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Stress affects salivary alpha-Amylase activity in bonobos

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ABSTRACT

Salivary alpha-Amylase (sAA) is a starch digesting enzyme. In addition to its function in the context of nutrition, sAA has also turned out to be useful for monitoring sympathetic nervous system activity. Recent studies on humans have found a relationship between intra-individual changes in sAA activity and physical and psychological stress. In studies on primates and other vertebrates, non-invasive monitoring of short-term stress responses is usually based on measurements of cortisol levels, which are indicative of hypothalamic–pituitary–adrenal activity. The few studies that have used both cortisol levels and sAA activity indicate that these two markers may respond differently and independently to different types of stress such that variation in the degree of the activation of different stress response systems might reflect alternative coping mechanisms or individual traits. Here, we present the first data on intra- and inter-individual variation of sAA activity in captive bonobos and compare the results with information from other ape species and humans. Our results indicate that sAA activity in the bonobo samples was significantly lower than in the human samples but within the range of other great ape species. In addition, sAA activity was significantly higher in samples collected at times when subjects had been exposed to stressors (judged by changes in behavioral patterns and cortisol levels) than in samples collected at other times. Our results indicate that bonobos possess functioning sAA and, as in other species, sAA activity is influenced by autonomic nervous system activity. Monitoring sAA activity could therefore be a useful tool for evaluating stress in bonobos.

1. Introduction

Salivary alpha-Amylase (sAA) is one of the major salivary proteins [1]. This enzyme digests starches by hydrolyzing α-1,4 linkages of starch, which then allows an organism to exploit starch as an energy source. Consequently, variation in starch intake correlates with the number of copies of the salivary amylase gene and amylase concentration in saliva in human populations [2,3].

Saliva products are released by acinar cells after neurotransmitter stimulation [4]. Acinar cells are innervated by sympathetic and parasympathetic branches of the autonomic nervous system [5]. While functioning as a digestive enzyme, sAA has turned out to be useful for detecting autonomic activity (reviewed in [6]). Recent studies in humans have found a relationship between intra-individual changes in sAA and physical [7–9] and psychological stressors [7,9–14].

Traditionally, non-invasive monitoring of short-term stress responses has primarily been performed by measuring salivary cortisol levels, which are indicative of hypothalamic–pituitary–adrenal activity [15]. Within the order Primates, this method has been used in studies on humans [15–21], old and new world monkeys [22–24], and great apes [25–29].

Some studies that measured both cortisol levels and sAA activity found that an increase in cortisol levels was paralleled by an increase in sAA activity as a response to different kinds of stressors [30]. In other studies, no correlation was found between changes in salivary cortisol levels and sAA activity, suggesting that these two stress markers act independently [9,11,30]. Indeed, behavioral interactions inducing the release of plasma norepinephrin may not affect cortisol levels while others are associated with low plasma norepinephrin and high cortisol release (reviewed in [31]). Therefore, the variation in the degree of activation of different stress response systems might reflect alternative coping mechanisms or individual traits (reviewed in [31]).

While some New World primate species do not produce sAA [32], all Old World primates thus far investigated for the production of sAA do, including macaques, baboons [32–35], and hominoidae such as chimpanzees, orangutans, and gorillas [36–39]. However, in comparison to chimpanzees, bonobos, the fourth African great ape, have increased AMY1 copy numbers, but AMY1 in bonobos has a distributed coding sequence. It was therefore suggested that bonobos might not produce sAA [3]. While differences in the expression and activity of sAA in nonhuman primates have been related to the amount of starch in the diet [36], no information is available on the applicability of sAA activity as a marker for stress.

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We therefore carried out a study to investigate sAA activity in captive bonobos. In the first part of the study, we explored whether or not bonobos do in fact secrete sAA and how sAA activity in bonobos compares to that of closely related species, such as chimpanzees, gorillas, orangutans, and humans. In the second part of the study, we explored sex and age related variation in bonobo sAA activity and how bonobo sAA reactivity is influenced by stress. Finally, to investigate whether sAA has the potential to serve as a marker for stress in bonobos, we compared sAA activity and salivary cortisol levels in samples from stressed and unstressed individuals.

