Pergamon 0031-9422(94)00141-7 Phytochemistry, Vol. 39, No. 5, pp. 1063–1070, 1995
Elsevier Science Ltd
Printed in Great Britain
0031-9422/95 $9.50 + 0.00

GUARANÁ (*PAULLINIA CUPANA*) REWARDS SEED DISPERSERS WITHOUT INTOXICATING THEM BY CAFFEINE

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(*Received* 9 *November* 1994)

Key Word Index—*Paullinia cupana*; Sapindaceae; guaraná; toucan; aril digestion; seed dispersal; caffeine; purine alkaloids; sugars.

Abstract—The fruit of the Amazonian guaraná liana (*Paullinia cupana*) looks like a human eye, and undoubtedly shows the 'bird dispersal syndrome'. The seeds were reported to be ingested by large birds such as toucans and guans. We determined the purine alkaloid content of the various fruit and seed parts. The two aspects of defence and dispersal are reflected in the differential seed alkaloid distribution: the seed kernel (embryo with bulky cotyledons) and the seed coat (testa) accumulate much caffeine, i.e. 4.28 and 1.64%, respectively, whereas the 'white of the eye', the aril, is virtually alkaloid-free, but contains glucose, fructose and sucrose up to almost 70% of aril dry weight. Furthermore, the aril is strongly hygroscopic and it is suggested that it extends germination power by preventing seed desiccation. Experiments simulating pH and temperature conditions in the avian stomachs showed rapid desintegration of the aril and no caffeine release by the intact seed at pH 4.5 (crop) during the first 30 min of 'digestion'. Only a tiny fraction (between 0.025 and 0.07%) of total seed caffeine left the intact seed after 60 min at pH 4.5 or during the incubation at pH 2.3 (gizzard), indicating the presence of a very powerful diffusion barrier in the seed coat which at least theoretically should prevent intoxication of the dispersing bird even after an assumed foraging bout of 50 seeds. The cracked seed, however, releases a considerable fraction of its caffeine, considered harmful to destructive birds, if a few seeds were processed in this way. Absence of caffeine in the aril could well be the result of a 'secondary' degradation during maturation, analogous to hypoglycin A in the aril of the closely related sapindaceous *Blighia sapida*.

INTRODUCTION

The genus *Paullinia* comprises approximately 180 species confined, with the exception of the widespread *P. pinnata*, to the neotropics. About 40 are known to be have been used for centuries by the indigenous people as fish poisons, medicine, stimulating beverages or for other purposes [1]. The species manufactured into activating, caffeine-rich beverages are *P. cupana* H.B.K. var. *sorbilis* (Mart.) Ducke and *P. yoco* Schultes & Killip. The latter is found in the westernmost Amazon of Colombia, Ecuador and Peru, and the Indios prepare the stimulating beverage by rasping the bark of the stem and squeezing the resulting mass into cold water [2]. *Paullinia cupana* var. *sorbilis*, also called guaraná, is possibly the cultivated variety of the wild type var. *cupana* Ducke repeatedly found in the Upper Amazon [3], but first collected by Humboldt in the Upper Orinoco basin more than 170 years ago. The traditional cultivation of the guaraná liana and the processing of its seeds is carried out by the Saterê-Maué Indians in the Central Amazon basin around Maués [4, 5]. They pick the eye-shaped (Fig. 1) seeds as soon as a few fruits are opening. The 'white of the eye', botanically an aril of mealy consistency, is rubbed off manually and the seeds are gently roasted to facilitate the removal of the glossy, tough and dark brown seed coat and the later grinding of the kernels in a hardwood mortar. While grinding, water is added to make a 'dough', which is portioned and shaped into the so-called bastão (Fig. 2). It looks like a peeled salami and serves as the storage form of guaraná after a slow drying process over the fire. As needed, the bastão is grated (Fig. 2) and the resulting powder suspended in water to give a highly stimulating drink not only appreciated by the Indians to withstand hunger and climate but also by others, especially and quite recently by young people in Europe to get through dancing all night at 'techno-parties'. The seed caffeine content of guaraná is reported [6] to range between 3.6 and 5.8% (dry wt), i.e. 3–5 times higher than in an Arabica coffee bean [7]. There is no doubt that the dehisced fruit's striking appearance made up of the deep yellow to orange-red pericarp, of the white, scentless aril and the glossy, almost black seed coat in the centre (Fig. 1) is to attract dispersing frugivorous vertebrates, and most likely matches the 'bird fruit (diaspore) syndrome' [8, 9]. Indeed, large birds such as toucans (*Ramphastos* spp.) and guans (*Penelope* spp.) have been reported to gulp guaraná seeds [10] which they may

