Interaction between food, saliva, and tooth surface

Marcus Mau, Achim Johann, Thomas M. Kaiser, and Karl-Heinz Südekum

Introduction

Saliva is an outstanding fluid, especially in terms of research and diagnostic possibilities. Its composition – namely electrolytes, hormones and especially its proteome – contains valuable information about feeding status, nutritional requirements as well as adaptation to diet and environment. The biggest advantage of saliva as a 'research tool', however, is the possibility to collect it on a non-invasive basis; and there is almost no need for special training. Therefore, the results of our analysis of salivary proteomes from five different herbivorous species (camel, cattle, gelada baboon, goat, hamadryas baboon) already ignited major interest in salivary research, with the future goal to maintain and improve livestock productivity on one hand and certainly zoo animal welfare on the other. Moreover, the comprehensive analysis and identification of salivary proteins is a necessary pre-requisite to better understand animal physiology and diet adaptation.

How to address food adaptation in animal saliva?

When starting to work on animal saliva, one soon realises the great variety of saliva within mammals. What we know about human saliva and its use in measuring hormone levels, or its ability to pre-digest high-molecular carbohydrates such as starch, shows only the limits of our current understanding of salivary functions. For example, how do we explain the huge amount of saliva produced by cattle – up to 180 litres per day? Bovine saliva lacks amylase or comparable digestive enzymes. On the other hand, free-ranging cattle feed on fibre-rich, "difficult-to-digest" plant diets all their lives. So, how do they manage? What role does saliva play in these herbivores?

The aim of this research project was to characterise salivary proteins in herbivores, and to find certain interactions between them and tooth enamel as well as plant phytoliths or grit, in order to protect teeth from abrasion. The answers to the following five questions produced the highest practical interest:

1. Does saliva composition correlate with feeding traits in herbivorous mammals, and is salivary composition adaptive to dietary changes?
2. Which saliva components interact specifically with tooth enamel and/or dentin and thus might play a certain role in the reduction of diet abrasiveness?
3. Do various herbivorous species, such as grazers, show similar salivary protein patterns and binding capabilities in adaptation to their fiber-rich and highly abrasive diet?
4. Are salivary proteins of grazing mammals able to bind and enclose plant phytoliths?
5. Which other, so-called accessory functions does saliva of herbivorous species provide?

Animal saliva sampling

Work on animal salivary proteins demands the collection of larger amounts of saliva and adequate frozen storage. However, full saliva – as it is obtained by using cotton swabs – always represents a mixture of oral secretions and proteins originating from different salivary glands as well as from gingival fluid. Saliva further contains cellular debris and food particles. Furthermore, salivary components might be altered or partially degraded by the oral microbiome.

Animal saliva sampling can be adapted from collecting human saliva, using cotton pads and centrifuge tubes. While goat and cattle can be easily handled during the procedure, camels turned out to be slightly more laborious. After the first tries ending up in the researcher being covered with camel saliva, well trained camels could be sampled like cows.

For the collection of whole saliva from goats, camels and cattle, a cotton pad was held into the animal's mouth, and allowing the animal to chew on the cotton wool to obtain a saliva sample.

Sampling in the primate species (hamadryas baboons, gelada baboons) was performed during routine checks or operations in zoological gardens, while the animals were unconscious.

All saliva samples were kept on ice and centrifuged according to a standard protocol, described in Mau et al. (2006), to precipitate food debris and other solid particles in the sample. The samples were kept frozen at −80 °C.

