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RESEARCH ARTICLE

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Salivary tannin-binding proteins are a pervasive strategy used by the folivorous/frugivorous black howler monkey

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Dietary tannins can affect protein digestion and absorption, be toxic, and influence food selection by being astringent and bitter tasting. Animals that usually ingest tannins may regularly secrete tannin-binding salivary proteins (TBSPs) to counteract the negative effects of tannins or TBSPs production can be induced by a tannin-rich diet. In the wild, many primates regularly eat a diet that contains tannin-rich leaves and unripe fruit and it has been speculated that they have the physiological ability to cope with dietary tannins; however, details of their strategy remains unclear. Our research details the salivary protein composition of wild and zoo-living black howler monkeys (*Alouatta pigra*) feeding on natural versus manufactured low-tannin diets, and examines differences in TBSPs, mainly proline-rich proteins (PRPs), to determine whether production of these proteins is dependent on the tannin content of their food. We measured the pH, flow rate, and concentration of total protein and trichloroacetic acid soluble proteins (an index of PRPs) in saliva. Howler monkeys produced slightly alkaline saliva that may aid in the binding interaction between tannin and salivary proteins. We used gel electrophoresis to describe the salivary protein profile and this analysis along with a tannin-binding assay allowed us to detect several TBSPs in all individuals. We found no differences in the characteristics of saliva between wild and zoo-living monkeys. Our results suggest that black howler monkeys always secrete TBSPs even when fed on foods low in tannins. This strategy of constantly using this salivary anti-tannin defense enables them to obtain nutrients from plants that sometimes contain high levels of tannins and may help immediately to overcome the astringent sensation of their food allowing howler monkeys to eat tanniferous plants.

KEYWORDS
anti-tannin defense, howler monkeys, physiologic strategy, primate feeding adaptation, salivary proteins

1 | INTRODUCTION

Almost all plants contain chemical defenses that play a variety of ecological roles in defense against herbivores and pathogens. Among them, tannins have historically received a great deal of attention; these polyphenolic compounds deter herbivore feeding through two principal effects: (1) making food unpalatable due their astringent and bitter taste (Horne, Hayes, & Lawles, 2002), and b) binding dietary proteins and digestive enzymes reducing food digestibility (Austin, Suchar, Robbins, & Hagerman, 1989; Moore, Andrew, Külheim, & Foley, 2014; Robbins, Hanley et al., 1987).

Although tannins may be present in most of the foods of herbivorous primates (Glander, 1982), we know little about how and in what extent these compounds impact primate feeding behavior. For many years researchers have explored the relationship between food choice and the concentration of tannins (both condensed and hydrolysable) of wild leaf-eating primates; however, results remain conflicting and conclusions unclear in many cases. Field investigations in Colobines (which are the most extensively studied leaf-eating primates with respect to the chemical content of their food) have shown that tannins inhibit feeding in several species including black and white colobus *Colobus guereza* (Oates, Swain, & Zantovska, 1977), black colobus *Colobus satanas* (McKey, Glasgow, Gartlan, Waterman, & Choo, 1981), and olive colobus *Procolobus verus* (Oates, 1988); on the other hand, tannins seems had little inhibitory effect on food selection of other Colobines including red colobus *Procolobus badius* (Chapman & Chapman, 2002), Tana River red colobus *Procolobus badius rufomitratus* (McKey et al., 1981), and the Asian colobine *Presbytis rubicunda* (Davies, Bennett, & Waterman, 1988).

Similar findings have been presented for apes, for example, Reynolds, Plumptre, Greenham, and Harborne (1998) found that the highly frugivorous chimpanzees (*Pan troglodytes*) did not select foods according their tannin levels and appeared to tolerate high concentrations of tannins. Also, mountain gorillas (*Gorilla beringei*) are not deterred by condensed tannins and include foods with high concentrations in their diet, as high as 20% on dry matter basis (Rothman, Dusinberre, & Pell, 2009). It is clear that there are species of primates that avoid tannins, and species that tolerate them (Ganzhorn, 1989); such behavioral differences are related with a dynamic and complex relationship between nutrients, chemical characteristics of tannins, and the physiological traits of animals for neutralizing them (Ganzhorn, 1989; Glander, 1982; Robbins, Mole, Hagerman, & Hanley, 1987).

One physiological adaptation to deal with tannins is to produce tannin-binding salivary proteins (TBSPs) (Robbins, Hanley et al., 1987). These proteins precipitate tannins that interfere with their binding to other more valuable proteins (Mehansho, Gutler, & Carslon, 1987), and minimize the astringent sensation of food (Dinnella, Recchia, Fia, Bertuccioli, & Monteleone, 2009; Horne et al., 2002). Salivary proline-rich proteins (PRPs) are the first line of defense against dietary tannins because they are the prevalent type of TBSPs, and readily bind tannins (Shimada, 2006). These proteins are ubiquitous in animals that regularly consume large amounts of tannins (Bennick, 2002). In some animals PRPs production may be up-regulated when the animals are consuming a diet with high tannin concentrations, but this up-regulation may require a few days (e.g., rodents can start producing PRPs only 3 days after eating tannin-rich foods (Mehansho, Asquith, Butler, Rogler, & Carlson, 1992). Macaques (genus *Macaca*), for example, continuously secrete PRPs in their saliva crab-eating macaque *Macaca fascicularis*, Oppenheim, Kousvelari, and Troxler (1979); rhesus monkey *Macaca mulata*, Sabatini, Warner, Saitoh, and Azen (1989); stump-tailed macaque *Macaca arctoides*, Schlesinger and Levine (1989), which may help them to show an immediate tolerance of bitter/astringent taste of potential foods. It has been shown that Barbary macaques (*Macaca sylvanus*) spend considerable annual feeding time eating leaves with high concentration of tannins (Hanya et al., 2011). On the other hand, the gelada baboon (*Theropithecus gelada*), a graminivorous species with a very restrictive range and narrow dietary spectrum (Beehner, Berhanu, Bergman, and McCann, 2007) lacks PRPs with tannin-binding capacity, which would be related with their diet largely restricted to monocots plants that lack tannins (Mau, Südekum, Johann, Sliwa, and Kaiser, 2009).

