JZAR JOURNAL OF ZOO AND AQUARIUM RESEARCH
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Koalas (*Phascolarctos cinereus*) utilise volatile compounds to choose preferred Eucalyptus leaves

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Keywords: Koala, Eucalypt, feeding preference, sniffing, volatile compound

Article history:
Received: 29 Jan 2018
Accepted: 19 Mar 2019
Published online: 30 Apr 2019

Abstract

The koala (*Phascolarctos cinereus*) is an arboreal folivore that specialises on the leaves of Eucalyptus spp. Although koalas have a strong preference for this diet, the cues used to discriminate between preferred and unpreferred leaves are still unclear. This study investigated the smelling behaviour of koalas using a food selection experiment to determine if volatile compounds were related to koala food selection. Two studies were conducted. In the first, 10 captive koalas housed in Higashiyama Zoo and Kanazawa Zoo, Japan, were offered various Eucalyptus leaves and observed for their sniffing behaviour. The subjects ate the leaves more after sniffing than without it, suggesting that volatile compounds may be an important cue when choosing preferred leaves. In the second study, four koalas in Higashiyama Zoo were fed a choice of six Eucalyptus leaf species simultaneously. The duration of the koalas' feeding behaviour and the Eucalyptus species on which they fed were recorded. The volatile compounds contained in the leaves were investigated using gas chromatography and gas chromatography-mass spectrometry. The subsequent multiple regression analysis showed that the amount of certain volatile compounds such as β-linalool, α-thujenal, geranial and so on, the majority of which were classified as a group of secondary metabolites, terpenes, was related to the foraging duration on leaves, but was expressed differently for each subject. These results from the behavioural and odorant analyses, namely koalas sniff leaves before eating or rejecting them and the feeding duration depends on the amount of specific volatile compounds, suggested that koalas smell the specific volatile compounds when determining their individually different preference from the available choices. Based on the amount of specific volatile compounds, koala preferences for Eucalyptus leaves can be predicted.

Introduction

The koala (*Phascolarctos cinereus*) is an arboreal marsupial that inhabits forests in eastern Australia. It is a specialised folivore that feeds exclusively on Eucalyptus spp. leaves (Martin and Handasyde 1999; Moore and Foley 2000). Although the genus Eucalyptus consists of over 600 species, koalas selectively feed on a restricted number of eucalypt species (Cork et al. 1983; Martin and Handasyde 1999). Generally, the leaves of plants contain low concentrations of energy as well as some important nutrients, such as nitrogen and phosphorous. This imposes constraints on body size of herbivores and it is difficult for a small animal to survive as a herbivore because they have high energy requirements (per unit of body weight) compared with large animals. The koala has a small body size for an herbivore, and thus must select the best quality foliage in order to maximise its energy intake (Martin and Handasyde 1999). The following factors have been suggested as candidates that dictate the feeding preferences of koalas (see a review by Moore and Foley 2000): eucalypt species (Warneke 1978; Pahl and Hume 1990; Reed et al. 1990; White and Kunst 1991; Martin and Handasyde 1999; Higgins et al. 2011), leaf freshness (Ogura et al. 2015), leaf age (Pahl and Hume 1990), koala habitat region (Pahl and Hume 1990), daytime or nighttime (Marsh et al. 2013), season (Martin 1985), available nitrogen (Stalenberg et al. 2014), sideroxylonals (secondary metabolites found exclusively in eucalypts) (Stalenberg et al. 2014), and the formylated phloroglucinol compounds, a group of lipophilic phenolic compounds (Lawler et al. 1998b; Moore et al. 2005). Individual differences in food preference are also observed (Martin and Handasyde 1999; Marsh et al. 2007; Higgins et al. 2011). However, the cues by which koalas differentiate factors involved in leaf preference are still unclear.

A number of koalas have been imported and reared in zoos in the United States, Europe, and Japan, which are not their original habitats and are areas where Eucalyptus trees do not proliferate in the wild. The koala is not the easiest of animals to feed (Martin and Handasyde 1999). Eucalypt foliage must be cultivated in order to meet the dietary requirement of koalas, to provide opportunities to express natural foraging behaviour and to build naturalistic exhibitions for the environmental education of zoo visitors. However, a number of leftover branches are disposed of due to a lack of understanding about how koalas discriminate their preferred eucalyptus. Some zoos use eucalypt species as a cue to predict koala preferences, but species preferred by each individual varies day to day even if combination of species fed simultaneously are same. Our preliminary study revealed that koalas housed in a zoo eat only 10 to 15% by weight of fed branches. This severely imposes many costs for the husbandry and also presents a risk to the welfare of captive koalas in the meaning of caring their natural behaviour for food selection. The exploration of these cues that allow for the selection of preferred foliage from various choices will help solve this problem.

