes after capture for weighing. Similarly, Cross and Rogers (2006) found a consistent decrease in salivary cortisol level in all marmosets after presentation of a snake-model stimulus, although their behaviour indicated this was a clear stressor for them. Why there are such differences in findings is not immediately clear, and demonstrates the complexity of using cortisol as a measure of stress. These studies highlight the importance of collecting contextual and behavioural data to assist with the interpretation of cortisol measurements.

Collecting and measuring cortisol

Cortisol can be collected from several different mediums, giving researchers options for how to measure the physiological stress response (Novak et al, 2013). Blood samples have traditionally been taken, often to determine acute reactions to stressors such as social separation (eg. Higley et al, 1992). However, this method is often confounded by the stress of restraint or sedation. Urine can instead be collected, which is not influenced by unplanned stressful events occurring shortly beforehand. However, individual differences in output, and the precise time lag for excreted cortisol to reach the urine, can make interpretation difficult (Novak et al, 2013). Furthermore, if 24 hour sampling is required, animals have to be individually housed (Setchell et al, 1977), which may confound the measurement, although primates have been trained using positive reinforcement to provide a urine sample on request (eg. *C. jacchus*: McKinley et al, 2003). Faecal cortisol can also be sampled (Romano et al, 2010), although like urine, lag time means pinpointing changes in relation to a specific stressor under study are imprecise, and levels depend on species, food availability and circadian variation (Touma and Palme, 2005; Smith et al, 2015). To examine chronic HPA axis activity, hair has been analysed in a variety of species, with significant relationships being found between hair cortisol and stressors or abnormal behaviour (eg. Carlitz et al, 2014; Davenport et al, 2008; Dettmer et al, 2012; Dettmer et al, 2014; Fourie and Bernstein, 2011; Fourie et al, 2015; Van Uum et al, 2008). Levels of cortisol in hair are not affected by time of day or associated restraint or isolation stress, although it can be difficult to measure the time frame represented and as it is a relatively new technique, there are potential issues in how to best process the hair, extract cortisol and measure it (Novak et al, 2013).

Saliva sampling is the preferred means for assessing HPA function. Salivary cortisol is thought to reflect the non-protein bound 'free' cortisol, which is the biologically active fraction of the hormone. It is highly correlated with plasma cortisol levels (*M. mulatta*: Davenport et al, 2003), with concentrations beginning to change within one minute of a bolus injection of cortisol (Laudenslager et al, 2006), indicating almost no lag time. Saliva can therefore provide a reflection

of acute changes in hormone level (*M. mulatta*: Higham et al, 2010), which could not be investigated using metabolites within excreta, and does not cause stress like restraint or isolation as animals can learn to chew voluntarily on collection devices without structured training (eg. *C. jacchus:* Cross et al, 2004; *M. mulatta*: Lutz et al, 2000). Previous studies have shown that coating a cotton bud in fruit is an effective method for saliva collection in the marmoset. Banana is the preferred flavour, reliably encouraging chewing, and variations in banana concentration are likely to have minimal effects on the assayed cortisol concentration (Cross et al, 2004). Samples can be obtained quickly and in a number of different settings, while animals remain in their social group. There has therefore been significant progress in non-invasive physiological welfare assessment using hormones in saliva (Higham et al, 2010).

Once samples are collected, the enzyme-linked immunosorbent assay (ELISA) can be used to quantify the cortisol response. Saliva assays are being increasingly used to measure cortisol levels, and have been validated in a number of primate species, including baboons (*Papio h. hamadryas*: Pearson et al, 2008), macaques (*M. mulatta*: Lutz et al, 2000) and marmosets (*C. jacchus*: Cross et al, 2004). Validation involves the assessment of four established criteria, specificity, accuracy, precision and sensitivity (see Reimers and Lamb, 1991), to ensure the reliability of the assay and the absence of any species-specific problems. Biological relevance of the results should also be examined. However, cortisol concentrations have differed between studies (eg. *C. jacchus*: Cross et al, 2004; Bowell, 2010), which may be due to methodological differences, including the collection and assay techniques used (Salimetrics, 2012). Improvement and validation of methods are therefore needed, to promote more widespread use of non-invasive cortisol sampling techniques (Pearson et al, 2008).

The aim of this study was to assess methods of collecting and analysing salivary cortisol samples in captive common marmosets. We explore how the use of different collection devices (cotton buds and Salimetrics® Oral Swabs, with and without banana coating) can affect results. We also validate the use of a commercially available enzyme-linked immunosorbent assay

(Salimetrics®), previously unused in this species, by assessing four typically used criteria, as well as looking at changes in cortisol level following the mild routine stressor of capture and weighing, which involved short separations from the pair-mate. As direction is difficult to predict based on previous research (eg. increases post stressor: Cross et al, 2004; decreases post stressor: Cross and Rogers, 2006), we hypothesise that cortisol concentration will change significantly from baseline levels following brief isolation and weighing. Those weighed last in the room may also have higher cortisol levels than those weighed first. Once validated, the method can be used to monitor stress levels of marmosets in the colony, in combination with behavioural observations, and the commercial availability of the assay will encourage uptake by other facilities, increasing valid comparisons across studies.