Besides PRPs, other salivary proteins have been described in several animal species as TBSPs such as histatins, mucins, sIgA, amylase, statherins, and cystatins (Mau, de Almeida, Coelho, and Südekum, 2011; Nayak & Carpenter, 2008; Perez-Gregorio, Mateus, and De Freitas, 2014; Sabatini et al., 1989; Shimada, 2006). Unfortunately, although it has been speculated that probably most primates produce TBSPs in saliva (Milton, 1999; Remis & Kerr, 2002; Rothman et al., 2009), only few primate species have been evaluated for such salivary proteins (Table 1) and mostly their presence has been inferred from identifying genes that encoded them.

Howler monkeys (genus *Alouatta*) are generalist herbivorous and have the most widespread geographical distribution of any New World primate; these monkeys regularly eat a diet that may contains tannin-rich leaves and unripe fruit (Garber, Righini, & Kowalevsky, 2015) which has allowed researchers to speculate about their ability to cope with dietary tannins. Some argue that tannins affect howler's feeding behavior mainly discouraging feeding (Glander, 1982; Welker, König, Pietsch, & Adams, 2007). Other studies have failed to demonstrate a consistent relationship between concentrations of tannins and food selection (Milton, 1979; Estrada, 1984). Milton (1979) identified food quality (protein to fiber ratio) over tannin concentration as the primary factor influencing howler's food choice and she recognized that physiology features appear to play an important role in their ability to cope with dietary tannins (Milton, 1998).

Recently it has been shown that mantled howler monkeys (*Alouatta palliata mexicana*) produce TBSPs (Espinosa-Gómez et al., 2015) when fed on natural diets, and that these monkeys maintain the same salivary total protein concentration across a diet varying considerably in condensed tannins (7 vs. 4 g/d dry matter). This suggests that TBSPs are not up-regulated for dietary tannin concentration, but are produced continuously. To increase understanding about howler dietary strategy, it was important to test whether production of TBSPs differs among howler monkey species and to determine if this anti-tannin defense is pervasive (e.g., the tannin-binding proteins are always present in the saliva) or if it is dependent on tannin concentration in diet.

TABLE 1 Salivary proteins identified in several primates species, related to anti-tannin defense

Species	Salivary proteins	Continuously produced	Up-regulated	Feeding strategy	References
Human (*Homo sapiens*)	PRPs Histatins Statherins Cystatins	Yes Yes - -	Yes No - -	Omnivorous	Oppenheim et al. (1979); Perez-Gregorio et al. (2014); Sabatini et al. (1989)
Crab-eating macaque (*Macaca fascicularis*)	PRPs Histatins Statherins	Yes Yes	- -	Omnivorous	Bennick (2002); Oppenheim et al. (1979)
Rhesus monkey (*Macaca mulata*)	PRPs Histatins Statherins Cystatins	Yes Yes - -	- - - -	Omnivorous	Sabatini et al. (1989)
Stump-tailed macaque (*Macaca arctoides*)	PRPs Statherins[a]	Yes Yes	- -	Omnivorous/mainly frugivorous	Schlesinger et al. (1989)
Golden snub-nosed monkey (*Rhinopithecus roxellana*)	Cystatins[a]	-	-	Folivorous-frugivorous	Zhou et al. (2014)
Gorilla (*Gorilla gorilla*)	Statherins[a,b] Cystatins[a,b]	- -	- -	Herbivorous	Protein knowledgebase (UniProtKB, 2017)
Northern white-cheek gibbon (*Nomascus leucogenys*)	Statherins[a,b] Cystatins[a,b]	-	-	Frugivorous	Protein knowledgebase (UniProtKB, 2017)
Chimpanzee (*Pan troglodytes*)	Statherins[a,b] Cystatins[a,b]	- -	- -	Omnivorous/mainly ripe frugivorous	Protein knowledgebase (UniProtKB, 2017)
Bonobo (*Pan paniscus*)	Cystatins[a,b]	-	-	Omnivorous/mainly frugivorous	Protein knowledgebase (UniProtKB, 2017)
Orangutan (*Pongo abeii*)	Statherins[a,b] Cystatins[a,b]	- -	- -	Frugivorous	Protein knowledgebase (UniProtKB, 2017)
Silvered leaf monkey (*Trachypithecus cristatus*)	Statherins[a,b]	-	-	Folivorous	Protein knowledgebase (UniProtKB, 2017)
Green monkey (*Chlorocebus aethiops*)	Statherins[a,b] Histatins[a,b]	-	-	Omnivorous	Protein knowledgebase (UniProtKB, 2017)
Hamadryas baboon (*Papio hamadryas*)	PRPs Cystatin Amylase	Yes Yes Yes	- -	Omnivorous	Mau et al. (2011)
Olive baboon (*Papio anubis*)	Cystatins[a,b]	-	-	Omnivorous	Protein knowledgebase (UniProtKB, 2017)
Squirrel monkey (*Saimiri boliviensis*)	Cystatins[a,b]	-	-	Insectivorous-frugivorous,	Protein knowledgebase (UniProtKB, 2017)
Marmoset (*Callithrix jacchus*)	Cystatins[a,b]	-	-	Exudativorous-insectivorous	Protein knowledgebase (UniProtKB, 2017)
Mantled howler monkey (*Alouatta palliata mexicana*)	Probably PRPs	-	No	Folivorous-frugivorous	Espinosa-Gómez et al. (2015)
Black howler monkey (*Alouatta pigra*)	Probably PRPs	Yes	No	Folivorous-frugivorous	This study

[a]Data obtained by identification of genes that encoded those proteins.
[b]Preliminary data.