Koala leaf selection is suggested to be made possibly based on odour (i.e. from volatile compounds) (Lee and Martin 1988; Pahl and Hume 1990; Martin and Handasyde 1999). These studies reported that koalas carefully smell foliage before they eat it. But Higgins et al. (2011) reported that koalas ate the Eucalyptus leaves that they initially approached in many cases regardless of whether they smelled them first, although they rarely ate them without first sniffing. They concluded that the role of sniffing leaves on feeding selection was ambiguous. Therefore, mixed results have been obtained regarding the relationship between smell and preference. The aim of this study is to understand the role of volatile compounds on koala feeding preferences. This research will contribute to the understanding of koala feeding ecology and to provide better husbandry for koala welfare in captivity. To investigate the effect odour has on koala Eucalyptus species preference, we examined the smelling behaviour in a food selection situation as well as the correlation between volatile compounds in Eucalyptus leaves and the duration of foraging.

Materials and methods

This research consists of two studies. First, the sniff reaction to various Eucalyptus species was investigated, such as sniff and eat, sniff without eating, eat without sniffing, and sniff and leave. Second, which volatile compounds were related to food selection in koalas were determined.

Sniff reaction to various Eucalyptus species

Subjects

The subjects in this study were 10 captive koalas (Table 1). Six were housed in Higashiyama Zoo and Botanical Gardens in Nagoya, Japan. Four were housed in Kanazawa Zoo in Yokohama, Japan. Kinship between the subjects is shown in Table 1. All subjects were reared by their biological mothers until at least approximately one year after birth. The subjects were housed in individual areas except for two (Vanilla and Yui) because they were sisters. But, during observations, these rarely shared the same feeding site simultaneously, so they were regarded them as two independent sampling units. The housing areas ranged from

Table 1: A description of the subjects.

Study site	ID	Sex	Birthday	Birthplace	Kinship	Study 1	Study 2
Higashiyama	Archer	M	2007.3.9	Taronga Zoo, Australia	Father of Artie	+	
	Taichi	M	2012.5.3	Saitama Children's Zoo, Japan	Half-brother of Koko (with a different mother)	+	
	Artie	M	2012.8.11	Higashiyama, Japan	Grandson of Clements, Son of Archer	+	+
	Clements	F	1997.11.20	Taronga Zoo, Australia	Maternal grandmother of Artie	+	+
	Tilly	F	2009.12.15	Taronga Zoo, Australia	None		+
	Koko	F	2010.5.5	Saitama Children's Zoo, Japan	Half-sister of Taichi (with a different mother)	+	+
Kanazawa	Teru	F	2008.11.22	Kanazawa, Japan	Mother of Vanilla and Yui, half-sister of Waka's mother (with a different mother)	+	
	Vanilla	F	2011.3.26	Kanazawa, Japan	Daughter of Teru, sister of Yui, half-sister of Waka (with a different mother)	+	
	Waka	F	2012.10.21	Kanazawa, Japan	half-sister of Vanilla and Yui (with a different mother)	+	
	Yui	F	2013.7.27	Kanazawa, Japan	Daughter of Teru, sister of Vanilla, half-sister of Waka (with a different mother)	+	

Figure 1: Experimental settings. Left: Higashiyama, Right: Kanazawa, a: koala, b: Eucalyptus branches with leaves in the pots/tubes. No barriers separated koalas and branches, thus the subjects could eat leaves while sitting on Eucalyptus branches.

28 m^2 to 57 m^2. Each area was equipped with wooden scaffolds and either three or four pots (in Higashiyama) or two or three PVC tubes (in Kanazawa) for feeding (Figure 1). The koalas were fed Eucalyptus leaves on branches put into the pots/tubes daily at about 13:00 h in Higashiyama and at 13:30 h in Kanazawa. Three species in Higashiyama and four to six species in Kanazawa were fed simultaneously during the study periods. The eucalypt species in this study consisted of *Eucalyptus amplifolia, E. camaldulensis, E. camaldulensis var. obtusa, E. camaldulensis var. obtusa petford, E. haemastoma, E. microcorys, E. propinqua, E. punctata, E. robusta, E. rudis, E. resinifera, E. tereticornis,* and *E. viminalis;* the species were identified following by the instruction from the farmers who cultivated these. All species had been fed to the koalas before the experiment as one of the repertoires of daily diets. The combinations of species were chosen based on the previous experience of the keepers. The amounts of branches fed in each day were approximately same among the subjects within each zoos. The experiments were performed in accordance with the permission of the Animal Experiment Committee of the School of Veterinary Medicine, Kitasato University.