2. Methods

2.1. Subjects

Saliva samples from 15 bonobos, nine chimpanzees, seven Sumatran orangutans, ten western lowland gorillas, and ten humans (see Table 1 for more information) were collected. All great apes were kept in social groups at all times; food was offered at least three times a day and consisted mainly of a mixture of fruits and vegetables. As a control for the specificity of the assay used in this study, we collected saliva samples from Gotland sheep, which are known to not produce sAA [40].

All experiments with animals were carried out in accordance with NIH published standards. All human participants provided informed consent at the time of participation.

2.2. Saliva sampling protocol

For the first part of the study, saliva was collected from apes at the Frankfurt Zoo between January 2006 and December 2008, and at the Frankfurt, Leipzig, and Berlin Zoos between August 2010 and February 2011. Samples from human volunteers were taken at the Max Planck Institute for Evolutionary Anthropology in November 2010. For the second part of the study, only saliva samples from bonobos at the Frankfurt and Berlin Zoos were used. All saliva samples and behavioral data used to investigate stress reactivity came from the bonobos at the Frankfurt Zoo.

The apes were trained to chew on cotton rolls that had been soaked in a sugar solution in order to enhance their acceptance by the subjects [10,25,36] and a food reward was given to motivate subjects to return the device to the experimenter. The sugar solution was prepared by dissolving three teaspoons of icing sugar in 1 L of water. Cotton rolls were briefly immersed in the sugar solution and then dried overnight in an oven set to 45 °C. To avoid interferences with food, saliva was only collected at least 3 h after feeding. Samples were never collected after a reward was given. For the human volunteers and Gotland sheep, the same sugar supplemented devices were used. To obtain saliva from the Gotland sheep, the experimenter placed sugar supplemented devices in the sheeps' mouths while they were masticating. To control for possible influences of the sugar solution in cotton rolls, one of the sugared cotton rolls was extracted with deionized water (MilliQ®) and analyzed together with the others. No amylase activity was detected in this control sample.

For humans, sheep, and bonobos in the Frankfurt Zoo, chewing duration was measured. The average chewing duration in bonobos was 1.1 min. (range from 15 s. to 2 min.), which was comparable to the 1 min chewing duration in humans and Gotland sheep. Salivette R (Sarstedt, Nümbrecht, Germany) collection devices were used to obtain fresh saliva. After collection, samples were placed into plastic containers and stored at −20 °C until analysis.

Table 1
Species, location, sex, age range, and number of animals involved in this study.

Species	Location	Sex		Sample number	Age range
		Male	Female		
Human	MPI EVA	5	5	20	27–46
Bonobo	Frankfurt Zoo	4	9	297	2–60
Bonobo	Berlin Zoo	1	1	10	16–30
Western lowland gorilla	Frankfurt Zoo	4	6	69	4–54
Sumatran orangutan	Frankfurt Zoo	3	4	94	6–45
Chimpanzee	Berlin Zoo	2	2	20	22–32
Chimpanzee	Leipzig Zoo	0	7	40	11–35
Gotland sheep	Berlin Tierpark	0	5	Pool sample	1–5

2.3. Sample preparation and cortisol- and alpha-Amylase assay

The frozen cotton rolls containing saliva were thawed and centrifuged (1500 g, 10 min.). Three microliters of the resulting saliva plus 47 μl of assay buffer were used for the cortisol-assay. For alpha-Amylase activity measurement, saliva samples were diluted with sample buffer from the assay kit and 10 μl of the diluted saliva were applied to the assay. Dilution depended on the species and individual levels and ranged from pure to 1:20 for bonobos, 1:50 for gorillas, from 1:20 to 1:50 for orangutans, from pure to 1:10 for chimpanzees, from pure to 1:2 for Gotland sheep, and 1:300 for humans.