2 Method

2.1 Animals and housing

Twenty-six adult common marmosets, housed in vasectomised male mixed-sex pairs in 3 rooms at Dstl, Porton Down, UK were studied (aged between 1 year 7 months and 2 years 7 months). All animals were purpose bred in captivity: 19 were family-reared, 7 received supplementary feeding from carestaff as infants, but remained with the family for the majority of time. All marmosets were socialised to humans from birth, with regular hand-feeding and positive interactions.

Marmosets were housed in cages measuring 100cm wide x 60cm deep x 180cm high, lined with wood chippings and furnished with a nestbox, wooden platforms, perches, ropes, suspended toys and a wire veranda. All marmosets had *ad libitum* access to water, and food was delivered twice a day. Primate pellets (40/pair) were fed in the morning, and a variety of fruit (1 piece/animal) was provided in the afternoon. This was supplemented with malt loaf, egg, rusk, mealworms, dates, peanuts and bread on alternating days. Gum arabic and milkshake (with added Vitamin D once a week) were also given twice a week, and a constant supply of forage mix was

192 available. Enrichment was introduced once a week, where paper parcels, cardboard boxes or
193 bottles were provided, with forage mixed into sawdust. Temperature and humidity were at 23-
194 24ºC and 55 +/- 10% respectively. Lighting was provided on a 12 hour light/dark cycle, with a
195 dawn and dusk phase. Methods were approved after review by the Stirling University Psychology
196 Ethics Committee and the facility involved, and complies with legal and ethical requirements in
197 the UK.
198
199 **2.2 Study 1: Assay validation criteria**
200 Initially, 4 marmosets (2 male, 2 female) provided 5 samples each, using Salimetrics®
201 Oral Swabs (SOS) coated in banana, to assess typical assay validation criteria.
202 *2.2.1 Saliva collection*
203 The monkeys were first habituated to the saliva collection device for 5 minutes on three
204 days prior to sampling. One end was presented through the wire wall of the home cage, with the
205 other held by the experimenter, and the marmoset allowed to lick and chew the end, depositing
206 saliva onto the swab (following Cross et al, 2004). After approximately 5 minutes, the collection
207 device was removed and the marmoset given a small piece of banana. All samples were taken
208 between 9:00-10:00.
209 The collection device was then taken for processing (any containing visible traces of
210 blood, which would affect the cortisol assay, were removed). The device was first cut to
211 approximately 3cm to fit into the storage tube, and sealed. Samples were marked with subject ID,
212 time and date. The tubes, with their contents, were frozen at -20ºC for less than one week. The
213 samples were then placed into a centrifuge and spun for 15 minutes at 1500 RPM, to separate the
214 saliva from the collection device. A minimum of 25μL of saliva is necessary for analysis
215 (Salimetrics, 2012a), which was typically collected. The saliva samples were then stored at
216 –80ºC, until being assayed within 6 months. Storage time should not exceed 9 months (Aardal
217 and Holm, 1995).

2.2.2 Cortisol assay

Samples were analysed using Salimetrics® Salivary Cortisol Enzyme Immunoassay Research Kits. The plate was run as per the manufacturer's instructions (Salimetrics®, 2012a), using the standards in the range 82.77, 27.59, 9.19, 3.06, 1.02, 0.33 nmol/L. Cross reactivities of the cortisol antibodies can be found in Salimetrics® (2012a). All SOS samples were run in duplicate at a dilution of 1:5000.

2.2.3 Assay validation

The Salimetrics® assay was validated for use in common marmosets, using standard techniques (Buchanan and Goldsmith, 2004). Serial dilutions of pooled SOS samples, detailed above, were run in conjunction with synthetic standards provided in the kit, to assess specificity. Accuracy was investigated by quantifying the recovery of increasing amounts of synthetic cortisol (0, 9.19, 27.59, 82.77 nmol/L), added to known quantities of sample measured from the pooled saliva (2.43 nmol/L). Coefficients of variation (CV) of low and high concentration quality controls were assessed within and between plates, to identify intra- (N= 3 plates) and inter-assay precision (N=3 plates). Sensitivity was determined as the smallest concentration of cortisol that could be detected in the working range (the point of 90% B/B0) of the assay (Reimers and Lamb, 1991).

2.3 Study 2: Collection method

Six marmosets (3 male, 3 female) provided 4 samples each to assess the collection method (Salimetrics® Oral Swabs vs cotton buds, with and without banana).