Procedure

The observations were conducted from 13th to 28th August 2014 in Higashiyama and from 25th August to 5th September 2015 in Kanazawa. In Higashiyama, the subjects' behaviours were recorded for 60 min immediately after feeding, resulting in eight hours of recording for each subject. In Kanazawa, in addition to the above-named periods, observations were conducted from 9:30 h to 10:30 h and again from 15:30 h to 16:30 h, resulting in 24 hours of recording for each subject. During these sessions, the subjects' responses to the branches were recorded when they approached a branch for the first time after feeding and when they returned to a branch after moving farther than their body length away from it. The other situations (e.g. the subjects stayed at one site, held a branch in their hand, and ate another leaf from same branch) were excluded because it is unclear whether the subjects made a new selection or relied on their past selection. The recorded behaviour was categorised into one of four groups per Higgins et al. (2011): (1) smell and eat, (2) smell and not eat, (3) eat and not smell, or (4) smell and not eat, but move to another pot. In the present study, smelling was defined as that koalas moved around and sometimes stopped their nose in a close distance to the leaves. The data were analysed using a Pearson's chi-squared goodness of fit test within individuals and a residual analysis using the standardised residuals to investigate their difference from chance level.

The relationship between foraging duration on each Eucalyptus species and the amount of volatile compounds

Subjects

The subjects were four captive koalas (Artie, Clements, Tilly, and Koko) housed in Higashiyama Zoo and Botanical Gardens in Nagoya, Japan. Housing environment and care condition were described above. Six Eucalyptus species such as *E. amplifolia, E. camaldulensis, E. punctata, E. camaldulensis var. obtuse petford, E. robusta* and *E. tereticornis* were simultaneously fed to the koalas during the study periods. They were put into three pots installed on wooded scaffolds. Each pot had two of above six species. The position of each species were randomly changed on day in order to avoid the effect of feeding site.

Behaviour observations

The behaviour observations were conducted from 4th–8th and 18th to 22nd August 2015. The subjects' foraging behaviours were recorded for 60 min immediately after feeding, resulting in eight hours of recording for each subject. During this period, the subjects' foraging behaviours and the Eucalyptus species were recorded using an instantaneous sampling method with 30-s intervals (Altmann 1974).

The number of sampling points at which the subjects engaged in foraging behaviour (hereafter, regarded as "foraging duration") was analysed using a Generalised Linear Model (GLM) (the freeware package R, Version 2.14.2; R Development Core Team 2012). The models were constructed using a Poisson distribution because the number of sampling points used consisted of non-negative count data (Dobson 2002). The model with both the Eucalyptus species and the subjects as explanatory variables was compared to the models with only one of these variables as the explanatory variable. The Akaike information criterion (AIC) (Akaike 1974) can be used to compare models with different numbers of fitted parameters (Dobson 2002) and the model with the lower AIC is considered the best model.

Sample collection

Eucalyptus leaves were collected from leftover branches the day after feeding. The position of the collected leaves was randomised within the branch.

Reagents

Methanol (purity: >99.8%), pentane (purity: >99.0%), diethyl ether (purity: >99.0%) and anhydrous sodium sulphate (purity: >98.5%) were obtained from Kanto Chemical Co., Tokyo, Japan. Methyl decanoate (purity: >99.0%) was purchased from Tokyo Chemical Industry Co., Tokyo, Japan.

The preparation of volatile concentrate from Eucalyptus leaves and the isolation of the volatile compounds by means of gas chromatography and gas chromatography-mass spectrometry analysis

The preparation of volatile concentrate under non-heated conditions and the isolation of volatile compounds by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) were carried out according to the described method of To Quynh et al. (2012) with several modifications. Fresh Eucalyptus leaves were briefly frozen using liquid nitrogen and then crushed. Ten grams of crushed Eucalyptus leaves and 1 µL of methyl decanoate aqueous solution (final concentration: 0.5 mg/mL) as an internal standard (IS) were added into 10% methanol (200 mL), stirred for 30 min at 4°C and then filtrated. The filtrate was passed through a glass column packed with 17 g of a porous polymer resin (Porapak Q, 50/80 mesh, Waters Co., Tokyo, Japan) and then the volatile compounds were adsorbed to Porapak Q. The volatile compounds were eluted with 200 mL of a mixture

Table 2: The number of sniff reactions under the selection situation.

