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1 Social relationship and hair cortisol level in captive male chimpanzees (*Pan troglodytes*)

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Abstract

Understanding how social relationships affect long-term stress is important because stress has a profound impact on the welfare of animals and social relationships often exert a strong influence on their stress responses. The purpose of this study was to investigate the relationship between social behaviors and long-term stress levels as assessed by hair cortisol (HC) concentration. The subjects were 11 chimpanzees living in an all-male group (divided into two sub-groups) in Kumamoto Sanctuary, Kyoto University, Japan. Behavioral data were collected between December 2014 and March 2015. The total observation time was 129 h. Hair samples were collected in late March and early April 2015, and cortisol was extracted from the hair and measured with enzyme immunoassay. The hair growth rate was estimated to be 1.33 ± 0.06 cm/month. The results revealed that there was a positive correlation between the rate of receiving aggression and HC levels. We also found a significant negative correlation between the balance between giving and receiving grooming (grooming balance index: GBI), which was calculated by subtracting the rate with which grooming is given from that with which it is received, and the rate of receiving aggression and between the GBI and HC levels. Thus, individuals receiving higher levels of aggression also tended to give grooming for relatively long periods compared to the time they were being groomed. In contrast, the rate of initiating aggression did not have a relationship with either HC levels or any measure of social grooming. We did not find social buffering effects, as there was no correlation between mutual social grooming and HC levels. These results show that not only aggressive interactions but also overall social situations in which animals do not have balanced relationships with others might result in the long-term elevation of cortisol levels in captive male chimpanzees.

44 **Keywords**

45 Social behavior; Hair cortisol; Chimpanzee; Long-term stress; Animal Welfare

46

47 Introduction

48 Social relationships can affect the physical and psychological health of animals
49 including rodents, non-human primates and humans (For review: Cohen 2004;
50 Hennessy et al. 2009). Many studies have suggested that social status and social bonds
51 can affect health, longevity, and stress responses of animals such as baboons, marmoset,
52 squirrel monkeys, rhesus monkeys and chimpanzees (Abbott et al. 2003; Sapolsky
53 2005; Silk et al. 2010; Wittig et al. 2016). Understanding how social relationships affect
54 long-term stress is important, as long-term stress profoundly affects the health and
55 behavior of animals (Broom and Johnson 1993) and social relationships often exert a
56 strong influence on their stress responses. However, the link between long-term stress
57 and social relationships is not well understood in primates, including our closest relative
58 the chimpanzee, partly due to the difficulty in estimating long-term stress efficiently.

59 Hair cortisol (HC) has been shown to be a useful measure of long-term
60 hypothalamic–pituitary–adrenal (HPA) axis activation in several species recently
61 (Davenport et al. 2006; Mastromonaco et al. 2014; Russell et al. 2012; Terwissen et al.
62 2013). The relationship between social relationships and HC in non-human primates has
63 recently been investigated using the novel technique for estimating long-term stress
64 (Tennenhouse et al. 2017; Wooddell et al. 2017). Our studies were the first to apply this
65 methodology to explore this relationship in captive chimpanzees, and suggested that
66 there is a link between aggressive behavior and HC levels in this primate (Yamanashi et
67 al. 2013; Yamanashi et al. 2016a). We found that the rate of receiving aggression
68 positively correlated with HC levels, while that of initiating aggression negatively
69 correlated with HC levels in male chimpanzees. Although a previous study reported that
70 single aggressive interactions in both directions (initiating or receiving) can increase
71 urinary glucocorticoid levels (Wittig et al. 2015), our study showed different effects of