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regurgitate after having digested the arils. Since birds are, like other organisms, sensitive to caffeine we should put the crucial question, why are avian seed dispersers not intoxicated by the ingested, caffeine-rich seeds of guaraná. To find an answer we determined the amount and concentration of purine alkaloids in the various fruit parts. Thereafter, we simulated the conditions in the avian digestive system [11] and studied caffeine release during aril 'digestion'. It became obvious that the guaraná plant has not only evolved a tissue-specific allocation of this purine to protect the seeds from destructive predation but also a strategy avoiding caffeine intoxication of the seed disperser digesting the arils rich in sugars.

RESULTS AND DISCUSSION

Distribution and concentration of purine alkaloids in the fruit

The size of globose seeds including the aril varies between 13 and 18 mm. Aril thickness is a maximum (up to 6 mm) at the adaxial side around the hilum (Fig. 3). Most of the seed tissue consists of the two cotyledons surrounded by the seed coat (testa). One entire infrutescence (from Costa Rica) carrying 35 fruits, with 36 seeds (only one fruit was 2-seeded), was separated into the various tissues or organs, and the parameters listed in Table 1 were determined. The aril is completely devoid of any purine alkaloid and its fresh weight is close to that of the seed (cotyledons plus testa), but, due to high water content (*ca* 86%), dry weight is considerably lower, i.e. 0.15 g versus 0.62 g for the aril and seed, respectively. The caffeine content (dry wt) is, as expected, highest in the cotyledons (4.28%), and distinctly lower in the testa (1.64%). Similarly, if we relate caffeine to tissue water in order to get a measure of the biologically relevant concentration, the cotyledons showed the highest value (*ca* 300 mM) followed by the testa (*ca* 110 mM). Both theobromine and theophylline are minor alkaloids in all tissues, with the exception of theobromine in the pericarp, where it is the main alkaloid as well as in leaves (not shown). Although within this 'bunch' maturation of one ovule in only one locule was the rule, the septa separating the ovary into 3 locules developed to their full size, as is typical for the genus *Guaraná* [3], and accumulated theobromine to the same extent as the pericarpic valves (= pericarp in Table 1). However, in the septa, the relative caffeine content (0.67%, 33 times that of the pericarp), and particularly the relative theophylline content, are surprisingly high (0.084%, 84 times that of the

Table 1. Purine alkaloids and weights of various fruit and seed parts of guaraná

Tissue analysed	Fr. wt (g)	Dry wt (g)	H_2O (%)	Caffeine		Theobromine		Theophylline	
				% d. wt	mM	% d. wt	mM	% d. wt	mM
Seed (Cotyl.)	1.07	0.62	42	4.28	303.5	0.015	1.15	0.007	0.53
Testa	0.32	0.18	44	1.64	108.6	0.027	1.93	0.005	0.35
Aril	1.14	0.15	86	0	0	0	0	0	0
Pericarp	1.97	0.37	81	0.02	0.24	0.203	2.57	0.001	0.01
Septa	0.17	0.03	82	0.67	7.40	0.217	2.58	0.084	1.00

