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Does diet influence salivary enzyme activities in elephant species?

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Abstract Asian elephants (*Elephas maximus*) and African elephants (*Loxodonta africana*) are herbivore generalists; however, Asian elephants might ingest a higher proportion of grasses than Africans. Although some studies have investigated nutrition-specific morphological adaptations of the two species, broader studies on salivary enzymes in both elephant species are lacking. This study focuses on the comparison of salivary enzymes activity profiles in the two elephant species; these enzymes are relevant for protective and digestive functions in humans. We aimed to determine whether salivary amylase (sAA), lysozyme (sLYS), and peroxidase (sPOD) activities have changed in a species-specific pattern during evolutionary separation of the elephant genera. Saliva samples of 14 Asian and eight African elephants were collected in three German zoos. Results show that sAA and sLYS are salivary components of both elephant species in an active conformation. In contrast, little to no sPOD activity was determined in any elephant sample. Furthermore, sAA activity was significantly higher in Asian compared with African elephants. sLYS and sPOD showed no species-specific differences. The time of food provision until sample collection affected only sAA activity. In summary, the results suggest several possible factors modulating the activity of the mammal-typical enzymes, such as sAA, sLYS, and sPOD, e.g., nutrition and sampling procedure, which have to be considered when analyzing differences in saliva composition of animal species.

Keywords Elephant · Salivary enzyme activity · Amylase · Nutrition · Evolution

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Introduction

Elephants are the only living representatives of the order Proboscidea. These herbivores descended from the 55-mya-old *Phosphatherium* (Gheerbrant 2009). There are two different genera: *Loxodonta*, including the African bush elephant (*Loxodonta africana*), and the African forest elephant (*Loxodonta cyclotis*), and *Elephas* which only includes the Asian elephant (*Elephas maximus*). Approximately 7.6 million years ago, the lineage of ancestors of African elephants separated from the ancestry leading to mammoths and Asian elephants (Rohland et al. 2007).

The biotope of the African bush elephant is at present sub-Saharan Africa (Blanc et al. 2007), whereas the Asian elephant lives in South and Southeast Asia (India, Bangladesh, Thailand, Myanmar, Cambodia) (IUCN 2008). Both elephant species have a similar genome; their sequences align to approximately 94 % (Dastjerdi et al. 2014). Furthermore, both elephant species are unspecialized generalists (Codron et al. 2012). In contrast to other mammals' herbivores, elephant's gastrointestinal physiology is represented by a short digestive tract (Shoshani and Eisenberg 1982; Clauss et al. 2003). In general, their digestion has been shown to be more inefficient in the exploitation of

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nutrients compared to other hindgut fermenting animals, e.g., horses (Clauss et al. 2003). Elephants consume a wide variety of plants depending on seasonal and regional abundance. Their diet consists of fruits, bulbs, and roots in addition to grasses, forbs, shrubs, and trees (Ullrey et al. 1997). Elephants tend to graze and browse which vary depending on the habitat and the season (Guy 1976; Koch et al. 1995; Ullrey et al. 1997; Codron et al. 2006). African as well as Asian elephants tend to graze during the 4-month wet season, feeding on forbs and sedges. In the dry months, trees, shrubs, forbs browse, barks, wood, and twigs dominate the diet of both species, characterizing them rather as browser (Wing and Buss 1970; De Boer et al. 2000; Mohapatra et al. 2013). Overall, both elephant species prefer browsing, although both lineages were formerly grazers (Cerling et al. 1999).

Studies on nutrition habits of African elephants recognized a diverse number of plant species (36–133) ranging from large trees to small herbs depending on the season (Guy 1976; Kabigumila 1993). A study on Asian elephants showed that they consume 95–112 different plant species, but mostly feed on only 25 of these plants, about 85 % of the whole food intake (Sukumar 1989; De Boer et al. 2000). The feeding behavior of Asian elephants is characterized by a highly seasonal selection of food, which is driven by the protein content of the available food (Guy 1976; Joshi and Singh 2008; Mohapatra et al. 2013). Besides, it has been shown that Asian elephants might ingest a higher proportion of grasses than Africans (Cerling et al. 1999).

In addition, the two elephant species differ in a few other aspects. Investigations on calcium concentration in serum and plasma of both species showed that Asian elephants have a lower calcium concentration in plasma than African elephants (van Sonsbeek et al. 2013). Therefore, there are obvious differences in anatomy and morphology (Dhindsa et al. 1972), i.e., the number of ribs and trunk fingers, size of ears, and shape of back and head (Laursen and Bekoff 1978; Shoshani and Eisenberg 1982). All these differences might have evolved, because elephants occupy slightly diverse ecological niches. For example, grass is more difficult to chew than plant material during browsing (reviewed by Shipley 1999; Sanson 2006), and requires longer retention times for its digestion (Hummel et al. 2006). Anatomical differences of the intestines between the two species have been interpreted as an adaptation to differential proportions of grass in the diet of the respective elephant species (Shoshani and Eisenberg 1982; Clauss et al. 2007). Besides the gastrointestinal organs, e.g., the stomach and intestine, accessory structures, such as molar teeth, and the salivary glands play an important role during the exploitation of nutrients. The morphological adaptation to different proportions of grass in their diet might also continue in the oral cavity. For example, the teeth of Asian elephants have more enamel ridges than African elephants (Todd 2010), probably for the mastication of this difficult to chew material. This leads to the assumption that further adaptations may have arisen due to their special diet, e.g., the saliva composition, which plays an essential role during initiation of digestion in mammals.