initiating and receiving aggressive interactions on HC levels in captive chimpanzees (Yamanashi et al. 2016a). Compared with urinary glucocorticoid levels (Bahr et al. 2000), HC levels can reflect long-term accumulation of cortisol over several months (Davenport et al. 2006; Russell et al. 2012). As individuals undergo various experiences daily in addition to aggression, one possible explanation for the different results is that not only aggressive interactions but also other aspects of social life, such as how individuals form affiliative relationships with others, can simultaneously affect long-term accumulation of stress. A previous study reported the stress-reducing effects of grooming with bonding partners (Wittig et al. 2016). Another study suggested that factors such as availability of social supports possibly influence the relationship between social status and measures of stress physiology (Abbott et al. 2003). Our previous study (Yamanashi et al. 2016a) included only aggressive interactions as a measure of social relationships and did not take relationships assessed by affiliative social behaviors into account. Thus, it is unknown whether other social behavior is also related to HC levels, and detailed mechanisms underlying the association between social relationship and long-term stress levels remain unknown. The quality of social relationships might differ between individuals receiving higher levels of aggression and those who often initiate aggressive interactions, when it is assessed by affiliative social behaviors such as social grooming and social play.

Understanding the above-mentioned points is important to improve the quality of care for captive chimpanzees. Wild chimpanzees form a multi-male and multi-female society, which is characterized as a fission–fusion society, and male chimpanzees form strong social bonds (Goodall 1986). Therefore, in animals kept in a captive environment, social groups that are comparable to their wild counterparts should be created, providing chimpanzees an opportunity to develop and exert their

social skills (AZA Ape Taxon Advisory Group 2010; Ross et al. 2009). Nevertheless, because males are aggressive in nature, keeping several adult males in a captive environment with females is difficult as it often results in escalated aggression. As a result, the formation of social groups including only one or two males and multiple females is more frequent. Unfortunately, because of this practice of group formation, surplus males often emerge and in the worst case, these surplus individuals can become socially isolated. Providing a social environment for all captive chimpanzees and methodologies by which this can be achieved are among the most important topics related to the welfare of chimpanzees. One approach to providing a social environment to surplus male chimpanzees is the formation of all-male groups, although in wild chimpanzees, such groups do not exist, unlike the case for wild gorillas, which sometimes form groups of this kind (Fritz and Howell 2001; Yamanashi et al. 2016a). However, the types of social relationships that males in all-male groups form and how this affects long-term stress responses in captive environments with limited opportunities for fission are not well understood. Investigating such points is useful for creating guidelines for the formation and management of social groups including multiple male chimpanzees and for discussing optimal strategies for the organization of social groups for captive chimpanzees.

The purpose of this study was to investigate the relationship between social behavior and long-term stress levels assessed by HC concentration. We tested the following three hypotheses. Our hypothesis is that individuals who receive higher levels of aggression need to invest more of their time in forming stable relationships and avoiding future aggression on a daily basis, which results in an increase in stress over a long period of time (the investment hypothesis). In contrast, individuals who often initiate aggression would not need to engage in such activities. We predicted that there

122 would be an association between aggression and giving grooming and thus also an
123 association between HC levels and the rate of giving grooming. We also hypothesized
124 that individuals who had good relationships with others would show lower levels of HC
125 based on social buffering theory (Kikusui et al. 2006). We predicted that the rate of
126 mutual grooming could have a negative relationship with HC levels and that the rate of
127 social play would not show any relationship with HC levels. This is because our
128 previous study (Yamanashi et al. 2017) showed that the rate of mutual grooming
129 implied an affiliative relationship with others, but social play did not imply such a
130 relationship in the same population of chimpanzees. Finally, we hypothesized that a
131 balance between giving and receiving grooming is also important (grooming balance
132 hypothesis). Costs accrue when individuals groom others, while they gain benefits from
133 receiving grooming (Machanda et al. 2014). If individuals invest time in giving
134 grooming, but also receive grooming, costs might be balanced to some extent by the
135 benefits gained from receiving grooming. Therefore, rather than only considering the
136 rate with which social grooming is given, the balance between giving and receiving
137 grooming can be more important. We used the grooming balance index (GBI) to test this
138 hypothesis. The GBI is simply the subtraction of the rate with which grooming is given
139 from that with which it is received. This value increases if an individual receives more
140 unilateral social grooming than what is given. When assessing the extent of dyad-level
141 grooming equality, previous studies have often utilized the grooming equality index
142 (GEI), which also includes differences in the length of time grooming is given and
143 received but that is adjusted by the total time of grooming given and received (e.g.
144 Mitani 2009; Silk et al. 2013). This index might also be useful for checking the
145 grooming balance at the individual level, but a problem arises when considering the
146 situation in captive chimpanzees. It is well known that like other primates, captive

