Title: Cortisol analysis of hair of captive chimpanzees (*Pan troglodytes*)

YumiYamanashi[ab]

[a]Primate Research Institute, Kyoto University

[b]Japan Society for Promotion of Science

NarukiMorimura[c]

[c]Wildlife Research Center, Kyoto University

Yusuke Mori[c]

[c]Wildlife Research Center, Kyoto University

Misato Hayashi[a]

[a]Primate Research Institute, Kyoto University

JuriSuzuki[a]

[a]Primate Research Institute, Kyoto University

Address

[a]Primate Research Institute, Kyoto University, 41-2, Kanrin, Inuyama City, Aichi, 484-8506, Japan

[b]Japan Society for Promotion of Science, Kojimachi Business Center Building, 5-3-1 Kojimachi, Chiyoda-ku, Tokyo, 102-0083, Japan

[c]Wildlife Research Center, Kyoto University, 3rd Floor, 2-24 Tanaka-Sekiden-cho, Sakyo, Kyoto, 606-8203, Japan

Present address

Yumi Yamanashi moved to [c]Wildlife Research Institute, Kyoto University

NarukiMorimura moved to [a]Primate Research Institute, Kyoto University

Correspondence: Yumi Yamanashi

yamanashi@wrc.kyoto-u.ac.jp

Wildlife Research Center, Kyoto University

3rd Floor, 2-24 Tanaka-Sekiden-cho,

Sakyo, Kyoto, 606-8203, Japan

Tel: +81-75-7714399

Abstract

In addition to behavioral evaluations, stress assessments are also important for measuring animal welfare. Assessments of long-term stress are particularly important given that prolonged stress can affect physical health and reproduction. The use of hair cortisol as a marker of long-term stress has been increasing, but there has not yet been any report on the use of such methods with chimpanzees. Therefore, the purpose of this study was to establish and validate a methodology for analyzing hair cortisol in captive chimpanzees. In the first experiment, hair was removed from the arms of nine chimpanzees living in the Kumamoto Sanctuary (KS) and the regrown hair was sampled 3 months later. Fecal samples were collected periodically during the hair-growth period. The results showed that hair cortisol level was positively correlated with the rate of receiving aggression. Although the correlation between hair and fecal cortisol levels was not significant, the individual with the highest hair cortisol concentration also had the highest fecal cortisol concentration. These results suggest that hair cortisol may reflect long-term stress in chimpanzees. In the second experiment, we investigated the physiological factors affecting hair cortisol concentrations. We cut hair from the arms, sides, and backs of 25 chimpanzees living at the KS and the Primate Research Institute. The results revealed that cortisol varied based on source body part and hair whiteness. Therefore, we recommend that hair should always be collected from the same body part and that white hair should be avoided as much as possible.

Keywords

Hair cortisol; Chimpanzees; Welfare; All-male group; Aggression

1. Introduction

Many chimpanzees are kept in captive environments around the world. A total of 325 chimpanzees were living in 51 Japanese facilities including zoos, sanctuaries, and laboratories as of 2013 [23]. In the US, 1,970 chimpanzees were living in zoos, sanctuaries, laboratories, and private houses as of 2012 [44]. A total of 1,032 chimpanzees were registered in the studbook of the European Associations of Zoos and Aquariums [12]. Chimpanzees live in a physically and socially complex environment in their wild habitats, but captive environmentsdiffer greatly from their original habitats [45]. Despite the large number of captive chimpanzees and increasing attention toward animal welfare, little is known about the link between stress and the environment of chimpanzees living in such captive environments. Understanding animal stress is important, and long-term assessments of stress are particularly important because prolonged stress often has deleterious effects on behaviors, reproduction, and physical and mental health [11, 13, 53]. However, practical constraints often prevent assessment of long-term stress in many individuals. Therefore, it is important to develop practical ways of estimating long-term stress in chimpanzees.

Cortisol is a steroid hormone released by the adrenal cortex, and its production usually increases in reaction to stressors [19]. Cortisol helps mobilize energy during stress by influencing the metabolism of sugar in the direction of increasing blood glucose levels [53]. Therefore, it is often used as a marker of stress. Cortisol assessment in chimpanzees has been performed using blood, urine, feces, and saliva [3-5, 17, 24, 30, 40, 57]. However, the use of these types of samples for estimating long-term stress presents several difficulties. For example, cortisol responds to circadian rhythms, and the peak cortisol level is found just before arousal from sleep [47]. Additionally, the

time course of cortisol excretion in chimpanzees is short: peak cortisol levels were detected in fecal samples 22 hrs after injection of ^{3}H-labeled cortisol [5]. Thus, samples should be collected at multiple times. Additionally, repeated blood-sample collection can itself be stressful for animals. Recently, an increasing number of studies have validated the use of hair for stress measurement [2, 9, 15, 18, 28, 32, 49, 58]. For example, Davenport et al. (2006) found a significant correlation between hair and saliva cortisol concentrations and showed that the hair cortisol concentrations in a group of rhesus macaques increased after their relocation to another institute. Hair cortisol levels have an advantage for the assessment of long-term stress insofar as it has been hypothesized that they reflect an accumulation over a period of time. Additionally, chimpanzees often form good relationships with humans [34], which may enable us to collect hair samples relatively easily. However, there have been no reports to date on hair cortisol measurements in chimpanzees. Considering that the manner in which substances such as cortisol are incorporated into hair is not fully understood [22, 25] and that there are variations in physiology and hair features between species, it is necessary to validate the use of hair cortisol as a stress marker for each species separately and to modify the methodology appropriately.

Hence, this study aimed to investigate the validity of hair cortisol as a marker of long-term stress in chimpanzees and to examine how physiological factors such as the body part from which the hair was taken influence hair cortisol concentrations.

2. General Method

The sample consisted of 26 captive chimpanzees (16 males and 10 females) living at the Primate Research Institute, Kyoto University (PRI) (N = 13) and the

105 Kumamoto Sanctuary, Kyoto University (KS) (N = 13). Basic information about each
106 subject is presented in Table 1.

107 At the PRI, the subject chimpanzees lived in two mixed-sex groups in an
108 outdoor enclosure that connected to several inside rooms. The outdoor enclosure was
109 separated into two compartments: one was a 700-m^2 outdoor compound with 15-m-high
110 climbing frames, a small stream and numerous trees; the other was a 250-m^2 outdoor
111 compound with climbing frames and two small streams [35, 41]. Chimpanzees could
112 freely access the outdoor enclosure and inside room at all times. The chimpanzees were
113 fed seasonal fruits and vegetables, along with monkey pellets three times per day.
114 During the experimental period, feeding-enrichment items were provided between
115 meals on a few occasions, and cognitive experiments were conducted on some
116 individuals [34, 36, 43].

117 The KS was the first chimpanzee sanctuary in Japan and is now operated by the
118 Wildlife Research Center, Kyoto University [38]. At the KS, the subject chimpanzees
119 lived in all-male groups. All-male groupingare not observed in wild chimpanzees, but
120 are often formed in captivity to solve the problem of surplus animals [10, 20]. There
121 were three all-male groups in the sanctuary, and each subject belonged to one of these
122 groups. These individuals lived in an outdoor cage that connected to inside rooms. All
123 outdoor cages were covered with iron mesh fences,and were about 120 m^2 in size and
124 3.8 m in height. Some grass and shrub vegetation was present on the ground. Climbing
125 structures and several feeding devices were present in the cages for environmental
126 enrichment. Indoor rooms were small cells 4 m^2 in size and 2.7 m in height. During the
127 experimental period, the subjects were isolated in indoor rooms from evening until the
128 next morning, but were able to communicate with neighboring individuals by visual and

physical contact through the iron bars [38, 39]. KS chimpanzees were fed seasonal fruits and vegetables three times a day. Additionally, various enrichment efforts were made every day [29]. During the period of this study, as part of a social enrichment program, group members were changed on a daily basis. For example, one day, 15 chimpanzees were divided into two groups of 10 and five members. On another day, 15 chimpanzees were divided into three groups of five members each. Additionally, Toon and Kenny, two of the subjects in this study, arrived from a zoo in June 2008 and were initially integrated into an all-male group on June 25th, 2008. Thus, the social stimulation available to chimpanzees at KS was greater than that at the PRI, which did not offer such social variation during the period of this study.