T. Martin & W. v. Koenigswald (eds.): Mammalian Teeth – Form and Function. Pp. 81–86, 3 figs., 2 tabs.

DOI: http://doi.org/10.23788/mammteeth.05 Published 22 December 2020

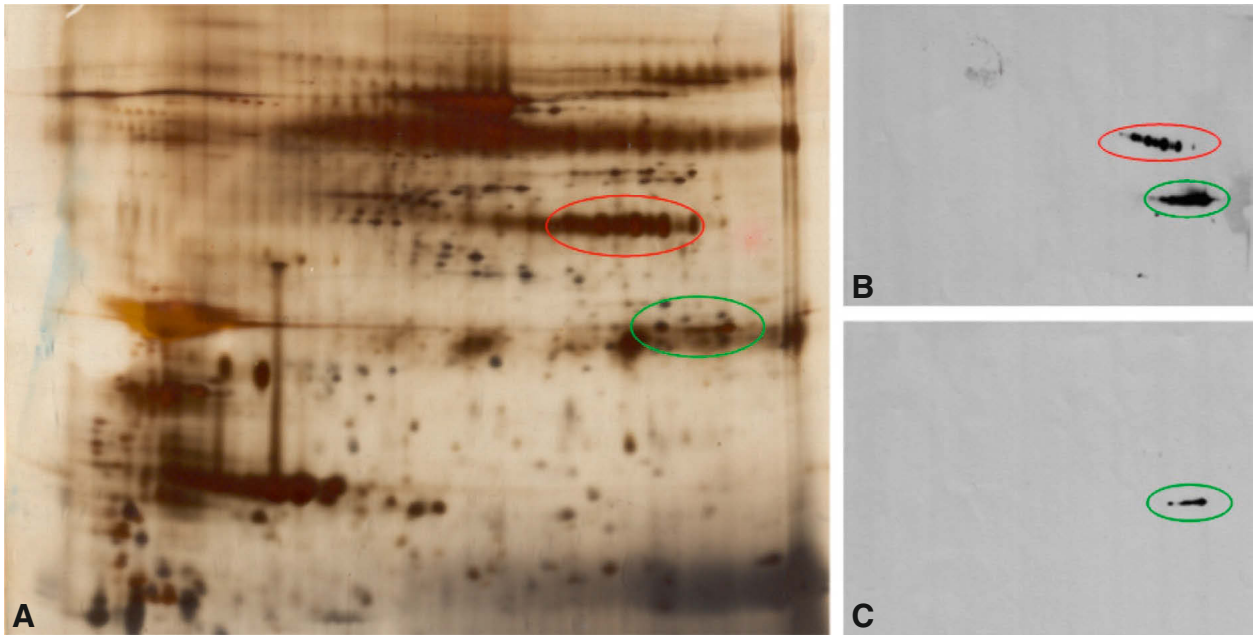

Fig. 5.1. A, 2D gel electrophoresis of an exemplary bovine saliva sample. The upper red marking shows the position of CA VI in the 2D gel. The lower green mark describes CA II spots. **B,** the anti-bovine CA antibody reacted with CA VI (upper red mark) and CA II (lower green mark). **C,** a second used anti-human CA II antibody did only cross-react with bovine CA II (green marking) in the same saliva sample. From Mau et al. (2010).

Saliva composition

To identify and also to quantify common salivary proteins from whole animal saliva of different herbivorous mammals and primate species, immunoblots were performed using standard Coomassie R250 staining as well as commercially available anti-bovine carbonic anhydrase VI (CA VI) and anti-human CA II antibodies. The anti-bovine CA VI antibody was found to further cross-react with bovine CA II as described in detail in Mau et al. (2009).

Furthermore, the detection protocols were improved using bovine saliva for the first time in 2D gel electrophoresis as shown by Mau et al. (2010). This enabled us to reproduce certain species-specific salivary protein patterns and to isolate and identify diverse protein spots, such as CA VI, in mass spectrometry (Fig. 5.1).

Salivary protein patterns of various grass-eating mammals

For the first time saliva samples originating from various ruminating mammals and primates were collected and analysed to find characteristic salivary protein patterns discriminating the various feeding traits in mammals.

In this experiment saliva samples of four grazing species were compared using 1D gel electrophoresis in order to describe common salivary proteins as well as distinct differences in the protein patterns. The mean protein concentrations of bovine, camel, goat and gelada saliva are presented in Table 5.1.

The lowest protein concentration was observed in bovine saliva, while gelada baboons presented saliva secretions very rich in protein. Camels and goats showed intermediate salivary protein concentrations.

Protein bands in 1D gel electrophoresis were visualised using Coomassie R250 staining. Protein masses ranged from 140 kD to 10 kD showing distinct species-specific patterns with differences not only in the range of the molecular weights, but also in the number of protein bands. Bovine and goat saliva consisted of 10 to 14 visible protein

Table 5.1. Mean salivary protein concentrations of four herbivorous mammal species. From Mau et al. (2009).

Species	Feeding trait	Digestive tract	n	Protein (mg/ml)
Bos primigenius f. *taurus*	grazer	foregut-fermenting	4	1.18 ± 0.202
Camelus ferus f. *bactrianus*	intermediate	foregut-fermenting	3	3.20 ± 0.929
Capra aegagrus f. *hircus*	intermediate	foregut-fermenting	11	3.20 ± 2.294
Theropithecus gelada	grazer	hindgut-fermenting	13	6.71 ± 2.693

bands, whereas camel saliva showed 12 distinct bands. Gelada saliva samples, on the other hand, contained up to 29 protein bands.