Saliva is mainly released by the three major glands (parotid, submandibular, and sublingual) and to a lesser degree by a great number of minor salivary glands (Shackleford and Klapper 1962; Veerman et al. 1996; Sreebny 2000). Its evolutionarily selected physiological functions range from the protection of teeth and pre-digestion of food to the lubrication and rinsing of the oral cavity. Saliva has been intensively investigated in humans. It consists of 99 % water and 1 % solid organic, and inorganic components (Lima et al. 2010). Important enzymatic components in human saliva are amylase, lysozyme, and peroxidase (Kaufman and Lamster 2000; Humphrey and Williamson 2001; Nater and Rohleder 2009). Salivary α-amylase (sAA, EC 3.2.1.1) is one of the key digestive enzymes in the saliva of many mammals, and is even the most abundant protein in human parotid saliva (Noble 2000; reviewed by Nater and Rohleder 2009; Carpenter 2013; Boehlke et al. 2015). This hydrolase initiates the polysaccharide digestion by cleaving their α-(1,4)-glycosidic bonds. Salivary lysozyme (sLYS, EC 3.2.1.17) has an antibacterial function based on its muramidase activity (Laible and Germaine 1985), which cleaves peptidoglycans of bacteria cell walls (Wang and Germaine 1993). Salivary peroxidase (sPOD, EC 1.11.1.7) has a major antioxidant and antimicrobial function (Battino et al. 2002; Ihalin et al. 2006). sPOD is responsible for the reduction of hydrogen peroxide (H_2O_2) and the oxidation of thiocyanate. In addition, it can prevent bacterial colonization in the oral cavity (Steele and Morrison 1969; Björck et al. 1975; Pruitt and Adamson 1977).

So far, elephant saliva composition is largely unknown. Only two studies pertaining to the saliva of African elephants have been published to date. One study focused on inorganic composition, and detected salivary chloride, magnesium, potassium, calcium, and phosphorus (Raubenheimer et al. 1988). Furthermore, urea, glucose, and creatinine were present in the saliva of African elephants, but surprisingly, sodium and albumin were absent (Raubenheimer et al. 1988). Beside this study, another investigation focused on the content of salivary steroid hormones, e.g., progestin, depending on menstrual cycle of female African elephants (Illera et al. 2014). In Asian elephants, salivary cortisol levels have been determined and were found to vary with season. The highest salivary cortisol concentrations have been observed at the end of the wet season, after decreasing to the lowest level at the end of dry season (Marcilla et al. 2012).

To our knowledge, broader investigations on salivary enzymes, i.e., lysozyme and peroxidase, in both elephant species are lacking, and only one study stated the lack of salivary α-amylase in African elephants (Raubenheimer et al. 1988), whereas no study investigated sAA in Asian elephants. Even more important, no study design compared directly the salivary profiles of African and Asian elephants. The different morphological adaptions of elephants to different proportions of grass in their diet, as described above, led to the possibility that the salivary composition of Asian and African elephants differs from each other, in addition to the question if the initiation of digestion in elephants is as important as in humans (reviewed by Touger-Decker and Van Loveren 2003; Butterworth et al. 2011) and in ungulates (Fickel et al. 1998; reviewed by Mason and Rushen 2006). Indications for a potential influence of a species-specific diet on the presence and activity of sAA have been reviewed previously. So far, high amounts of the enzyme have been detected in the saliva of many omnivorous animals in contrast to negligible up to low amounts in a few herbivores, and almost absent in carnivores (reviewed by Boehlke et al. 2015). sLYS was only investigated in domestic cattle (*Bos primigenius f. taurus*) (Ang et al. 2011) and two macaque species (*Macaca mulatta* and *Macaca fascicularis*) (Polyzois et al. 1976). sPOD has been found in bovines (*Bosprimigenius f. taurus*) (Banerjee and Datta 1986; Wheeler et al. 2011; Mau et al. 2013) as well as in non-human primates (*Macaca mulatta* and *Macaca fascicularis*) (Mäkinen et al. 1978). The highest amounts of exogenous peroxidase in animals were found in domestic cats (*Felis silvestris f. catus*) (Barabash et al. 1979). Due to the paucity of studies, further investigations are required to explore the salivary composition of mammals.

This study compares the activity profiles of salivary enzymes of African and Asian elephants, which are relevant for protective and digestive functions in humans. In detail, it was aimed to determine (1) whether sAA, sLYS, and sPOD show similar activities in these two herbivorous species, or if salivary composition has changed in a species-specific manner during evolutionary separation and adaptation of the elephant genera. In addition (2), the lack of sAA which has been determined for some herbivorous species (Raubenheimer et al. 1988; reviewed by Boehlke et al. 2015) should be verified for elephants.

Methods

Subjects and their nutrition

In July 2015, saliva samples ($n = 22$) were collected from six male and eight female Asian elephants as well as from one male and seven female African elephants kept in three different zoos in Germany (Table 1).