3. Experiment 1

In Experiment 1, we examined whether the cortisol concentration in hair was correlated with that in feces and with aggressivebehaviors.

3.1 Materials and Methods

3.1.1 Study site and subjects

The sample consisted of nine male chimpanzees at the KS (Table 1). Six of these chimpanzees lived in a group with daily membership changes (represented as (f) in Table 1), whereas the other three chimpanzees lived in a stable male group (represented as (s) in Table 1).

3.1.2 Sample collection

Samples were collected at the KS between May and August 2009. Hair was cut

153 from the arms of the chimpanzees at the KS for the first time in May 2009 (Table 1, Fig.
154 1). We then waited for the growth of the new hair and collected hair from the same
155 place in August 2009. Hair was cut by a caregiver (YM) with whom the subject
156 chimpanzees had had extensive experience. We used scissors to reduce the stress of the
157 sample collections. The chimpanzees voluntarily stretched out their arms and enabled us
158 to cut near the bottom part of the hair, close to the skin surface. Fecal samples were
159 collected periodically from individual indoor rooms in the mornings between May and
160 July 2009. Behavioral data were obtained for 48 days between May 28th and July 31st
161 2009 by a researcher (NM) observing the group while group members changed daily.
162 Sixty-minute observations were performed once per day from around 9:00–9:30 a.m.
163 when the chimpanzees were introduced into the outdoor compound. The occurrence of
164 aggressive behaviors including chase, charge, charging display, and attack were
165 recorded using the focal-animal sampling method. The focal individuals were scored
166 when initiating or receiving aggressive behaviors. The chimpanzees who were the
167 recipients of aggression often showed responses such as scream and flee [39]. The
168 number of aggressions was calculated as the number of bouts in which aggressive
169 behaviors described above were seen. If an aggression ceased and started again after a
170 while, we considered it to be two different bouts.
171
172 ***3.1.3 Sample preparation***
173 *3.1.3.1 Hair samples*
174 We analyzed the hair samples collected in August. Hair samples were preserved
175 at ambient temperature and covered in aluminum foil until assayed. The method of
176 washing the hair and extracting cortisol from it was based on Davenport et al. [15]. Hair

was placed in a 15-ml tube with isopropanol and shaken gently for 2 min. This washing procedure was repeated three times. After washing, samples were placed in a clean hood for approximately 5 days. The hair was ground using aPrecelly 24 (Bertin Technologies, 6500 rpm for 4 min), and 40 mg of powdered samples were weighed and placed in a 2-ml tube. One milliliter of methanol was added to each tube and incubated at ambient temperature for 24 hrs while being shaken gently with a petite shaker (Waken B Tech). After extraction, the samples were centrifuged for 15 min, and 0.6 ml of supernatant was aliquoted into a clean 5-ml tube. The extracted samples were then dried at 38 °C under nitrogen gas. The samples were reconstituted with PBS buffer before assay.

3.1.3.2 Fecal samples

After collection, 1 g of fecal sample (wet weight) was placed in a 15-ml tube with 4 ml of ethanol (80 %) and shipped from KS to the PRI. Following Khan et al. [26], the samples were not kept at ambient temperature for more than 2 weeks. As soon as the samples arrived at the PRI, the samples were stored in a biomedical freezer at –30 °C. The samples were dried at 38°C under nitrogen gas just before extraction. The extraction was performed based on the protocol for orangutan fecal cortisol extraction created by Yamazaki et al. [59]. Dried samples were weighed and thoroughly mixed with 5 ml of PBS buffer for 45 min. After being centrifuged for 15 min, 1.5 ml of supernatant was transferred to a clean glass tube. Three milliliters of di ethylether was added and then mixed for 15 min. Two milliliters of supernatant was evaporated using a vacuum dryer and then reconstituted with PBS buffer before assay.

3.1.4 Cortisol Assay

Concentrations of fecal and hair cortisol were determined by enzyme-linked immunosorbentassay (ELISA) based on Suzuki et al. [54]. Reagents including cortisol (hydrocortisone) used as a standard, peroxidase-labeled cortisol, and O-phenyilenediamine were purchased from Sigma (Tokyo, Japan). The antibody to cortisol was obtained from Cosmo Bio Co. (Tokyo, Japan). Microplates were obtained from Gleiner (Tokyo, Japan). The software used for the calculation was LS-Plate manager 2000 and 2004. Any duplicates with a CV greater than 20% were removed from analysis. This resulted in some variability in the number of samples used for each individual (7–10 fecal samples per each subject). Intra- and inter-assay variability for fecal cortisol assays were 15.7 % and 28.3 %, respectively, and those for hair cortisol were 6.8 % and 14 %, respectively.

3.1.5 Statistical Analysis

Parallelism was determined by analysis of covariance (ANCOVA). When the interaction between absorbance and group (standard or sample) was not significant, we considered parallelism confirmed. For fecal samples, cortisol concentration per 1 g dry matter was calculated by dividing cortisol concentration by dry matter weight. The numbers of initiated and received aggressions per day were calculated and used for the analysis. To check the correlations between fecal and hair cortisol concentrations and between hair cortisol concentration and aggression, we used Spearman's rank correlation test. The software used for analysis was R 2.15.1 [46] .

3.2 Results

3.2.1 Parallelism test

The results of the parallelism test are shown in Figure 2. Data on the serially diluted fecal and hair cortisol concentrations were both very similar to those on the cortisol standards (Fig. 2-a, fecal samples: $F = 0.058$, $p = 0.814$; Fig. 2-b, hair samples: $F = 0.030$, $p = 0.286$).

3.2.2 Correlation between hair and fecal cortisol concentration

There was no significant correlation between overall hair and mean fecal cortisol concentration (Fig. 3-a: $r_s = 0.167$, $p = 0.678$, n =9). However, the individual with the highest hair cortisol concentration also had the highest fecal cortisol concentration.

3.2.3 Correlation between behaviors and cortisol concentrations

A significant correlation between number of received aggressions and hair cortisol concentration was observed (Fig. 3-b: $r_s = 0.841$, $p = 0.036$, $n = 6$). However, no significant association between number of initiated aggressions and hair cortisol concentration was found ($r_s = -0.559$, $p = 0.249$, $n = 6$). There was no significant correlation between the rate of aggressive behavior and fecal cortisol concentration (received aggressions: $r_s = 0.319$, $p = 0.538$, $n = 6$; initiated aggressions: $r_s = -0.588$, $p = 0.219$, $n = 6$).

4. Experiment 2

To establish a practical methodology, we investigated the effects of body part, hair length, hair color, age, sex, and institutions on hair cortisol concentrations in Experiment 2. Additionally, we compared two different assay methodologies.

249

250 **4.1 Materials and Methods**

251 ***4.1.1 Study sites and subjects***

252 The sample consisted of 25 chimpanzees at the PRI and the KS (Table 1).

253

254 ***4.1.2 Sample collection***

255 *4.1.2.1 Hair sample collection*

256 From November to December 2011, hair was cut from three parts of the bodies
257 (arm, side, and back) of subjects at the PRI and the KS (Fig. 1). We chose these three
258 body parts because hair loss was least evident on the back and the side, and the arm was
259 the easiest part from which to collect hair. The hair loss level by body part are shown in
260 supplementary materials. Samples were collected in essentially the same way as they
261 were in Experiment 1.It was not possible to collect samples from all three parts of all
262 individuals at KS (Table 1). To check the length of hair, YY measured three randomly
263 chosen hairs from each sample before washing. The average length of these three hairs
264 was used as the mean length of each hair sample.