Although both species are grass-eaters, cattle and geladas had very distinct salivary protein patterns, with geladas having up to three times more protein bands in their saliva. This difference is apparently linked to the evolutionary origins of the two species, with the gelada baboon being a primate and the cattle an artiodactyl. Interestingly, in all four species there were two prominent proteins at 42 kD and 29 kD, identifying again as CA II and VI by immunoblotting as well as in mass spectrometry.

Carbonic anhydrases had been described before as important enzymes that guarantee oral homeostasis. With the help of the established immunoblot for CA II and CA VI, we detected CA II in saliva of different mammals such as cattle, goat, camel and gelada baboons. In comparison, human saliva only contained very small quantities of CA II. As a primarily cytoplasmatic enzyme, the CA II in ruminant saliva might be actively secreted by apocrine secretion from parotid glands as described earlier in cattle by Stolte & Ito (1996).

The presence of two highly active CA isoenzymes (CA II and CA VI) in ruminant saliva might indicate a co-operation of the two in regulating oral pH, thus maintaining constant conditions for the fermentation of grass matter in the rumen. Based on their proposed function, a decline in either CA II or CA VI salivary concentrations could result in impaired digestion or might increase the risk for acidosis in ruminants. Could CA II in ruminant saliva therefore act as a potential marker for acidosis risk in livestock animals?

Furthermore, CA II concentrations in primate and also human saliva seemed comparably marginal. However, based on our data we postulated a descriptive inverse correlation between salivary CA II concentrations and CA II levels in the brains of various taxa, such as cattle, primates and humans. According to the expensive tissue hypothesis this means, the higher the CA II level in saliva of an animal species, the lower is the CA II level in the brain of the same species and vice versa as published by Mau et al. (2009b). This finding might demonstrate the "evolutionary balance" between two otherwise energetically expensive tissues (here: brain versus gut).

Visualising feeding adaptations in animal saliva

Tannin-binding capacity

Additionally, we performed direct electrophoretic comparisons between gelada saliva and saliva samples of various primate species (*Papio hamadryas*, *Pan troglodytes*, *Homo sapiens sapiens*) with distinct feeding traits. It was hypothesised that gelada baboons might lack certain proteins, not necessary for grass diet. Other primate species were chosen for comparison, because primates are a diverse group with various feeding adaptations.

However, animal nutrition and feeding are only half of the evolutionary story. Many plants "learned" to prevent excessive feeding by the development of certain counter-measures. In grasses one will find so-called phytoliths embedded into the cell structure of the leaves to wear down animal teeth. Many trees and herbs, on the other hand, protect themselves by producing tannins, which precipitate proteins and enzymes, thereby influencing digestion.

Answering to these counter-measures, primates developed different adaptations to circumvent pytoliths or tannins. For example, in adaptation to a highly abrasive grass diet, the gelada baboon developed high-crowned molar teeth. *Papio hamadryas*, a primate species feeding primarily on tannin-rich foliage, possibly developed specialised proline-rich, tannin-binding proteins to bind and to neutralise plant tannins.

In order to investigate, whether such feeding adaptations are present in saliva of hamadryas and gelada baboons, 1D gel electrophoresis of native saliva was performed. The results supported the idea of special salivary proteins, which are characteristic for certain dietary niches in primate species. While saliva of hamadryas baboons showed a rich pink staining with Coomassie – indicative for proline-rich proteins (PRP), which also include the so-called tannin-binding proteins (TBP) – these bands were completely absent in gelada saliva (Mau et al., 2009a; Fig. 5.2). Protein identification as hamadryas baboon PRP and TBP was subsequently managed using mass spectrometry (Mau et al., 2011).

The absence of salivary TBP is common in grazing mammals, such as gelada baboons and cattle. Therefore it seems that the lack of TBP demonstrates a direct adaptation of a species to a diet based on grasses, which do not contain tannins. Diet obviously influences saliva composition.

In order to further test this interaction hypothesis, we analysed the amylase protein expression in primate saliva. Earlier studies have shown that the expression and activity of salivary amylase in primates is correlated with the level of starch present in a certain diet.