Study site	ID	Smell and eat	Smell and not eat	Eat and not smell	Smell and not eat, but move to another pot
Higashiyama	Archer	7	5	4	3
	Taichi	12[a]	1[b]	4	8
	Artie	13	8	1[b]	11
	Clements	2	8[a]	0[b]	2
	Koko	14[a]	7	1[b]	12
Kanazawa	Teru	3	10[a]	0[b]	4
	Vanilla	7	10[a]	0[b]	1
	Waka	7	8	0[b]	4
	Yui	5	9[a]	1	3

a: observed significantly more than chance level (P < 0.05)
b: observed significantly less than chance level (P < 0.05)

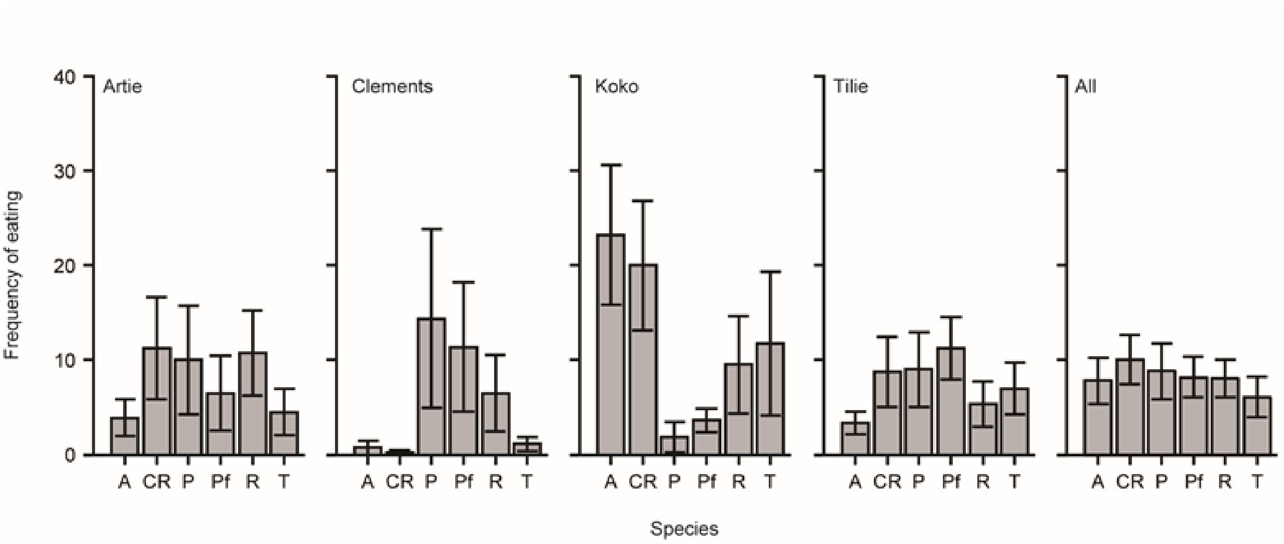

Figure 2: The mean (±SE) foraging duration for each Eucalyptus species for each subject. The number of sampling points at which the subject engaged in foraging behaviour was regarded as foraging duration. A: *Eucalyptus amplifolia, CR: E. camaldulensis, P: E. punctata, Pf: E. camaldulensis var. obtusa petford, R: E. robusta and T: E. tereticornis.*

Table 3: The amounts of the volatile compounds and corresponding odour qualities of the Eucalyptus leaves fed to koalas.