Elephant's diet depended on the zoos (Table 2). In all zoos, herbage, carrots, and apples were offered at least two times a day to elephants. In addition, hay and branches were available nearly the whole day. Main differences in the composition of diet are the components of the herbaceous plants. In Heidelberg and Dresden Zoo, the herbaceous diet comprised dandelion (*Taraxacum* spp.), clover (*Trifolium* spp.), and ribwort (*Plantago lanceolata*). In contrast, alfalfa (*Medicago sativa*) was the main component of herbaceous diet parts in Berlin's Animal Park. Bananas (*Musa* spp.) and more different types of pellets (high starch content; see Appendix Table 4) were fed at Heidelberg Zoo, but were not part of the diet of elephants in Berlin's Animal Park and Dresden Zoo.

Saliva sampling and preparations for analysis

For each elephant, two saliva samples were collected and pooled to achieve a sufficient saliva volume using Salivette® (Sarstedt, Nümbrecht, Germany), which includes a synthetic swab as an absorbing material. The elephants were in no way forced to participate in saliva collection, but voluntarily participated; therefore, the animals did not suffer or they were stressed by the collection procedure. Due to this fact, the saliva collection procedure was not evaluated as an animal experiment referring to the institutional Animal Care and Use Committee or by the German law.

Saliva collection was performed by the responsible animal caretaker of each zoo. All samples have been collected between 10:00 and 12:00 h; however, saliva samples from African elephants in Dresden Zoo were collected at 16:00 h due to management reasons. In addition to their daily routine, elephants are trained to respond to different commands, including the command to open their mouth. Saliva was collected by wiping the absorbent material inside the whole oral cavity for at least 30 s. In detail, absorbent material was wiped for a few seconds under the tongue as well as left and right to the tongue to ensure mixed saliva of different salivary glands.

At Berlin's Animal Park, sampling started after both species had been provided with food, and saliva collection was performed within 1 h for all elephants. The caretaker sampled a different elephant approximately every 3 min. Therefore, we can exclude that feeding during saliva collection affected one individual but not another. However, the study design started with saliva collection in Asian elephants, and continued approximately 30 min later with African elephants. During the saliva collection procedure, bread was available for the individuals in Berlin's Animal Park and Dresden Zoo; however, unfortunately, it was not

Table 1 Information about Asian and African elephants from different zoos

Species	Zoo	Sex	Individual number	Age at collection in years (year of birth)
Asian elephant (*Elephas maximus*)	Zoo Heidelberg GmbH (Heidelberg Zoo)	♂	1	10 (2005)
		♂	2	9 (2006)
		♂	3	6 (2009)
		♂	4	4 (2011)
	Tierpark Berlin-Friedrichsfelde GmbH (Berlin's Animal Park)	♀	5	3 (2012)
		♀	6	7 (2008)
		♀	7	35 (1980)
		♀	8	35 (1980)
		♀	9	22 (1993)
		♀	10	32 (1983)
		♀	11	42 (1973)
		♀	12	20 (1995)
		♂	13	3 (2012)
		♂	14	9 (2004)
African elephant (*Loxodonta africana*)		♂	15	8 (2007)
		♀	16	9 (2006)
		♀	17	44 (1971)
		♀	18	34 (1981)
		♀	19	27 (1988)
	Zoo Dresden GmbH (Dresden Zoo)	♀	20	25 (1990)
		♀	21	19 (1996)
		♀	22	20 (1995)

Sex is denoted by ♀ (female) and ♂ (male). Individual's ages are shown in years at collection time and the year of birth

documented if bread was eaten before or after the saliva collection. In contrast to the other zoos, in Heidelberg Zoo, elephants had not been provided with food before sample collection, but bread was used to reward the individuals during sampling. In general, no other feed was given in any zoos to reward elephants than described above.

After collection, all samples were stored on dry ice until centrifugation (4 °C, 20 min, and 4.000 rpm). After centrifugation, saliva was pooled for each elephant and stored at −80 °C at the Institute of Zoology, Dresden until analysis. The analysis of sAA, sLYS, and sPOD was performed in triplicates for each saliva sample.

Statistical analyses

To evaluate differences in sAA, sLYS, and sPOD activities between the elephant species and the influence of sex, we used the two tailed Wilcoxon–Mann–Whitney test (WMW-TEST) and a paired *T* test. To investigate the impact of "feed intake lengths", i.e., the time between food was available for elephants and the start of sample collection, and age-related effects on different salivary enzymes, we ran Spearman correlations (SP). Analyses were run in SPSS 22.0 (IBM Corporation, Chicago, Illinois, USA). After normal distribution of the data was tested via Kolmogorow–Smirnow test and Shapiro–Wilk test combined with a Q–Q-plot, the paired *T* test was used when the data were normally distributed. WMW-TEST was used when data were not normally distributed. The significance level was defined as $p < 0.05$ for all used tests. Zero values for enzyme activity (0 U/ml) were considered in the same way as other values during statistical analyses, because they indicate a very low or no detectable enzyme activity.