chimpanzees often show social impairment due to the environment in which their early rearing took place. The level of engagement in social grooming decreases if they had less experience with conspecifics as infants (Freeman and Ross 2014). The GEI is between 0 and 1, and information on the absolute amount of time individuals invest in grooming is eliminated. As a result, the value can be similar between individuals who are social and invest much time in giving grooming but who do not receive grooming and those who are less social and devote less time to giving grooming and who do not receive grooming. Therefore, we used the GBI, which is similar but differs in the value not being adjusted by the total time of grooming given and received. We collected equal amounts of data from each individual, so possible biases arising from inter-individual differences in observation time could be minimized. We used this simple, but new, index to test the final hypothesis. We predicted that individuals who receive high levels of aggression also receive less grooming while they give more grooming.

Methods

The subjects were 11 chimpanzees living in an all-male group in Kumamoto Sanctuary, Kyoto University, Japan (KS). Males in the all-male groups of chimpanzees were biologically unrelated except for one dyad that had an uncle-nephew relationship. Ages of the individuals were written in (Supplementary1). KS is the sanctuary for chimpanzees and bonobos in Japan (for details of the sanctuary, please see Morimura et al. (2010)). All individuals had access to 14 indoor and six outdoor cages. Each indoor cage was 7 m² in area and 2.6 m in height. The six outdoor cages included four small cages (approximately 23–34 m² in area and approximately 4 m in height) and two large cages (120 m² in area and 12 m in height; 110 m² in area and 8 m in height). All these cages were connected with each other and could be connected or disconnected at any

time. Based on these flexible joint cages, the 11 chimpanzees were divided into two subgroups during the observation. The members of each subgroup were periodically changed to provide social stimulation and prevent escalated aggression, particularly directed toward immigrant individuals (Yamanashi et al. 2016a). Therefore, the number of chimpanzees in each subgroup was between 4 and 7, depending on the day during this study period. The chimpanzees had free access to water at any time, and regular meals (consisting mainly of fruits, vegetables, and monkey pellets) were provided three times per day. Additionally, routine feeding enrichment (e.g., juice feeders, puzzle feeders, browsing opportunities, and foods concealed in boxes or newspapers) were changed daily. Other types of environmental enrichment were also provided. For example, fire hoses, ropes, hammocks, climbing structures, and substrate materials were installed, and natural vegetation was planted to increase the complexity of the physical environment. Spaces were also available for the chimpanzees to escape from rain, strong sunlight, and cold, and they were provided with comfortable bedding materials for day- and night-time sleep. Materials that they could manipulate freely were also provided, such as toys, buoys, and sacks.

This study was approved by the ethics committee of the Wildlife Research Center, Kyoto University (No. WRC-2014KS001A). We have complied with the ethical standards for the treatment of the chimpanzees according to the guidelines of the Primate Society of Japan, which adhered to the legal requirement of Japan.

Data collection

Behavioral data were collected between December 2014 and March 2015. The total observation time was 129 h. All data were collected by YY, using the iOS application “ISBOapp” and a notebook to complement (Ogura 2013). Between 11:00

and 15:00, YY conducted 30 min of focal observations of 11 chimpanzees in a randomly assigned order and recorded behaviors of the chimpanzees in their enclosures (Martin and Bateson 2007). At least 10.5 h (21–23 sessions of focal observation) of focal animal sampling were conducted for each individual, and the observation sessions for each individual were almost evenly distributed between 11:00 and 15:00. During the focal observations, YY recorded social grooming (Fig 1), pant-grunt and social play every 30 s and all aggressive interactions that occurred within the groups (Table 1). YY stopped making focal observations and began recording aggressive interactions when a scream was heard or when there were any other signs of aggression. YY recorded the aggressors and receivers of each aggressive interaction.