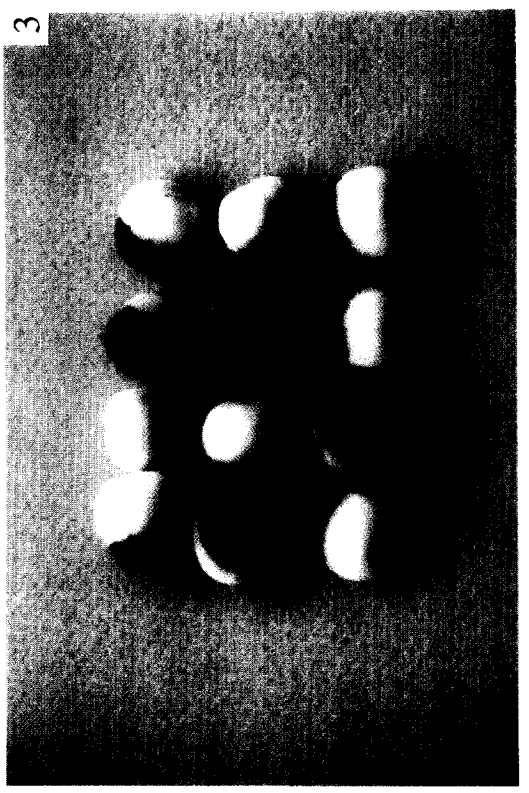
Fr. wts of all tissues, and dry wts as well as alkaloids of the aril, pericarp and septa are average values obtained from $n = 35$. Dry wts and alkaloids of the seed (cotyledons) and of the seed coat (testa) were determined in a smaller sample ($n = 4$) of average fr. wt.

Fig. 1. Part of a 'bunch' (botanically, a thyrsus) of guaraná (*Paullinia cupana*) showing septifragal fruit dehiscence. The striking resemblance of the individual fruit to the human eye has evoked many legends, see ref. [33].

Fig. 2. The rod-like bastão is the storage form of guaraná. The bone inside the tongue of a large freshwater fish, the pirarucú (*Arapaima gigas* Cuvier) is used as a rasp to pulverize the amount of guaraná needed to prepare a drink.

Fig. 3. Guaraná seeds collected from the 'Brazilian' infrutescence and furnished with a white aril bulged at the adaxial side. Seeds are either globose, hemispherical or have the shape of a third of a sphere depending on the development of one, two or three seeds per fruit, respectively. Both the seed coat and the kernel are rather hard.