Determination of enzyme activity

Salivary α-Amylase (sAA)

For the investigation of sAA activity, the low-molecular-weight substrate 2-chloro-4-nitrophenyl-4-O-β-D-galactopyranosylmaltotrioside ($GalG_2CNP$) was used as described previously (Hannig et al. 2004). Briefly, this trisaccharide is linked at the reducing end via an α-glucosidic bond to the chromophore 2-chloro-4-nitrophenol. Without any auxiliary enzyme, sAA hydrolyzes $GalG_2CNP$ directly yielding the free aglycone 2-chloro-4-nitrophenolate (CNP) (e.g., Morishita et al. 2000) at a constant rate without a lag phase. CNP was determined photometrically.

Table 2 Information about the specific diet composition of elephants in different zoos

		Asian elephant *(Elephas maximus)*		African elephant *(Loxodonta africana)*
		Zoo Heidelberg GmbH	Tierpark Berlin-Friedrichsfelde GmbH	Zoo Dresden GmbH
Nutrition	Herbage	hay, herbaceous plants (clover, ribwort, dandelion), branches	hay, herbaceous plants (alfalfa), branches (poplar)	hay, herbaceous plants (ribwort, clover, dandelion), branches (oak)
	Fruits and Vegetables	bananas - apples, carrots	- cabbage apples, carrots	broccoli, turnip cabbage apples, carrots
	Cereals	bread	bread	bread
	Pellets	SALVASTAR E-Selen-Pellets, Pre Alpin® Wiesencobs; SALVANA elephant mineral; wild life park pellets	SALVANA Leckerli® for horses; Pre Alpin® Wiesencobs	SALVANA Leckerli® for horses
	Powder	-	SALVANA elephant mineral powder	-

Salivary lysozyme (sLYS)

The investigation of sLYS activity was performed via the hydrolysis of fluorescein-labelled *Micrococcus lysodeicticus* (EnzCheck Lysozyme assay kit; E-22013, Molecular Probes, Leiden, The Netherlands) as described previously (e.g., Vray et al. 1980).

Salivary peroxidase (sPOD)

For the investigation of sPOD activity, the fluorogenic 2′,7′-diacetlchlorofluorescin (LDCF) was used. In the presence of peroxidase and hydrogen peroxide, the substrate was converted to the fluorescing dichlorofluorescin (DCF) as described previously (e.g., Black and Brandt 1974). The sensitivity of the assay was enhanced by thiocyanate (Proctor and Chan 1994).

Results

First, we analyzed the effect of sex and age on the salivary enzymes. Sex had no impact on any of the tested enzymes: sAA [WMW: $p = 0.647$, $U(15,7) = 46$], sLYS [WMW: $p = 0.307$, $U(15,7) = 38$], and sPOD [WMW: $p = 0.215$, $U(15,7) = 42$]. Age was not correlated with any of the enzyme activities [sAA (SP: $r = 0.221$; $n = 22$; $p = 0.322$), sLYS (SP: $r = 0.126$; $n = 22$; $p = 0.578$), and sPOD (SP: $r = 0.001$; $n = 22$; $p = 0.997$)].

Amylase

Considering all zoos, sAA activity was significantly lower in African elephants ($n = 8$; average 16 ± 6.35 U/ml) in comparison with Asian elephants ($n = 14$; average 127 ± 105.78 U/ml) (WMW-TEST: $U(14,8) = 1$, $p = 0.0002$) (Fig. 1; see

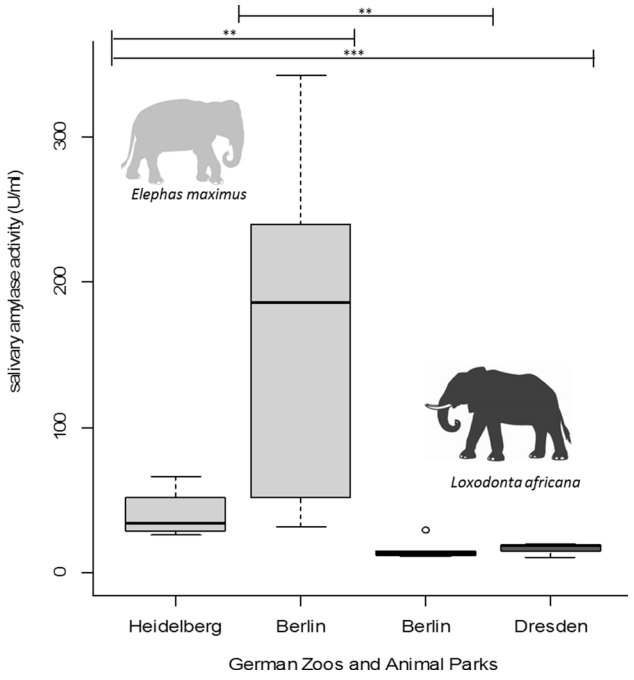

Fig. 1 Salivary alpha amylase (sAA) activity of two elephant species from three different zoos. sAA activity (U/ml) of African elephants (*Loxodonta africana*) from Berlin's Animal Park and Dresden Zoo are shown by *dark grey-colored boxes*. *Light grey boxes* indicate the enzyme activity of Asian elephants (*Elephas maximus*) from Berlin's Animal Park and Heidelberg Zoo. The *boxes* illustrate the 25th and 75th percentiles, *bars* show medians, and outliers are expressed by *circles*. The highest significance is displayed by ***($p < 0.001$), and **($p < 0.01$) illustrates a highly significant result. Total sample sizes ($n = 22$): $n_{\text{African elephants}} = 8$, $n_{\text{Asian elephants}} = 14$