This research describes the salivary protein patterns of black howler monkeys (*Alouatta pigra*) with different diets: zoo-living individuals fed a low-tannin diet, and free-ranging monkeys feeding on their natural diet that was determined to include tannin-rich food items. The black howler monkey's diet is characterized by a mix of fruit and leaves, but they experience periods when either fruit or leaves are the predominant dietary component (Pavelka & Knopff, 2004); correspondingly, the levels of condensed tannin varies (Righini, Garber, & Rothman, 2017). If TBSPs, specifically PRPs are identified in saliva of monkeys feeding on diets with high and low tannin concentration, this would suggest that PRPs production is pervasive in howler monkeys. This pattern may explain an instantaneous flexibility to eat tannin-rich foods that does not require transition periods between diets of varying tannin content. By contrast, differences in

PRPs concentration in saliva of wild and zoo monkeys may suggest that an up-regulation by dietary tannins is required, which would benefit howlers during seasonal variations of dietary tannin concentrations because the continuous production of TBSPs may cause losses of endogen nitrogen (Skopec, Hagerman, & Karasov, 2004).

Building on this, our research on black howler monkeys had two objectives: (1) describe the protein profile, identify TBSPs, and quantify PRPs in whole saliva from free-ranging black howlers, in which the condensed tannin content of their diet was determined, and (2) examine whether there are differences in salivary protein profile of zoo-living monkeys eating a diet low in tannin.

2 | METHODS

2.1 | Composition and tannin content in diet of wild monkeys

A group of howler monkeys (two adult males, three adult females, one juvenile) in a 2.2 ha fragment near Balancan, Mexico (17°44′05″N; 91° 30′17″W) was studied in October 2014 and focal observations of adults were made over 8 continuous days to determine their diet and to calculate the tannin content of their food (Table 2). We collected samples of each food eaten by the monkeys that comprise more than 1% of their feeding time to determine their condensed tannin content. Samples were obtained from feeding trees on the day the group ate them; when the group fed from more than one tree of the same species, we sampled from each tree. Samples were dried in the field and then oven-dried (<45 °C) to constant weight. We analyzed samples of leaves and fruits of six plant species and all analysis were made in duplicate; a seventh species was not analyzed due to failure in its drying process. Tannin analysis was done in the Faculty of Veterinary Medicine of the Universidad Autonoma of Yucatan (FMVZ-UADY). Dried food was ground through a 1 mm sieve in a mill (Knifetec, FOSS Analytical, Hillerød, Denmark). We determined dry matter by drying 2 g samples at 110 °C for 6 hr. Samples were analyzed for CT with the Vanillin Assay (Price, Van Scoyoc, & Butler, 1978) using catequin as a standard. However, we recognize that using commercial standards as "catequin" instead "internal standards" may overestimate the tannin concentration in samples, because that commercial tannin may not be similar to those in plants sampled (Rothman et al., 2009). The group had a diet with leaves and fruits of seven plant species and the average CT of the analyzed foods was 6.3 ± SD 6.7 mg/g dry matter (catequin equivalent, Table 2).

2.2 | Zoo-living individuals and diet

Saliva samples of *Alouatta pigra* were supplied from two Mexican zoos. Samples were obtained from Zacango Zoological Garden in February 2015, one adult female (4.2 kg body weight) and one adult male (6.2 kg) and from Chapultepec Zoological Garden in December 2015 (two adult females and one adult male; mean body weight 7.8 ± SD 1.4 kg). In both zoos, diets were constant for the 2 months before saliva sampling, including ripe banana and fresh cultivated vegetables (lucerne, celery, squash, chayote, spinach, lettuce, green beans, carrot) and were supplemented with mini-biscuits for leaf-eating primates (Mazuri, Purina Mills, LLC, Arden Hills, MN). We were unable to analyze the CT in zoo diets, but cultivated foods typically have little tannins (e.g., legumes used usually have less of 30 mg/kg fresh weight) (King & Young, 1999); ripe bananas have low concentration of tannins (Von Loesecke, 1950).

2.3 | Chemical immobilization of study subjects

In the field two adult males and two adult females (mean body weight 7.13 ± SD 1.9 kg) were darted and immobilized with ketamine hydrochloride (8 mg/kg estimated body mass, Ketaset, Fort Dodge Animal Health, IA, Overland Park, KS) by a veterinarian experienced in immobilization of wild animals following established protocol to minimize stress and physical injury (Rodríguez-Luna, García-Orduña, & Canales-Espinosa, 1993). We used ketamine because problems with respiratory and circulatory depression are low (Green, Knight, Precious, & Simpkin, 1981) and it does not cause excessive muscular relaxation so it is possible to reach the anesthetized monkey that is fastened to a tree branch by its prehensile tail by climbing the tree. Once monkeys were sedated we determined their body weight before saliva sampling.

Zoo-living howler monkeys were anesthetized (Ketaset, Fort Dodge Animal Health, 6 mg/kg), by the zoo's veterinarian as part of their annual medical health survey. This research complied with the guidelines of the IUCN (1998) and of the Mexican authorities (Diario Oficial de la Federación, 1999), as well as the American Society of Primatologists Principles for the Ethical Treatment of Primates and techniques adhered to the guidelines of Zacango and Chapultepec Zoo. All research protocols reported here were reviewed and approved by the government of Mexico (SEMARNAT SGPA/DGVS/10426/14).