Fig. 5. Fruit and arillate seed of the West African ackee tree (*Blighia sapida*). The firm and greasy aril called 'seso vegetal' (vegetable brain) is, when ripe, virtually free of the toxic principle, hypoglycin A. The triloculate fruit has the size of a small apple.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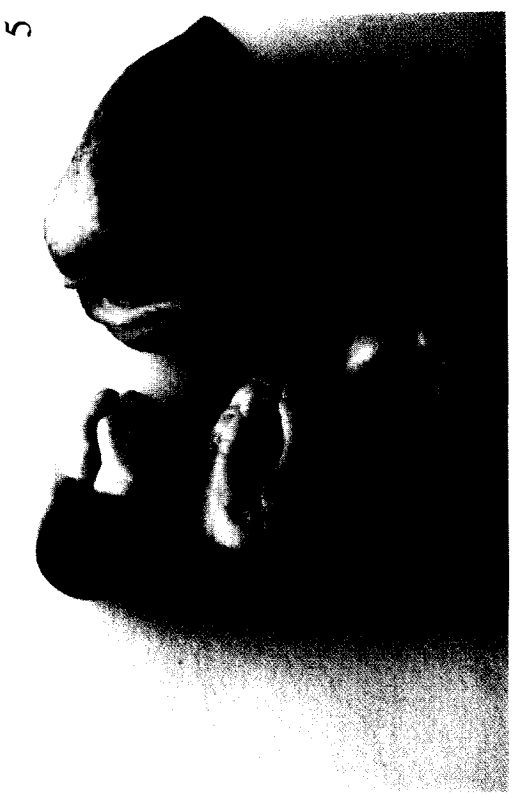
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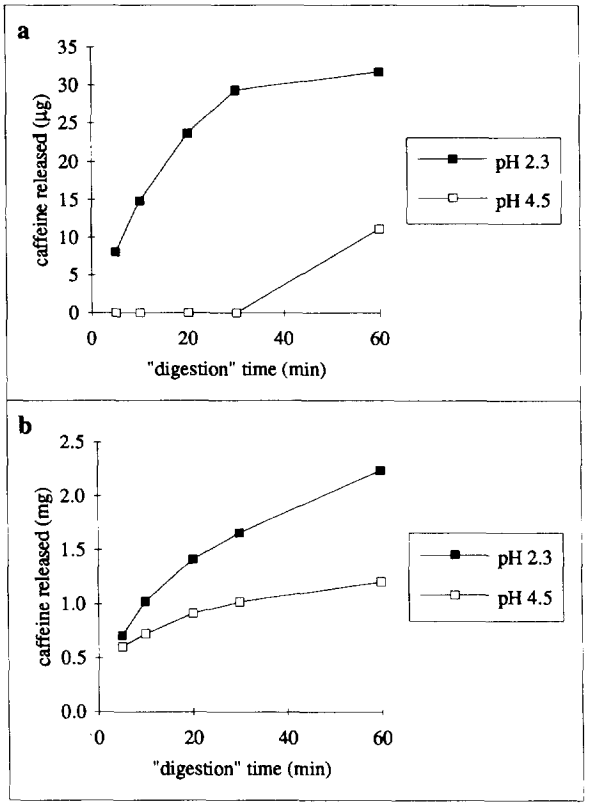

Fig. 4. Release of caffeine from (a) intact and (b) halved seeds (from Brazil) at both pH 2.3 and 4.5. Seeds with arils ($n = 3$) were incubated at 42°, and samples for caffeine analysis were taken at intervals. Data were corrected for removal of caffeine by sampling. In order to achieve almost equal plant fr. wts (between 3.90 and 4.08 g for $n = 3$), globose seeds were not included in these experimental sets. At the end of the experiment, caffeine was determined in the seed-set ($n = 3$) used in (a) and at pH 2.3 (4.08 g with arils; 2.40 g after aril digestion) and was 43.0 mg in total. This value correspondingly served as the basis for the calculation of initial seed caffeine content in the other experimental sets.

pericarp). The latter is, to our knowledge, the highest relative value for theophylline ever found in nature. Finally, we should mention that the peduncles and the central axis of the ripe 'bunch' also contain purine alkaloids in order of magnitude and pattern similar to the pericarp. Conclusively, the purine alkaloids are allocated in a tissue- or organ-specific manner, and it is conceivable that their complete absence from the guaraná aril and their abundant presence in the other seed parts mirrors the opposing aspects of dispersal and predation [12] inherent to the diaspore.

The aril and its nutritional value

The bulky, soft and mealy aril (Fig. 3) firmly connected to the large scar is coated by a thin, membranous layer. The aril remains white and turgescent for at least 10 days after fruit dehiscence. Obviously it prevents desiccation of the seed and thereby loss of germination power known to occur rapidly after harvest [5]. The highly hygroscopic nature of the aril was also experienced during routine dry weight determination: the dried tissue quickly absorbed water from the air. Since the aril tissue turns reddish within a few seconds upon injury, sampling of fresh portions from several seeds (from the Brazilian fruit) was done rapidly to give 1 g aril tissue for carbohydrate and protein analyses. The enzymatic analyses revealed exactly equal amounts of glucose and fructose, i.e. 4.9% (fr. wt) for each, and 0.44% for sucrose. On the basis of 85% tissue water, we found that, altogether, 68.5% of the aril dry weight was glucose, fructose and sucrose. Starch was not present, and the protein content was very low, i.e. 0.53% (dry wt). Unfortunately, we missed the chance to do lipid analysis. In conclusion, we suggest that the aril not only preserves the germinating ability of the seed but also provides a rich reward for the dispersal agent.