To measure sAA activity, we used the "Salivary alpha Amylase Enzymatic Assay" (IBL International, Hamburg, Germany), a commercial enzyme kinetic assay designed to measure alpha-Amylase activity in human saliva. Ten microliters of each pre-diluted standard, control, or sample was pipetted into each well and 200 μl of substrate solution was added per well with an 8-channel micropipetter and incubated at room temperature (18–25 °C) for 3 min. After incubation, one measurement was taken using a photometer with 405 nm and a reference of 650 nm. A second measurement was taken 5 min after incubation. All standards, controls, and samples were measured in duplicate. Intra- and inter-assay coefficients of variation of low and high value quality controls were 2.8% and 2.9% (N = 25) and 7.1% and 5.5% (N = 25), respectively. Average recovery of spiked sAA in bonobo saliva was 83.9% (N = 7, SD = 3.35%), in gorilla saliva 82.36% (N = 3, SD = 3.86%), in orangutan saliva 81.3% (N = 3, SD = 4.22%), and in chimpanzee saliva 75.51% (N = 3, SD 5.24%). Recovery for human saliva was provided by the instruction of the assay as 94.33 (N = 3, SD = 4.17%).

Using the same saliva samples, cortisol was measured with an enzyme immunoassay (EIA) previously described by Palme and Möstl [41]. Briefly, we used BSA cortisol-3-CMO linked to diamino-3.6-dioxaoctane-biotin as a label and cortisol as a standard. Intra- and inter-assay coefficients of variation of high and low value quality controls were 8.6% and 14.5% (N = 37) and 10.4% and 14.1% (N = 93), respectively. We re-assayed measurements if bindings were outside of a 30–70% range or if divergence of duplicates was greater than 10%.

2.4. Diurnal variation in cortisol levels and salivary alpha-Amylase activity

To avoid potentially confounding effects of diurnal variation in sAA activity [43] and cortisol levels [16,21,42–46], all saliva samples used in this study were collected during a narrow time window: between 14:00 h and 16:00 h for humans and between 15:00 h and 16:00 h for apes, respectively.

For the second part of the study in which we explored the impact of stress on sAA in bonobos, we selected saliva samples collected under conditions that were expected to impose stress, including the day when (a) one female gave birth for the first time (*birth*), (b) subjects were transferred from their familiar enclosure to an entirely new building (*transfer*), and (c) a new female was integrated into the resident group (*integration*). Although sampling was constrained by both the cooperation of subjects and logistical conditions (for number of samples and animals for each event, see Table 2), we succeeded in collecting multiple samples after the presumed onset of elevated stress (sampling

Table 2
Number (No.) of bonobos and saliva samples for each condition and location of sampling.

Condition	No. animals	No. samples	Location
Unstressed	15	236	Frankfurt/Berlin
Birth	9	27	Frankfurt
Transfer	11	22	Frankfurt
Integration	11	22	Frankfurt

times: 30, 60, and 180 min. for birth, transfer and integration). In humans, an increase in sAA activity after a single stressful event is observed immediately after the onset of the stressor and increased activity returns to baseline levels after 20 min. [47]. However, our sampling regime for stressful situations in bonobos differed fundamentally in that after the onset of the stressful event, excitement in the group (defined as continuous exaggerated vocalization and motor activity) occurred for several hours, which suggests that sAA activity did not return to baseline levels as fast as in a single stressor event experiment. Drastic increases in scores of motor activity in combination with increases in self grooming and vocalizing are typical behavioral indicators of stress [48,49] and correlate with cortisol levels [45] measured after the onset of the stressful event. Therefore, during the chosen events (transfer, birth and integration), we tested whether scores of motor activity, self grooming, and vocalizing indeed increased and would thus allow us to verify that these situations were indeed perceived as stressful by the animals.

2.5. Behavioral data

Behavioral data from the bonobo group at the Frankfurt Zoo was collected between November 2006 and December 2008. This period included 68 days during which no stressful incidents occurred. Behavioral data collected on these days were used to calculate individual baseline behavioral levels. Group size increased during the study period from 10 animals at the beginning to 13 individuals at the end. Behavioral observations were conducted from 11:00 h to 13:00 h and from 14:00 h to 16:00 h, respectively. Focal animals were chosen randomly and observed for 10 min., allowing for one observation of each group member during each observation window. The behavior of the bonobos was recorded at each minute mark. During periods with stressful events, such as during a birth (August, 3, 2011), an integration (February 14, 2008), and a transfer (August 15, 2008), behavioral data were collected as described above. We used three behavioral categories, daily activity, vocalization, and self grooming, to investigate changes in behavior in relation to stress. Vocalization included all described bonobo vocalizations e.g. hoots, barks, peeps, and screams. Daily activity was calculated by summing up the frequencies of the following behaviors per day: carrying other group members, nest building, fecal smearing, displaying, locomotion, feeding, urinating, playing, vocalizing, sexual behavior, and interacting with humans. The third category, self-grooming behavior, included scratching, self-grooming, and hair plucking. In addition, all occurrences of diarrhea were continuously recorded whenever observed.