2.4 | Saliva collection and total protein quantification

We collected the saliva samples immediately after the animal stabilized subsequent to sedation using identical methods in the field and zoos. Before saliva collection, salivary pH was recorded using pH-indicator strips (109502 Merck-Milipore Darmstadt, Germany). An intramuscular administration (upper limb) of the parasympathomimetic compound pilocarpine-hydrochloride (5 mg/kg BW) stimulated saliva flow (Da Costa et al., 2008) and in the first 10 min 5.2 ± SD 2.5 ml of saliva was collected ($N = 9$). The saliva flow rate (ml/min) and the relative saliva secretion (ml min^{-1} kg^{-1} body weight) were calculated from the volume of saliva collected during these 10 min. We collected whole saliva (e.g., secretion from all salivary glands) directly from the mouth using a micropipette and placed it in a tube. The saliva was immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Prior to protein quantification, frozen saliva was thawed and then it was centrifuged at 16,000 g for 10 min at 4 °C to remove particulate matter. We used only the supernatant for analyses. We determined the salivary total protein concentration by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. Absorbance was measured at 595 nm with a microtiter plate reader (SpectroMAX 340, Molecular Devices, Union City, CA).

TABLE 2 Diet composition and condensed tannin concentration in plant species consumed by wild black howler monkeys *Alouatta pigra* ($N = 4$) during the 8 consecutive days before the saliva sampling

Plant specie/family	Item	Diet contribution (% feeding time)	Condensed tannin (mg/g dry matter)[a]
Maclura tinctoria/Moraceae[b]	MF	14	0.6
	IF	20.5	0.9
	YL	7.8	0.8
Inga edulis/Fabaceae[b]	YL	10.5	15.9
	ML	3.7	19.9
	IF	9	17.7
Cellobium lanceolatum/Leguminoseae	MF	5.8	4.1
	YL	9.6	2.0
Ficus sp./Moraceae[b]	MF	2.0	3.4
	IF	4.4	5
	YL	1.9	4.9
Enterolobium cyclocarpum/Mimosaceae	YL	5.6	2.3
Tabebuia rosea/Bignoniaceae	YL	1.9	No analyzed
	ML	1.2	No analyzed
Psidium guajaba/Myrtaceae[b]	IF	1.6	5.5

ML, mature leaves; YL, young leaves; MF, mature fruits; IF, immature fruits.
[a]CT were determined with the Vanillin method (Price et al., 1978) using catequin as standard. Data are shown as mg/g dry matter (catequin equivalent).
[b]Fruit items were analyzed complete, including seeds.

2.5 | Protein profile and identification of TBSPs by gel electrophoresis

We analyzed the salivary proteins using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following Laemmli (1970). We mixed the whole saliva (calculated volumes containing approx. 30 μg of total protein with SDS loading buffer 4:1 (0.125 M Tris–HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 20% glycerol with traces of bromophenol blue). We then incubated the mixture in a boiling water bath (5 min) to denature the proteins before cooling the mixture to room temperature. We separated the proteins on 12% SDS gels over 2 hr at 75 V using a Mini-Protean III Cell apparatus (Biorad, Hercules, CA) with a running buffer (0.03 M Tris, 0.144 M glycine, 0.1% [w/v] SDS, pH 8.3). Molecular mass markers (Precision Plus Protein Dual Color Standards, BioRad 1610374) were run in each gel to calibrate the molecular masses of the salivary proteins. Detection limits meant that we were unable to identify salivary proteins smaller than 10 kDa. Following electrophoresis, we fixed the proteins in the gels with a mixture of 26% ethanol, 14% formaldehyde, and 60% water for 3 hr, followed by 3 hr in a mixture of 50% methanol and 12% acetic acid (Steck, Leuthard, & Bürk, 1980). To detect PRPs protein bands, we used a PRP-specific stain/de-stain procedure (Beeley et al., 1991); gels were stained overnight with a 0.25% Coomassie brilliant blue R-250 solution (Biorad 1610400) in 40% (v/v) methanol and 10% (v/v) acetic acid. Finally, we de-stained the gels with several changes of 10% acetic acid; under these conditions it is assumed that only PRPs stained pink or pink-violet, unlike other non proline-rich proteins that stained blue.

We identified TBSPs using tannic acid (hydrolysable tannin) in the tannin-binding assay; this compound binds readily PRPs in rodents (Glendinning, 1992) and it is used commonly as standard to show the tannin-binding affinity of salivary proteins (Austin et al., 1989; Mau et al., 2009; Ventura-Cordero, Sandoval-Castro, Torres-Acosta, & Capetillo-Leal, 2017). We mixed samples of whole saliva (30 μl) with 10 μl of a tannic acid solution (0.5 and 2.5 μg/μl; Sigma–Aldrich, St Louis, MO) prepared in 50% methanol and then incubated and mixed the sample by continuously shaking for 6 hr at 4 °C (Austin et al., 1989). Samples were centrifuged at 800*g* for 10 min at 4 °C and we separated the resulting pellets and supernatants and ran them in SDS-PAGE, including control samples mixed with 10 μl of 50% methanol without tannic acid. In the presence of tannic acid, tannin-binding proteins were precipitated.