We compared amylase protein expression in saliva samples of humans, gelada baboons, hamadryas baboons and additionally in a few chimpanzee samples. The two baboon species showed a higher expression level than the hominid primate species or humans (Mau et al., 2010a).

Chimpanzees primarily feed on fruits, which are poor in starch. On the other hand, baboons and geladas seasonally refer to seeds and plant roots that are naturally rich in starch, which explains the different expression levels of salivary amylase.

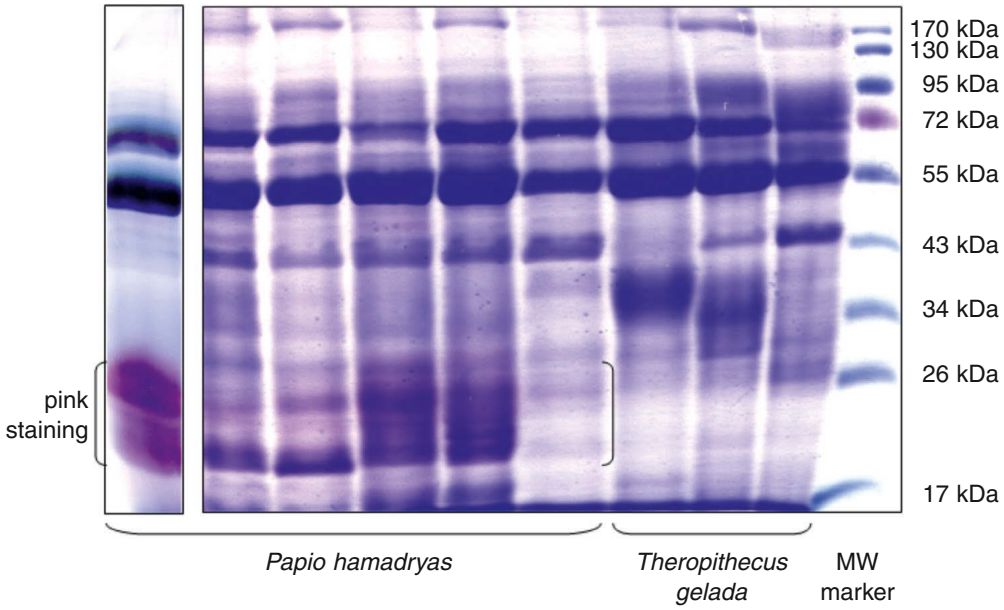

Fig. 5.2. Salivary protein patterns of hamadryas baboons (*Papio hamadryas*) und gelada baboons (*Theropithecus gelada*) stained with Coomassie R250. The original protocol to identify proline-rich proteins (PRP) in saliva was published by Beeley et al. (1991). All potential PRP shown as pink bands in hamadryas saliva. Gelada saliva totally lacks the PRP as demonstrated by the absence of any pink staining protein bands. The protein group of proline-rich proteins amongst others includes the tannin-binding proteins (TBP) to neutralise dietary tannins. From Mau et al. (2009a).

Table 5.2: Use of mass spectrometry to identify proteins from bovine saliva that bound to phytoliths, bovine dental enamel or dentin. From Mau et al. (2013).

Protein name	Matching species	Protein accession no. (NCBI)	Total protein score	Peptides matched in MS/MS	Molecular weight [kDa]	Sequence coverage [%]
Lactoperoxidase	*Bos taurus*	P80025	171	DSLQKVSFSR EQINAVTSFLDASLVYGSEPSLASR FGHMEVPSTVSR IIKDGGIDPLVR IVGYLDEEGVLDQNR TPDNIDIWIGGNAEPMVER	80.59	13
carbonic anhydrase VI (CA-VI)	*Bos taurus*	P18915	175	DLDIQDMLPGDLR DYAENTYYSNFISHLEDIR QGEFPMTNNGHTVQISLPSSMR VVEANFVSHPHQEYTLGSK YGSYEEAQNEPDGLAVLAALVEVK	36.98	30
BSP30	*Bos taurus*	P79124	260	GLGTFDSTIEIIQNLK KLIGEPQVTTQQEI LDLNVDLQTSVSIETDAETGDSR LIGEPQVTTQQEI RLISGLER RPGLLNDVVDFGVNLVR	26.37	33
odorant-binding protein (OBP)	*Bos taurus*	P07435	555	AQEEEAEQNLSELSGPWR ATKQDDTYVADYEGQNVFK ELVFDDEKGTVDFYFSVK LNVEDEDLEK LNVEDEDLEKFNK NVVNFLENEDHPHPE QDDGTYVADYEGQNVFK THLVAHNINVDK TVYIGSTNPEK	18.49	67
Haemoglobin β	*Bos taurus*	P02070	354	AAVTAFWGK EFTPVLQADFQK FFESFGDLSTADAVMNNPK LLVVYPWTQR NFGKEFTPVLQADFQK VKVDEVGGEALGR VLDSFSNGMK VVAGVANALAHR	15.94	61