265

266 *4.1.2.2 Effects of white hair*

267 To check the effects of white hair on cortisol concentration, we chose six
268 mixed-color samples that had been collected from the backs or sides of PRI individuals.
269 We divided one sample into black and white sections and then processed and analyzed
270 them separately. Additionally, YY rated the hair color of each individual by direct
271 observation (see supplementary materials).

272

273 *4.1.2.3 Cortisol in distal and proximal sections of the hair shaft*

274 To check whether cortisol concentrations in the distal and proximal sections of
275 the hair shaft differed, we randomly chose 10 hair samples collected from the arms or
276 sides of KS and PRI chimpanzees. We divided each hair into two parts by approximate
277 distance from skin surface and then processed and analyzed them separately.

278

279 ***4.1.3 Sample preparation***

280 Hair sample storage and preparation methods were the same as those described
281 for Experiment 1.

282

283 ***4.1.4 Cortisol Assay***

284 We used a commercially available EIA kit (expanded range, high-sensitivity
285 salivary cortisol EIA, Salimetrics LLC) and followed the manufacturer's instructions for
286 the assay. We changed the assay method because the antibody used for Experiment 1
287 was no longer commercially available, and the Salimetric kit can reduce the time needed
288 for assay. Intra- and inter-assay variability were 6.9% and 6.0%, respectively.

289

290 ***4.1.5 Statistical Analysis***

291 Parallelism was determined by analysis of covariance (ANCOVA). When the
292 interaction between OD and group (standard or sample) was not significant, parallelism
293 was confirmed. We used analysis of variance (ANOVA) to test for variations in the hair
294 lengths of the three body parts. To check the effect of physiological factors and home
295 institutes on cortisol concentrations, we used arepeated-measures ANCOVA and
296 included body part, hair length, hair color, age, sex, and institute as fixed factors.To

297 compare the hair cortisol concentration between white and black hair obtained from the
298 same body region, we used the paired t test. When data were not normally distributed,
299 we log-transformed and then *Z*-transformed them to achieve approximate normality.
300 Spearman's correlation was used to check the correlations among the hair cortisol levels
301 in the three parts of the body. The software used for analysis was R 2.15.1 [46].
302
303 **4.2 Results**
304 ***4.2.1 Parallelism***
305 Data on serially diluted hair cortisol concentrations closely matched those for
306 the cortisol standards ($F = 0.024$, $p = 0.883$).
307
308 ***4.2.2 Comparison of the assay methodologies used in Experiment 1 and Experiment 2***
309 To check the correlation between the two assay systems used for Experiments 1
310 and 2, we analyzed cortisol concentrations in the same hair samples using the two
311 methods. We found a significant correlation between the hair cortisol concentrations
312 calculated using the two systems ($r = 0.98$, $p < 0.001$, $n = 8$), although the exact mean
313 values obtainedusing the two systems differed: 22.7pg/mg for the Salimetric kit and
314 51.5 pg/mg for the assay system used in Experiment 1.
315
316 ***4.2.3 Basic hair information***
317 Mean hair length differed among the three parts of the body (arm: 5.86 ±1.03
318 cm, side 5.52±0.75 cm, back: 4.67 ± 1.25 cm, $F = 11.68$, $p < 0.001$). Tukey's HSD
319 revealed that hairs taken from the arm and side were longer than were those taken from
320 the back (arm: diff = 1.19, $p < 0.001$; side: diff = 0.85, $p < 0.01$). Hairs taken from the

back varied more in length than did hairs taken from the arm and side. Hair color information is summarized in Supp. 1-b.

4.2.4 Effects of body part, sex, age and environmental differences on hair cortisol concentration

There was a significant difference in the cortisol concentrations in hair samples taken from the three parts of the body (Fig. 4: $F = 7.83$, $P < 0.01$). Tukey's HSD revealed that the cortisol concentrations in hairs taken from the side were significantly higher than were those taken from the arm (diff = 0.593, $P < 0.01$), and the cortisol concentrations in hairs taken from the back tended to be higher than those in hairs taken from the arm (diff =0.414, $P = 0.051$). The cortisol concentrations in hairs taken from the back and side did not differ significantly (diff = 0.179, $P = 0.557$). Correlations between the cortisol concentrations in different body parts were significant, but correlations between those in hairs taken from the back and those in hairs taken from other parts were weaker than was the correlation between those in hairs taken from the arm and those taken from the side (arm–side: $r_s = 0.708$, n = 20, $p < 0.001$; side–back: $r_s = 0.641$, $n = 19$, $p < 0.01$; arm–back: $r_s = 0.610$, n = 20, $p < 0.01$). Cortisol concentrations were higher in the KS than in the PRI chimpanzees ($F = 6.14$, $p = 0.018$). Hair length, hair color, age, and sex did not significantly influence hair cortisol (hair length: $F = 0.878$, $p = 0.355$; hair color: $F = 0.003$, $p = 0.954$; age: $F = 2.688$, $p = 0.11$; sex: $F = 0.478$, $p = 0.494$).

4.2.5 Effects of white hair on hair cortisol concentration

Comparisons of the cortisol concentrations in white and black hairs showed

that the concentrations in white hairs were consistently higher than those in black hairs (white hair: 26.5 ± 6.91 pg/mg hair; black hair: 12.6 ±3.40 pg/mg hair; $t = 5.39$, $p = 0.003$, $n = 6$).

4.2.6 Cortisol in distal and proximal sections of hair shaft

There were variations in the direction of the differences in the cortisol concentrations found in proximal and distal sections of hair shafts. Cortisol concentration in the distal section was higher than that in the proximal section in four of 10 samples, whereas the difference was in the reverse direction or not significant in the remaining six samples (Fig. 5).

4.2.7 Comparison of hair cortisol concentrations between 2009 and 2011

The cortisol concentration for Toon, the chimpanzee with the highest hair cortisol level, decreased in 2011 from its level in 2009 (51.1pg/mg in 2009; 21.5 pg/mg in 2011). In contrast, the cortisol concentration for George increased (8.25pg/mg in 2009; 28.6 pg/mg in 2011). Analysis of veterinary and daily chimpanzee care records revealed that Toon did not sustain any injury requiring medical treatment after 2010. Additionally, the increased cortisol concentration in George matched a period during which he refused to leave his night cage. The concentration in other individuals did not vary so muchover time(average 12.7 ±5.29 pg/mg in 2009; average 16.6 ±3.87 pg/mg in 2011).

5. Discussion

The hair cortisol concentrations of chimpanzees can be assessed in a

meaningful way. A significant correlation between hair cortisol concentration and number of received aggressions was observed here. Muller and Wrangham[40] found that urinary cortisol level and rate of aggressive behavior were positively correlated. They also found that aggressive behavior and rank were positively correlated. This is opposite to the trend found in the current study, but it is not improbable because variations in the relationship between rank and stress have been reported among primate species, and these variations may be related to factors such as availability of social supports [1]. The subject group in the present study was relatively new, and the individual with the highest hair cortisol level was one of the two individuals introduced into the group a year before the study. Therefore, hair cortisol may be a good measure of long-term stress in chimpanzees as well as in previously studied species. Nevertheless, it was difficult to precisely determine the particular stressful period that was reflected in hair cortisol levels. More detailed studies are needed to confirm the accuracy of hair cortisol as a marker of stress and to precisely determine the period of stress reflected by hair cortisol.

