Current Zoology, 2018, 64(1), 63–68
doi: 10.1093/cz/zox012
Advance Access Publication Date: 27 February 2017
Article

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Taste responsiveness to two steviol glycosides in three species of nonhuman primates

Sandra Nicklasson[a], Desirée Sjöström[a], Mats Amundin[a,b], Daniel Roth[c], Laura Teresa Hernandez Salazar[d], and Matthias Laska[a,*]

[a]IFM Biology, Linköping University, Linköping, SE-581 83, [b]Kolmården Wildlife Park, Kolmården, SE-681 92, [c]Borås Zoo, Borås, SE-501 13, Sweden, and [d]Instituto de Neuro-Etologia, Universidad Veracruzana, Xalapa, Veracruz, C.P. 91000, Mexico

*Address correspondence to Matthias Laska. E-mail: malas@ifm.liu.se.

Received on 23 December 2016; accepted on 21 February 2017

Abstract

Primates have been found to differ widely in their taste perception and studies suggest that a co-evolution between plant species bearing a certain taste substance and primate species feeding on these plants may contribute to such between-species differences. Considering that only platyrrhine primates, but not catarrhine or prosimian primates, share an evolutionary history with the neotropical plant *Stevia rebaudiana*, we assessed whether members of these three primate taxa differ in their ability to perceive and/or in their sensitivity to its two quantitatively predominant sweet-tasting substances. We found that not only neotropical black-handed spider monkeys, but also paleotropical black-and-white ruffed lemurs and Western chimpanzees are clearly able to perceive stevioside and rebaudioside A. Using a two-bottle preference test of short duration, we found that *Ateles geoffroyi* preferred concentrations as low as 0.05 mM stevioside and 0.01 mM rebaudioside A over tap water. Taste preference thresholds of *Pan troglodytes* were similar to those of the spider monkeys, with 0.05 mM for stevioside and 0.03 mM for rebaudioside A, whereas *Varecia variegata* was slightly less sensitive with a threshold value of 0.1 mM for both substances. Thus, all three primate species are, similar to human subjects, clearly more sensitive to both steviol glycosides compared to sucrose. Only the spider monkeys displayed concentration-response curves with both stevioside and rebaudioside A which can best be described as an inverted U-shaped function suggesting that *Ateles geoffroyi*, similar to human subjects, may perceive a bitter side taste at higher concentrations of these substances. Taken together, the results of the present study do not support the notion that a co-evolution between plant and primate species may account for between-species differences in taste perception of steviol glycosides.

Key words: taste preference thresholds, stevioside, rebaudioside A, Western chimpanzees, spider monkeys, black-and-white ruffed lemurs

Comparative studies on the sense of taste in primates found marked differences between species in their ability to perceive a given taste substance and in their sensitivity to a given taste substance (Glaser 1986; Laska and Hernandez Salazar 2004; Wielbass et al. 2015). Possible explanations for the observed interspecific differences in the perception of substances described as sweet by humans, for example, include, but are not restricted to: the degree of frugivory (Laska et al. 1996), body mass (Simmen and Hladik 1998), and phylogenetic relatedness (Glaser et al. 1995; Nofre et al. 1996). The latter explanation implies that species that are phylogenetically closely related to each other should share a higher proportion of taste receptors specialized for the detection of certain taste substances compared to species that are less closely related. Both psychophysical tests of primate sweet-taste perception and genetic studies lend some support to this notion: only Old World primates (catarrhines) display taste responses to the sweet-tasting proteins thaumatin and monellin, whereas all prosimians and New World primates (platyrrhines) tested so far are indifferent to these two substances (Glaser et al. 1978). Thaumatin is found in the west African katemfe fruit *Thaumatococcus daniellii*, and monellin in the fruit of the west African serendipity berry *Dioscoreophyllum cumminsii*, suggesting that a co-evolution between these two plant species and Old World primates feeding on their fruits might have occurred which ultimately led to the expression of a taste receptor that is responsive to thaumatin and monellin (Hladik 1993). This notion is supported by genetic studies reporting that the ability of the mammalian T1R2–T1R3 sweet-taste receptor to bind proteins of high molecular weight such as thaumatin and monelllin required considerable modifications of its recognition sites which are likely to have taken place during primate evolution (Temussi 2002; Li et al. 2011).