3. Statistical analyses

For the first part of the study, in which we aimed to investigate species differences in sAA levels, we used a one-way ANOVA applied to log transformed averages per individual. For subsequent post-hoc comparisons, we used a pairwise Bonferroni-corrected *t*-test.

For the second part of the study, we analyzed sAA and cortisol levels in the samples from the bonobos at the Berlin and Frankfurt Zoos. Data were log transformed to achieve normal distribution and homogeneous residuals (assessed by visual inspection of residuals plotted against fitted values). We used Spearman correlations to correlate age with sAA activity for bonobos from both zoos. Furthermore, we used Spearman correlations to correlate chewing duration and cortisol levels with sAA activity in the Frankfurt Zoo bonobos. We used Mann–Whitney U tests to explore differences in sAA activity between sexes (bonobos from both zoos). These two tests were applied to averages per individual. We used a repeated measures ANOVA to analyze the interaction between sex (between subjects factor), stress marker (within subjects factor; levels: sAA activity and cortisol levels, respectively), and context (within subjects factor; levels: three stressed (integration, birth, and transfer) versus an unstressed condition). We also used repeated measures ANOVA for paired comparisons of stress level measures between the unstressed condition, on the one hand, and the three stressed conditions, on the other. This analysis included only samples from the Frankfurt Zoo bonobos because only these were observed in both stressed and unstressed conditions. To compare the rates of behaviors potentially indicative of stress between stressed and unstressed conditions, we used exact Wilcoxon matched pair tests on means per individual and condition; we did this separately for each behavior. In these, we included only data from the nine bonobos from the Frankfurt Zoo that were observed in both conditions. To analyze the time course data, General Linear Mixed Models (GLMM) [50] were conducted using the function "lmer" of the R-package lme4 [51]. In this, we included time as a fixed effects factor with three levels and subject identity as a random effect. Approximate normality and homogeneity of error variances were assessed by visual inspection of residuals plotted against fitted values. To achieve this, the response variable "amylase" was log transformed, and "cortisol" was square root transformed. Tests of significance were calculated using a Markov Chain Monte Carlo (MCMC) analysis [50]. These were run using the functions "pvals.fnc" and "aovlmer.fnc" of the R package languageR [52]. We ran a total of six such models, one for each combination of stress measure (cortisol and amylase) and stressor (birth, transfer, and integration). To prevent form spurious significances due to multiple testing, we used Fisher's Omnibus test. This procedure combines a number of P-values into a single chi-square distributed variable with degrees of freedom equaling twice the number of P-values [53]. Combining the P-values using Fisher's omnibus test revealed a clearly significant result ($\chi^2 = 25.566.3$, $df = 12$, $p = 0.01235$). However, only one of the individual tests revealed significance [see results]. Sample sizes partly differed between different analyses because some bonobos were born during the study or transferred.

We calculated one-way ANOVAs, exact Wilcoxon and Mann–Whitney U tests with SPSS 14.0 and repeated measures ANOVA with R (R Development Core Team 2009). We calculated Spearman correlations with a software program written by R. Mundry. All non-parametric tests were exact tests, as is required for small sample sizes [54].

4. Results

4.1. Species differences in salivary alpha-Amylase activity

For the first part of the study, we collected 550 saliva samples from humans, sheep, and apes. In all samples from great ape and human subjects, sAA activity was within the sensitivity range of the assay (Fig. 1). As expected, there was no sAA activity in the samples from the Gotland sheep. We found significant differences between the sAA activity of humans and great ape species [$F(4,51) = 80.66$, $p < 0.001$]. Pairwise comparisons of sAA activity (see Table 3) revealed that activity was highest in humans (mean 202.12 ± 109.25 U/mL), followed by gorillas (mean 50.45 ± 49.26 U/mL), orangutans (mean 18.81 ± 32.64 U/mL), bonobos (mean 7.30 ± 5.83 U/mL), and chimpanzees (3.45 ± 2.16 U/mL).

