

Howler Monkeys (*Alouatta palliata mexicana*) Produce Tannin-Binding Salivary Proteins

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Abstract Dietary tannins are ubiquitous in woody plants and may have serious negative effects on herbivores by inducing a loss of dietary protein and producing toxins if they are hydrolyzed in the gut. Many herbivorous mammals counter the negative effects of tannins through tannin-binding salivary proteins (TBSPs) that inactivate tannins by forming insoluble complexes and prevent them from interacting with other more valuable proteins. Howlers are the most folivorous New World primates and ingest foods with varying tannin content. We studied the presence of TBSPs in six wild mantled howlers (*Alouatta palliata mexicana*) immediately after capture and in captivity when fed on two diets composed of natural ingredients: a mixture of fruit and leaves or only leaves. Protein concentration was determined in whole saliva samples, followed by gel electrophoresis. We identified two protein bands

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of 17 and 25 kDa that have tannin-binding capacity. Although the monkeys ate almost twice as much condensed tannins in the leaf diet than in the fruits and leaves diet (7 vs. 4 g/d dry matter) the salivary protein concentration did not differ between the two diets (leaf diet: $3.29 \pm \text{SE } 0.82$ vs. fruit and leaves diet: $3.42 \pm \text{SE } 0.62$ mg/ml) and we found no additional protein bands in response to either diet. We suggest that the continuous expression of TBSPs is part of a dietary strategy that enables howlers to consume diets with variable tannin contents, thus partly explaining their dietary flexibility. Although the importance of salivary proteins to arboreal primates is broadly accepted, to our knowledge this is the first report of TBSPs in any Neotropical primate.

Keywords Condensed tannins · Mantled howler monkeys · Plant secondary metabolites · Saliva · SDS-PAGE · Tannins

Introduction

Plant secondary metabolites influence how folivorous primates select their diets (Glander 1982; Welker *et al.* 2007). Among these, tannins are perhaps the most widespread group, occurring in most parts of most woody plants (Waterman and Mole 1994). There are two main subgroups of tannins: condensed (not readily susceptible to degradation) and hydrolyzable tannins (susceptible to acid, base, or enzyme-catalyzed hydrolysis) (Hagerman 2011). Hydrolysis of tannins in the mammalian digestive tract releases phenolic acids that may be locally toxic to the gut microbiota and may damage the gastrointestinal mucosa and epithelium (McLean and Duncan 2006; Meiser *et al.* 2000). These products of hydrolysis may also be absorbed from the intestine and cause systemic harm (Niho *et al.* 2001).

Researchers attribute the defensive role of tannins against herbivorous vertebrates to the tannins' ability to precipitate proteins and to inhibit gastrointestinal enzymes, thereby reducing the digestibility of dietary proteins (Austin *et al.* 1989; McArthur *et al.* 1995; Robbins *et al.* 1987). This loss of protein may lead to a loss of body condition, reduced fecundity, and slow offspring growth in arboreal animals, e.g., the common brushtail possum (*Trichosurus vulpecula*: DeGabriel *et al.* 2009). Feeding tannins to experimental animals may depress growth and cause weight loss (North American pika (*Ochotona princeps*: Dearing 1997), rats (*Rattus norvegicus*: Smith *et al.* 2001), red jungle fowl (*Gallus gallus*: Featherstone and Rogler 1975), and Japanese wood mouse (*Apodemus speciosus*: Shimada *et al.* 2006).

Mammalian herbivores adopt various behavioral and physiological counter-measures against dietary tannins, including simple avoidance (Fashing *et al.* 2007; Takemoto 2003), microbial degradation of tannins in the gut (Shimada *et al.* 2006), activating detoxifying enzymes (Guengerich 2004; Hoffmann and Kroemer 2004), and the modification of saliva composition or flow rate (Fickel *et al.* 1998; Lamy *et al.* 2010b; Shimada *et al.* 2011). Saliva contains a complex mixture of proteins with different biological roles in digestion, host defense, and lubrication, and their plasticity represents a rapid mechanism that allows animals to adapt to dietary changes, for example, to tannin-rich diets (da Costa *et al.* 2008). Some mammals that frequently ingest tannins produce tannin-binding salivary proteins (TBSPs). As their name suggests, these proteins

bind tannins and the resultant tannin–salivary protein complex passes through the gut to the feces (Austin *et al.* 1989; Skopec *et al.* 2004). In addition, the major amino acids that comprise the principal TBSPs are nonessential in mammals. This means that the animal sacrifices nonessential amino acids from TBSPs (low-quality protein) to protect the essential amino acids in high-quality dietary or endogenous proteins (Clauss *et al.* 2005; McArthur *et al.* 1995; Mehansho *et al.* 1987).