Stevia rebaudiana is a neotropical plant species belonging to the family Asteraceae and native to Paraguay (Soejarto 2002). Its leaves contain intensely sweet-tasting *ent*-kaurene diterpene glycosides among which two, stevioside and rebaudioside A, have been identified as quantitatively predominant and are commercially interesting as low-calorie sweeteners (DuBois and Prakash 2012). Both substances have been reported to be 100–300 times sweeter than sucrose as perceived by humans although numbers may vary depending on the psychophysical method and reference concentration used (DuBois et al. 1991; Carakostas et al. 2012; Upreti et al. 2012). However, there is consensus that both stevioside and rebaudioside A have a bitter side taste for humans when presented at higher concentrations (Schiffman et al. 1995) which may limit their use as sugar substitutes.

Considering that only platyrrhine primates, but not catarrhine or prosimian primates, share an evolutionary history with *Stevia rebaudiana*, we decided to investigate whether members of these three primate taxa differ in their ability to detect and/or in their sensitivity to the two predominant sweet-tasting substances found in this neotropical plant. It was therefore the aim of the present study to assess the taste responsiveness of a prosimian primate species, the black-and-white ruffed lemur *Varecia variegata variegata*, a platyrrhine primate species, the black-handed spider monkey *Ateles geoffroyi*, and a catarrhine primate species, the Western chimpanzee *Pan troglodytes verus* to the two major steviol glycosides found in *Stevia rebaudiana*. More specifically, we determined taste preference thresholds for stevioside and rebaudioside A, and assessed whether high concentrations of these tastants are rejected by the animals. To this end, we employed a two-bottle preference test of short duration (Richter and Campbell 1940). This method allows to directly measure absolute preferences and largely rules out the influence of postingestive factors on the animals' ingestive behavior.

Materials and Methods

Animals

We assessed the taste responsiveness to two steviol glycosides in two adult female and one adult male Western chimpanzees *Pan troglodytes verus*, four adult female and one adult male black-handed spider monkeys *Ateles geoffroyi*, and three adult male black-and-white ruffed lemurs *Varecia variegata variegata*. The chimpanzees were housed, together with two other individuals, at Borås Zoo, Sweden, in a 261 m^3 indoor exhibit with access to a 560 m^2 outdoor island with natural vegetation. They were 27, 33, and 48 years old at the start of the study. The spider monkeys were housed at the field station of the Universidad Veracruzana, near the town of Catemaco, in the province of Veracruz, Mexico. The animals were housed as part of social groups in outdoor enclosures of 50–200 m^2 that were adjacent to indoor enclosures of 20 m^2. They were 7, 8, 9, 10, and 14 years old at the start of the study. The black-and-white ruffed lemurs were housed at Kolmården Wildlife Park, Sweden, in a 117 m^3 indoor exhibit with access to a 100 m^2 outdoor island with natural vegetation. They were 11, 12, and 20 years old at the start of the study.

With all three species, we performed the tests in a smaller room adjacent to the indoor exhibit which held several compartments in which the animals were tested separately to avoid competition and distraction. All animals were trained to voluntarily enter the test compartments and were completely accustomed to the procedure described below. The animals were fed fresh fruit and vegetables (spider monkeys: one time per day; black-and-white ruffed lemurs: two times per day; chimpanzees: three times per day). The chimpanzees and the black-and-white ruffed lemurs were additionally provided with commercial primate chow pellets and all three species had permanent access to water. Thus, no water deprivation schedule was adopted. The amount of food offered daily to the animals was such that leftovers were still present on the floor the next morning. Thus, it was unlikely that ravenous appetite affected the animals' ingestive behavior.