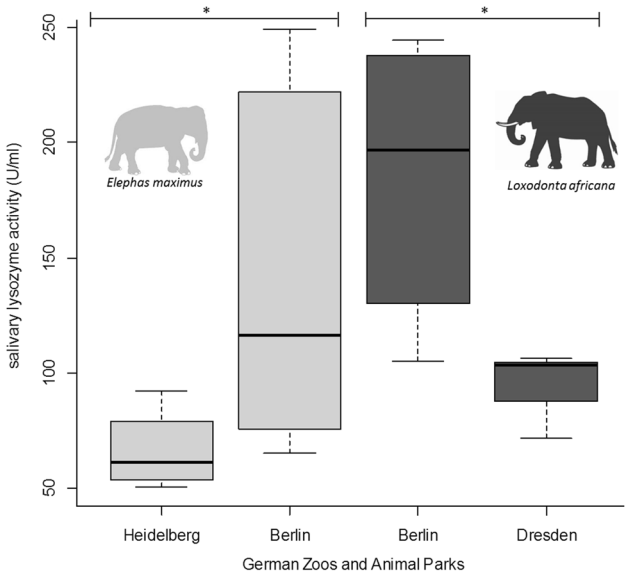

Fig. 2 Salivary lysozyme (sLYS) activity of two elephant species from three different zoos. sLYS activity (U/ml) of African elephants (*Loxodonta africana*) from Berlin's Animal Park and Dresden Zoo is shown by *dark grey-colored boxes*. *Light grey boxes* indicate the enzyme activity of Asian elephants (*Elephas maximus*) from Berlin's Animal Park and Heidelberg Zoo. The *boxes* illustrate the 25th and 75th percentiles, *bars* show medians, and outliers are expressed by *circles*. Significance is displayed by *($p < 0.05$). Total sample sizes ($n = 22$): $n_{\text{African elephants}} = 8$, $n_{\text{Asian elephants}} = 14$

Appendix Table 3). In Asian elephants, sAA activity differed significantly between zoos (*T* test: $t(10.175) = 3.483$, $p = 0.006$) with higher levels in Berlin's Animal Park in comparison with Heidelberg Zoo (Heidelberg Zoo_$n = 4$; average: 40 ± 18.01 U/ml; Berlin's Animal Park_$n = 10$; average: 162 ± 106.61 U/ml). However, sAA showed great variability in Asian elephants from Berlin's Animal Park, e.g., in four out of ten individuals' sAA activity was as low ($n = 4$; average 50 ± 15.50 U/ml) as in the four elephants from Heidelberg Zoo ($n = 4$; average 40 ± 18.01 U/ml). In contrast, in African elephants, sAA activity was independent of zoo (Berlin's Animal Park_$n = 5$; average 16 ± 7.64 U/ml; Dresden Zoo_$n = 3$; average 16 ± 4.93 U/ml) (WMW-TEST: $U(5,3) = 7$, $p = 0.881$). To test for species effects, we only considered elephants from Berlin's Animal Park due to the same nutrition and husbandry conditions. Asian elephants had significantly higher sAA activity (162 ± 106.61 U/ml) than African elephants (16 ± 7.64 U/ml) (WMW-TEST: $U(10,5) = 0$, $p = 0.002$) (Fig. 1).

However, we found that the time of food provisioning until sample collection and sAA activity was negatively correlated in elephants in Berlin's Animal Park (SP: $r = -0.696$; $n = 15$; $p = 0.004$). In other words, elephants that were sampled first and fed only a few minutes before sample collection had higher sAA activity in comparison with elephants that had been fed approximately 30 min before saliva collection took place. sAA activity was not different in saliva samples collected from African elephants in the forenoon in comparison with samples from the afternoon (Fig. 1). Therefore, time of the day at sampling had no impact on the sAA activity.

Lysozyme

The comparison of sLYS activity in African and Asian elephants (see Appendix Table 3) revealed that they do not differ significantly ($n = 8$; average African 149 ± 67.11 U/ml and $n = 14$; average Asian 117 ± 70.60 U/ml) (WMW-TEST: $U(14,8) = 38$, $p = 0.219$) (Fig. 2). In Asian as well in African elephants, we found high inter-individual variability in sLYS activity ranging from 50 to 249 U/ml in Asian elephants and from 72 to 245 U/ml in African elephants. Feed intake length was not correlated with sLYS activity in Asian and African elephants in Berlin's Animal Park (SP: $r = 0.129$; $n = 15$; $p = 0.648$).

Zoo identity had a significant impact on sLYS activity in Asian elephants. Individuals from Heidelberg Zoo had a significantly lower sLYS activity ($n = 4$; average 66 ± 18.44 U/ml) than individuals from Berlin's Animal park ($n = 10$;

138 ± 43.84 U/ml) (*T* test: $t(11.208) = 2.855$; $p = 0.015$). sLYS activity differed between African elephants from different zoos (Dresden Zoo_$n = 3$, average 94 ± 19.26 U/ml; Berlin's Animal Park_$n = 5$, average 183 ± 62.97 U/ml) (*T* test: $t(5.096) = 2.947$; $p = 0.031$). By testing differences between elephant species, we found that the predictor had no impact (*T* test: $t(13) = 1.162$; $p = 0.266$) on the sLYS activity of Asian elephants ($n = 10$; average 138 ± 73.84 U/ml) and African elephants ($n = 5$; average 183 ± 62.97 U/ml).