Hair cortisol analysis

The methods to quantify HC levels were based on our previous study (Yamanashi et al. 2016a; Yamanashi et al. 2016b). In these studies, we validated HC analysis methods and investigated various analysis methods to obtain stable results. To collect hair samples, EN cut the arm hairs of the 11 chimpanzees with scissors. Although the hair was attempted to cut at the skin surface as much as possible, few mm of hair often remained. Moreover, small variations existed in terms of the location of hair sample collection across the chimpanzees. Our previous study indicated that we can obtain consistent results if hair samples are collected from similar body parts (Yamanashi et al. 2016b). Hair samples were collected in late March and early April 2015 after completing the behavioral data collection. We cut the arm hair once and waited the regrowth of hair to evaluate the hair growth rate in five captive male chimpanzees in the KS. We cut the regrown hairs twice for each individual after approximately one and two months from the first cut. We randomly selected 10 hairs

from each sample and measured the length. The growth rate was adjusted by the number of days following the haircut and we took the individual average from data obtained after one and two months. The average hair growth rate was 1.33 ± 0.06 cm/month (mean $\pm$ SD). Our previous study showed that the average hair length was 5.86 ± 1.83 cm (mean $\pm$ SD) and no effect of degradation along the hair shaft (Yamanashi et al. 2013). Therefore, we determined that cortisol was accumulated for previous 4-5 months in the full hair length and that the period of behavioral data collection roughly corresponded to the hair sampling period. Due to the slight individual variations in the hair growth rate and hair length, we used full hair length for analysis rather than using identical hair lengths across the animals. Additional details of the HC analysis methods are described in our previous study (Yamanashi et al. 2016b). Cortisol concentrations were measured with a salivary cortisol enzyme immunoassay (EIA) kit (Salimetrics, Philadelphia, PA, USA). The sensitivity of the kit was < 0.03 ng/ml, and the coefficient of variation within a plate was 4.08%.

Data analysis

We used the average rate of each social behavior to investigate the relationship with HC levels. We also used the difference between grooming given and received as a measure of the level of investment toward others (Grooming Balance Index, GBI: rate of grooming received minus rate of grooming given). Although we tried to quantify the ranks of the individuals by the direction of pant grunting (Nishida et al. 2010; Noe et al. 1980), we could identify only the two highest- and two lowest-ranking individuals. The group members directed pant grunts toward the alpha male, and the lowest-ranking individuals directed pant grunts toward several individuals. However, there were many blank relationships and other individuals did not pant-grunt at each other. Thus, we were

unable to quantify the rank of every individual. Nevertheless, aggression was seldom directed at highest-ranking individuals and frequently directed at lowest-ranking individuals (Supplementary 1, 2). Therefore, the rate of being subjected to aggression was closely related to the dominance rank of these chimpanzees.

Statistical analysis

Relationships between aggressive behaviors and affiliative behaviors and between each social behavior and HC levels were tested by Spearman's rank correlation test. All statistical testing was conducted with R. 3.3.3 (R Development Core Team 2017). We used the package "coin" to calculate p -values for Spearman's rank correlation test (Hothorn et al. 2008). The level of significance was set at $\alpha = 0.05$.

Results

Association between aggression and other social behaviors

There was a significant negative correlation between the rate of receiving aggression and GBI ($\rho = -0.65$, $Z = -2.04$, $p = 0.0412$). The rates of initiating aggression, mutual grooming, giving and receiving unilateral grooming, and social play were not significantly correlated with the rate of receiving aggression (initiating aggression: $\rho = 0.187$, $Z = 0.591$, $p = 0.555$; giving unilateral grooming: $\rho = 0.436$, $Z = 1.38$, $p = 0.168$; receiving unilateral grooming: $\rho = -0.227$, $Z = -0.719$, $p = 0.472$; mutual grooming: $\rho = -0.182$, $Z = -0.575$, $p = 0.565$; social play: $\rho = 0.178$, $Z = 0.562$, $p = 0.574$).