We did not find any significant correlation between hair and fecal cortisol concentrations. There was no significant correlation between aggressive behavior and fecal cortisol concentration. Although no previous reports on the relationship between fecal cortisol and aggressive behavior in chimpanzees have been published, such an association has been found in other animal species [e.g. 7, 14]. Additionally, changes in fecal cortisol have been reported to be associated with stressful events (anesthesia, relocation) in captive chimpanzees [48, 57]. Given that the collection period of fecal samples matched with the period of the hair growth, sampling procedures are not likely to influence cortisol concentrations. Therefore, it may have been also due to our method

393 of fecal cortisol analysis that there was no significant correlation between hair and fecal
394 cortisol concentrations. The method of fecal cortisol measurement should be refined to
395 obtain more accurate results, given that the inter-assay variability was relatively higher
396 than that in previous studies. Nevertheless, the individual with the highest hair cortisol
397 concentration also had the highest fecal cortisol concentration. In addition, the
398 differences between individuals were greater than inter-assay variability. Thus, an
399 increased sample size may have resulted in more positive results, as in a study in dogs
400 and cats [2]. However, considering the practical limitation on the number of fecal
401 samples available and daily changes in cortisol level, our results might mean that hair
402 cortisol would be a better tool to assess long-term accumulation of stress.

403 We cut hairs from chimpanzees rather than shaving them to reduce the stress of
404 sample collection. Chimpanzees voluntarily stretched their arms or showed their arms,
405 sides, or backs after being asked to do so by the person who cut their hair. In contrast
406 with shaving, which usually requires anesthesia in non-human primates, this method
407 allowed us to collect samples more frequently without compromising subject welfare
408 [15, 18, 31]. Although hair cortisol has been reported to reflect long-term accumulation
409 of cortisol in many animal species, some studies reported the possibility of short-term
410 production of hair cortisol in hair follicles, which might indicate that sample collection
411 can influence the hair cortisol level [25, 52]. Thus, it is important to reduce the stress of
412 sample collection as much as possible.

413 We found that hair cortisol concentration varied depending on the body part
414 from which hairs were cut. Cortisol concentrations in hairs taken from the side were
415 higher than were those in hairs taken from the arm.However, a strong correlation was
416 found between the cortisol concentrations of hairs taken from the side and those taken

417 from the arm; thus, the trends characterizing changes in the cortisol levels measured
418 from these two body parts may be similar. Macbeth et al. (2010) also found
419 intra-individual variations in hair cortisol concentration in grizzly bears. They
420 considered that this variations was due to differences in timing and patterns of hair
421 growth in individuals. The cortisol concentrations in hairs taken from the side and back
422 did not differ significantly, but the strength of this correlation was weaker than that
423 between hairs taken from the side and those taken from the arm. Hair length varied
424 among individuals, and the variations between individuals were most evident on the
425 back. Thus, similarly to grizzly bears, chimpanzees might also show intra-individual
426 variations in hair cortisol concentration due to such differences in timing and patterns of
427 hair growth.

428 In addition, variations in hair color across body parts can also influence the
429 regional differences. This is because white hair was more evident on the back and
430 higher cortisol concentrations were found in white than in black hair, even when
431 collected from the same body part. Although hair color was not a significant factor in
432 the final model, it may be that the rating system used to assess black hair levels was not
433 sensitive enough to detect a relationship between hair cortisol and hair color. Supporting
434 the importance of this factor, Bennett et al. [9] also found that cortisol concentrations
435 differed in relation to coat color in dogs. They discussed the possible roles of
436 stress-associated inhibition of hair growth as well as of melanocyte development and
437 differentiation. However, it is also possible that white and black hair differ with respect
438 to the efficiency with which cortisol can be extracted given that it is well known that
439 melanin can inhibit the efficiency of DNA extraction [60]. However, because the exact
440 nature of this variation in chimpanzees was not clear, it is better to avoid white hair as

much as possible when using hair samples as a method of cortisol measurement.

We chose these three body parts for hair cortisol analysis based on the practical convenience of collection as well as on the stable presence of hair on those sites across many individuals. Previous studies in humans and other animals have used hair from the posterior vertex region, where the lowest coefficient of variation has been found in humans [15, 22]. However, in chimpanzees, hair loss was most evident in this region, and it was not a feasible sample-collection site. Based on the results of this study, the sides or arms may provide the best hair samples for chimpanzees.

Proximal–distal comparisons revealed that individuals varied in terms of the direction of the differences between the cortisol concentrations in hairs taken from the proximal section and those taken from the distal section of the same hair samples. It is hypothesized that because cortisol accumulates during hair growth, different sections of hair differ in this regard. The results of this study support this hypothesis because the direction of difference would be more consistent if cortisol concentrations varied according to more basic factors such as absolute amount of time elapsed. Other animal studies found no significant differences in hair cortisol level between proximal and distal sections of hair [9, 15, 32]. Although some studies in humans also did not find a time-dependent change in hair cortisol level as in animal studies, other studies found a continuous decrease in hair cortisol in older hair segments [21, 27, 33]. However, in general, a significant decrease in hair cortisol concentration is most obvious beyond 6cm distal from scalp [49]. Chimpanzee hairs examined were not as long as those in humans, which might be one reason that there were no systematic change in hair cortisol according to the distance from the scalp.Sex, age, and hair length did not significantly affect cortisol concentration. However, different results may have been

obtained if we had included samples from younger individuals. A previous study found differences in hair cortisol concentrations among different age groups of vervet monkeys [31]. Future research should analyze hair samples from more individuals across a wider range of age groups.

We found that cortisol levels tended to be higher in the KS group, which is not surprising considering the fact that these individuals lived in an all-male group, and the social composition of the group changed on a daily basis. However, this does not necessarily mean that the welfare of the chimpanzees at the KS was worse. Indeed, all-male groups can be an alternative way of supplying social stimulation when it is difficult to keep several males in a mixed-sex group. In addition, wild chimpanzee societies are characterized by patterns of fission and fusion according to which group companions change. For this reason, the manipulation of group membership may be a good way to provide chimpanzees with a more stimulating life in a captive environment. Nevertheless, effects of these husbandry procedures on stress in chimpanzees have rarely been documented and it was not clear whether the challenges posed to the chimpanzees by these social management schemes were at an appropriate level [11, 37, 42]. Toon's cortisol concentration in 2009 was much higher than that of other individuals, but it decreased to a more typical level in 2011. This large change over time suggests that long-term analysis of hair cortisol and comparisons among multiple individuals from different social environments are necessary to understand the effects of all-male group and social variability. From this perspective, hair cortisol has potentially greater value than other methods that have reported stability over several years [8, 56].

Assessment of long-term stress is important from the view of animal welfare, because long-term stress can have negative effects on welfare including behavioral

changes, suppression of reproduction and increase of the likelihood of becoming sick [11, 13, 53]. The link between long-term stress and those welfare parameters has rarely been investigated in chimpanzees. However, there are some problems in captive chimpanzee care which can be related to long-term stress. For example, one of the leading causes of death in captive great apes is heart disease, of which the actual cause is not well understood [51, 55]. In humans, it is said that both short-term and long-term stress can increase the risk of developing heart disease [16]. Furthermore, maternal rejection and inappropriate care of infants are major problems in captive chimpanzees, and studies have shown that stress might impair maternal behaviors [6, 50]. Therefore, investigating long-term stress might shed light on the unknown causes of these important issues.

In conclusion, hair cortisol measurement is a promising tool for estimating long-term stress in chimpanzees. Hair collection should be made from the arm or side while avoiding white hair as much as possible. In combination with behavioral measures, this approach can contribute to understanding the long-term effects of husbandry procedures on the welfare of chimpanzees.

6. Acknowledgement

This study was financially supported by grants from MEXT Japan (#20002001 and #24000001) to Tetsuro Matsuzawa. It was also supported by grants from the Japan Society for Promotion of Science (#10J05294 to YY, #19700245 and #23700313 to MH) and by the Grants to Excellent Graduate Schools program of MEXT.