Taste stimuli

We used the following two steviol glycosides: stevioside (CAS# 57817-89-7), and rebaudioside A (CAS# 58543-16-1). The substances were obtained from Shanghai Xunxin Chemical Co. (Shaoxing, China), and Xinghua Green Biological Preparation Co. (Jiangsu, China), respectively, and were of the highest available purity ($\geq$ 99.5%).

Procedure

We used a two-bottle preference test of short duration (Richter and Campbell 1940). The animals were allowed to drink for 1 min from a pair of simultaneously presented graduated cylinders (of 120 ml volume for the spider monkeys and the black-and-white ruffed lemurs, and of 700 ml volume for the chimpanzees) with metal drinking spouts. We performed four such 1-min trials per day and animal, two of them in the morning, and two in the afternoon.

To determine taste preference thresholds the animals were given the choice between tap water and defined concentrations of a steviol glycoside dissolved in tap water. With both substances, testing started at a concentration of 1 mM and proceeded in the following steps (0.1, 0.01 mM, etc.) until an animal failed to show a significant preference. Subsequently, they were presented with intermediate concentrations (between the lowest concentration that was preferred and the first concentration that was not) in order to determine the preference threshold value more exactly. To maintain the animals' motivation and willingness to cooperate, testing of the different concentrations did not follow a strict order but was pseudo-randomized. This was true for both within a given session (morning or afternoon) or between sessions. We presented each pair of stimuli 10 times per individual animal, and the position of the stimuli was pseudo-randomized in order to counterbalance possible position preferences. Care was taken that an animal sampled both stimuli at least once during each trial. The order in which the two steviol glycosides were tested was the same for all three species: (1) stevioside, (2) rebaudioside A.

Data analysis

For each animal, we recorded the amount of liquid consumed from each bottle, summed it for the 10 trials with a given stimulus combination, converted it to percentages (relative to the total amount of liquid consumed from both bottles), and took 66.7% (i.e., 2/3 of the total amount of liquid consumed) as the criterion of preference. We chose this rather conservative criterion for reasons of comparability of data as the same criterion had been used in previous studies on sweet-taste responsiveness with other primate species (Larsson et al. 2014; Laska 1996, 1997, 2000; Laska et al. 1996, 1998, 1999, 2001; Wielbass et al. 2015), and in order to avoid misinterpretation due to a too liberal criterion. Additionally, we performed binomial tests, and regarded an animal as significantly preferring one of the two stimuli if it reached the criterion of 66.7% and consumed more from the bottle containing the preferred stimulus in at least 8 out of 10 trials (binomial test, $P < 0.05$).

Thus, we defined taste preference threshold as the lowest concentration at which the animals met both criteria mentioned above. Preliminary analyses of the data indicated that there were no systematic differences in choice behavior and liquid consumption between the first and the second presentation of a session, or between the morning and the afternoon session, respectively. Intraindividual variability of the amount of liquid consumed across the ten trials with a given stimulus combination was low and averaged <20%. Thus, a theoretically possible bias in the overall preference score due to excessive drinking in aberrant trials did not occur.

Compliance with ethical standards

The experiments reported here comply with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication no. 86-23, revised 1996) and also with current Swedish and Mexican laws. This study was approved by Gothenburg's Animal Care and Use Committee (Göteborgs djurförsöksetiska nämnd, protocol #75-2016, for the chimpanzees), the Swedish Board of Agriculture (Jordbruksverket, protocol #5.2.19-5974/15, for the black-and-white ruffed lemurs), and the Ethical Board of the Federal Government of Mexico's Secretariat of Environment and Natural Resources (SEMARNAT; Official permits no. 09/GS-2132/05/10, for the spider monkeys).