Caffeine release

As mentioned above, large birds native to the Upper Amazon [13], such as toucans or guans, are known to forage on and disperse the arillate seeds of the guaraná liana [10]. The related activity of toucans was thoroughly investigated for the neotropical wild nutmeg *Virola surinamensis* [14]. (Processing at a resting point several hundred metres from the feeding spot takes 15–25 min and is terminated by regurgitation; birds approach the maximum number they can swallow, i.e. 4–7% of body mass). Since we are not sure about the manner of processing of guaraná seeds, we also considered defecation in this experiment with a doubled retention time, compared to regurgitation [15]. Therefore, both the crop (regurgitation) and the gizzard (defecation) conditions were simulated [11], and the sampling time was extended to 60 min. Figure 4(a) shows the release of caffeine from three entire, arillate seeds. At both pHs the arils were completely broken apart within 30 min to form a fine suspension. At pH 2.3, for the gizzard, only 32 µg of caffeine had left the seeds after 60 min, i.e. 0.07% of the total caffeine content (= 43.0 mg) of the seeds. Absorption of released caffeine by phenolics present in the aril tissue can be ruled out, since the additional and strongly acidified sample taken after 60 min had the same caffeine content as the untreated sample. At pH 4.5, representing the crop, caffeine release was nil during the first 30 min and at the low level of 11 µg after 60 min. Thus, in the crop, which is presumably the target organ, only 0.025% of the seed caffeine would become free after digestion extended to 60 min. In contrast to intact seeds, cracked seeds (Fig. 4(b)) readily released caffeine, 2.2 mg or 5.4% of total seed caffeine within 60 min at pH 2.3 (gizzard). The figures are somewhat lower at pH 4.5 (crop) i.e. 1.2 mg or 2.9%.

Caffeine toxicity

The seeds used in this study originated from fruits (from Brazil) that predominantly carried two to three

seeds correspondingly flat or angular at the contact surfaces (Fig. 3). On average, they were smaller (0.8 g fr. wt without aril) than the globose seeds from Costa Rica (1.4 g) of Table 1. Overall (seed plus testa) relative caffeine content of the Brazilian seeds was 3.1%, and 3.7% for the globose seeds from Costa Rica. Therefore, and in the following, we took, for comparison, an average coated seed with a fr. wt of 1.1 g (corresponding to a dry wt of 0.63 g) and with a total caffeine content of 21 mg (3.4%).

Since we did not find systematic data for caffeine toxicity in birds, we may relate our findings to a human subject with a body weight 100 times that of a toucan (500–800 g). Thus, the caffeine dose (5 μg) released into the crop by one intact seed (0.025% of 21 mg) would correspond to *ca* 0.5 mg in a human, and the theoretical, non-destructive processing of 50 seeds by the bird at a time (theoretical bolus dose achieved during an extensive foraging bout) would result in the liberation of 0.25 mg, which corresponds to half of the caffeine content (25 mg) of a pleasant cup of tea for humans! However, the amount of caffeine (1.1 mg; 5.4% of 21 mg) 'liberated in the gizzard' by one cracked seed, which in this experiment was simply split into two halves and therefore exhibited a relatively small surface area for diffusion, would already correspond to one very strong cup of 'espresso' (110 mg). A few seeds ingested and properly cracked at one time would be very harmful to a seed-destroying bird, and also intermittent but repeated foraging would not lower intoxication, since the biological half-life of caffeine may be in the range of one to a few hours, as in mammals [16].