We are very grateful to the following people who supported our study: Tetsuro Matsuzawa, Masaki Tomonaga, Ikuma Adachi, Satoshi Hirata, Shohei Watanabe,

513 KiyonoriKumazaki, Norihiko Maeda and the members of the Language and Intelligence
514 Section and the Center for Human Evolution Model Research of the PRI. We thank
515 Tomoko Takashima and Etsuko Ichino for their help in assessing basic hair information.
516 We thank Chris Martin, Masayuki Tanaka, Michael Seres and Elizabeth Nakajima for
517 checking an earlier draft of this manuscript.

References

[1] D.H. Abbott, E.B. Keverne, F.B. Bercovitch, C.A. Shively, S.P. Medoza, W. Saltzman, et al., Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. Hormones and Behavior. 43 (2003) 67-82.

[2] P.A. Accorsi, E. Carloni, P. Valsecchi, R. Viggiani, M. Garnberoni, C. Tarnanini, et al., Cortisol determination in hair and faeces from domestic cats and dogs. General and Comparative Endocrinology. 155 (2008) 398-402.

[3] S.F. Anestis, R.G. Bribiescas, Rapid changes in chimpanzee (*Pan troglodytes*) urinary cortisol excretion. Hormones and Behavior. 45 (2004) 209-213.

[4] S.F. Anestis, R.G. Bribiescas, D.L. Hasselschwert, Age, rank, and personality effects on the cortisol sedation stress response in young chimpanzees. Physiology & Behavior. 89 (2006) 287-294.

[5] N.I. Bahr, R. Palme, U. Mohle, J.K. Hodges, M. Heistermann, Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. General and Comparative Endocrinology. 117 (2000) 427-438.

[6] N.I. Bahr, C.R. Pryce, M. Döbeli, R.D. Martin, Evidence from urinary cortisol that maternal behavior is related to stress in gorillas1. Physiology & Behavior. 64 (1998) 429-437.

[7] G.M. Barrett, K. Shimizu, M. Bardi, S. Asaba, A. Mori, Endocrine correlates of rank, reproduction, and female-directed aggression in male Japanese macaques (*Macaca fuscata*). Hormones and Behavior. 42 (2002) 85-96.

[8] T.Ø. Bechshøft, F.F. Rigét, C. Sonne, R.J. Letcher, D.C.G. Muir, M.A. Novak, et al., Measuring environmental stress in East Greenland polar bears, 1892–1927 and 1988–2009: What does hair cortisol tell us? Environment International. 45 (2012) 15-21.

[9] A. Bennett, V. Hayssen, Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. Domestic Animal Endocrinology. 39 (2010) 171-180.

[10] M.A. Bloomsmith, K.C. Baker, Social management of captive chimpanzees. In: L. Brent, (Ed.), The care and management of captive chimpanzees, American Society of Primatologists, San Antonio, 2001, pp. 205-242.

[11] D.M. Broom, K.G. Johnson, Stress and animal welfare, Chapman & Hall: London, 1993.

[12] F. Carlsen, European studbook for the chimpanzees. (2009).

553 [13] K. Carlstead, Shepherdson, D., Effects of environmental enrichment on
554 reproduction. Zoo Biology. 13 (1994) 447-458.
555 [14] S.A. Cavigelli, Behavioural patterns associated with faecal cortisol levels in
556 free-ranging female ring-tailed lemurs,Lemur catta. Animal Behaviour. 57
557 (1999) 935-944.
558 [15] M.D. Davenport, S. Tiefenbacher, C.K. Lutz, M.A. Novak, J.S. Meyer, Analysis
559 of endogenous cortisol concentrations in the hair of rhesus macaques. General
560 and Comparative Endocrinology. 147 (2006) 255-261.
561 [16] J.E. Dimsdale, Psychological stress and cardiovascular disease. Journal of the
562 American College of Cardiology. 51 (2008) 1237-1246.
563 [17] M. Emery Thompson, M.N. Muller, S.M. Kahlenberg, R.W. Wrangham,
564 Dynamics of social and energetic stress in wild female chimpanzees. Hormones
565 and Behavior. 58 (2010) 440-449.
566 [18] L.A. Fairbanks, M.J. Jorgensen, J.N. Bailey, S.E. Breidenthal, R. Grzywa, M.L.
567 Laudenslager, Heritability and genetic correlation of hair cortisol in vervet
568 monkeys in low and higher stress environments. Psychoneuroendocrinology. 36
569 (2011) 1201-1208.
570 [19] D. Fraser, Understanding animal welfare: The science in its cultural context,
571 Wiley Blackwell: Chichester, 2008.
572 [20] J. Fritz, S. Howell, Captive chimpanzee social group formation. In: L. Brent,
573 (Ed.), The care and management of captive chimpanzees, American Society of
574 Primatologists, San Antonio, 2001, pp. 173-204.
575 [21] W. Gao, Q. Xie, J. Jin, T. Qiao, H. Wang, L. Chen, et al., HPLC-FLU detection
576 of cortisol distribution in human hair. Clinical Biochemistry. 43 (2010) 677-682.
577 [22] R. Gow, S. Thomson, M. Rieder, S. Van Uum, G. Koren, An assessment of
578 cortisol analysis in hair and its clinical applications. Forensic Science
579 International. 196 (2010) 32-37.
580 [23] Great Ape Information Network. http://www.shigen.nig.ac.jp/gain/top.jsp.
581 [24] M.R. Heintz, R.M. Santymire, L.A. Parr, E.V. Lonsdorf, Validation of a cortisol
582 enzyme immunoassay and characterization of salivary cortisol circadian rhythm
583 in chimpanzees (*Pan troglodytes*). American Journal of Primatology (2011)
584 903-908.
585 [25] N. Ito, T. Ito, A. Kromminga, A. Bettermann, M. Takigawa, F. Kees, et al.,
586 Human hair follicles display a functional equivalent of the
587 hypothalamic-pituitary-adrenal (HPA) axis and synthesize cortisol. FASEB J.
588 (2005) 04-1968fje.

589 [26] M.Z. Khan, J. Altmann, S.S. Isani, J. Yu, A matter of time: evaluating the
590 storage of fecal samples for steroid analysis. General and Comparative
591 Endocrinology. 128 (2002) 57-64.
592 [27] C. Kirschbaum, A. Tietze, N. Skoluda, L. Dettenborn, Hair as a retrospective
593 calendar of cortisol production—Increased cortisol incorporation into hair in the
594 third trimester of pregnancy. Psychoneuroendocrinology. 34 (2009) 32-37.
595 [28] L. Koren, E. Geffen, Androgens and social status in female rock hyraxes.
596 Animal Behaviour. 77 (2009) 233-238.
597 [29] Kumamoto Sanctuary. http://www.wrc.kyoto-u.ac.jp/kumasan/indexE.html.
598 [30] N. Kutsukake, K. Ikeda, S. Honma, M. Teramoto, Y. Mori, I. Hayasaka, et al.,
599 Validation of salivary cortisol and testosterone assays in chimpanzees by liquid
600 chromatography-tandem mass spectrometry. American Journal of Primatology.
601 71 (2009) 696-706.
602 [31] M.L. Laudenslager, M.J. Jorgensen, L.A. Fairbanks, Developmental patterns of
603 hair cortisol in male and female nonhuman primates: Lower hair cortisol levels
604 in vervet males emerge at puberty. Psychoneuroendocrinology (2012).
605 [32] B.J. Macbeth, M.R.L. Cattet, G.B. Stenhouse, M.L. Gibeau, D.M. Janz, Hair
606 cortisol concentration as a noninvasive measure of long-term stress in
607 free-ranging grizzly bears (*Ursus arctos*): considerations with implications for
608 other wildlife. Canadian Journal of Zoology-Revue Canadienne De Zoologie. 88
609 (2010) 935-949.
610 [33] L. Manenschijn, J.W. Koper, S.W.J. Lamberts, E.F.C. van Rossum, Evaluation
611 of a method to measure long term cortisol levels. Steroids. 76 (2011) 1032-1036.
612 [34] T. Matsuzawa, The Ai project: historical and ecological contexts. Animal
613 Cognition. 6 (2003) 199-211.
614 [35] T. Matsuzawa, Sociocognitive development in chimpanzees: a synthesis of
615 laboratory work and fieldwork. In: T. Matsuzawa, M. Tomonaga, M. Tanaka,
616 (Eds.), Cognitive development in chimpanzees., Springer, Tokyo, 2006, pp.
617 3-33.
618 [36] T. Matsuzawa, M. Tomonaga, M. Tanaka, Cognitive development in
619 chimpanzees, Springer: Tokyo, 2006.
620 [37] C.L. Meehan, J.A. Mench, The challenge of challenge: Can problem solving
621 opportunities enhance animal welfare? Applied Animal Behaviour Science. 102
622 (2007) 246-261.