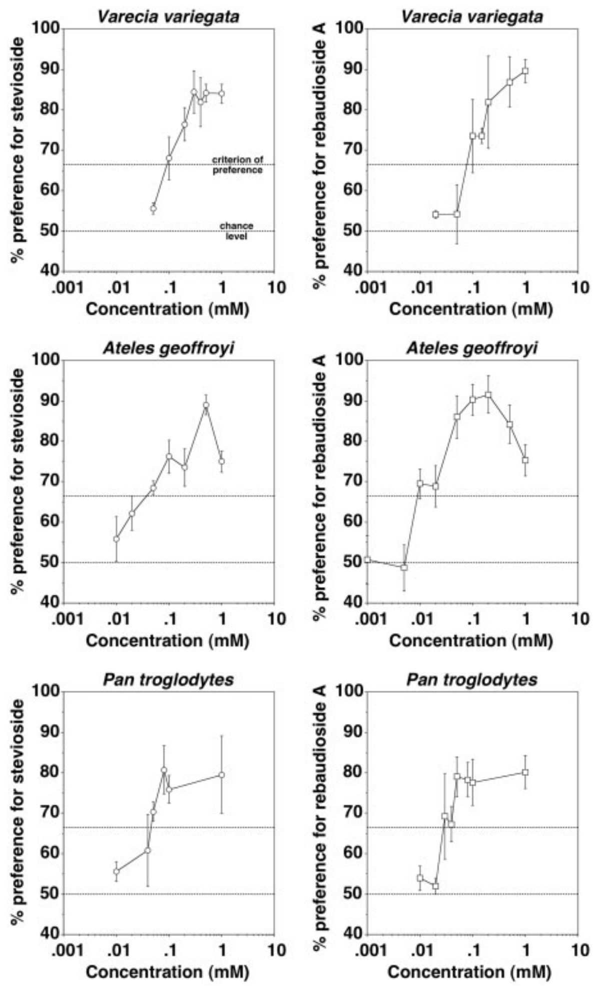

Figure 1. Mean taste responses (± *SD*) of three black-and-white ruffed lemurs *Varecia variegata,* five black-handed spider monkeys *Ateles geoffroyi,* and three Western chimpanzees *Pan troglodytes* to aqueous solutions of stevioside and rebaudioside A tested against tap water. Each data point represents the mean value of 10 trials of 1 min per animal. The dotted horizontal lines at 66.7% and at 50% indicate the criterion of preference and the chance level, respectively.

Results

Taste preference thresholds for stevioside were found to be 0.1 mM in the black-and-white ruffed lemurs, and 0.05 mM in the spider monkeys and the chimpanzees, respectively (Figure 1, left panels). Taste preference thresholds for rebaudioside A were 0.1 mM in the black-and-white ruffed lemurs, 0.01 mM in the spider monkeys, and 0.03 mM in the chimpanzees (Figure 1, right panels). All animals failed to show a significant preference for the lowest concentrations presented, suggesting that the preference for higher concentrations was indeed based on the chemical nature of the stimuli. In most cases, interindividual variability of scores was low for both sub- and suprathreshold concentrations tested (Figure 1) and with only few exceptions all animals of a given species either reached the criterion of preference (> 66.7% of total consumption, plus binomial test, $P < 0.05$) with a given stimulus combination or all animals failed to do so.

Only the spider monkeys, but not the black-and-white ruffed lemurs and the chimpanzees, displayed a marked decrease of >10% in their preference with the highest concentration of both stevioiside and rebaudioside A tested (1 mM) relative to at least one of the lower and detectable concentrations tested (Figure 1). Accordingly, the concentration-response function with the best goodness-of-fit for the spider monkeys can be described as an inverted U-shaped function (third-order polynomial regression: $R^2 = 0.86$ with stevioside, and $R^2 = 0.90$ with rebaudioside A), whereas the corresponding functions for the black-and-white ruffed lemurs and the chimpanzees can be described as a sigmoid function (symmetric sigmoid regression: $R^2 = 0.98$ and 0.94 with stevioside, and $R^2 = 0.96$ and 0.92 with rebaudioside A).

Discussion

The results of the present study demonstrate that black-and-white ruffed lemurs, black-handed spider monkeys, and Western chimpanzees are clearly able to perceive the two quantitatively predominant sweet-tasting diterpene glycosides of the neotropical plant *Stevia rebaudiana*. Further, they show that all three primate species are able to detect concentrations as low as 0.1 mM or even lower with both substances and are thus, similar to human subjects, clearly more sensitive to both stevioside and rebaudioside A compared to sucrose.