Proline-rich proteins, in which proline constitutes 20–40% of the protein, are the prevalent type of TBSPs and the first line of defense against tannins in some mammals because they readily bind them (Shimada 2006). Since the first identification of tannin-binding proline-rich proteins in human saliva (Mandel *et al.* 1965), researchers have discovered these proteins in 23 of the 34 species of mammal studied, including rodents, lagomorphs, and marsupials (Beeley *et al.* 1991; Bennick 2002; Boze *et al.* 2010; Mau *et al.* 2009; McArthur *et al.* 1995; Mehansho *et al.* 1985; Shimada *et al.* 2006). Reports also describe these proteins in other nonhuman primates: crab-eating macaque (*Macaca fascicularis*: Ann and Lin 1993; Bennick 2002; Oppenheim *et al.* 1979; Sabatini *et al.* 1989; Yan and Bennick 1995) and hamdrys baboon (*Papio hamadryas*: Mau *et al.* 2009, 2011).

Some animals can produce proline-rich salivary proteins in response to dietary change. For instance, some rodents can adjust to dietary tannins and produce proline-rich proteins 3–12 days after exposure to tannin-rich diets (Asquith *et al.* 1985; Mehansho 1992; Mehansho *et al.* 1985). Because the production of TBSPs incurs a metabolic cost (Clauss *et al.* 2005), such induction would benefit an animal whose natural diet varies in tannin content. This often occurs when diets vary seasonally, as in rodents such as *Apodemus* spp. (Shimada *et al.* 2011), brown howlers (*Alouatta guariba*: Leitão *et al.* 1999), and saki monkeys (*Chiropotes* spp.: Norconk 1996).

Of the nine species of howler (*Alouatta* spp.) found in Central and South America, the mantled howler, *Alouatta palliata*, has the widest distribution, from southeast Mexico to the north east of Peru (Cortés-Ortiz *et al.* 2015). These arboreal primates are also the most folivorous Neotropical monkeys and occur in a variety of habitats (Baumgarten and Williamson 2007). They have an extremely flexible diet (Crockett 1998), feeding on a high proportion of foliage, while their physiological traits determine their capacity to cope with diets with variable concentrations of nutrients and plant secondary metabolites, including tannins (Glander 1978, 1981; Milton 1978). Several authors report that the favored food species of howlers contain high levels of condensed tannins (Leitao *et al.* 1999; Milton 1979; Righini 2014). These findings, however, should be treated with caution because the results are mostly qualitative.

This study had two aims. The first was to investigate whether wild mantled howlers produce TBSPs. In view of their nutritional ecology of howlers, we predicted that they would do so as a first line of defense against tannins. Our second aim was to compare the total protein concentration and the protein profile of whole saliva from captive monkeys fed on two natural diets differing in their concentrations of tannins. The first diet consisted of fruits and leaves while the second was exclusively leaves. We also determined the daily intake of condensed tannins by howlers on each diet. We predicted that saliva obtained from monkeys fed the leaf diet would 1) have a higher total protein concentration and 2) show more protein bands with tannin-binding capacity than saliva from monkeys fed on fruit and leaves because leaves contain more tannins.

Methods

Subjects

We captured a group of six wild howlers, four females (three adults and one subadult, aged by the smoother and rounder face and a small and closed vulva according to Domingo Balcells and Veà Baró 2009) and two adult males. A field veterinarian anesthetized the monkeys with ketamine hydrochloride (8 mg/kg estimated body mass, Ketaset, Fort Dodge Animal Health, Fort Dodge, IA). Immediately after the capture, we placed the monkeys in individual cages (3 × 2 × 2.5 m) supplied with tree trunks for climbing to encourage normal behavior and fed them with a mixed diet of fruit and leaves from trees of the neighboring area. These enclosures were located in a protected natural area in Catemaco, Veracruz, Mexico (Espinosa Gómez *et al.* 2013). At the end of the quarantine period, we released the group of howlers into a protected private natural area in San Juan Evangelista, Veracruz, Mexico.