Correlation between the rate of initiating aggression and social play was marginally significant ($\rho = 0.605$, $Z = 1.91$, $p = 0.0557$). Other behaviors (the rates of mutual grooming, giving and receiving unilateral grooming, and GBI) did not show any

correlation with the rate of initiating aggression (mutual grooming: $\rho = -0.356$, $Z = -1.12$, $p = 0.261$; giving unilateral grooming: $\rho = -0.0456$, $Z = -0.144$, $p = 0.885$; receiving unilateral grooming: $\rho = -0.105$, $Z = -0.331$, $p = 0.740$; GBI: $\rho = -0.0091$, $Z = -0.0288$, $p = 0.977$).

Association between social behaviors and hair cortisol

We found that the rate of receiving aggression was positively correlated with HC levels (Fig 2-a: $\rho = 0.71$, $Z = 2.23$, $p = 0.0255$). We also found a significant negative correlation between HC levels and GBI (Fig 2-c: $\rho = -0.78$, $Z = -2.38$, $p = 0.0175$). Correlation between HC levels and the rate of giving unilateral grooming was marginally significant ($\rho = 0.59$, $Z = 1.86$, $p = 0.0631$). The rates of initiating aggression, receiving unilateral social grooming, mutual grooming, and social play were not significantly correlated (Fig 2-b: initiating aggression: $\rho = -0.22$, $Z = -0.708$, $p = 0.479$; receiving unilateral grooming: $\rho = -0.18$, $Z = -0.56$, $p = 0.574$; Fig 2-d: mutual grooming: $\rho = 0.28$, $Z = 0.89$, $p = 0.372$; Fig 2-e: social play: $\rho = -0.03$, $Z = -0.10$, $p = 0.920$). There was no significant correlation between age and HC levels ($\rho = 0.04$, $Z = 0.13$, $p = 0.896$).

Discussion

The results of our study supported our prediction for the grooming balance hypothesis. We found a positive correlation between the rate of receiving aggression and HC levels, as in the previous studies in male chimpanzees (Yamanashi et al. 2013; Yamanashi et al. 2016a). In this study, we also investigated the relationship between affiliative behaviors and HC levels for the first time. We found a significant negative correlation between GBI and the rate of receiving aggression, and between GBI and HC

levels. In contrast, the rate of initiating aggression did not have a relationship with either HC levels or social grooming. Thus, individuals receiving higher levels of aggression also showed skewed patterns of social grooming, in which they have to invest their time in giving grooming while they do not receive grooming from others so often. Situations like these might cause long-term elevations in cortisol levels in such individuals. Although we investigated the relationship between the rate of giving grooming and HC levels to test the investment hypothesis, we found the trend to be only marginally significant. Therefore, although it is still possible that it is costly to spend much time on giving grooming to maintain relationships with others, the balance is more important. As there was a significant correlation between the rate of receiving aggression and the GBI, overall social situations in which chimpanzees often receive aggression and have to pay a higher cost for maintaining social relationships might result in increased stress levels in such individuals in the long term.

A study in a wild population of chimpanzees showed a positive correlation between dominance and fecal glucocorticoid metabolites (Muller and Wrangham 2004). The finding of our present study in terms of the correlation between aggression and cortisol is opposite, considering the fact that the study of Muller and Wrangham assessed dominance relationships based on aggressive interactions, and the close relationship between dominance rank and the rate of being subjected to aggression in our study chimpanzees. Studies have suggested that the relationship between dominance and stress can differ even in the same species; the possible explanations offered were social stability, physical environment, and controllability of the surrounding environment (Abbott et al. 2003). During the period of the present study, there were no obvious hierarchical changes in the studied group. The results suggested that there was no need for dominant individuals to make efforts to maintain their rank, while

individuals who received high levels of aggression needed to work hard on a daily basis. The nature of the social relationship that dominant individuals form with others can vary depending on the situation and group characteristics. Therefore, to understand the effects of social environment on long-term stress responses, it is necessary to investigate multiple social behaviors and quantify detailed situations of individuals.