2.3.1 Saliva collection

Salimetrics® Oral Swabs are made of a polymer, have verified recoveries of salivary cortisol, and do not cause a change in sample pH. Saliva was collected using the method outlined in section 2.2.1. Each marmoset was presented with both collection devices (cotton bud first, followed by SOS 5 minutes later), firstly without banana. Approximately 30 minutes later, they

were then presented with each collection device again (cotton bud first, followed by SOS 5 minutes later), after rubbing it into a banana for 5 seconds to coat it with the fruit. This order avoided contamination of the first samples, and has been used previously by Cross et al (2004). Cortisol was assayed using the above method (see section 2.2.2).

2.3.2 Statistical analysis

As no transformation was successful in making data normally distributed (assessed using Kolmogorov-Smirnov tests), non-parametric tests were used to assess the saliva collection method. Mann Whitney tests were used to compare cortisol concentration between cotton buds and SOS with and without banana. Spearman's rank correlations were also conducted, to look at the relationship with and without banana for each collection device. Two-tailed tests were used, with $P<0.05$ considered to be statistically significant. All analyses were conducted in SPSS Version 19.

2.4 Study 3: Biological validation

Twenty-one marmosets (12 male, 9 female) provided baseline (same time period on normal, undisturbed days in the lab) and post stressor samples on one weighing occasion, to assess biological validity of the assay. All marmosets provided 3 baseline samples each. Eighteen marmosets provided 2 post stressor samples, while the remaining 3 individuals provided only one post stressor sample. In 5 cases, the same animal was sampled in both the biological validation and the collection device studies.

2.4.1 Weighing procedure

Weighing is a necessary routine event, carried out each month, which provides a good opportunity to assess how individuals cope with a mild stressor, without imposing any stress for the sole purpose of the study. Weighing took place between 9:00 and 10:00. The marmoset was caught by grasping the base of the tail and then holding the animal around the chest. After a brief health check, the animal was placed into a small, plastic box and weighed on the scales. They had

no visual or olfactory contact with their pair member while in the weighing box, although they were within auditory contact. The box was opened in the new clean cage and the animal allowed to leave at will. The old cage was then removed for washing. The whole process lasted approximately 5 minutes/marmoset. While in the home cage, the marmosets were in view of other pairs in the room being weighed. Order of weighing (comparing 12 individuals weighed first in the room with 9 individuals weighed last in the room (see Ash et al, in prep) was counter balanced between males and females.

2.4.2 Saliva sampling

Saliva was sampled on three baseline days between 9:00 and 10:00 in the week prior to weighing, with similar timings for each individual animal, to ensure compatibility and avoid variation due to circadian rhythm (Cross and Rogers, 2004). Two saliva samples were collected after capture and weighing, at 0-5 mins and 25-30 mins. Saliva was collected using the method in section 2.2.1, using SOS with a banana coating, and the assay was conducted as outlined in section 2.2.2.

2.4.3 Statistical analysis

To look at biologically meaningful changes in cortisol level, means were calculated from the three baseline cortisol values for each individual, to obtain one baseline value for use in the analysis, in attempt to reduce variability. As no transformation was successful in making data normally distributed, Friedman tests were conducted to look at differences in cortisol concentration over the time points (baseline, post 0-5 mins and post 25-30 mins). Follow-up Wilcoxon tests were conducted to find where the difference lay. Mann Whitney tests were used to look at sex differences at baseline. As data was approximately normally distributed within order of weighing, differences in cortisol between those weighed first and last in the room were analysed at baseline (using all 3 values), post 0-5 mins and post 25-30 mins using Independent samples t tests.

3 Results

3.1 Study 1: Assay validation criteria

Displacement curves of serial dilutions of the commercial standards and the pooled saliva samples over the 10-90% binding range did not differ significantly (ANCOVA: $F(1,16)=0.944$, NS), inferring parallelism between the standards and samples, and so assay specificity. Recovery of the commercial standards (3.06, 1.02, 0.33 nmol/L) added to a low concentration (1:2000 dilution) mixed saliva pool was 101.71% +/- 6.26 ($r=0.998$, $P<0.0001$), and a high concentration (1:1000 dilution) mixed saliva pool was 92.64% +/- 5.41 ($r=0.999$, $P<0.0001$), suggesting good accuracy at both dilutions. Intra-assay coefficients of variation for low and high concentration quality controls were 2.39% and 2.39% respectively (N=3 plates). Inter-assay coefficients of variation for low and high concentration quality controls were 4.54% and 7.28% respectively (N=3 plates). Sensitivity, computed from the pooled saliva samples, was 0.86nmol/L.

3.2 Study 2: Collection method

A dilution of 1:1000 was necessary for pooled samples collected by cotton buds to fall within the linear range of the standard curve (i.e. B/B_0 of around 50%), while a 1:5000 dilution was necessary for samples collected by SOS. For cotton bud samples, those without banana had significantly higher cortisol concentrations than those with banana (Mann Whitney tests: $U=0.00$, $N=16$, $P=0.001$). A highly significant positive correlation was also found between cortisol concentrations collected with and without banana (Spearman's rank correlation: $r=0.98$, $P<0.001$). The relationship fit the following equation: without banana=with banana/0.55. However, for SOS samples, those with banana had significantly higher cortisol levels than those without banana ($U=1.00$, $N=11$, $P=0.011$; Figure 1). SOS samples with and without banana were not significantly correlated ($r=0.70$, $P=0.188$).