2.6 | Estimation of PRP concentration

Once we identified PRPs in the saliva, we measured their concentration (mg/ml). PRPs were extracted from the monkeys' saliva as trichloroacetic acid (TCA) soluble proteins (Austin et al., 1989; Mole, Butler, & Iason, 1990; Robbins, Mole et al., 1987; Shimada, Saitoh, Sasaki, Nishitani, & Osawa, 2006), since PRPs are soluble in TCA, but other salivary proteins are typically not (Muenzer, Bildstein, Gleason, & Carlson, 1979). We mixed 500 μl of saliva from each monkey with an equal volume of 10% TCA. After 20 min of incubation at 4 °C, the TCA-saliva solution was centrifuged at 17,000*g* for 20 min at 4 °C, to partially purify the PRPs by removing the TCA-insoluble material. The supernatant (TCA-soluble material) was collected and adjusted to pH 7 with NaOH, before dialysis against 3–4 changes of deionized water for 20 h at 4 °C using dialysis tubing (D9277 Sigma–Aldrich) with a molecular mass cutoff

of 12–14 kDa (Robbins, Mole et al., 1987). This pore size retains PRPs identified in other primates including mantled howler monkeys *Alouatta palliata* (Espinosa-Gómez et al., 2015), hamadryas baboons *Papio hamadryas* (Mau et al., 2009), and macaques *Macaca fascicularis* (Oppenheim et al., 1979). Following dialysis the TCA-soluble fraction was frozen at −40 °C and then lyophilized. We re-dissolved the dry material in 100 µl of distilled water and measured the protein concentration (proline rich proteins—TCA soluble proteins, mg/ml) by the Bradford method. We ran a tannin-binding assay followed by SDS-PAGE gels to confirm that the TCA-soluble fraction consisted primarily of PRPs and that these proteins have tannin-binding capacity. Finally, we calculated the percentage of total salivary protein retained in TCA-soluble fraction and used this as a secondary index of tannin-binding capacity (Robbins, Mole et al., 1987).

2.7 | Data analysis

We tested for differences in salivary flow rate, relative saliva secretion, total protein concentration, quantity of PRPs, and percent of total salivary protein retained in TCA-soluble fraction between wild and zoo-living monkeys using a Student *t*-test in R 3.0.2 for Windows (www.r-project.org). We present results in the text as mean ± SD.

3 | RESULTS

3.1 | Salivary pH, saliva flow rate, and total protein content

The salivary pH range was 7–8 and all zoo-living individuals had a pH of 8. Wild and zoo-living monkeys had similar salivary flow rates (0.4 ± SD 0.2 and 0.6 ± SD 0.2 ml/min, respectively, $t = -1.09$, $p = 0.31$). The mean relative saliva secretion was 0.08 ± 0.04 ml min^{-1} kg^{-1} BW and there were no differences between wild and zoo-living monkeys (wild, 0.07 ± SD 0.05; zoo, 0.09 ± SD 0.04; $t = -0.92$, $p = 0.40$). Salivary total protein concentration averaged 0.8 ± 0.3 mg/ml, but interestingly, wild individuals tended to produce saliva with a marginally higher protein content (1.1 ± 0.3 vs. 0.6 ± 0.1 mg/ml; Figure 1; $t = 2.60$, $p = 0.06$).

3.2 | Salivary protein profile, PRP identification, and other tannin-binding proteins

In one-dimensional SDS-PAGE gels, the salivary proteins of *A. pigra* showed multiple bands ranging from 10 to 250 kDa. In spite of slight differences between individuals, we identified 13 major protein bands in the saliva from wild and zoo-living individuals. In all individuals we identified a main protein band with an apparent molecular mass between 22 and 30 kDa that stained pink with Coomassie R-250 and thus can be considered a PRP according to Beeley et al. (1991) (Figure 2, brackets). Interestingly, the saliva of monkeys from Zacango Zoo had a pink-violet stain, rather than pink, which may indicate the presence of non-PRP proteins in the same molecular weight. A clear unidentified protein band with apparent molecular mass of 22 kDa

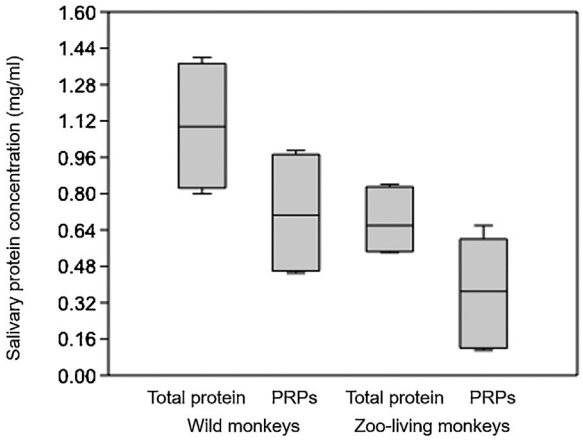

FIGURE 1 Concentration of total protein and PRPs (TCA-soluble proteins) in saliva of wild ($N = 4$) and zoo-living ($N = 5$) black howler monkeys *Alouatta pigra* after stimulation of saliva flow by a parasympathomimetic compound at forest fragment in Balancan, Mexico, and two Mexican Zoological Gardens (Zacango and Chapultepec Zoo)

occurs in saliva collected from a female at Chapultepec Zoo (Figure 2, white arrow). The PRP that we identified coincides with similar sized PRPs of mammals, including primates (10–45 kDa). In both wild and zoo-living animals we found three strong protein bands between 10 and 17 kDa that stain blue.

During the tannin-binding assay a reddish-white protein precipitate appeared in all samples a few minutes after adding tannic acid. The assay confirmed that a precipitating protein band (22–30 kDa) was a PRP because after running the precipitated fraction in SDS-PAGE, it developed a pink color whereas this band was either absent or much less evident in the supernatant (Figure 3, line C marked with brackets). This was less obvious in saliva from zoo-living monkeys. In addition, the three strong protein bands (blue stain) at 10–17 kDa were identified as other TBSPs as they precipitated with tannic acid solution (Figure 3, line C marked with light arrows). Again the precipitates of these three protein bands were clearer in saliva from wild monkeys than in saliva from zoo-living monkeys.