Diets and Determination of Condensed Tannin Intake

We fed the howlers wild plants that form part of their natural diet in the areas near the field station. The fruits and leaves diet consisted of young leaves, mature leaves, petioles, and sprouts of *Ficus maxima*, *Ficus aurea* (both family Moraceae) and *Bursera simaruba* (Burseraceae) and ripe fruits of *Spondias radlkoferi* (Anacardiaceae) and *Manilkara zapota* (Sapotaceae). The leaf diet consisted of young leaves, mature leaves, petioles and sprouts of *F. maxima*, *F. aurea*, *B. simaruba*, and *Cecropia obtusifolia* (Cecropiaceae). We offered the monkeys the fruit and leaves diet in the first trial to encourage them to feed (Espinosa Gómez *et al.* 2013).

We accustomed the monkeys to their experimental diets for 8 days before starting a 7-day trial period to determine their intake of dry matter and thus of condensed tannins. We collected foods early in the morning and offered each monkey equal amounts of each dietary item at 07:00 h, in excess of its normal intake. At the same time we dried a subsample of each plant species to constant mass at 50°C for analysis. The following morning, we removed and weighed the uneaten food before offering the new food. We dried a subsample of rejected food from each plant species to constant mass at 50°C; we adjusted for the loss of mass over the 24-h period by weighing representative samples of each plant species (Espinosa Gómez *et al.* 2013). We analyzed subsamples of food to determine the nutrient composition of the diet (methods are described in Espinosa Gómez *et al.* 2013). We calculated the dry matter (DM) and tannin intake (g/d) by subtracting the total DM refused from the DM offered for each plant species. The composition of the fruit and leaf diet (% DM) was 27.5% DM, 8.1% crude protein, 19.3% soluble sugars, 32.7% acid detergent fiber, and 1.4% condensed tannins. The leaf diet consisted of 25.4% DM, 11.4% crude protein, 6.5% soluble sugars, 39.8% acid detergent fiber, and 2.4% condensed tannins.

Saliva Samples

We collected saliva samples from the six howlers on three occasions: sample 1 was taken immediately after capture and was considered to come from free ranging howlers.

We collected samples 2 and 3 on day 8 of each feeding trial. We did not feed the monkeys for *ca.* 10 h (all night) before saliva collection to prevent the contamination of saliva by food. In the early morning (*ca.* 06:00 h) on the morning of saliva collection, a veterinarian sedated the monkeys with ketamine hydrochloride (4 mg/kg body mass of Ketaset). Using a micropipette we collected a sample (1.0–1.5 ml) of whole saliva (secretion mixture of all salivary glands) directly from the mouth of each monkey. We placed the sample in a tube, froze it immediately in liquid nitrogen, and stored it at -80°C until analysis. Occasionally we induced saliva production by placing 0.05 ml of lemon juice on the individual's tongue. All individuals recovered consciousness almost immediately after sampling when we offered them the appropriate diet. There were no changes in feeding behavior with this protocol.

Protein Quantification

Before protein quantification, we centrifuged saliva samples at 16,000 *g* for 10 min at 4°C to remove particles (Lamy *et al.* 2010b). We used only the supernatant fraction for further analyses. We determined the total protein concentration using a modification of the Lowry method (Hartree 1972), using bovine serum albumin as a standard. We measured the absorbance at 650 nm with a microtiter plate reader (SpectroMAX 340, Molecular Devices, Union City, CA).

Gel Electrophoresis

We analyzed salivary proteins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Briefly stated, we mixed saliva (30 μl) with 10 μl of 4X-loading buffer (0.125 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 20% glycerol with traces of bromophenol blue), and then incubated the mixture in a boiling water bath for 5 min to denature the proteins. We loaded and separated the samples on 12% SDS gels using a Mini-Protean III Cell apparatus (Bio-Rad, Hercules, CA) with a running buffer [0.03 M Tris, 0.144 M glycine, 0.1% (w/v) SDS, pH 8.3 (Bio-Rad 161-0772, Hercules, CA)]. We ran molecular mass markers (Precision Plus Protein Dual Color Standards, Bio-Rad 1610374, Hercules, CA) in each gel to calibrate the molecular masses of the salivary proteins.