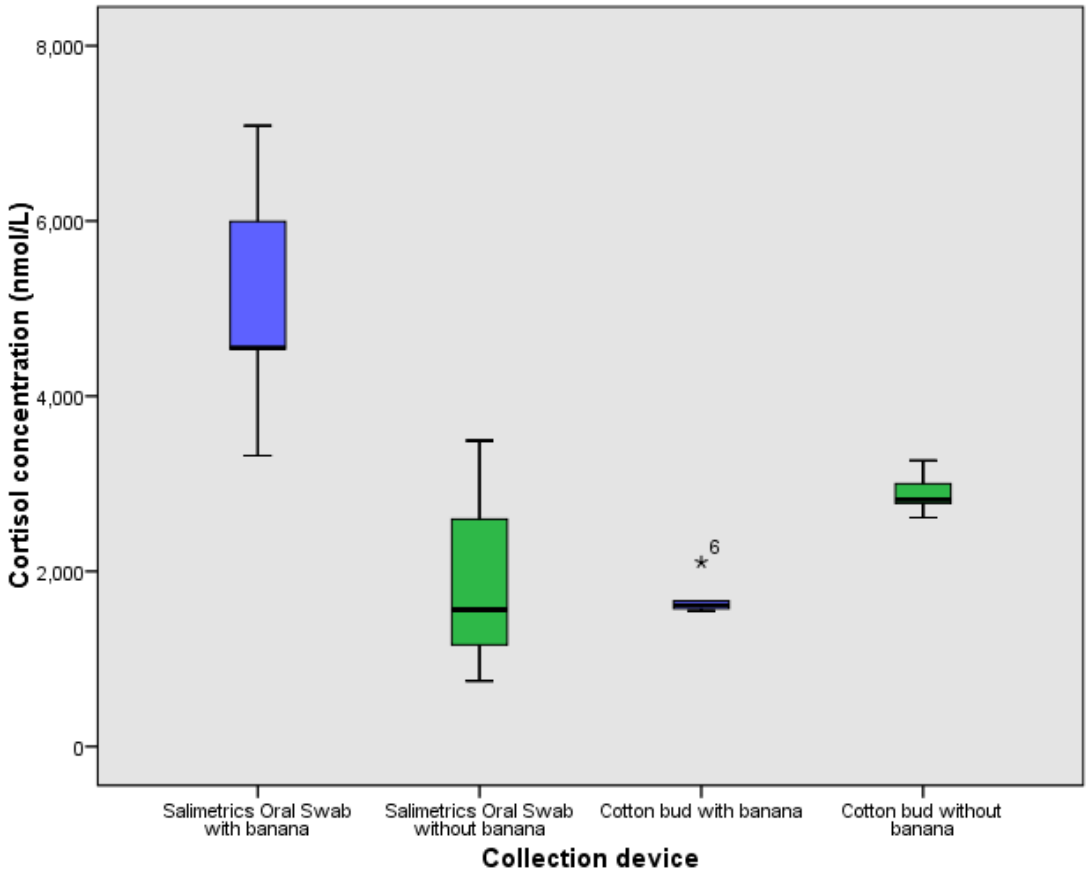

Figure 1: Median cortisol concentration (nmol/L) for each collection device, with and without banana. Median: solid line; Interquartile range: boxes; Minimum and Maximum value: whiskers; Outliers: stars.

3.3 Study 3: Biological validation

In total, 95.06% of samples were successfully collected and analysed. As a banana correction factor for SOS was difficult to identify (see section 3.2), all data presented were uncorrected for banana. Variation across baseline cortisol measurements was high, ranging from 614.10-28917.10 nmol/L. Although not significant, females had higher baseline cortisol values than males (mean 9473.34+-7833.69nmol/L v. 6388.47+/-5530.48nmol/L).

There was a significant difference in cortisol concentrations across the three time points ($X^2(2)=19.86$, $P<0.001$). Cortisol significantly decreased from baseline to post-capture 0-5 mins

(Z=-3.82, P<0.001), and from baseline to post-capture 25-30 mins (Z=-3.36, P<0.001; Figure 2). Those weighed last in the room had significantly higher cortisol values than those weighed first, both at baseline (t=2.79, P=0.007) and at post-capture 25-30 mins (t=2.86, P=0.013; Figure 3).