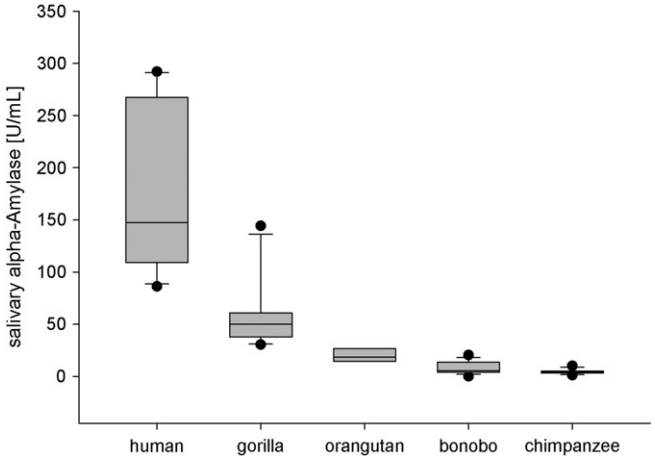

Fig. 1. Boxplot of average salivary alpha-Amylase activity in humans and great apes. The boxes illustrate the 10th and 90th percentiles, bars indicate median and range, and circles indicate outliers. Sample sizes were: humans N = 10, bonobos N = 15, western lowland gorillas N = 10, Sumatran orangutans N = 7, and chimpanzees N = 11.

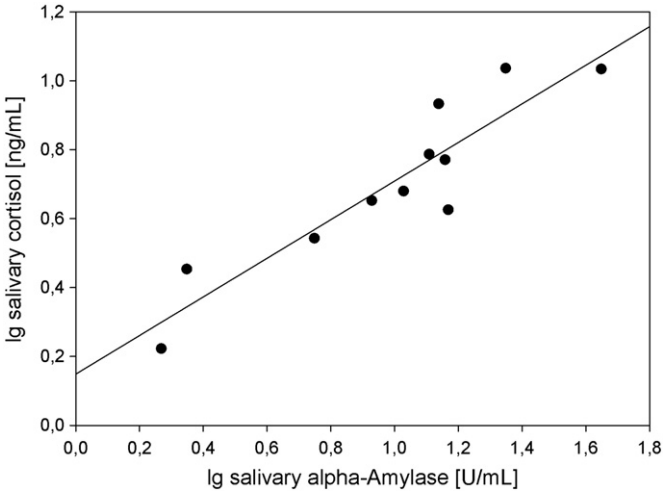

Fig. 2. Correlation of lg salivary alpha-Amylase activity and lg salivary cortisol levels in bonobos in the unstressed condition.

Table 3
Pairwise comparisons (Bonferroni corrected t-tests) of salivary alpha-Amylase activity for all tested species.

Pair-wise	t	p
Human vs. chimpanzee	15.67	<0.001
Human vs. bonobo	13.73	<0.001
Human vs. orangutan	8.07	<0.001
Human vs. gorilla	4.91	<0.001
Gorilla vs. chimpanzee	10.87	<0.001
Gorilla vs. bonobo	8.64	<0.001
Gorilla vs. orangutan	3.67	<0.001
Orangutan vs. chimpanzee	5.99	<0.001
Orangutan vs. bonobo	3.69	<0.001
Bonobo vs. chimpanzee	3.06	0.035

4.2. Effect of sex, age, and chewing duration on salivary alpha-Amylase activity in bonobos

There was considerable variation in basal sAA activity between individuals. sAA activity ranged from 1.83 U/mL to 13.22 U/mL in females and 3.67 U/mL to 19.67 U/mL in males (Table 4). Female bonobos had lower sAA activity (6.44 U/mL (±4.73)) than males (13.42 U/mL (±6.32.)) [Mann–Whitney *U*-test: U = −3; N_{males} = 5, $N_{females}$ = 10, p<0.01]. There war no linear correlation between sAA activity and age [Spearman correlation: females: r_S = 0.31; N = 10; p = 0.34; males: r_S = 0.1; N = 5; p = 0.95]. However, the three adolescents had higher sAA levels than infants and adults (not tested due to small sample size; Table 4). No correlation between sAA activity and chewing duration was found [females r_S = 0.371; N = 9; p = 0.497; males: r_S = 0.278; N = 4; p = 0.333; only the Frankfurt Zoo bonobos were considered].