623 [38] N. Morimura, G. Idani, T. Matsuzawa, The first chimpanzee sanctuary in Japan:
624 an attempt to care for the "surplus" of biomedical research. American Journal of
625 Primatology. 73 (2010) 226-232.
626 [39] N. Morimura, Y. Mori, Effects of early rearing conditions on problem-solving
627 skill in captive male chimpanzees (*Pan troglodytes*). American Journal of
628 Primatology. 72 (2010) 626-633.
629 [40] M.N. Muller, R.W. Wrangham, Dominance, cortisol and stress in wild
630 chimpanzees (*Pan troglodytes schweinfurthii*). Behavioral Ecology and
631 Sociobiology. 55 (2004) 332-340.
632 [41] T. Ochiai, T. Matsuzawa, Planting trees in an outdoor compound of
633 chimpanzees for an enriched environment., Third International Conference on
634 Environmental Enrichment., Orlando, 1997, pp. 355-364.
635 [42] T. Ogura, S. Yamazaki, Y. Yamanashi, S. Mitsuya, Future prospects of animal
636 welfare research. Japanese Journal of Animal Psychology. 61 (2011) 115-124.
637 [43] Primate Research Institute. http://langint.pri.kyoto-u.ac.jp/ai/.
638 [44] Project ChimpCARE. http://www.chimpcare.org/.
639 [45] J.D.E. Pruetz, W.C. McGrew, What does a chimpanzee need? Using natural
640 behavior to guide the care and management of captive populations. In: L. Brent,
641 (Ed.), Care and Management of Captive Chimpanzees., American Society of
642 Primatologists, San Antonio, 2001, pp. 16-37.
643 [46] R Development Core Team, R: A language and environment for statistical
644 computing. R Foundation for Statistical Computing, Vienna, 2011.
645 [47] D.M. Reeder, K.M. Kramer, Stress in free-ranging mammals: integrating
646 physiology, ecology, and natural history. Journal of Mammalogy. 86 (2005)
647 225-235.
648 [48] M. Reimers, F. Schwarzenberger, S. Preuschoft, Rehabilitation of research
649 chimpanzees: Stress and coping after long-term isolation. Hormones and
650 Behavior. 51 (2007) 428-435.
651 [49] E. Russell, G. Koren, M. Rieder, S. Van Uum, Hair cortisol as a biological
652 marker of chronic stress: Current status, future directions and unanswered
653 questions. Psychoneuroendocrinology. 37 (2012) 589-601.
654 [50] W. Saltzman, D.H. Abbott, Effects of elevated circulating cortisol
655 concentrations on maternal behavior in common marmoset monkeys (Callithrix
656 jacchus). Psychoneuroendocrinology. 34 (2009) 1222-1234.

[51] B.M. Seiler, E.J. Dick Jr, R. Guardado-Mendoza, J.L. VandeBerg, J.T. Williams, J.N. Mubiru, et al., Spontaneous heart disease in the adult chimpanzee (Pan troglodytes). Journal of Medical Primatology. 38 (2009) 51-58.

[52] C.F. Sharpley, K.G. Kauter, J.R. McFarlane, An initial exploration of in vivo hair cortisol responses to a brief pain stressor: latency, localization and independence effects. Physiological Research. 58 (2009) 757-761.

[53] J.E. Squires, Applied animal endocrinology: 2nd edtion, CAB International: Oxfordshire, 2010.

[54] J. Suzuki, S. Ohkura, K. Terao, Baseline and stress levels of cortisol in conscious and unrestrained Japanese macaques (*Macaca fuscata*). Journal of Medical Primatology. 31 (2002) 340-344.

[55] N. Varki, D. Anderson, J.G. Herndon, T. Pham, C.J. Gregg, M. Cheriyan, et al., Heart disease is common in humans and chimpanzees, but is caused by different pathological processes. Evolutionary Applications. 2 (2009) 101-112.

[56] E. Webb, S. Thomson, A. Nelson, C. White, G. Koren, M. Rieder, et al., Assessing individual systemic stress through cortisol analysis of archaeological hair. Journal of Archaeological Science. 37 (2010) 807-812.

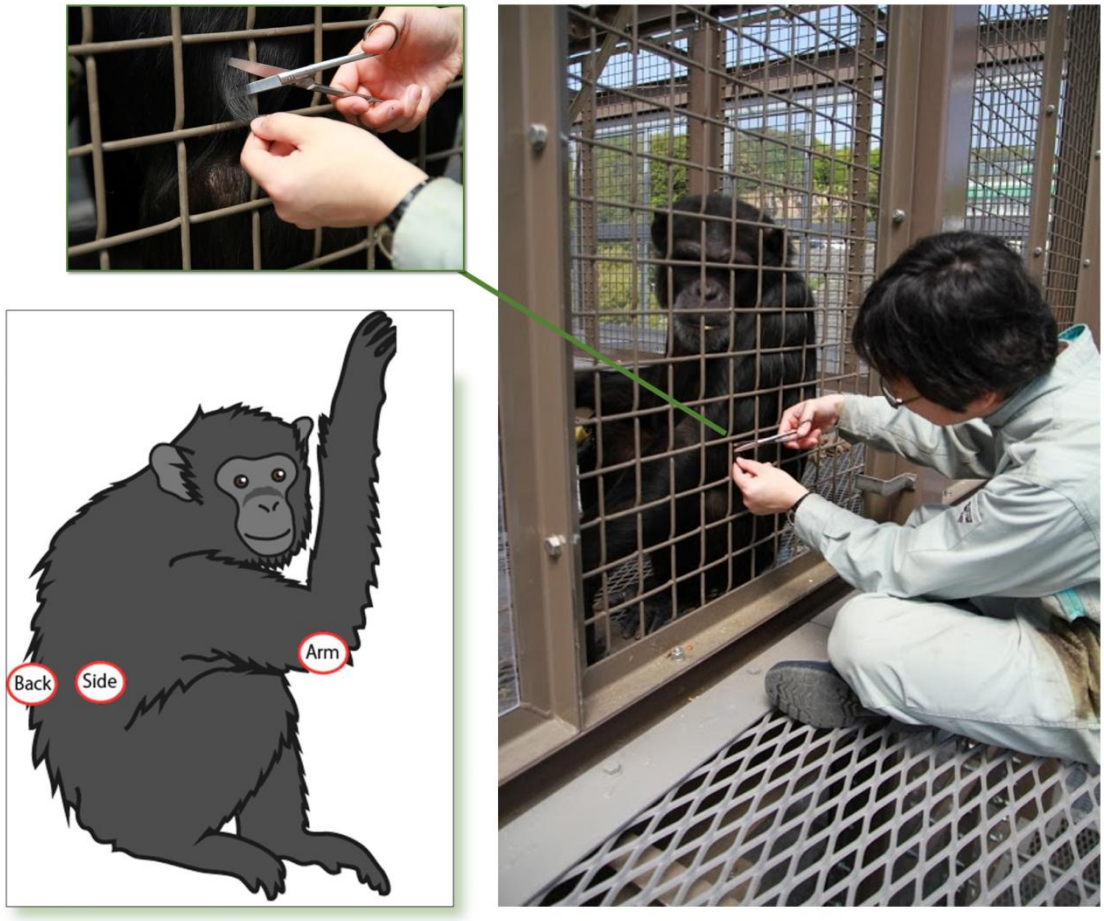
[57] P.L. Whitten, R. Stavisky, F. Aureli, E. Russell, Response of fecal cortisol to stress in captive chimpanzees (*Pan troglodytes*). American Journal of Primatology. 44 (1998) 57-69.