In 1859, Alfred Mitscherlich [17] fed 125 mg of either caffeine or theobromine to young pigeons. The caffeine (bolus) dose was toxicologically out of range and the animal rapidly died within three hours, whereas a further pigeon narrowly escaped from the first dose of theobromine, but did not survive the second one applied 24 hours later. Thus, the lethal dose of theobromine for birds may be in the range of 1 g per kg body weight (BW). In mammals (mouse), theobromine is *ca* 10 times less toxic than caffeine [18]. If we apply this factor to birds, the lethal caffeine dose would be in the range of 100 mg per kg BW. This value corresponds fairly well to caffeine toxicity in humans, where the total lethal dose is estimated to range between 5 and 10 g. Finally, for humans, untoward reactions are expected after ingestion of 15 mg caffeine per kg BW [19], which by analogy, should be true for birds too.

The following considerations suggest that guaraná seeds are chemically protected to prevent destruction by the avian agent. Caffeine is known to pass easily through biological membranes, presumably due to its dual, hydrophilic and lipophilic character. Therefore, after consumption of a caffeine-containing beverage, this purine is rapidly distributed within (the water of) the body to reach peak plasma levels after 15–45 min [20]. In the pertinent living plant species, however, a large fraction of caffeine is usually fixed by complexation with phenols, such as chlorogenic acids in coffee [21, 22]. The complex in equilibrium with the free complex partners is in solution and compartmented together with the phenols in the vacuole [23]. Complexation drastically decreases with increasing temperature [24]. In seed-destroying birds, uncomplexed caffeine will leave the broken seeds (as shown in Fig. 4(b)) and, depending on liquid volume and temperature in the crop, additional caffeine will be released to reach a new equilibrium. Moreover, caffeine removed from the equilibrium by intestinal absorption will be quickly replaced. However, because of caffeine toxicity we believe that seed destruction may occur rarely and that in such instances intoxication may be reduced, since caffeine released into the digestive system may shorten retention time in the same manner as suggested for the mild toxicity present in the pulp of some temperate species [25].

Caffeine diffusion barrier

Our studies clearly show that the guaraná seed is equipped with a diffusion barrier most likely to be effective in the testa, which efficiently hinders caffeine from leaving the intact seed. Similarly, coffee seeds (*Coffea arabica*) lose less than 1% of their caffeine during the seven-day period of imbibition in water-soaked cotton wool [26]. The caffeine barrier is not 'testa-mediated' and must be assigned to the outermost cell layers of the endosperm, since the testa (silver skin) is fragile and rapidly decays. We suppose that such a diffusion barrier in caffeine-containing plant species is furnished by a peripheral zone rich in phenolics. Indeed, histochemical analyses of the coffee bean revealed compact deposits of chlorogenic acids in the epidermal cell walls and adjacent cells [27]. Guaraná seeds have been reported to be rich in (+)-catechin (6%) and (−)-epicatechin (3.8%) amounting to approximately 80% of total tannin [28]. Although in the cited study the seed coat was not analysed separately, we assume a physiological barrier in this tissue consisting of catechins which may also act as caffeine complexors in this species.

Primary or secondary absence of caffeine in the aril?

The aril of guaraná is like that of yew [25], *Taxus baccata*, devoid of the toxic principle. Is its absence the result of a lack of primary allocation or of 'secondary' breakdown during fruit maturation? Although we were not able to show, in preliminary experiments, a caffeine degradation potential for mature aril tissue (unripe arils were not available), we cannot exclude 'pre-allocation' of caffeine. In this respect it is interesting to mention the closely related ackee tree (*Blighia sapida* Koenig), with fruits and seeds similar to guaraná (Fig. 5). This species is notorious for the Jamaican 'vomiting sickness' caused by careless consumption (by humans) of unripe arils [29] containing the toxic principle hypoglycin A [30] at a concentration of *ca* 1% (fr. wt) and which decreases, as recently shown [31], during maturation below the detection limit of 0.0012%. Although we did not find literature data on seed dispersal of this West African tree, we assume that specialized frugivorous birds [32] may pull

out the arillate seeds of the dehisced fruit, which is borne terminally and firmly attached in the canopy. Later, they may eat only the appending aril at a favourite perch.