When we compared the salivary proteins on SDS-PAGE gels after incubation with 5 and 25 µg of tannic acid, the TBSPs precipitates were clearer with the higher tannic acid concentration (Figure 4, light arrows). Also, two new protein bands with apparent molecular masses of 37 and 75 kDa showed tannin-binding affinity, being present in the pellet fraction and absent from the supernatant (Figure 4, dark arrows). Again, this suggests that these proteins have tannin-binding capacity.

3.3 | TCA-soluble proteins (PRPs)

The protein concentration of the TCA-soluble fraction averaged 0.52 ± SD 0.1 mg/ml and interestingly the concentration was almost twice as high in the wild monkeys than in the zoo-living monkeys (0.71 ± SD 0.28 vs. 0.36 ± SD 0.24 mg/ml), but there was no significant difference ($t = 1.97$, $p = 0.096$; Figure 1). Of the total

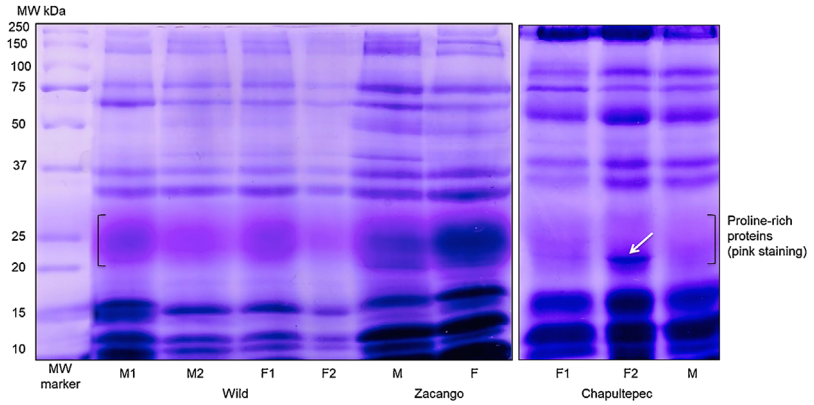

FIGURE 2 Electrophoretic profiles of salivary proteins from four wild black howlers compared with five individuals from two Mexican Zoos. Molecular weights (MW) of protein markers are shown in kDa on the left. Despite dietary differences, we observed similar protein patterns from 10 to 250 kDa. A main protein band between 22 and 30 kDa was identified as PRP according to Beeley et al. (1991) by pink staining with Coomassie-R250 (brackets); in saliva samples from Zacango Zoological Garden this band showed a pink-violet stain rather than the typical pink in most samples. An unidentified protein band with apparent molecular mass of 22 kDa (light arrow) was evident in females from Chapultepec Zoological Garden. M, male; F, female

salivary protein, 55.5 ± SD 7% was TCA-soluble protein and there was no difference between saliva from wild and zoo-living individuals (wild 63.5 ± SD 5.1; zoo 49.17 ± SD 11.7%; $t = 1.12$, $p = 0.31$). Electrophoresis shows that the TCA-soluble fraction of the salivary proteins had several strong pink bands and several weak blue ones, indicating that the TCA-soluble proteins in howler monkeys' saliva must be mainly PRPs.

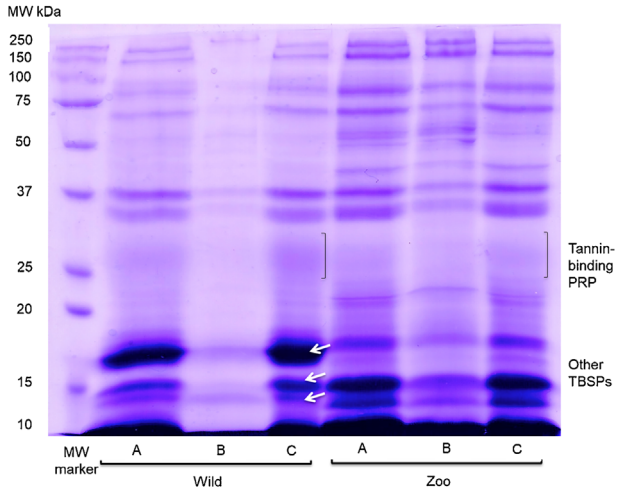

FIGURE 3 Whole saliva of wild and zoo-living black howler monkeys shows the presence of PRPs with tannin-binding capacity. Here we show the SDS-PAGE of samples after a tannin-binding assay. (A) Control, saliva mixed with 50% methanol and no tannin. (B) The supernatant fraction of saliva mixed with tannic acid solution (5 µg in 50% methanol) shows very weak PRP between 22 and 30 kDa. (C) The pellet fraction shows proline-rich proteins (marked with brackets) with a molecular weight between 22 and 30 kDa that precipitated during incubation with acid tannic. Also three strong protein bands between 10 and 17 kDa, were recognized with tannin-binding capacity and identified as other TBSPs (light arrows); their blue staining suggests that they are not proline-rich

4 | DISCUSSION

We documented that black howler monkeys routinely produce TBSPs. It is the presence of these proteins in the saliva of zoo-living monkeys ingesting a low tannin diet that suggests that TBSPs are produced at all times. For many decades researchers have speculated about the ability of howler monkeys to cope with dietary tannins, and this study demonstrate a major physiological adaptation. These results add to our previous findings in mantled howler monkeys (Espinosa-Gómez et al., 2015) who still produce TBSPs when facing a low tannin diet. The relevance of our study is that it shows a salivary anti-tannin defence