[58] J. Yamada, B. Stevens, N. de Silva, S. Gibbins, J. Beyene, A. Taddio, et al., Hair cortisol as a potential biologic marker of chronic stress in hospitalized neonates. Neonatology. 92 (2007) 42-49.

[59] S. Yamazaki, J. Suzuki, H. Kurotori, S. Takeda, Y. Ueno, Behavioural and physiological impacts on Borneo Orangutans in response to introduction to a new exhibit as structural enrichment. Proceedings of the Nineth International Conference on Environmental Enrichment (2009) 132.

[60] T. Yoshii, K. Tamura, I. Ishiyama, Presence in a PCR-inhibitor in hairs. Japanese journal of legal medicine. 46 (1992) 313-316.

688 **Fig 1. Illustration of sample collection and the parts of the body from which hair**
689 **samples were collected**



690

691

Fig2. Parallelism for Experiment 1 and 2

Parallelism between cortisol standards and serially diluted samples.

Fig 2-a. The results of parallelism test of fecal cortisol and cortisol standards for Experiment 1.

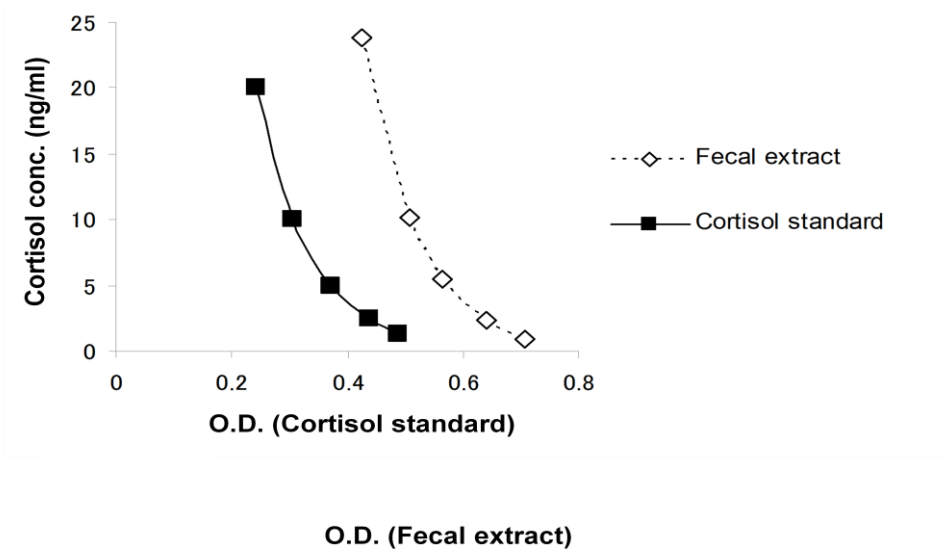


Fig 2-b. The results of parallelism test of hair cortisol and cortisol standards for Experiment 1.