After electrophoresis we fixed the proteins in the gels with a mixture of 26% ethanol, 14% formaldehyde, and 60% water for 1 h, followed by 1 h in a mixture of 50% methanol and 12% acetic acid (Steck *et al.* 1980). The formaldehyde fixation step is essential to retain small proteins in the gel throughout the staining procedure (Austin *et al.* 1989). We stained the protein bands overnight with a solution of 0.1% Coomassie Brilliant Blue R250 (Bio-Rad 1610400, Hercules, CA) in 40% (v/v) methanol and 10% (v/v) acetic acid. Finally, we destained the gels for 2–3 days with several changes of 10% acetic acid to detect proline-rich proteins, according to Beeley *et al.* (1991).

Assay for Tannin-Binding Proteins

We examined all saliva samples for proteins with tannin-binding capacity by mixing samples of whole saliva (30 μl) with 10 μl of a tannic acid solution (0.5 $\mu\text{g}/\mu\text{l}$; Sigma-Aldrich, St. Louis, MO) prepared in 50% methanol. We then incubated the mixture

with continuous shaking for 2 h at 4°C followed by centrifugation at 800 g for 10 min at 4°C. We separated the resulting pellets and supernatants and then separated their proteins in SDS-PAGE. We mixed control samples with 10 µl of 50% methanol without tannic acid. In the presence of tannic acid, tannin-binding proteins do not enter the gel or have altered electrophoretic mobility (Austin *et al.* 1989). We used samples of whole saliva without purification or concentration to get a realistic measure of the tannin-binding capacity of the proteins in the whole saliva of howlers; the assay is based on the property of proteins to unspecifically bind to tannins and do not consider ion concentration, degree of glycosylation of the protein, or pH values.

Statistical Analysis

We compared the total protein concentration in the saliva of monkeys eating the experimental diets and the condensed tannin intake (g/d DM) using a paired *t*-test (paired by individual) in R 3.0.2 for Windows (www.r-project.org). We did not include female 4 in this analysis because we did not have sufficient saliva from this individual.

Ethical Note

This research followed all institutional guidelines of the Universidad Veracruzana and the ethical and legal requirements of Mexican laws (Diario Oficial de la Federación 1999). The government of Mexico approved our protocols (permit SEMARNAT SGPA/DGVS/02315/07). We followed the capture and handling techniques described by Rodríguez-Luna *et al.* (1993) and we maintained the individuals in captivity following IUCN guidelines (IUCN 1998). Because the monkeys were being translocated, we conducted the study during the 90-day quarantine stage required by the Mexican authorities. We endeavored, during both the translocation and the study, to monitor the individuals closely while minimizing human contact.

Results

Protein Profile and Tannin-Binding Proteins in Howler Saliva

The mean total protein concentration (mg/ml) in saliva collected from free-ranging howlers was $5.9 \pm \text{SE } 1.04$. We found polymorphic patterns in salivary proteins ranging from 10 to 150 kDa. We identified 16 major protein bands that occurred in the saliva of all howlers, although detection limits prevented us from identifying proteins smaller than 10 kDa (Bennick 1982). We found two protein bands that might be proline-rich proteins (Fig. 1) according to their pink-violet staining with Coomassie R250 and their apparent molecular masses (17 and 25 kDa), which are within the molecular weight range reported for proline-rich proteins in humans and other mammals (10–45 kDa) (Mau *et al.* 2009; Shimada 2006).

After the tannin-binding assay, we identified two protein bands with tannin-binding capacity that occurred in all saliva samples. In these samples a reddish-white protein

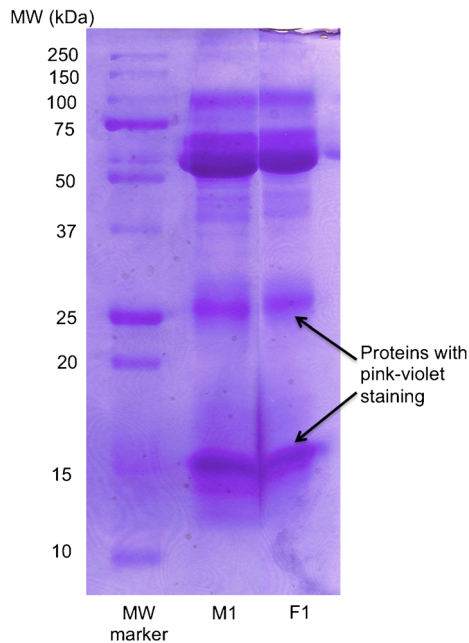


Fig. 1 Electrophoretic profiles of the salivary proteins of two wild mantled howlers from samples collected immediately after capture. Molecular weights (MW) of protein markers are shown in kDa on the left. M1 = male 1; F1 = female 1. The saliva shows strong protein bands between 10 and 150 kDa. Proteins with apparent MW of 17 and 25 kDa (indicated by arrows) might be salivary proline-rich proteins according to Beeley *et al.* (1991) by pink-violet staining with Coomassie R250.

precipitate formed a few minutes after addition of tannic acid. On SDS-PAGE gels, the pellet fractions showed two major protein bands with apparent molecular masses of 17 and 25 kDa that precipitated by incubation with the tannin (Fig. 2, line 2), which were either absent or less obvious in the supernatant fractions (Fig. 2, line 1). This suggested that they bound to tannin.