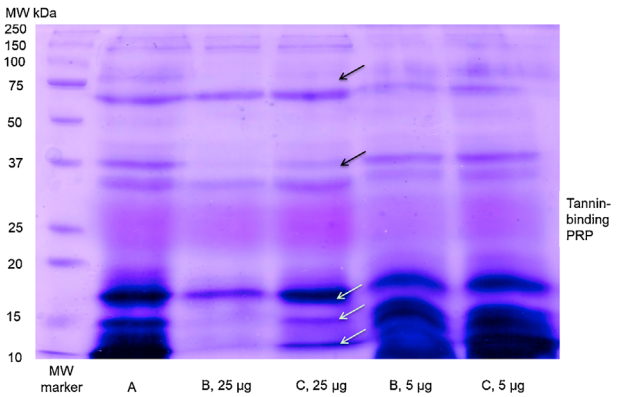

FIGURE 4 Comparison of electrophoretic profile of whole saliva sample from a wild black howler monkey after a tannin-binding assay using a 50% methanol solution with 25 and 5 µg of tannic acid. After the assay with 25 µg of tannin, the interactions between tannic acid and PRPs (at 22–30 kDa), and with other TBSPs at 10–17 kDa (light arrows) were clearer. Also two additional TBSPs at 37 and 75 kDa (dark arrows) were observed. (A) Control, saliva mixed with 50% methanol without tannin shows the typical pattern. (B) The supernatant fraction of saliva shows weak TBSPs bands. (C) The pellet fraction shows proteins that precipitated during incubation with tannic acid, indicating tannin-binding capacity

being always present, which helps howler monkeys to overcome the detrimental effects produced by tannins (astringent taste and digestibility reduction) without requiring transition periods between diets of varying tannin content (Robbins, Hagerman, Austin, McArthur, & Hanley, 1991).

The strategy of routinely producing TBSPs likely facilitates dietary flexibility allowing howler monkeys species to persist in disturbed habitats where they sometimes feed on leaf-based diets and include novel, possibly tannin-rich, food items when available (Chapman, 1987, 1988; Cristobal-Azkarate & Arroyo-Rodriguez, 2007; Glander, 1978). It has been suggested that many herbivores cannot afford to reject bitter and astringent tastants, as this would restrict their diet too greatly (Glendinning, 1994). Besides the role of protecting protein digestibility, TBSPs play a role inhibiting astringency of unpalatable tannin-rich foods (Horne et al., 2002). It has been proposed that the high taste inhibition threshold for tannins observed in captive western lowland gorillas (*Gorilla gorilla g.*) is result of the presence of TBSPs, but it remains unverified (Remis & Kerr, 2002).

Omnivorous hamadryas baboons (*Papio hamadryas*) produce PRPs as indicated by the presence of pink-staining protein band on SDS-PAGE (Mau et al., 2009). These proteins were identified by mass spectrometry as a basic-PRP (Mau et al., 2011). In a similar fashion, using gel electrophoresis and the PRP-specific stain/destain procedure (Beeley et al., 1991), we found a pink-staining protein band at 22–30 kDa with tannin-binding capacity, which we suggest is proline-rich. This method allowed empirical observations to conclude that PRPs stained pink under certain conditions on gels, but confirmation would only be obtained by analysis based on amino acid composition of salivary proteins (Ann Hagerman, personal communication) and proteomics/genomics techniques (Perez-Gregorio et al., 2014; Zhou et al., 2014). We were unable to determine in saliva samples the proportion of proline of total amino acids, as main indicator of PRPs, mainly due to the small quantity of saliva collected. Instead, we calculated the amount of PRPs as TCA-soluble proteins (Shimada et al., 2006).

It has been hypothesized that animals whose natural diet varies in tannin content would benefit from being able to vary the production of TBSPs as necessary (McArthur, Sanson, & Beal, 1995). In some animals TBSPs production are up-regulated by dietary tannin concentration for example, black rhinoceros *Diceros bicornis* (Clauss et al., 2005) and sheep *Ovis aries* (Pech-Cervantes et al., 2016; Vargas-Magaña et al., 2013). Wild monkeys ate food items rich in condensed tannins during the 8 days prior to the saliva sampling, for example, *Inga edulis* (young leaves, 15.9 mg/g DM; unripe fruit, 17.7 mg/g DM, catequin equivalent) and *Psidium guajaba* (unripe fruit, 5.5 mg/g DM, catequin equivalent) and tended to produce saliva with higher concentrations of total protein and of PRPs. Although results might suggest that howler monkeys increase the production of these proteins according to tannin intake, the data obtained are not enough to confirm this and tests involving diets with a wide range of tannin concentrations are needed.

Salivary histatins are another group of well-characterized proteins with high tannin-binding affinity. These small proteins (MW < 5 kDa) have been identified only in saliva of humans (Yan & Bennick, 1995) and crab-eating macaques (Sabatini et al., 1989) and their relevance have been linked to their strong binding capacity to tannic acid and condensed tannins (Yan & Bennick, 1995). On our electrophoresis gels we were unable to identify salivary proteins smaller than 10 kDa, which may influence our results for the identification of other TBSPs as histatins because this it is possible that black howler monkeys secrete these small proteins.

Unfortunately, few reports of TBSPs in primates have been published, and those that are available are on species used in medical research, for example, rhesus monkey (Sabatini et al., 1989), stump-tailed macaque (Schlesinger et al., 1989), and crab-eating macaque (Bennick, 2002; Oppenheim et al., 1979; Sabatini et al., 1989; Yan & Bennick, 1995). Most of the information on New World monkeys is preliminary and has been obtained by identification of genes that encoded those proteins (Zhou et al., 2014). Interestingly, in primates with an omnivorous feeding strategy, there are several types of salivary proteins with tannin affinity such as PRPs, histatins, statherins, cystatins, and amylase, and some of them are secreted continuously in humans and macaques (Table 1).

In general, howler monkeys produced slightly alkaline saliva, which suggests a strong buffering capacity to minimize dental demineralization (which occurs at pH ≤ 5.5) caused by eating unripe fruits (Cuozzo et al., 2008). Another advantage of producing alkaline saliva with a pH above 6.5 is that it may facilitate the binding of tannins (McArthur et al., 1995).