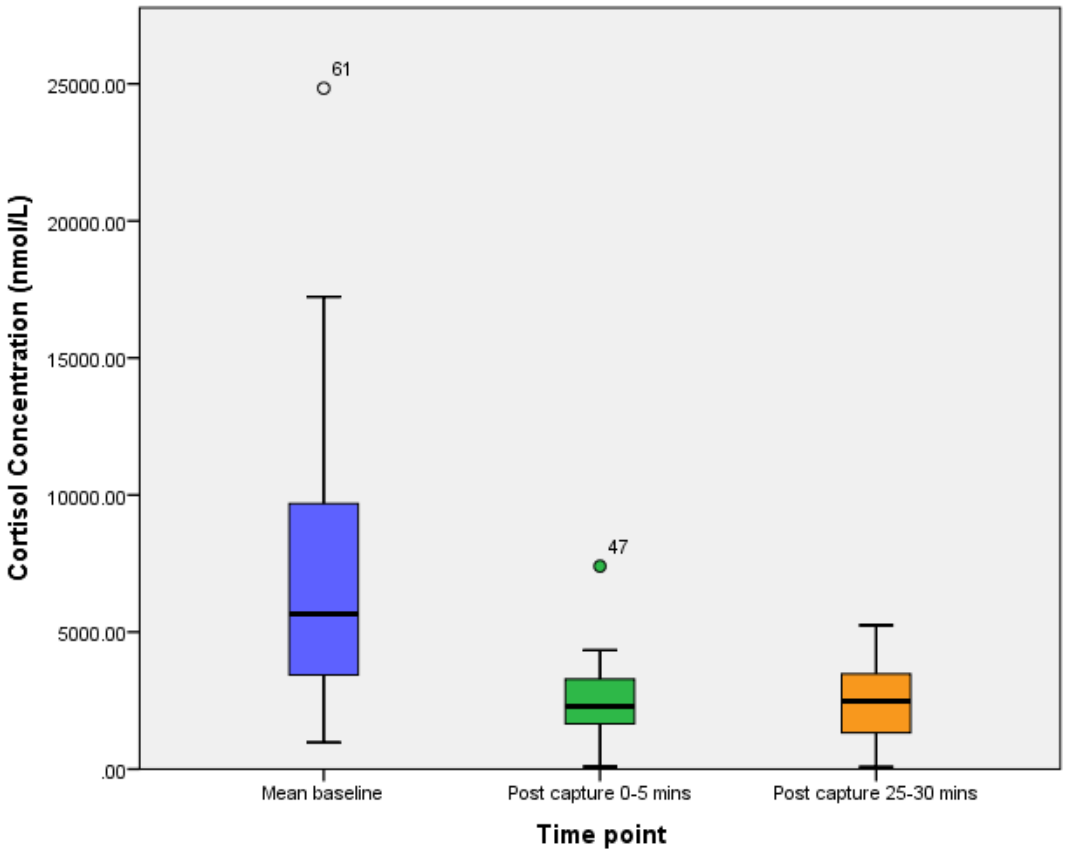

Figure 2: Median cortisol concentration (nmol/L) at each time point (average baseline, post capture 0-5 mins, post capture 25-30 mins). Median: solid line; Interquartile range: boxes; Minimum and Maximum value: whiskers; Outliers: circles.