Compound	Odour quality assessed by GC-Olfactometry[a]	Internal standard ratio[b] of each volatile compound in Eucalypt species					
		A[c]	CR[d]	P[e]	Pf[f]	R[g]	T[h]
monoterpene compounds							
α-phellandrene	citrus, black pepper	1.41	12.66	11.72	0.12	0.08	28.49
γ-terpinene	herbal, citrus, lemon, lime	8.75	1.27	1.15	10.18	0.20	2.50
o-cymene	camphor	2.28	5.26	18.23	2.81	0.75	17.11
Piperitone	herbal, minty, camphor, woody	n. d.[i]	n. d.	11.00	n. d.	n. d.	n. d.
γ-elemene	woody	n. d.	3.10	22.51	n. d.	n. d.	n. d.
1,8-cineole	eucalyptus, herbal, camphor	3593.88	438.53	1321.14	2355.01	224.11	604.71
5-isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol	woody, leaves, herbal	21.08	83.19	54.72	3.88	0.25	37.27
β-linalool	citrus, woody	58.85	52.14	2.90	0.79	2.91	3.95
cis-β-terpineol	woody, pungent, earthy	34.10	44.23	104.15	6.82	0.42	28.46
trans-1-methyl-4-(1-methylethyl)-2-cyclohexen-1-ol	sweet, floral	4.32	12.71	19.53	2.71	0.20	9.34
δ-terpineol	woody, pine	70.57	36.95	35.70	84.47	11.29	37.00
cis-1-methyl-4-(1-methylethyl)-2-cyclohexen-1-ol	citrus, lemon, orange	6.16	64.59	130.89	2.23	0.27	26.92
α-terpineol	woody, floral, citrus	466.63	64.39	33.68	51.21	89.91	45.20
1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6-ol	sweet, floral	5.01	2.84	3.57	6.48	2.18	3.23
Geranial	lemon, citrus	7.04	0.24	5.02	24.83	3.70	0.88
α-thujenal	woody, herbal, citrus	1.74	1.13	8.59	2.17	0.73	1.08
α-terpineol acetate	herbal, sweet, bergamot, pine	13.44	n. d.	n. d.	n. d.	n. d.	n. d.
2-acetoxy-1,8-cineole	herbal, minty, fresh eucalyptus	16.32	n. d.	n. d.	6.23	n. d.	6.58
sesquiterpene compounds							
(-)-globulol	floral, rose	34.74	17.98	6.26	5.41	1.82	2.59
other compounds							
cis-jasmone	floral, woody, herbal, citrus	3.52	0.62	3.78	0.69	1.29	1.49
2,5-diethylphenol	alcohol	3.97	1.82	25.55	3.47	0.88	1.87
Eugenol	spicy, sweet, clove, woody	1.73	5.40	0.09	1.34	0.11	2.59
dodecanoic acid	oily	5.63	6.17	40.25	2.13	1.60	7.69
unknown[j]	-[k]	1.75	6.99	39.18	0.62	0.31	27.57

a: In the GC-Olfactometry (GC-O) system, the GC eluate of the volatile concentrate is split to a flame-ionisation detector and a sniffing port. Analysts then assess the odour quality of each volatile compound at the same time as isolation; b: GC peak-area ratio for each component relative to an internal standard; c: *Eucalyptus amplifolia*; d: *E. camaldulensis*; e: *E. punctata*; f: *E. camaldulensis var. obtusa petford*; g: *E. robusta*; h: *E. tereticornis*; i: Peak not detected; j: The peak was detected but not identified by GC-MS; k: The odour was not detected by the noses of analysts.

of diethyl ether and pentane (1:1), the eluate was dried over anhydrous sodium sulphate and the solvent was evaporated at 40°C at atmospheric pressure to obtain the volatile concentrate. The volatile compounds were concentrated in a nitrogen stream to 20 µL just before analysing for GC and GC-MS.

GC analysis performed with a Shimadzu GC2010 Plus gas chromatograph equipped with a flame-ionisation detector, and GC-MS analysis was performed with a Shimadzu QP-2010 mass spectrometer combined with a Shimadzu GC-2010 gas chromatograph. The fused silica capillary column was DB-Wax (60 m 0.25 mm i.d., 0.25-µm film thickness, J&W Scientific, Wilmington, USA) for GC and GC-MS analyses. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The oven temperature was maintained at 60°C for the first 4 min, then increased to 220°C at the rate of 2°C /min and then held at 220°C for 20 min. The injector and detector temperatures were 220°C. Mass spectrometry (MS) was performed in the electron-impact mode with an ionisation voltage of 70 eV and an ion source temperature of 200°C. Compounds were identified by their agreement with retention times and mass spectra with authentic standards or Wiley library. The IS ratio for each compound was determined by the ratio of the corresponding GC peak area versus the peak area of the IS.

The determination of the volatile compounds related to food selection in koalas

A multiple regression analysis was performed with PASW Statistics in order to determine which volatile compounds related to the foraging duration on each Eucalyptus species. The independent variable was the IS ratio of each volatile compound detected from the provided Eucalyptus leaves and the dependent variable was the foraging duration of each koala.