Table 4
Average salivary alpha-Amylase (sAA) activity in individual female (f) and male (m) bonobos.

Age	Sex	Ave rage sAA [U/mL]	SD.	N.
57	f	12.49	17.91	15
45	f	12.47	9.57	15
29	f	4.28	0.11	15
25	f	10.42	0.26	15
23	f	3.05	2.54	15
13	f	2.84	8.24	15
13	f	1.84	0.16	15
10	f	13.23	4.38	10
4	f	4.02	9.05	10
3	f	4.78	1.43	10
26	m	11.27	6.49	15
16	m	17.15	10.82	15
10	m	15.27	13.50	15
7	m	19.78	18.90	15
4	m	3.67	4.18	10

4.3. Relationship between salivary alpha-Amylase activity and cortisol levels in stressed versus unstressed condition

For the second part of the study, only samples from the Frankfurt Zoo bonobos were used. When subjects were not stressed, there was a significant correlation between individual sAA activity and salivary cortisol concentration means [r_S = 0.827; N = 11; p<0.001; Table 2; Fig. 2]. However, there was no correlation when subjects were stressed [r_S = 0.109; N = 11; p = 0.734]. Salivary cortisol levels and sAA activity differed significantly between the unstressed condition and the three stressed conditions [F(3,24) = 6.26, p<0.003] (Fig. 3). In the full model, none of the interactions revealed significance [sex*marker F(1,8) = 0.41, p = 0.54; sex*event F(3,24) = 1.16, p = 0.34; marker*event F(3,24) = 0.71, p = 0.55; sex*marker*event F(3,24) = 0.78, p = 0.52]. This indicates that differences between stress levels in the three conditions were similar for both markers and both sexes. Pairwise comparison showed that salivary cortisol levels and sAA activity were significantly higher during *integration*

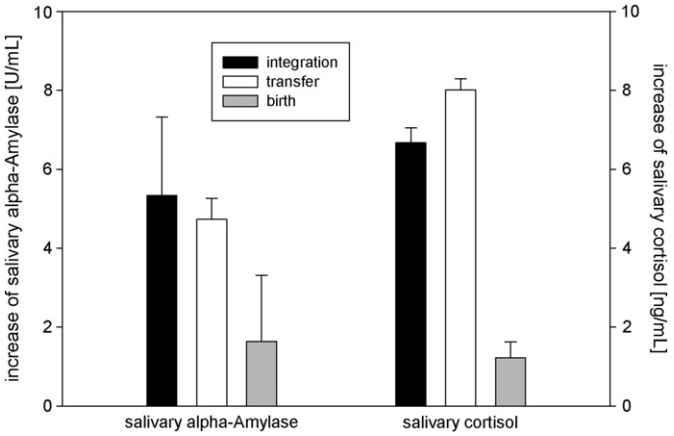

Fig. 3. Increase in salivary alpha-Amylase activity and salivary cortisol levels (with SD) in bonobos from the unstressed condition to the three different stressed conditions.

Table 5
Results of a pairwise repeated measure ANOVA comparing the unstressed condition with each of the three stress events with regard to the levels of salivary alpha-Amylase activity and salivary cortisol levels (within subjects factor). Gender was included as an additional between subjects factor. Reported statistics are those of the main effect of stressor which was the same for both stress measures and both sexes as indicated by non-significant interactions (all $p>0.07661$).

	Comparison unstressed vs. stressed			
Stress event	Numerator df	Denominator df	F	p
Birth	1	8	4.13	0.07
Integration	1	8	8.25	0.02**
Transfer	1	8	30.88	<0.001***

and *transfer*, which was not the case at the time when one female gave birth (Table 5). We did not find significant differences in sAA activity across the three sampling times (30, 60, and 180 min. after the onset of the stressor) for all three potentially stressful events (GLMMs with MCMC, all $p>0.1$) Furthermore, there were no significant differences in salivary cortisol levels across the three sampling times for integration and transfer (GLMMs with MCMC, $p>0.2$). However, salivary cortisol levels significantly decreased from 30 to 180 min. after the birth of an infant (GLMM with MCMC, $p=0.0006$). A Fisher's omnibus test combining the p-values of six GLMMs was significant ($\chi^2=25.566.3$, $df=12$, $p=0.01235$), which indicates that the observed significance in the cortisol patterns after birth was not due to multiple testing.