Our finding that not only spider monkeys, but also black-and-white ruffed lemurs and Western chimpanzees are able to perceive the two steviol glycosides tested here is not trivial considering that other plant-derived high-potency sweeteners such as thaumatin and monellin have been found to be perceptible only for catarrhine primate species that co-evolved with the plant species in which these proteins are found, but not for prosimian or platyrrhine primates (Glaser et al. 1978). At least two possible explanations may account for the finding that not only a representative of the New World primates, but also representatives of the prosimian and Old World primates, respectively, are able to perceive steviol glycosides that have so far exclusively been found in a neotropical plant species: first, it may simply be that between-species differences in the ability or inability to perceive sweet-tasting substances do not necessarily require that a co-evolution between animal and plant species has occurred. This notion is supported by the finding that only catarrhine primates, but not prosimian and platyrrhine primates have been found able to detect the artificial sweeteners aspartame and neotame (Glaser et al. 1992). Similarly, only prosimian and catarrhine primates, but not platyrrhine primates display taste responses to the artificial sweeteners AMPA (*N*-α-L-aspartyl-(R)-α-methylphenethylamine) and ASME (*N*-α-L-aspartyl-L-(*O*-tert-butyl)serine methyl ester) (Glaser et al. 1996). In all these cases, any co-evolution between primate and plant species as a driving force for between-species differences in sweet-taste perception can be excluded as the taste substances in question do not occur in nature.

Second, it might be that prosimian and catarrhine primates have co-evolved with plant species bearing taste substances that are structurally similar to the steviol glycosides tested here which might have led to modifications at the sweet-taste receptor allowing for the binding and recognition of stevioside and rebaudioside A. This notion is supported by the finding that two intensely sweet-tasting diterpene glycosides named rubusoside and suavioside A which are structurally closely related to stevioside and rebaudioside A have been identified in the leaves of a paleotropical plant, the Chinese blackberry *Rubus suavissimus* (Tanaka et al 1981; Hirono et al. 1990; Ohtani et al. 1991). Thus, the possibility that prosimian and catarrhine primates may have been exposed to these sweet-tasting substances over an evolutionarily relevant period of time and thus may have evolved a correspondingly modified sweet-taste receptor that is also responsive to the sweet-tasting compounds of *Stevia rebaudiana* cannot be excluded.

In this context, it is also interesting to note that a recent genetic study assessing variation in the sweet-taste receptor protein T1R3 reported that the sweet-tasting protein brazzein which is found in the fruits of the West African plant *Pentadiplandra brazzeana* is likely to be perceptible for most catarrhine primates, but not for gorillas (Guevara et al. 2016). The authors speculate that gorilla-specific mutations at this receptor might be a counter-adaptation to the deceptive, that is: non-caloric, sweet signal of brazzein. Thus, the possibility that co-evolution between animals and plants might affect even phylogenetically closely related species in a different manner should also be considered.

Table 1 compares the taste preference threshold values for stevioside and rebaudioside A obtained in the present study with those obtained in the same primate species for sucrose and with taste detection threshold values for the same three substances obtained in human subjects. Similar to humans, all three primate species tested here were found to be considerably more sensitive to the two steviol glycosides compared to sucrose. However, the differences in threshold values between the disaccharide and the two steviol glycosides is generally smaller in the nonhuman primates (factor of 60–400 with stevioside, and 250–667 with rebaudioside A) compared to the human subjects (factor of 1900 with stevioside, and 2200 with rebaudioside A). Small between-species variations in the binding domains of the T1R2-T1R3 sweet-taste receptor are thought to account for such differences in the receptor's affinity to certain ligands and thus in an organism's sensitivity to different sweet-tasting substances (Liu et al. 2011).