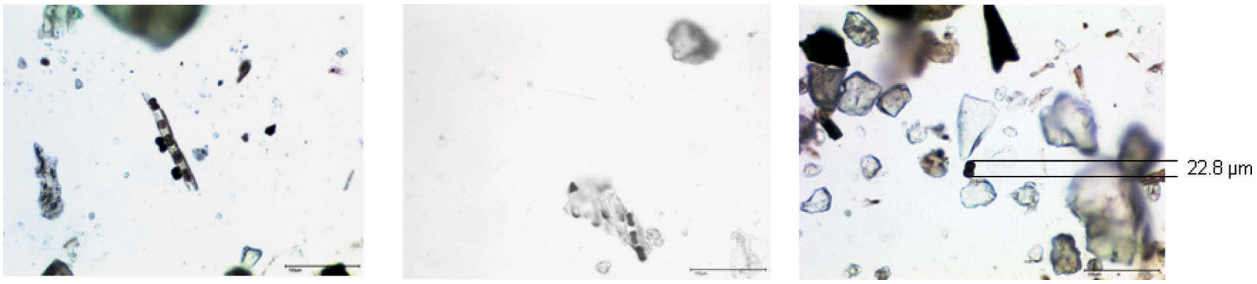

Fig. 5.3. Microscopic evidence for the presence of phytoliths in grass preparations. **A, B,** showing the characteristic bilobate short cell-form of phytolith; **C,** a single phytolith in comparison to the irregular appearance of environmental silicates. The mean length of the phytoliths prepared was 22.8 µm. Scale bar = 100 µm. From Mau et al. (2013).

From saliva proteins to feeding guidelines

As a practical outcome of our research, we developed new feeding guidelines for captive gelada baboons that are bred in accordance with the worldwide gelada breeding programme in zoological gardens. Saliva was collected from various zoo animals to determine certain dietary adaptations on enzymatic and physiological levels that might change our current understanding of animal nutrition and the feeding in zoo environments. The interest was primarily focused on species with specialized nutritional needs or fermentation strategies such as geladas, chimpanzees and camels.

The work produced highly valuable data especially on geladas, which, despite their adaptation to grass diet (= main food source), revealed to have the enzymatic precondition (namely salivary amylase) to use starch-rich food items very effectively, especially in seasons, when fresh grass is lacking in the wild. Therefore, in order to prevent obesity in captive geladas, the reduction of starchy food in their daily rations is mandatory (Mau & Johann 2011a).

Accessory salivary proteins

Furthermore, distinct salivary proteins were hypothesized to prevent enamel abrasion and adverse chemical effects on teeth of herbivorous mammals. To detect potential candidates for protecting proteins, whole bovine and goat saliva was incubated with dental enamel or glass powder. Salivary proteins, bound to dental enamel and glass, were analysed by 1D gel electrophoresis. Two salivary proteins were found to adhere to bovine enamel and were isolated by MS/MS mass spectrometry and subsequently identified by protein sequencing. Both the bovine odorant-binding protein (bOBP) and the CA VI were present in these protein layers.

The bovine CA VI adhering to teeth may serve as a local pH-regulator to protect bovine enamel and dentin from bacterial acids. The bOBP on the other hand may bind odorant particles right in the oral cavity and thus could enhance their transmission to the vomeronasal organ (VNO), as for example during flehmen. The signals of the VNO are important determinants in sexual behaviour of mammals. These results suggested that salivary proteins may support olfaction and protect enamel of ruminants from mechanical and chemical destruction as postulated by Mau et al. (2006).