Comparison of Salivary Protein Traits After Feeding Trials

The monkeys ate similar amounts of dry matter from both diets (range 275.4–455.8 g/d), but ingested almost twice as much as condensed tannin when eating the leaf diet compared with the fruits and leaves diet (20.8 vs. 11.9 mg condensed tannin per g DM/d) (Table I). Despite these differing amounts of dietary tannin, we found no differences between diets in the total salivary protein ($t = 0.90$, $P = 0.60$; Table II). We found similar protein patterns, and 16 protein bands, in all saliva samples: those corresponding to wild individuals and those collected during the feeding trials (Fig. 3).

Discussion

Our key finding was the discovery of TBSPs in wild mantled howlers, which, to our knowledge, is the first description of such salivary proteins in any Neotropical primate. All saliva samples obtained from howlers, including those obtained immediately after

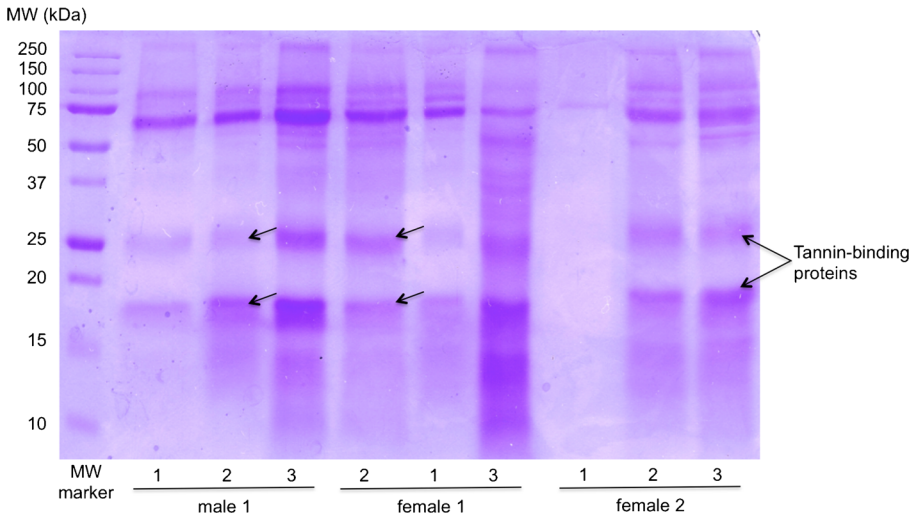


Fig. 2 The supernatant (line 1) and pellet (line 2) fractions of whole saliva collected from mantled howlers fed on a leaf diet. M1 = male 1; F1 = female 1; F2 = female 2. The pellet fraction shows TBSPs (marked with arrows) with molecular weights of 17 and 25 kDa that precipitated by incubation with a tannic acid solution, indicating tannin-binding capacity. These bands are weaker or absent in the supernatant fraction. The control (line 3), saliva sample mixed with 50% methanol and no tannin, shows the typical pattern.

capture and those taken during the feeding experiments, showed strong protein bands of 17 and 25 kDa that precipitated in the tannin-binding assay. We may have underestimated the number of such proteins, for two reasons. First, there may also have been smaller proteins (<10 kDa) present that we could not identify because of analytical limitations in our SDS-PAGE gels (Bennick 2002). Second, using standards that represent a wider variety of tannins than the tannic acid we used may have identified more proteins with affinity to tannins (Hagerman and Robbins 1987).