Peroxidase

sPOD activity was tested in 22 samples; however, sPOD activity was detectable with the assay in only three samples (13.6 %) from two Asian and one African elephant (see Appendix Table 3). The enzyme activity varied slightly within the two Asian elephants (ranging from 2 to 6 mU/ml). The sPOD activity of the African elephants (3 mU/ml) was within the range of the Asian elephants. sPOD activity was not correlated with differences in feed intake lengths in Berlin's Animal Park in Asian and African elephants (SP: $n = 15$; $r = 0.084$; $p = 0.765$).

Discussion

The analyses showed that sex and age had no impact on sAA, sLYS, and sPOD activities. For the first time, sAA and sLYS have been determined in an active conformation in elephants' saliva which is in opposition to a previous study showing a lack of sAA activity in African elephants (Raubenheimer et al. 1988). These differences might result from the small sample size of only two elephants in the previous study, varying sampling conditions, and most of all usage of different assays.

In our study, species-specific differences in enzyme activity have been verified by considering only individuals from both species in Berlin's Animal Park. The results indicate a species-specific difference in sAA activity, which was significantly higher in Asian elephants compared with Africans. In humans, sAA activity is related to the salivary amylase copy number (AMY) (Mandel et al. 2010; Falchi et al. 2014), which is also shown for non-human primates (Mau et al. 2010). Furthermore, a starch rich diet is related to high AMY copy numbers in humans (Perry et al. 2007; Luca et al. 2010). Therefore, it could be possible that the significantly higher sAA activity in Asian elephants compared to African elephants is related to the amount of starch in their natural diet. Compared to Africans, Asian elephants might have a higher starch content diet, i.e., grass, including seeds, leaves, bulbs, and roots (reviewed by Robbins 1983; Watt 2005; Rodiek 2010). So far, the literature on differences in sAA activity between browsing and grazing animals is rare. Based on a few studies stating uniformly low sAA activity in herbivores (reviewed by Boehlke et al. 2015), no difference in sAA activity between these feeding patterns can be assumed. However, it has been hypothesized that due to the tannin binding affinity of sAA, sAA activity is possibly higher in species which feed on a tannin-rich diet (da Costa et al. 2008). In contrast to grasses as monocotyledons, dicotyledons contain tannins (Shimada 2006; Mau et al. 2009). We expected a low sAA activity in monocotyledon feeding species; however, our data show the opposite, i.e., the more grass feeding Asian elephants exhibited a higher sAA activity, concluding potential other reasons for different sAA activities in the saliva of Asian and African elephants.

We found that feeding before sample collection affected sAA activity in Asian and African elephants, because the lag time from feeding until sample collection was negatively correlated. Previously, it was determined that salivary enzymes and proteins can be induced by feeding (Bird et al. 1977; Mäkinen et al. 1978; Mehansho et al. 1985; Clements et al. 1985; Clauss et al. 2005). It is likely that sAA was activated to increase the exploitation of nutrients in the saliva samples of the Asian elephants which were collected first. After feeding, sAA activity decreased. Because the African elephants already finished feeding, this might explain their significantly lower sAA activity. Unfortunately, each elephant was only sampled once. Therefore, to distinguish between species-specific and sampling effects on sAA activity, future studies should collect samples in a randomized order in both species at several different times, i.e., before, at the start, and after feeding.

While sAA activity increases in response to stress, due to the activation of the sympathetic nervous system reflecting alterations in the body (Nater et al. 2005; reviewed by Nater and Rohleder 2009; Koh et al. 2014), the elephants in our study were familiar with the sample collection, due to sampling during their daily routine. Therefore, we consider that stress had only a minor or no effect on sAA activity in these elephants.

The time of day at sampling collection had no impact on sAA activity in the elephants of our study. In humans, sAA activity levels show a circadian pattern, with low activity in the morning, an increase during the course of the day, with the highest levels in the late afternoon (reviewed by Rohleder and Nater 2009). Overall, differences in feeding pattern of omnivores (humans) and herbivores (elephants) potentially affect the sAA activity. Beside this, on the basis of this study, no conclusion can be drawn relating to the diurnal rhythm of elephants' sAA, given that animals were sampled only once a day.

Contrary to sAA activity, the results for sLYS and sPOD activities indicated no species-specific difference. In sLYS, high inter-individual and intra-individual variations in the enzyme activity within Africans and Asian elephants have

to be considered. Intra-specific differences may, in fact, be larger than inter-specific differences. However, sPOD was rarely detectable in saliva of any elephant species. sPOD activity was possible to measure only in 14 % of the elephant samples with this assay. While we were able to detected sPOD in three samples, we conclude that the assay is not sensitive enough to measure the low sPOD activity in elephants. Moreover, all zero values in sPOD have to be seen in context of an only moderately standardized sample acquisition in the elephants. In addition, feeding during sample collection might have affected sPOD activity by inhibition. Strong and sustainable inhibitory effects on sPOD have been shown for polyphenols in humans (Hannig et al. 2008).