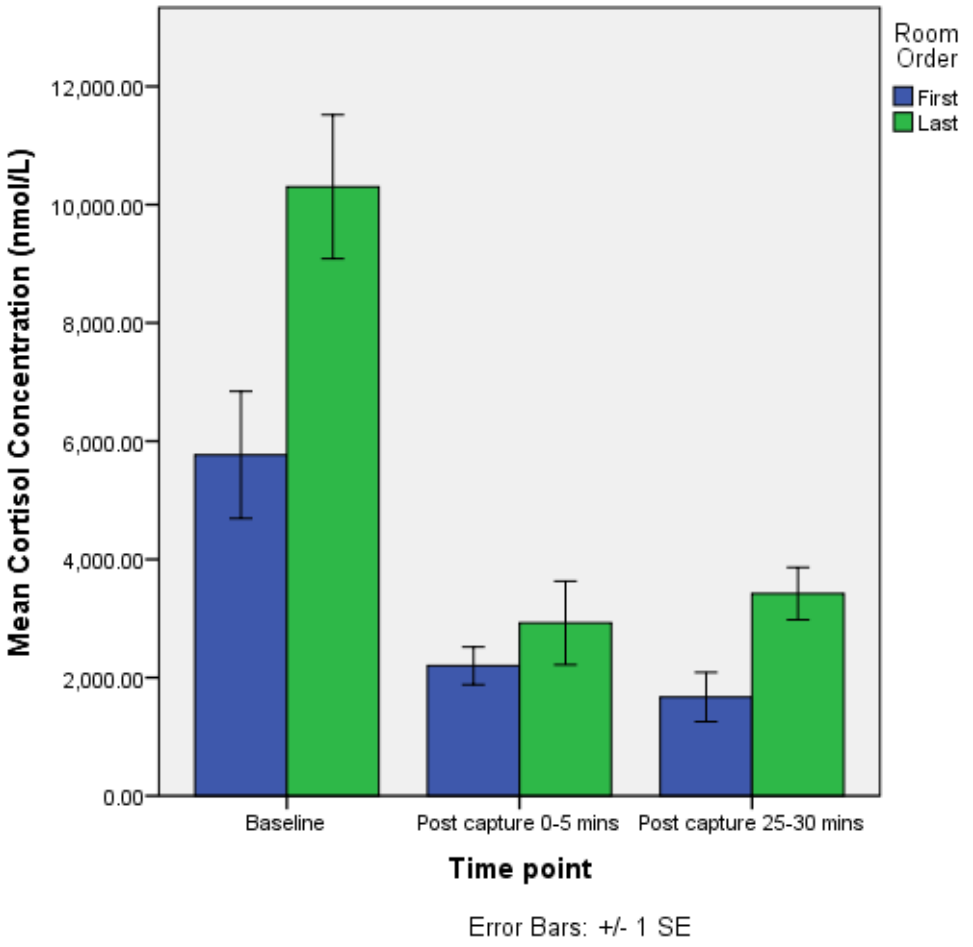

Figure 4: Mean cortisol concentration (nmol/L) for animals weighed first (n=12) and last (n=9) in the room, at each time point (3 baseline values, post capture 0-5 mins, post capture 25-30 mins).

4 Discussion

Assay validation criteria

The Salimetrics® ELISA performed well on typical tests used to validate an assay in a new species. It was found to have high specificity, demonstrating that cortisol in the samples and standards reacted in a similar manner with the antibody (Reimers and Lamb, 1991), with minimal cross reactivity from other molecules present in the saliva or banana. As the measurement obtained in the assay agreed with the actual amount of the substance when known amounts of cortisol were added to dilutions of the sample, accuracy was also high. Target values of less than

5% for intra-assay and 10% for inter-assay CVs were met (Schultheiss and Stanton, 2009), and so there was excellent agreement between replicate measures of a known sample, assayed within and between plates. Lastly, as the assay is able to detect even small concentrations of cortisol (computed at 90% B/B0%), sensitivity was high. Comparison of values with a further assay following a chromatographic procedure to purify the cortisol could however confirm validity (Cekan, 1979).

Collection method

Levels of cortisol have been reported in callitrichids using saliva (Cross et al, 2004; Bowell, 2010), blood plasma (eg. Torii et al, 1998; Johnson et al, 1996), urine (Torii et al, 1998; Smith et al, 1998), faeces (Sousa and Ziegler, 1998; Sousa et al, 2005) and hair (Clara et al, 2008), with cortisol measurements varying between methods of collection and even between studies using the same collection method (reviewed in Bowell, 2010). For example, blood plasma concentrations have been reported in adult female *C. jacchus* to range from 182.07μg/dl (Schultz-Darken et al, 2004) to 3858μg/dl (Whitehouse and Abayasekara, 2000).

Mean baseline cortisol level in the present study, using Salimetrics® Oral Swabs, was 7710.56+/-6735.65 nmol/L. Although not statistically significant, females had approximately one-third higher baseline levels than males, as reported previously in marmosets (*C. jacchus:* Johnson et al, 1996: blood cortisol; *Callithrix kuhli*: Smith and French, 1997: urinary cortisol), which may be due to the impact of reproductive steroids on HPA axis function (Saltzman et al, 1998). A considerably higher amount of salivary cortisol was therefore recovered in the current study, compared to previously published data. For example, Cross et al (2004) used cotton buds to collect saliva, finding mean concentration at undisturbed baseline periods to be 561nmol/L. However, this rose to almost 4500nmol/L in disturbed periods in certain individuals (mean 1198+/-179 nmol/L). Differences between studies may be due to time of sample collection, with Cross et al (2004) collecting their samples later in the day, at 16:00-17:00, when cortisol has

decreased significantly from morning levels. Cross and Rogers (2004) found that salivary cortisol in marmosets peaked upon waking (to as high as 1200nmol/L), then gradually declined throughout the day. They also found high variation in morning samples, during undisturbed periods, which is similar to our baseline findings. Direct comparisons between published studies may therefore not be useful, although relative differences can be found within studies.

Results from the current study showing that a 1:5000 dilution was necessary for SOS, compared to a 1:1000 dilution for cotton buds, suggest that polymer collection devices can recover 5 times more cortisol than cotton collection devices, which is similar to findings by Salimetrics® (2012b) and Groschl and Rauh (2014: Salivette). This finding is likely because SOS are designed for the collection of saliva samples for analysis, being made of a material that filters mucins, cells and other aggregates in the saliva, allowing for greater recovery. Therefore, the use of SOS is recommended over cotton buds.