Wild and zoo-living howler monkeys showed a similar salivary protein pattern producing PRPs with tannin-binding capacity between 22 and 30 kDa—within the range reported for PRPs from humans and other mammals (10–45 kDa) (Beeley et al., 1991; Bennick, 2002; Espinosa-Gómez et al., 2015; Mau et al., 2009; McArthur et al., 1995; Mehansho, Clements, Sheares, Smith, & Carlson, 1985). The identification of other non-proline-rich TBSPs in both wild and zoo-living individuals indicates that monkeys have a complex defense against tannins. Mole et al. (1990) reported a similar finding in the saliva of white-tailed deer (*Odocoileus virginianus*), in which non-PRPs with a molecular mass exceeding 10 kDa had an affinity to tannins that was six times that of BSA. Adding further to the tannin defenses of our howler monkeys are the larger TBSPs proteins identified when we incubated saliva with more tannin, suggesting that black howler monkeys' saliva has different types of tannin-binding proteins that bind different to tannins.

Interspecific comparison among salivary patterns is difficult because of differences in the analysis. Animals may be sedated or not, and when they are sedated different compounds, such as xylazine, zolazepam, tiletamine, and ketamine, may be used. The latter compound is known to cause hyper-salivation, but there is wide variation in response between species and between individuals (Green et al., 1981). In this study wild and zoo-living howler monkeys were sedated with different doses of ketamine (wild 8 vs. captive 6 mg/kg BW) and although the dose is low, zoo-living individuals produced more saliva. Therefore, it is difficult to predict the degree to which the anesthesia affected our results. In black bears (*Ursus americanus*),

tiletamine-zolazepam sedation did not affect saliva composition (Robbins, Hanley et al., 1987). Frequently saliva secretion has to be stimulated by administration of parasympathomimetic compounds, such as pilocarpine, carbachol (Beal, 1989; Shimada et al., 2006), or acetylcholine (McArthur et al., 1995), which vary in doses and administration route and such difference may affect salivary patterns. We stimulated salivary flow by using pilocarpine because it better stimulates parotid glands and it is a standard for most studies on TBSPs secretion (Da Costa et al., 2008; Fickel et al., 1998; Lamy et al., 2010; McArthur et al., 1995; Mole et al., 1990; Muenzer et al., 1979).

Although comparisons have to be carefully made, howler monkeys showed a salivary flow rate lower (0.51 ± 0.2 ml/min) than that measured for several ruminant species in which pilocarpine (6 mg/per animal by intra-glandular dose) stimulated saliva secretion (sheep *Ovis ammon*, 6.3 ± 1.6 ml/min; fallow deer *Cervus dama dama* 6.4 ± 0.9 ml/min; roe deer *Capreolus capreolus* 7.5 ± 0.6 ml/min) (Fickel et al., 1998). Also, saliva production by howler monkeys was lower than calculated for some marsupials forgut fermenters (1.22–7.46 ml/min) and hindgut fermenters (0.88–4.02 ml/min), with animals being sedated using ketamine or pentobarbital and saliva flow stimulated by pilocarpine or acetylcoline (McArthur et al., 1995).

For ruminants, high flow rates probably enhance buffering and provide ingesta with water, proteins, and electrolytes. They also provide a fluid medium for particle separation in the forestomach that is a prerequisite for rumination (Clauss & Hofmann, 2014). Also, the high fluid throughput may enhance microbial yield from the forestomach (Müller et al., 2011). The comparatively low salivary flow rate in our howler monkeys corresponds to a low degree of digesta washing in this and other howler monkey species, as indicated by a low ratio of the mean retention time of particles to that of fluids (0.7–1.2) (Edwards & Ullrey, 1999; Espinosa-Gómez et al., 2013) as compared to a range of 1.1–2.6 in artiodactyl foregut fermenters (Müller et al., 2011). In this respect, howler monkeys are basically similar to any other primate species investigated with ratios of 0.6–1.2 (Müller et al., 2011), and it has been suggested that all primates might be characterized by low saliva production (Müller et al., 2011). However, a high concentration of salivary proteins may compensate the low flow rate and still provide an anti-tannin defense. Surprisingly, the salivary protein concentration we reported for black howler monkeys was several times lower than concentration values reported for protein in saliva of other primates for example, *Alouatta palliata* (5.9 ± 1.04 mg/ml) (Espinosa-Gómez et al., 2015) and *Papio hamadryas* (6.7 ± 2.7 mg/ml) (Mau et al., 2009).

When we compared the percentage of total salivary protein retained in TCA-soluble fraction (a secondary index of tannin-binding capacity of salivary proteins) of black howler monkeys with data available from herbivorous occupying different feeding niches, we can observed that a folivore-frugivore as howler monkeys secrete more PRPs (average 55% from salivary total protein), compared with grazers, such as sheep (26%) and cattle (23%), and similar to browsing deer (45%) (Robbins, Hanley et al., 1987), which may indicate a high tannin-binding capacity (Mole et al., 1990).

In conclusion, our research contribute with valuable data to understand howler monkeys' dietary strategies because we have demonstrated a major adaptation to their arboreal diet that may allow them to eat foods with different levels of dietary tannins. The continuous production of TBSPs in saliva in our study subjects may explain why tannins seem to have little inhibitory effect on food selection in howler monkeys (Milton et al., 1980) due to TBSPs are helping overcome the astringent and bitter taste of tannins. We do not know either the taste inhibition threshold for tannins related to the concentration of TBSPs or whether these proteins binds other plant secondary compounds, but this anti-tannin defense in their saliva may allow howler monkeys to eat a high variety of tanniferous plants like other herbivorous generalist species, helping them to expand their feeding niche.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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