Investigating the influence of zoo on the different salivary enzyme activities revealed a significant impact of zoo on the activity of sAA and sLYS. In contrast to individuals from Berlin's Animal Park, enzyme activity was significantly decreased in the saliva in elephants from Heidelberg Zoo. These variations in enzyme activity within Asian elephants are probably a result of different sampling procedures or diets at the two zoos. Salivary enzymes respond immediately to gustatory and mechanical stimuli in the mouth (Oberg et al. 1982; Becerra et al. 2003; Neyraud et al. 2006; Rohleder and Nater 2009), and the stimulation of salivary flow rate is conceivable due to bread (Mackie and Pangborn 1990), which was used to reward the elephants in Heidelberg Zoo for their cooperation during sample collection. However, it has been shown that the stimulation of salivary flow rate does not necessarily result in elevated sAA activity in humans (Mackie and Pangborn 1990). The two zoos also differed in the dietary supplements offered to the elephants. Heidelberg Zoo fed SALVANA elephant mineral, wild life park pellets, and SALVASTAR E-Selen-Pellets in contrast to Berlin's Animal Park (SALVANA elephant mineral powder and SALVANA Leckerli® for horses) (Table 2). Therefore, dietary supplements in Heidelberg Zoo contained more starch compared with Berlin's Animal Park (see Appendix Table 4). The lower sAA activity in elephants in Heidelberg Zoo could be explained by the food intake and the possible resultant sAA activation in Asian elephants of Berlin's Animal Park before sampling, as mentioned above. In all zoos, no fruits were used to reward the elephants for cooperation during sample collection. Therefore, direct influence of fruit components on the measurement of enzyme activities can be disregarded, e.g., ascorbic acid (Abell et al. 1998).

We found significant differences in sLYS activities within in African elephants from Dresden Zoo and Berlin's Animal Park. In contrast to the elephants from Berlin's Animal Park, which were provided with fresh poplar branches (*Populus* spp.), Dresden Zoo provided branches from oak trees (*Quercus* spp.). Both tree species contain tannins and thereby possibly interfere with sLYS activity (Green 1995), but potentially to a different extent. In addition, herbaceous plants offered to the elephants in Berlin's Animal Park comprised alfalfa, whereas the herbage fed to elephants in Dresden Zoo comprised ribwort, clover, and dandelion. Otherwise, the similar low sAA activity in African elephants from Dresden Zoo and Berlin's Animal Park possibly occurred irrespective of the nutrition but more due to sampling conditions.

The enzymes investigated in this study were chosen primarily due to their high abundance and secondarily, because they are relevant for protective and digestive functions in human saliva (Battino et al. 2002; Hannig et al. 2005; Carpenter 2013). In humans, sAA activity in unstimulated saliva ranges between 3 and 63 U/mL (Schlueter et al. 2012), which is rather similar to sAA activity in saliva of Asian elephants from Heidelberg Zoo (26–67 U/mL) and all African elephants (12–30 U/mL). sPOD activity of elephants (0–6 mU/mL) is similar to humans (1–6 mU/mL) (Schlueter et al. 2012). In contrast, sLYS activity might be lower in elephants (50–246 U/mL) compared to results from humans (1.4–10 kU/mL) (Schlueter et al. 2012). Due to the use of identical assays by Schlueter et al. (2012) and this study, it appears that this enzyme activity in human and elephant saliva might be different, which easily can result from very different feeding patterns.

Taken together, the results indicated multiple possible factors influencing the activity of the mammal-typical enzymes, such as sAA, sLYS, and sPOD, i.e., diet and sampling procedure, which have to be considering when preparing the study design or when analyzing differences in saliva composition of animal species in zoos.

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Compliance with ethical standards

This article does not include studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animals did not experience pain or suffering, and were not distressed by the saliva collection procedure. Therefore, the saliva collection is not an animal experiment according to German law.

Conflict of interest The authors declare that they have no conflict of interest.

Appendix

See Tables 3 and 4.

Table 3 Synopsis of different salivary enzyme activities of Asian and African elephants from different zoos

Species	Zoo	Individual number	Enzyme activity					
			U/ml				mU/ml	
			sAA		sLYS		sPOD	
			Mean	SD	Mean	SD	Mean	SD
Asian elephant (*Elephas maximus*)	Zoo Heidelberg GmbH (Heidelberg Zoo)	1	66.67	1.85	66.36	2.83	0	0
		2	36.89	2.14	56.39	3.36	0	0
		3	26.18	1.91	92.07	3.77	0	0
		4	32.10	3.23	50.23	4.88	0	0
	Tierpark Berlin-Friedrichsfelde GmbH (Berlin's Animal Park)	5	44.49	3.16	249.44	23.59	0	0
		6	51.79	0.19	65.02	4.10	0	0
		7	245.20	2.80	221.99	94.84	0	0
		8	222.31	0.74	111.13	4.52	5.93	2.34
		9	188.21	4.57	245.84	8.48	0	0
		10	184.99	5.08	69.82	22.98	0	0
		11	240.39	0.58	75.42	4.82	0	0
		12	342.61	0.43	82.35	1.13	0	0
		13	31.39	1.39	121.83	13.41	0	0
		14	68.57	1.78	136.71	15.86	1.68	0.58
African elephant (*Loxodonta africana*)		15	11.62	0.57	244.64	9.04	2.68	0.95
		16	11.90	2.00	130.21	38.87	0	0
		17	13.49	0.14	238.04	2.54	0	0
		18	29.77	2.43	196.67	3.39	0	0
		19	14.68	0.80	105.18	12.68	0	0
	Zoo Dresden GmbH (Dresden Zoo)	20	18.88	0.51	103.40	2.83	0	0
		21	19.74	1.73	106.23	4.95	0	0
		22	10.81	0.04	71.55	1.70	0	0