4.4. Changes in behavioral activity levels during potentially stressful events

Daily activity in the Frankfurt Zoo bonobos increased significantly on days with potentially stressful events compared to days without potentially stressful events [Wilcoxon test: $T^+=72$; $N=11$; $p=0.007$; Table 2]. Furthermore, there was a significant increase in self-grooming [$T^+=74$; $N=11$; $p=0.003$] and vocalization rates [$T^+=78$; $N=11$; $p<0.001$] on days with potentially stressful events (Table 6). In healthy bonobos, diarrhea was observed only seven times in 68 days; in contrast, diarrhea was observed 14 times during the day of transfer and four times during the day of integration. Diarrhea was not observed during the day of birth.

5. Discussion

Using a highly sensitive commercial kit specifically designed to detect alpha-Amylase activity in human saliva, we found that saliva samples from bonobos showed significantly lower sAA activity than samples from humans, gorillas, and orangutans, but significantly higher sAA activity than samples from chimpanzees. Some patterns of sAA activity variation in samples from bonobos resembled those reported for humans, including our finding that sAA activity was higher in samples from males than from females, despite considerable variation across individuals. Compared to the unstressed condition, samples collected at times when subjects appeared to be stressed showed higher sAA activities. Thus, sAA activity in bonobos provides biologically meaningful information and, therefore, offers a new perspective for research exploring stress.

Table 6
Daily average proportions of activity, vocalization, and self-grooming during scans in 9 bonobos from Frankfurt zoo.

Behavior / Condition	Activity (%)	Vocalization (%)	Self-grooming (%)	N
Unstressed	57.63	0.89	2.96	9
Stress	66.76	6.26	7.33	9

So far, very little is known about sAA activity patterns in the great apes and, to our knowledge, the relevance of sAA activity in the context of stress has never been explored in nonhuman primates. Traditionally, changes in salivary cortisol levels have been used as an indicator of stress and this marker has proven invaluable for investigating stress in great apes [25–29]. However, interpreting cortisol profiles can sometimes be complicated as cortisol levels may change in response to both social and environmental stress [55,56] and under certain conditions, the contributions of different stressors to the overall stress response might be difficult to disentangle. In human studies, stress responses in salivary cortisol concentration and sAA activity can be additive, interactive, or complementary [30], depending on such variables as sex or life history of the subject, for example [8,30,57]. A relationship has been found between variation in sAA activity in humans and cognitive abilities, health status [30], and psychological phenomena such as anxiety [14], perception of test conditions, and the type of involvement in a stressful interaction [10]. Therefore, simultaneously monitoring sAA activity and cortisol levels in experimental studies with great apes and humans might be useful for identifying sex, species, and context specific variation in stress response.

5.1. Sex differences and inter-individual variation in salivary alpha-Amylase activities

We found that female bonobos had significantly lower sAA activity than males. While some studies on humans found no sex differences in sAA activity [43,58], others reported consistent sex differences, with men having higher sAA levels than women [9,59]. This disparity might be partly due to the high inter-individual variation in sAA values [14,60]. The sex differences in sAA activity mirror sex differences in salivary cortisol levels [27] and indicate that, at least in captivity, males may experience higher social stress than females.

5.2. Influence of age and chewing duration

In humans, sAA activity and expression is influenced by age [13,30]. While newborns do not produce sAA [30], a sharp rise occurs during the first year of life and adult levels are reached by the age of eight [61]. We found a similar profile in bonobos, with adolescents (7–10 years of age) having the highest sAA baseline activity and infants (1–7 years of age) and adults (10+ years) having low sAA activity.

Our results confirm what other studies have found: chewing duration did not affect sAA activity in humans [62,63] and sAA activity was not affected by the sampling procedure (passive drooling vs. cotton roll [30]). Therefore, a direct comparison of sAA activity of unstimulated and stimulated collected saliva samples might be problematic [47]. Given that all subjects involved in our study chewed on the cotton rolls for saliva collection, the variation reported above is unlikely to reflect variations in saliva collection methodology.