Furthermore, in a pilot study phytoliths from common feeding plants were isolated for in vitro incubation with bovine saliva samples. One gram of commercially available grass pellets was incinerated according to the method originally published by Parr et al. (2001). Acid-insoluble ash contained microscopic environmental grit (dust and soil particles) and biogenic, dumbbell-shaped phytoliths (medium length: 22.8 µm; Fig. 5.3). To detect potential candidates for silicate-binding proteins, bovine whole saliva was then incubated with isolated grass-derived phytoliths and silicates. Interactions of salivary proteins with pulverized bovine dental enamel and dentin were additionally analysed. After intense washing, the powder fractions were loaded onto 1D-polyacrylamide gels, most prominent adhesive protein bands were cut out and proteins were identified by mass spectrometry within three independent replicates (Table 5.2). All materials were mainly interacting with bovine OBP, BSP-30 and CA VI. The phytolith/silicate fraction showed additionally stronger interactions with haemoglobin β and lactoperoxidase (Mau et al., 2013).

Conclusions

Saliva sampling is a cost-effective and non-invasive procedure, the use of which is both practical and attractive in livestock research and also in a zoo environment. We demonstrated that saliva proteins might fulfil a great number of different tasks in oral homeostasis and may further protect teeth from abrasion in correlation to distinct feeding adaptations. In the future, the emerging field of salivary research will give birth to new applications of saliva as a tool to detect biomarkers of physiology and diseases, not only in animal husbandry. It might further help to develop therapeutic methods which regulate pH-levels and/or abrasion at the occlusal surface.

Acknowledgments

We thank the reviewers Marcus Clauss (Zurich) and Carsten Staszyk (Gießen) for their constructive review comments and Aditya Kombra for checking the English.

References

Beeley, J. A., Sweeney, D., Lindsay, J. C. B., Buchanan, M. L., Sarna, L. & Khoo, K. S. (1991): Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human parotid salivary proteins. Electrophoresis 12: 1032–1041.

Mau, M. & Johann, A. (2011): Saliva research in zoos – An aid for appropriate feeding of nutritional specialists. Zoologischer Garten N.F. 80: 262–270. [Article in German]

Mau, M., De Almeida, A. M., Coelho, A. V. & Südekum, K.-H. (2011): First identification of tannin binding proteins in saliva of *Papio hamadryas* using MS/MS mass spectrometry. American Journal of Primatology 73: 896–902.

Mau, M., Kaiser, T. M. & Südekum, K.-H. (2009): Evidence for the presence of carbonic anhydrase 29-kDa-isoenzyme in salivary secretions of three ruminating species and the gelada baboon. Archives of Oral Biology 54: 354–360.

Mau, M., Kaiser, T. M. & Südekum, K.-H. (2010): Carbonic anhydrase II is secreted from bovine parotid glands. Histology and Histopathology 25: 321–329.

Mau, M., Kaiser, T. M. & Südekum, K.-H. (2013): Pilot study on binding of bovine salivary proteins to grit silicates and plant phytoliths. Zoological Research 34 (E3): E87–E92.

Mau, M., Müller, C., Langbein, J., Rehfeldt, C., Hildebrandt, J. P. & Kaiser, T. M. (2006): Adhesion of bovine and goat salivary proteins to dental enamel and silicate. Archives of Animal Breeding 49: 439–446.

Mau, M., Südekum, K.-H., Johann, A., Sliwa, A. & Kaiser, T. M. (2009a): Saliva of the graminivorous *Theropithecus gelada* lacks proline-rich proteins and tannin-binding capacity. American Journal of Primatology 71: 663–669.

Mau, M., Südekum, K.-H., Johann, A., Sliwa, A. & Kaiser, T. M. (2010a): Indication of higher salivary α-amylase expression in hamadryas baboons and geladas compared to chimpanzees and humans. Journal of Medical Primatology. https://doi:10.1111/j.1600-0684.2010.00407.x

Mau, M., Südekum, K.-H. & Kaiser, T. M. (2009b): Why cattle feed much and humans think much – new approach to confirm the expensive tissue hypothesis by molecular data. Bioscience Hypotheses 2: 205–208.

Parr, J. F., Lentfer, C. J. & Boyd, W. E. (2001): A comparative analysis of wet and dry ashing techniques for the extraction of phytoliths from plant material. Journal of Archaeological Science: Reports 28: 875–886.

Stolte, M. & Ito, S. (1996): A comparative ultrastructural study of the parotid gland acinar cells of nine wild ruminant species (Mammalia, Artiodactyla). European Journal of Morphology 34: 79–85.