The vast majority of samples with banana were successfully analysed, and those with no readings were likely because not enough saliva was collected. However, while the relationship between cotton buds with and without banana was comparable to that found by Cross et al (2004) for *C. jacchus (*without banana=with banana/0.55), as expected due to dilution of the samples with banana, there was unexpectedly no consistent effect of banana on cortisol concentration over collection devices. Although the impact of using sequential presentation of cotton buds then SOS is not known for saliva samples, it is possible that previous exposure to the banana on the cotton bud increased cortisol levels for the subsequent SOS sample, either due to food (humans: Toda et al, 2004) or excitement. To further assess any effect of banana on SOS, recovery of samples with banana could be compared to samples without banana. However, given that banana may confound the data in some way, and that marmosets often chewed on the swabs with no banana, using SOS without fruit coating is the preferred option.

406 *Biological validation*

407 Biological validation is necessary to assess whether the assay can accurately reflect
408 biologically meaningful changes in hormone levels in the species (Heistermann et al, 2006).
409 Changes in cortisol concentration were detected following a stressor, with levels significantly
410 decreasing in the marmosets after they had been hand-captured, weighed and placed in a new
411 cage. As habituation to the swabs was carried out, it is unlikely the higher cortisol levels at
412 baseline were due to stress during saliva collection, although may have been related to positive
413 excitement, as, with rare exceptions, the marmosets were always willing to chew on the swabs.
414 Elevated baseline levels could also be due to greater activity (*Homo sapiens*: Stupnicki and
415 Obminski, 1992), with positive correlations being found between cortisol concentration and levels
416 of locomotion in *C. kuhli* (Smith et al, 1998), or because food was more freely available at this
417 time (Toda et al, 2004). Behavioural observations would therefore aid in interpretation (Ash et al.,
418 in prep).

419 While some studies have found significant elevations in salivary cortisol following social
420 isolation and a period of noise and human activity in the animal house (Cross et al, 2004; Kaplan
421 et al, 2012), others have found similar reductions in cortisol post-stressor. For example, all
422 marmosets had a significant decrease in salivary cortisol following presentation of a model snake
423 (Cross and Rogers, 2006). This response was unexpected, given the increase in stress related
424 behaviours, including tsik calls, agitated movement and mobbing responses. In a further study,
425 cortisol levels doubled in magnitude when marmosets were isolated from peers in an unfamiliar
426 room, although playback of mobbing (tsik) calls from a familiar conspecific when isolated lead to
427 decreases in cortisol (Cross and Rogers, 2006). Increases in these vocalisations were noted
428 following capture for weighing in the current study (Ash et al., in prep), which may help to
429 explain the decrease in cortisol.

430 Such stress reduction could be due to social buffering, the ability of a companion to ease
431 the stress of challenging situations (Gilbert and Baker, 2010), resulting in a reduced cortisol peak

and faster recovery (Novak et al, 2013), compared to when facing the situation alone. Much physiological evidence has been found for this, such as Smith et al (1998), who found no change in urinary cortisol levels in *Callithrix kuhli* after 4 day separations from their group when placed in close proximity to a pair-mate, although cortisol levels rose significantly when they were alone. Alternatively, 'blunting' of the HPA axis may have occurred following a prolonged period of stress (Tiefenbacher et al, 2004; Lolman and Gunnar, 2010), due to increased negative feedback sensitivity to glucocorticoids. In a study of humans, Gallagher et al (2016) found that although unemployed people reported higher levels of stress, they unexpectedly had lower cortisol output than employed people. Such down regulation may be an adaptive mechanism to protect the individual from exposure to high cortisol levels. Overall, these results suggest that decreases in cortisol associated with stress may be a common feature across primates.

Order of weighing in the room also appeared to have an effect on salivary cortisol levels. Cortisol concentration was significantly higher 30 minutes after capture in marmosets weighed last in the room, compared to marmosets weighed first, perhaps as they had been anticipating capture for longer. Previous research has found a positive relationship between order of blood sampling in a room and plasma cortisol concentrations (*M. fascicularis:* Flow and Jaques, 1997), suggesting that watching other monkeys undergo routine husbandry or procedures, or lengthy anticipation of a negative event, can be stressful. While this fits the predicted results, it is a little unexpected given the overall decrease in cortisol following weighing. As those weighed last had significantly higher baseline levels than those weighed first (which was not ideal), the result may simply be due to levels returning to these higher baseline concentrations at 30 minutes post capture. It is possible that as there was no disturbance 30 minutes after the last marmosets were weighed, compared to those weighed first (when weighing was still occurring 30 minutes after their capture), the mobbing calls were then reduced, having less diminishing effect on cortisol levels. However, there was a consistent pattern of results, with both those weighed first and last showing the same decrease in cortisol levels following capture for weighing.