5.3. Stress response in salivary cortisol levels and salivary alpha-Amylase activity

One goal of our study was to explore if sAA activity changes in response to stressful events. Increases in self-grooming or scratching rates have been described as displacement activities in primates [48,64,65] and a result of stressful situations in bonobos [66]. Furthermore, both diarrhea and increases in activity are reported as responses to stressors in humans [67]. Vocalizations can be stress-induced and their levels have been proven to be a valid measure of the emotional status of many mammals [49]. Indeed, we found that rates of these behaviors increased significantly during potentially stressful events as we had defined them, therefore indicating that these investigated events were in fact stressful for the bonobos. In

addition, results of the times course analyses suggest that after potentially stressful events, sAA activity did not return to baseline levels as fast as they did after a single stressful event in humans [47]. This is in agreement with the behavioral observations that indicated that arousal did not diminish rapidly after stressful events but was consistently high for at least three hours. Samples collected during these stressful events had higher sAA activities and higher cortisol levels than samples collected during periods without stressful events. In a number of human studies, sAA activity and salivary cortisol increased as a response to a stressor [30,57] while other studies found no association between the response of the two markers to stressful events [7,10,30,44,47,57]. Although both cortisol levels and sAA activity were higher during two of the three stressful conditions in our studies, the observed correlation between salivary cortisol levels and sAA activity in unstressful situations was absent during periods of stress. This indicates that, similar to humans, the reactivity of the specific stress axis might depend on a variety of parameters, including the nature of the stressor as well as the subjects' perception of the stressful event. Cross-species comparisons of the stress response to competitive situations and other social stimuli [68] could especially benefit from a higher resolution in physiological reactivity towards stress by simultaneously monitoring sAA activity and salivary cortisol levels.

5.4. Genetic implications of the existence of salivary alpha-Amylase activity in bonobos

The activity of sAA depends on the amount of gene product, which, in turn, is related to the mRNA expression of the sAA gene. When the region coding for sAA is duplicated (i.e., present more than twice in the diploid genome sequence), its mRNA expression and activity may increase. Compared to chimpanzees, bonobos have a larger number of AMY1 copies and appear to be polymorphic, at least in Exon 3 [3]. If, as has been suggested, amylase activity increases with copy number [2], bonobo saliva should have higher sAA activity. However, molecular modifications in coding regions of the AMY1 gene (a frame shift on AMY1 Exon 7 and a stop codon on AMY1 Exon 3) suggest that bonobos may be unable to produce functioning sAA [3]. Our results show that sAA activity in bonobos is comparable to other great ape species and humans and, thus, it might be possible that other genes became functional in amylase production. Furthermore, while Perry et al. [3] show evidence for pseudogenization of AMY1 in bonobos, it would be premature to exclude the possibility that some copies of the gene remain active in at least some individuals.

Species and population differences in sAA expression and sAA activity are generally related to the amount of starch in the diet. Primates and humans with high-starch diets have been found to have high sAA expression [3,36] and human populations with high-starch diets have more AMY1 copies than those with low-starch diets [3]. Bonobos are highly frugivorous and their starch intake is likely to be lower than that of species dependent on starch-rich food items such as grains and underground storage organs, like baboons [36,69]. However, bonobos occasionally consume plants that are high in starch, such as the seeds of African breadfruit, which contain up to 30% starch, and the pith of terrestrial herbs such as *Afromomum*, which contain about 40% starch (own unpublished data). Although starch intake by frugivorous apes is probably always lower than that of most human populations, starch remains an important source of highly accessible energy.

6. Conclusions

In this study, we found that sAA activity in bonobos is within the range of that of other great apes. Sex differences in sAA activity in bonobos matched the pattern reported by studies on humans and sAA activity in bonobos was higher during stressful events. Experimental studies on social and physical stress in great apes and other primates might benefit from exploring variation in sAA together with other markers to appreciate individual and species variation in behavior and physiology. This study has shown that sAA in bonobos is sensitive to experienced stress. Future studies can further investigate differences in the reactivity of these two stress markers, cortisol and sAA, to different stressors.

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