"sAA" refers to salivary alpha amylase, "sLYS" means salivary lysozyme, and "sPOD" means salivary peroxidase. "Mean" is the average enzyme activity of triple determination of the pooled saliva sample per animal. Standard deviation is denoted by "SD"

Table 4 Composition and ingredients of dietary supplements fed to the elephants during the study period

Dietary supplement	Fabricator	Ingredients	Percentage	Feeding stuffs additives per 1 kg in mg		Composition
SALVASTAR E-Selen-Pellets	SALVANA Tiernahrung GmbH Rosenstr. 9 25365 Klein Offenseth-Sparrieshoop	Crude protein	8.1	7150	Vitamin E	Apple pomace, Corn(decomposed), Wheat Bran, Sugar beet molasses
		Crude fat	3.3	7.1	Selenium	
		Crude fiber	16.2			
		Ash	7.8			
		Calcium	0.3			
		Phosphor	0.3			
		Sodium	0.7			
SALVANA elephant mineral		Crude protein	5.2	17.2	Vitamin A	Apple Pomace, Corn (decomposed), Wheat Bran, Sugar beet Molasses, Calcium Carbonate, Sodium Chloride, Dicalcium phosphate, Wheat flour, Wheat starch, Palm grease, Magnesia, Monocalcium, Phosphate
		Crude fat	6.5	1.4	Vitamin D3	
		Crude fiber	7.8	31,428	Vitamin E	
		Ash	30.0	57,142	Vitamin C	
		Calcium	4.6	3000	Iron	
		Phosphor	1.1	914	Cupper	
		Sodium	3.4	4057	Zinc	
		Magnesium	0.6	2400	Manganese	
				20	Iodine	
				17.7	Cobalt	
				35.4	Selenium	
SALVANA elephant mineral powder		Crude fat	2.4	150	Vitamin A	Glucose, Calcium carbonate, Sodium chloride, Monocalcium, Phosphate, Magnesia
		Ash	25.5	1.25	Vitamin D3	
		Calcium	4.0	25,000	Vitamin E	
		Phosphor	1.0	50,000	Vitamin C	
		Sodium	3.0	100	Vitamin K3	
		Magnesium	0.5	300	Vitamin B1	
				250	Vitamin B2	
				125	Vitamin B6	

Table 4 continued

Dietary supplement	Fabricator	Ingredients	Percentage	Feeding stuffs additives per 1 kg in mg		Composition
				650	Vitamin B12	
				400	Nicotinic acid amide	
				250	Calcium pantothenate	
				65	Folic acid	
				7250	Biotin	
				1850	Choline chloride	
				2650	Iron	
				875	Cupper	
				3950	Zinc	
				2100	Manganese	
				17.5	Iodine	
				15.5	Cobalt	
				31.0	Selenium	
SALVANA Leckerli® for horses		Crude protein	9.2	Not specified		Wheat, Apple pomace, Corn, Sugar beet molasses, Wheat starch, Carrots (dried), Calcium carbonate, Dicalcium, Sodium chloride
		Crude fat	2.1			
		Crude fiber	8.2			
		Ash	6.3			
		Calcium	1.2			
		Phosphor	0.4			
		Sodium	0.7			
Pre Alpin® Wiesencobs	St. Hippolyt, Vertriebs-GmbH, Im Grund 52, 36110 Schlitz, Germany	Crude protein	9.9	9.6	Cupper	More than 60 different herbs and grasses of the Bavarian Alpine foothills
		Crude fat	2.4	36	Zinc	
		Crude fiber	27.0	171	Manganese	
		Ash	9.6	0.01	Selenium	
		Calcium	0.9			
		Phosphor	0.3			
		Magnesium	0.3			

Table 4 continued

Dietary supplement	Fabricator	Ingredients	Percentage	Feeding stuffs additives per 1 kg in mg		Composition
Wild life park pellets	Raiffeisen-Kraftfutterwerk GmbH, Westst. 29, 77694 Kehl am Rhein, Germany	Crude protein	11.0	1.35	Vitamin A	Oat bran, Wheat bran, Alfalfa grass meal, Corn, Wheat gluten, Barley, Calcium carbonate, Molasses shavings, Sugar beet molasses, Cattle salt
		Crude fat	2.7	0.025	Vitamin D3	
		Crude fiber	15.0	15	Vitamin E	
		Ash	8.8	0.2	Selenium	
		Calcium	1.6			
		Phosphor	0.45			
		Sodium	0.2			

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