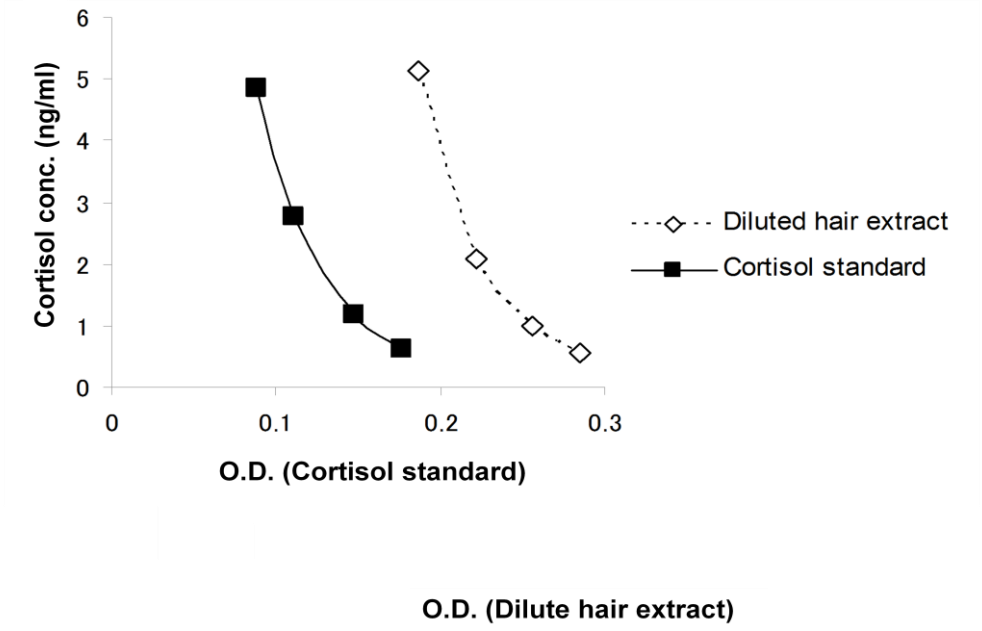


Fig 3. Correlation between hair cortisol concentration and other measures

Fig 3-a. Correlation between fecal and hair cortisol concentration

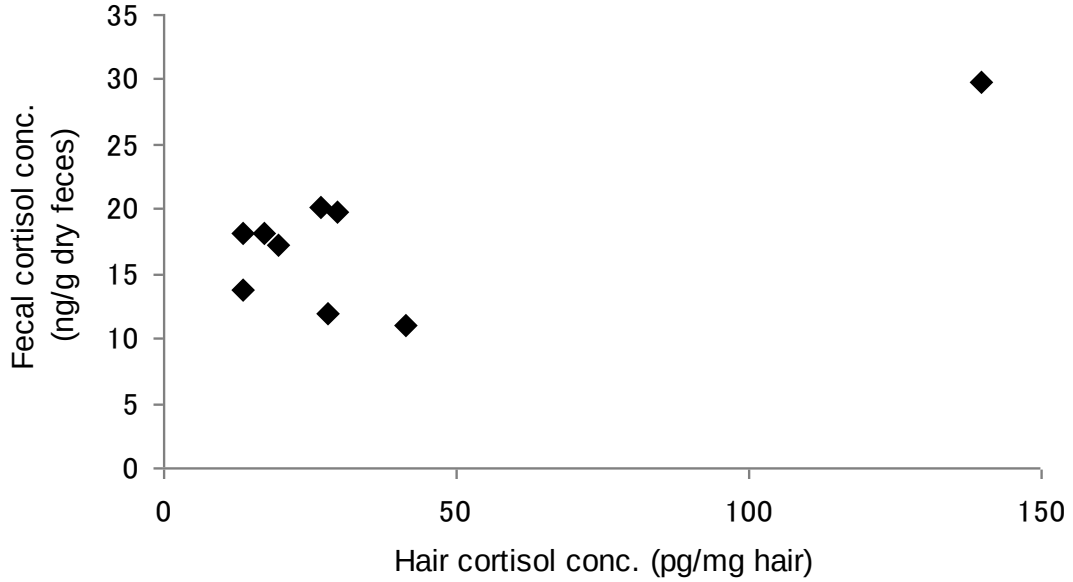
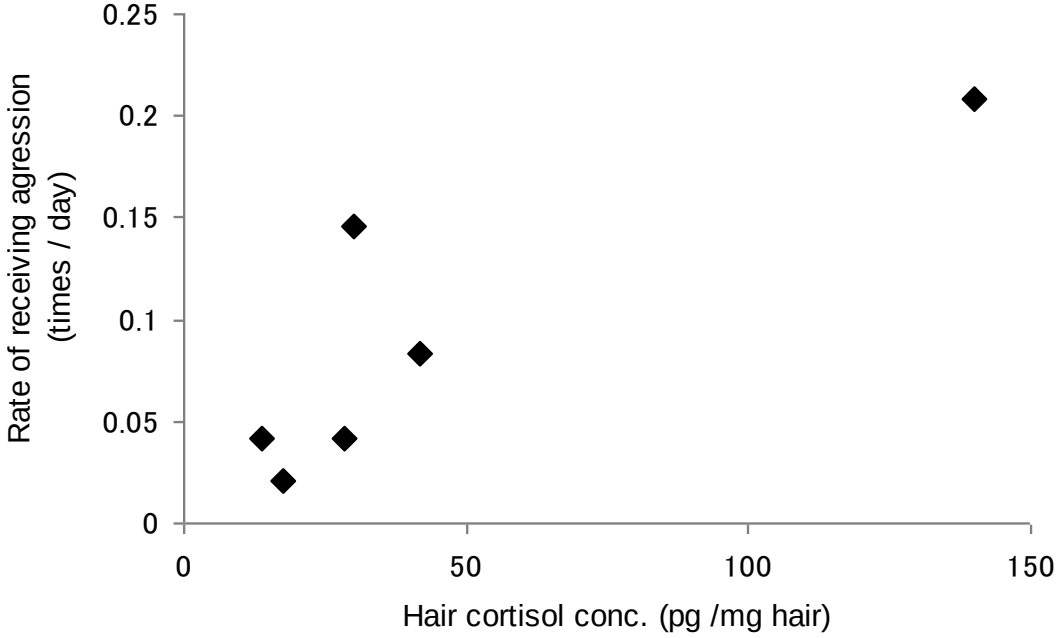
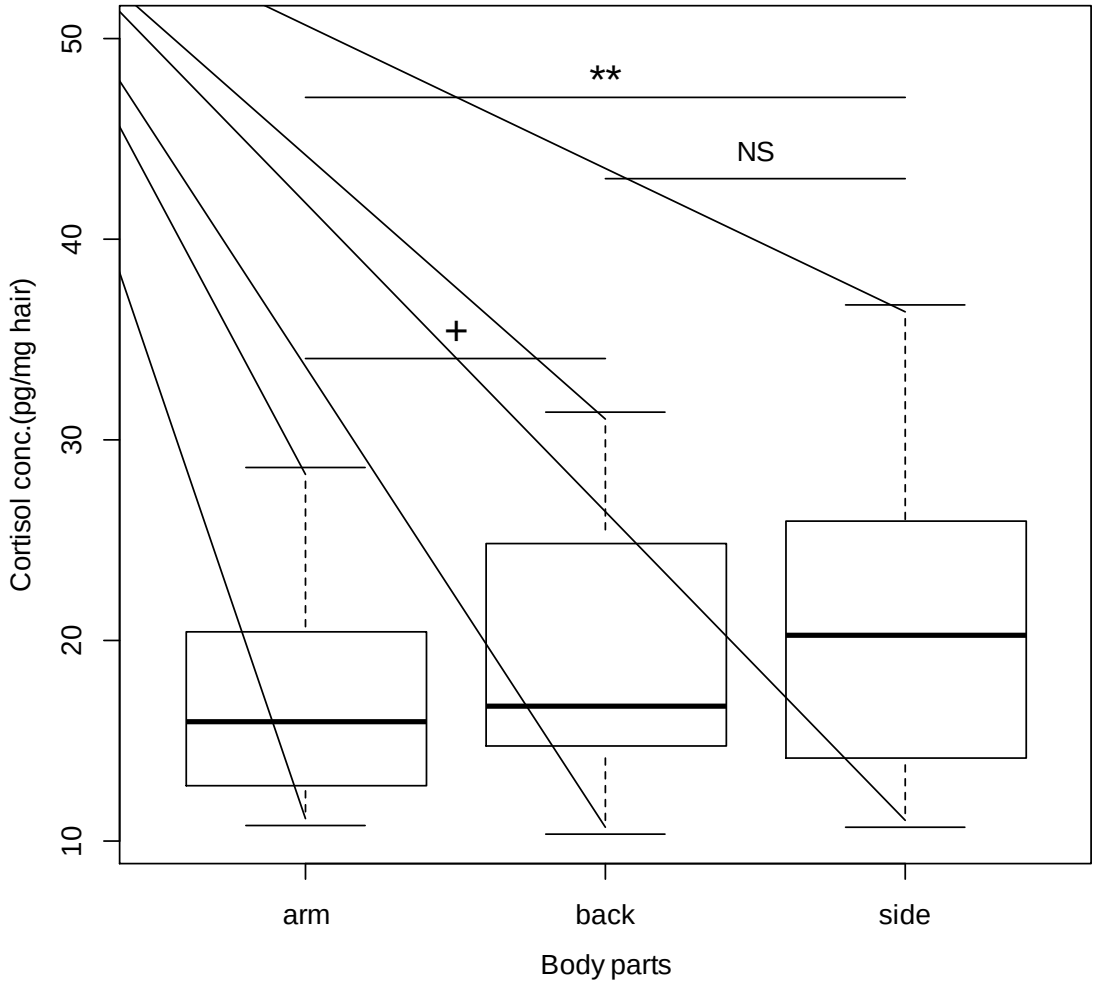


Fig 3-b. Correlation between hair cortisol concentration and the rate of receiving aggression



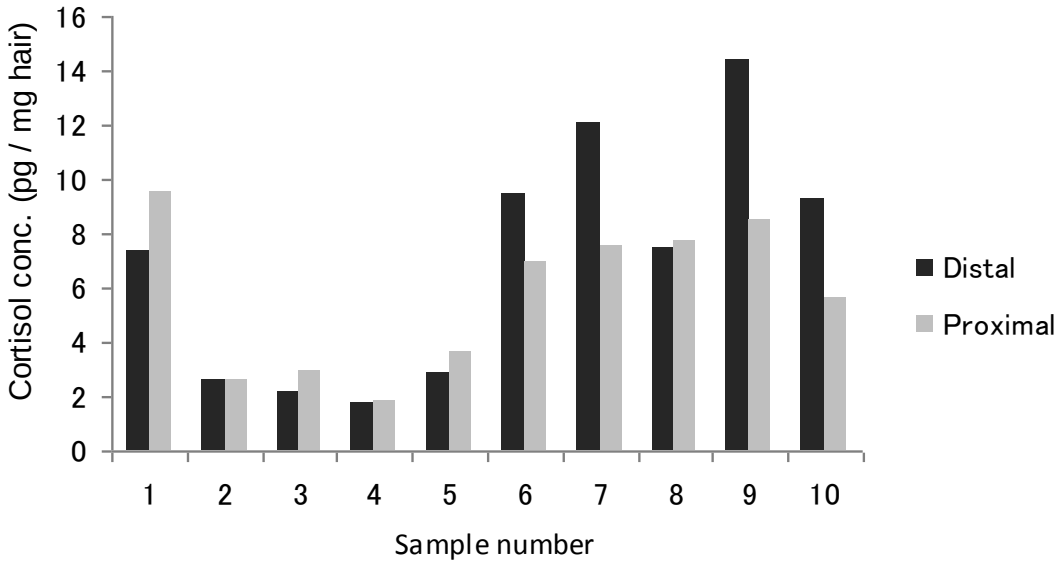
708 **Fig 4. Cortisol concentration in three parts of body**



709

710

711 **Fig 5. Proximal-distal comparison of cortisol concentration**



712