EXPERIMENTAL

Plant material. The two freshly plucked 'bunches' of guaraná were studied immediately on arrival in the laboratory and originated from Costa Rica (Finca La Fortuna) and Brazil (Fazenda Itarare).

Purine alkaloids. After drying at 80° for 24 hr the plant parts were finely pulverized in a mortar and aliquots of 100 mg were extracted with 2–10 ml 0.1 N HCl at 40° for 30 min by sonication. An aliquot of 0.5 ml extract was applied on Extrelut (Kieselgur, Merck) column (Pasteur pipette). Purine alkaloids were eluted with 6 ml CH_2Cl_2. The eluate was dried under a stream of N_2, and the residue dissolved, for HPLC analysis, in 0.5–2 ml H_2O.

HPLC separation of purine alkaloids. This was carried out on a Nucleosil-100 C18 column (5 μm; 2.1 × 100 mm; pre-column 2.1 × 20 mm) with water (A) and MeOH–MeCN (1:1) (B) at a total flow of 0.4 ml min^{-1} and by the following gradient (% B over A): 0–5 min (0–15); 5–12 min (15–50). Parameters were controlled by a Hewlett Packard liquid chromatograph HP 1090 equipped with diode array detector set at 272 nm. Column temp. was 40° and injection volume usually 10 μl. R_t values of theobromine, theophylline and caffeine were 3.9, 5.3 and 7.6 min, respectively.

Sugars. Fresh aril tissue (1 g; H_2O content 85%) was boiled in 3.4 ml EtOH (to give 80% EtOH in the extract) for 10 min while sonicating. After centrifugation for 5 min at 3000 g, the pellet was re-extracted (× 3) with 10 ml boiling EtOH (80%), and thereafter (× 2) with 10 ml water at 4°. The ethanolic, as well as the aq. extracts, were combined, separately, dried by evapn under red. pres. and for sugar analyses the residues were dissolved in 10 ml H_2O. To test for starch, the pellet left after ethanolic and aq. extraction was suspended in H_2O (final vol. 5 ml), autoclaved for 1 hr at 121° (1.8 bar) and then enzymatically (Boehringer) digested for 3 hr at 37° in 0.05 M acetate buffer (pH 4.7) in a final vol. of 10 ml with 80 units (60 μl) of α-amylase (*Bacillus subtilis*) and 40 units (280 μl) of amyloglucosidase (*Aspergillus niger*). Thereafter, the soln was deproteinized by boiling (5 min), centrifuged and analysed for sugars. All analyses were done enzymatically (Boehringer) whereby the ethanolic extract was diluted 1:10.

Protein determination. This was carried out using the Bio-Rad protein assay and 100 mg of fresh aril tissue, to which 915 μl 5 mM DTT was added. The sample was frozen, thawed and extracted for 10 min in a sonication bath. Aliquots of 100 μl served for protein determination. BSA was used as standard.

Caffeine release studies. To simulate physical parameters of the avian crop and gizzard, entire seeds ($n = 3$) furnished with intacts arils were put into 5 ml 0.1 M citrate buffer at pH 4.5 and 2.3, respectively. The Erlenmeyer vessels (25 ml) containing the seeds were gently (100 rpm) and reciprocally shaken at 42°. At intervals, aliquots of 0.5 ml were taken, centrifuged and analysed by HPLC for alkaloids. After 60 min, one extra aliquot was strongly acidified by adding 15 μl conc. HCl to liberate caffeine, if masked by complex formation with aril phenolics. To show caffeine release potential, fresh seeds were cut into half and incubated in the same way as intact seeds.

Acknowledgement—We thank Prof. Dr V. Ziswiler, Zoological Museum, University of Zurich, for critically reading the manuscript and for helpful comments.

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