The metachromatic patterns (pink-violet protein bands) and the molecular masses of the TBSPs we describe suggest that they are proline rich. For example, basic proline-rich proteins identified on MW 15–45 kDa (Boze *et al.* 2010) occur in evolutionarily diverse animal species such as nonhuman primates, rodents, lagomorphs, and marsupials (Shimada *et al.* 2006). Azen and Maeda (1988) classified the multigene family of proline-rich proteins as acidic (calcium binding, inhibit crystal growth, and involved in dental pellicle), basic (interact with plant tannins), and glycosylated (oral lubrication and binding oral bacteria). In human saliva, the three classes of proline-rich proteins produce different bands on electrophoretic gels (Austin *et al.* 1989). The core-glycosylated group shows bands of 69 kDa and *ca.* 50 kDa, the acid type between 27 and 39 kDa, and the basic proteins show several bands (37–43 kDa, 27–31 kDa, 24 kDa, and 14–22 kDa). Other reports define smaller proline-rich proteins: 5 kDa for humans (Kauffman *et al.* 1991; Robinson *et al.* 1989) and up to 25 kDa for rats (Muenzer *et al.* 1979). Further work will determine which of these classifications are appropriate for the howler proteins.

Howlers have large salivary glands, particularly the parotids (Hill 1972), suggesting that one of their main functions is the production of large volumes of fluid to neutralize plant secondary metabolites (Milton 1998). We tested for TBSPs in mixed (whole)

Table I Mean dry matter and condensed tannin intake of experimental diets fed to *Alouatta palliata mexicana* over 7-day trial

Subject	Fruits ^a and leaves ^b diet (1.4% condensed tannins)		Only leaves ^b diet (2.4% condensed tannins)	
	Dry matter intake (g/d)	Condensed tannin intake (g/d)	Dry matter intake (g/d)	Condensed tannin intake (g/d)
Male 1	340.6	3.7	366.8	7.5
Male 2	419.4	5.3	455.8	8.9
Female 1	324.2	3.3	316.8	7.1
Female 2	275.4	3.6	274.7	5.7
Female 3	302.9	4.1	312.2	7.2
Female 4	353.2	4.1	290.7	5.4
Mean	335.9	4.0	336.2	7.0
Standard error	20.1	0.3	27.1	0.5
Paired <i>t</i> -test on dry matter intake	$t = 0.01; P = 0.98$			
Paired <i>t</i> -test on condensed tannin intake	$t = 6.99; P = 0.0009$			

Diets were from plant species that have previously been reported as diet items of howlers.

^a Ripe fruits.

^b Leaves include the petiole, young and mature leaves, and leaf buds.

saliva rather than parotid saliva for two reasons: first, because it is whole saliva that is present during mastication (Fickel *et al.* 1999), and second, because the collection of parotid saliva is highly invasive, requiring either death of the animal or surgical or endoscopic methods. In other animals, the parotid glands are mainly responsible for the

Table II Concentration of total proteins (mg/ml) in the saliva collected from mantled howlers (*Alouatta palliata mexicana*) at capture (free-ranging sample) and when fed two natural diets with different condensed tannin concentrations

Individual	Free-ranging sample	Fruits ^a and leaves ^b diet (1.4% condensed tannins)	Only leaves ^b diet (2.4% condensed tannins)
Male 1	9.72	5.7	5.72
Male 2	5.7	4.78	4.8
Female 1	4.66	3.25	1.48
Female 2	5.87	2.02	2.5
Female 3	3.56	1.86	1.99
Female 4	No sample	2.92	No sample
Mean ± SE	5.9 ± 1.04	3.42 ± 0.62	3.29 ± 0.82

Comparisons of salivary proteins were made between the two experimental diets.

^a Ripe fruits.

^b Leaves include the petiole, young and mature leaves, and leaf buds.