Our prediction for the social buffering hypothesis was not supported. We did not find any relationship between the rate of mutual social grooming and HC levels. We also did not find any significant relationship between the rate with which grooming was received and HC levels. Social play also did not show a significant correlation with HC levels. Although there is still a possibility that mutual social grooming or receiving grooming buffer stress for short periods, as suggested by a previous study (Wittig et al. 2016), the results of this study indicated that receiving aggression and grooming balance had a stronger effect when the accumulation of stress over long periods was considered.

Some aspects of this study limit understanding the relationship between social situations and long-term stress levels. For example, we used the GBI to analyze the association between hair cortisol level and grooming balance. The GBI only considers differences in the overall rates with which grooming is given and received. Therefore, we could not explain how the social relationship with every other individual contributed to the stress responses of these chimpanzees. It might be sufficient to have a strong social bond with one partner from whom the individual can receive grooming, but having multiple moderate affiliative bonds might be better for having a less stressful life. The small sample size in this study did not allow us to investigate this point as the situation of each chimpanzee varied a lot. In the future, social relationships and stress levels should be investigated in multiple captive social groups.

In conclusion, social relationships affected hair cortisol levels in captive male

chimpanzees living in an all-male group. Not only aggressive interactions but also social situations in which chimpanzees do not have balanced relationships with others with whom they interact on a daily basis might result in long-term elevations in cortisol levels in captive male chimpanzees. Chimpanzees which had to invest their time in giving grooming without being groomed showed higher levels of stress. Aggression and incompatible combinations of individuals are often inevitable in captive chimpanzee groups, particularly when the group size is large. Therefore, group formation should be decided on taking the situation of each chimpanzee into consideration to ensure that each chimpanzee can have balanced relationships with others. In our sanctuary, we were aware of this and rotated the members of the all-male group to prevent escalated aggression, particularly directed toward immigrant individuals and excessive stress on such individuals. They are provided with opportunities to occasionally split up and maintain an appropriate distance from each other. Nevertheless, social situations had a strong influence on the stress level in male chimpanzees. Future studies should investigate solutions to alleviate the stress of such individuals and monitor such high HC individuals to check the effects later in life.

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465

466 Figure Caption

467 Fig 1. Photograph of social grooming among three males.

468 Mutual social grooming (a); Unilateral social grooming (b)

469

470 Fig 2. Relationship between social behaviors and HC concentration (pg/mg hair)

471 Relationship between the rate of receiving aggression and HC levels (a); the rate of

472 initiating aggression and HC levels (b); GBI (difference between grooming received and

473 given; Gr received minus Gr given) and HC levels (c); the rate of mutual social

474 grooming and HC levels (d); and the rate of social play and HC levels (e).

475

476

477

Fig1

(a)

(b)

Fig2

Table 1. Definition of behaviors

Behaviors	Definitions
Aggressive interactions	Behaviors that included chasing, hitting, biting, kicking, and charging displays directed toward group members; individuals who were targets of such behavior showed screaming, escaping, or counterattacking behaviors.
Social play	Playing with conspecifics. The behaviors included play push, play bite, play slap, tickle, play run, grab hands or legs repeatedly and rhythmically, social object play, rough and tumble and play stamping. These behaviors sometimes appeared sequentially. Social play is distinguishable from other social behaviours since all the social play sessions in this study included play face or play pants.
Social grooming	Groom another with hands or mouth. Mutual grooming included the grooming which two chimpanzees groom each other simultaneously. Groom unilaterally included the grooming another without reciprocation.