Methodological considerations

Use of SOS and the commercially available Salimetrics® assay did prove to be a valid way of monitoring salivary cortisol in pair-housed marmosets, confirming this is a promising non-invasive method of measuring acute changes in cortisol- an important tool in animal welfare assessment. However, we do not yet have a full understanding of time course and variation in responses to different stressors in most species of non-human primate (Novak et al, 2013). Previous research has found that the salivary cortisol response to an ACTH injection stressor in chimpanzees started to increase from 15 minutes and peaked at 45 minutes (Heintz et al, 2011), which is similar to humans. However, New World monkeys have low corticosteroid-binding globulin (CBG) capacity and affinity, leading to exceptionally high levels of cortisol compared to other primates (Klosterman et al, 1986), and so salivary cortisol response and half-times in marmosets may be different from other species. Despite this, studies looking at the response to capture and weighing in marmosets have detected significant changes in cortisol concentration from 0-30 minutes post stressor (eg. Bowell, 2010). Therefore, 30 minutes, as used in the current study, should be sufficient to find any changes in cortisol concentration.

Differences in early life history could have also contributed to the range in baseline levels (see Dettling et al, 2002). Twins are the usual litter size in wild marmosets, but triplet litters are common in captivity (Ash and Buchanan-Smith, 2014), and so intra-uterine stress or supplementary feeding of large litters to improve survival may have influenced cortisol reactivity. Other factors could have affected concentrations, such as ovulation in females (Saltzman, 1998) or undetected blood contamination, which will increase cortisol levels (Davenport et al, 2003).

While validation of a biochemical nature may be beneficial to confirm the validity of the assay, such as ACTH challenge, which is followed by significant elevations of glucocorticoid metabolites (Romero and Wingfield, 2001), purely non-invasive measures were selected in the present study, which also piggybacked on unavoidable, potentially stressful husbandry events. Similarly, plasma matching would require venepuncture, which is likely to be stressful in itself

and so influence cortisol levels (Reinhardt, 2003). Studies do however consistently report correlations between plasma and salivary cortisol levels, both in nonhuman (eg. *M. mulatta*: Davenport et al, 2003) and human primates (eg. Calixto et al, 2002; Galard et al, 1991), suggesting that salivary cortisol levels can reliably indicate plasma cortisol levels.

Using cortisol to assess welfare

Despite potential complexities, there is widespread use of cortisol level as a measure of physiological stress in the captive environment, with HPA axis activity being assessed in a variety of contexts, including management practices, social experiences and abnormal behaviour (eg. Reinhardt et al, 1995; Cross et al, 2004; Davenport et al, 2008). However, studies of similar stressors have yielded inconsistent results, with some studies finding reduced HPA axis activity and others finding no differences or increased cortisol levels (eg. abnormal behaviour: reviewed in Novak et al, 2013), making it difficult to draw firm conclusions about animal welfare. Further, particular conditions which are thought to be inherently stressful have led to lowered cortisol levels, including capture and weighing in the present study, and the HPA axis response to positive stimuli, such as winning a social interaction, can be as large as the response to aversive stimuli, such as social defeat (Koolhaas et al, 1997). These results suggest that the magnitude of the response is often simply a reflection of metabolic requirements of behavioural activity (Koolhaas et al, 2011).

The conventional use of the stress concept does therefore have its problems. However, the difference in responses to stressors may be due to the psychological, rather than physical, nature of the situation. For example, increased perception of predictability or controllability, could lead to a decline in the magnitude of the stress response or quicker recovery (Koolhaas et al, 2011). It is possible in the current study that by the time the marmosets were back in their home cage, the danger had passed, control had been regained, and the parasympathetic nervous system had dampened the stress response (eg. Arnhold et al, 2009). Alternatively, while a passive response is associated with increased activation of the parasympathetic system, resulting in

greater fluctuations of cortisol, more active responses involve activation of the sympathetic system, which releases adrenaline (Cross and Rogers, 2006). This again highlights the need for contextual and behavioural data.

With accumulating evidence that lower concentrations of cortisol may not always be good and higher concentrations may not always be bad (Novak et al, 2013), care is needed when using cortisol as an index of wellbeing, particularly when comparing studies using different collection methods. Measuring cortisol may however be a useful addition to other assessments of primate welfare (Dawkins, 1998), to provide a more holistic insight into their wellbeing.

5 Conclusion

This study demonstrated that Salimetrics® Oral Swabs and Salimetrics® Enzyme Immunoassays are reliable means of recovering salivary cortisol, to assess physiological stress in marmosets. The swabs recovered a much greater range of cortisol than traditionally used cotton buds, improving its measurement. The assay was also validated for use in marmosets, and could be used to monitor acute changes in free cortisol levels, including those associated with capture and brief separation from partners. There is now much empirical data showing decreases in cortisol following a stressor, along with increases in cortisol in response to positive stimuli, challenging traditional views on cortisol as an index of stress. The techniques presented may however aid researchers in deciding the optimal strategy for their work, and when used with other measures such as behavioural observations, could enhance our understanding of primate welfare.

Acknowledgements

We thank the National Centre for the Replacement, Refinement and Reduction of Animals in Research for their funding and support of the project. The authors are grateful to all staff at the colony for their valuable assistance, and thank Robert Hubrecht and Phyllis Lee for constructive discussions on the content, as well as 2 anonymous reviewers for their helpful comments.

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