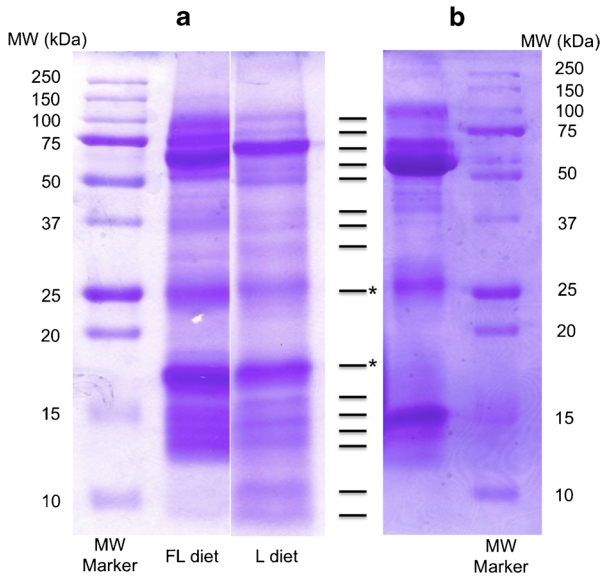


Fig. 3 Gel electrophoresis of whole saliva from mantled howlers. **(a)** The same individual feeding on a diet of fruits and leaves (FL, 1.4% condensed tannins on a dry matter basis) or only leaves (L, 2.4% CT DM). **(b)** A wild animal. Despite the dietary differences, all samples showed a similar pattern of 16 protein bands (marked by lines on the right side of panel). We identified proteins indicated by asterisks as tannin-binding salivary proteins. We used Coomassie R250 staining to visualize the proteins. Molecular weights (MW) are in kDa.

secretion of proline-rich TBSPs (Mehansho *et al.* 1985), and parotid saliva bound almost twice as much tannin as did mixed saliva in roe deer (*Capreolus capreolus l.*) (Fickel *et al.* 1998), suggesting that the enlarged parotid glands in howlers may produce the TBSPs.

McArthur *et al.* (1991) proposed a model to predict the production of TBSPs based on feeding niche. They proposed that animals that frequently encounter tannins in their diets, such as mule deer (*Odocoileus hemionus*), consistently produce TBSPs as part of normal salivary proteins (Robbins *et al.* 1987). In contrast, other animals that do not or that rarely encounter tannins, such as sheep (*Ovis aries*), domestic dogs (*Canis familiaris*), or cats (*Felis catus*), do not produce these proteins under any circumstances, even after feeding them high levels of tannins (Ammar *et al.* 2011; Austin *et al.* 1989; Lamy *et al.* 2010b; Mole *et al.* 1990). Further, as the production of TBSPs presents a metabolic cost, some mammals may regulate their expression. For example, some rodents such as rats and mice (*Mus musculus*) produce TBSPs only after 3–12 days of exposure to a high intake of tannins (Lamy *et al.* 2010a; Mehansho *et al.* 1983, 1985). TBSP secretion either constitutively or in a more regulated manner confers advantages to the mammal that depends on seasonal concentration of tannins in the diet (Clauss *et al.* 2005). According to this model, we predicted that the production of TBSPs in mantled howlers would vary with the tannin content of the diet. However, we found that although monkeys on the leaf diet consumed almost twice the amount of tannins as they did when eating the leaves and fruits diet, the salivary protein concentration did not differ and we found no additional protein bands on the SDS-PAGE gels. This contrasts with findings that tannin-rich diets cause overexpression of proline-rich proteins and other proteins, increasing the total protein content in the saliva of other

animals (da Costa *et al.* 2008; Gho *et al.* 2007), and that black rhinoceros (*Diceros bicornis*) increases salivary protein concentration and tannin-binding capacity when fed on diets supplemented with 11 mg of tannin per g of ingested dry matter (Clauss *et al.* 2005). The likely explanation for this difference is that both dietary concentrations of tannins were relatively low and that any responses occur at much higher concentrations of tannin. The highest total salivary protein concentrations occurred in samples collected immediately after capture, suggesting that wild monkeys may have been eating a diet more concentrated in tannins, as suggested by qualitative (Aristizábal Borja 2013; Glander 1982; Milton 1979; Righini 2014) and quantitative (Leitao *et al.* 1999) results for different species of howlers. We may also have overestimated tannin intake by using quebracho as a standard (Rothman *et al.* 2009). Further studies should determine the content of condensed tannins in foods by using internal standards (tannins purified from the plant species of interest) (Rothman *et al.* 2009).

Mantled howlers are generalist herbivores that presumably select their food by balancing nutritional demands with avoidance of plant secondary metabolites (specific presence/absence of condensed tannins and alkaloids) (Glander 1982; Milton 1981). Some researchers suggest that howlers avoid trees containing condensed tannins, arguing that detoxification is nutritionally demanding and that animals might best avoid foods that are metabolically costly (Glander 1981; Milton 1998). Tannins, however, are ubiquitous and our findings suggest that howlers may use a different strategy. By producing TBSPs they can ingest tannin-rich foods provided the nutritional benefits outweigh the cost of producing the salivary tannin-binding proteins. This helps to explain the dietary flexibility of these primates. Further studies should identify the types of TBSPs, their amino acid composition and functions, and details of any other proteins present in the saliva of the howlers (Lamy *et al.* 2010b).

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