Table 1. Comparison of taste thresholds (mM) for steviol glycosides and sucrose in primates

Species	Stevioside	Rebaudioside A	Sucrose
Prosimian primates			
Varecia variegata variegata	0.1[a]	0.1[a]	25[b]
Platyrrhine primates			
Ateles geoffroyi	0.05[a]	0.01[a]	3[c]
Catarrhine primates			
Pan troglodytes verus	0.05[a]	0.03[a]	20[d]
Homo sapiens	0.0053[e]	0.0046[e]	10[e]

Please note that taste *preference* thresholds in nonhuman primates are compared with taste *detection* thresholds in human subjects.
[a]Present study.
[b]Wielbass et al. (2015).
[c]Laska et al. (1996).
[d]Sjöström (unpublished data).
[e]van Gemert (2011).

A comparison of the taste threshold values between human and nonhuman primates suggests the former to be more sensitive for the two steviol glycosides tested here than the latter. However, it should be considered that the sophisticated psychophysical signal detection procedures employed with human subjects are commonly regarded to yield lower threshold values compared to the simple two-bottle preference test used with the nonhuman primates which provides only a conservative approximation of an animal's taste sensitivity (Spector 2003). As the differences between the human taste *detection* threshold values and the taste *preference* threshold values in the spider monkeys and chimpanzees were only a factor of 10 or even less than that, one can assume the two nonhuman primate species to have a similar sensitivity for the taste of stevioside and rebaudioside A as human subjects. With both steviol glycosides, *Varecia variegata* had slightly higher threshold values compared to *Ateles geoffroyi* and *Pan troglodytes* which is in line with the finding that the black-and-white ruffed lemur is also slightly less sensitive for sucrose compared to the spider monkey and the chimpanzee (Table 1). Nevertheless, all three species of nonhuman primates tested here are clearly sensitive enough to detect the average concentrations of stevioside (21.5 mM) and rebaudioside A (11.2 mM) found in fresh leaves of *Stevia rebaudiana* (Woelwer-Rieck et al. 2010). To the best of our knowledge, no study so far reported on behavioral observations of nonhuman primates ingesting *Stevia* leaves in the wild. Similarly, no study so far assessed behavioral responses of captive primates to the presentation of *Stevia* leaves. Such studies would allow for a first, tentative conclusion as to whether nonhuman primates may reject *Stevia* leaves due to the possible bitter side taste of stevioside and rebaudioside A at high concentrations.

Finally, our finding that the spider monkeys displayed concentration-response curves with both stevioside and rebaudioside A which can best be described as an inverted U-shaped function

(Figure 1, middle panels) suggests that *Ateles geoffroyi*, similar to human subjects (Schiffman et al. 1995), may perceive a bitter side taste at higher concentrations of these substances. This notion is supported by electrophysiological findings in another New World primate, the common marmoset *Callithrix jacchus*: in this species, not only sweet-best fibers, but also bitter-best fibers of both the chorda tympani and the glossopharyngeal nerve responded to stimulation with stevioside (Danilova et al. 2002). In a two-bottle preference test, stevioside presented at a concentration of 0.62 mM was clearly rejected by the marmosets, suggesting the bitter side taste even to prevail at this concentration (Danilova and Hellekant 2004). Based on the data of the present study, it is not possible to decide whether the other two primate species tested here, black-and-white ruffed lemurs and Western chimpanzees, may also perceive a bitter side taste with the two steviol glycosides when presented at concentrations higher than 1 mM. In order to exclude any risk for the health of the animals, and based on the current knowledge about the toxicology of stevioside and rebaudioside A, we decided to restrict the presentation of both substances to concentrations not higher than 1 mM (Huxtable 2002). However, the notion that at least catarrhine primates may also perceive a bitter side taste when presented with higher concentrations of steviol glycosides is also supported by eletrophysiological studies which reported that bitter-best taste fibers in rhesus macaques *Macaca mulatta* were found to respond to stimulation with stevioside (Hellekant et al. 1997).

Taken together, the results of the present study do not support the notion that a co-evolution between plant and primate species may account for between-species differences in taste perception of steviol glycosides.

Acknowledgments

The primate caretakers at Borås Zoo and at Kolmården Wildlife Park are gratefully acknowledged for their support.

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