Results

Sniff reactions to the various Eucalyptus species

The subjects sniffed more before eating the leaves in the selection situation. Table 2 shows the numbers of each response to branches using the four categories. In six out of nine koalas, smelling responses were observed significantly more than the chance level calculated as frequencies if the subjects randomly choice the four behavioural categories. All subjects also exhibited the "smell and not eat" response. On the other hand, six out of nine subjects exhibited the "eat and not smell" response at a significantly lower level than that of the chance level. Smelling behaviour was observed more than chance level during the selection situation.

The relationship between foraging duration on each Eucalyptus species and the amount of volatile compounds

The duration of foraging behaviour on each Eucalyptus species was different for each subject (Figure 2). The model including both the Eucalyptus species and the subjects (AIC: 3939.6) showed a lower AIC than both the model without the Eucalyptus species (AIC: 3978.3) and the model without the subjects (AIC: 4202.5) as fixed factors. The likelihood ratio test showed significant differences among the models (the species: $P<0.001$, the subjects: $P<0.001$). Both species type and individual differences among subjects affected the duration of foraging behaviour.

The volatile compounds from the leaves of each Eucalyptus species were investigated (Table 3). A total of 24 characteristic peaks were detected, of which 23 compounds were identified. It was revealed that most of them were terpene compounds. As shown in Table 3, some peaks were detected commonly for all Eucalyptus species, but the IS ratio of each compound differed depending on the species. There were several peaks specific to certain species.

The result of a multiple regression analysis is shown in Table 4. The independent variable was the IS ratio of detected volatile compounds and the dependent variable was foraging duration. It was indicated that positive and negative factors were different in each individual. In Artie, the positive factors were β-linalool and α-thujenal and the negative factor was 2-acetoxy-1,8-cineole. In Clements, the positive factor was α-thujenal and the negative factors were 2,5-diethylphenol and piperitone. In Tilly, the positive factor was geranial and the negative factor was α-terpineol. In Koko, the positive factors were 2-acetoxy-1,8-cineole and β-linalool and the negative factor was o-cymene.

Table 4: Multiple regressions of factors used to predict the foraging duration of koalas.

ID	factor	partial regression coefficient[a]	significance probability (P)
Artie	2-acetoxy-1,8-cineole	-0.826	< 0.001
	β-linalool	0.273	0.001
	α-thujenal	0.559	0.010
Celements	α-thujenal	3.874	0.001
	2,5-diethylphenol	-0.515	0.001
	piperitone	-9.397	0.001
Tilly	α-terpineol	-0.004	0.006
	geranial	0.019	0.014
Koko	2-acetoxy-1,8-cineole	0.086	0.014
	β-linalool	0.194	0.003
	o-cymene	-0.802	0.007

a: The independent variable in the regression expression.

Discussion

Sniff reactions to the various Eucalyptus species

When given the choice to select their preferred leaves for foraging, the subjects exhibited sniffing behaviour more than the "eat and not smell" response. All subjects exhibited "smell and eat" and "smell and not eat". These results indicate that volatile compounds may be important cues when choosing which leaves to forage. According to the optimal foraging strategy, the animals must use the lowest cost (usually energy and time) to take the best diet containing more nutrients and fewer toxic components (MacArthur and Pianka 1966; Pyke et al. 1977). However, koalas are unable to determine the amount of dietary components in a leaf without eating it. Therefore, smell might function as a cue that can be used before eating.

Contrary to the results of this study, Higgins et al. (2011) concluded that roles of sniffing leaves on feeding selection were ambiguous. This discrepancy might be due to differences in counting the frequency of the behaviour. In this study, sniffing behaviour was counted when the subject approached the branches, and again after leaving them as well as when the subject first approached the branches, whereas in Higgins et al. (2011), counting was limited to the first reaction when the foliage was first presented even though koalas seem to select which leaves to eat or not successively after starting to eat as well. The counting method employed in this study may better reflect selection behaviour in koalas because it included more various situations of food selection.

The relationship between foraging duration on each Eucalyptus species and the amount of volatile compounds

It was possible to obtain the multiple regression equations for each individual from the IS ratio of volatile compound as the independent variable and foraging duration as the dependent variable in this study, and the positive and negative factors influencing each koala's foraging time were determined from the multiple regression equations. That is to say, the higher IS ratio of the compound shown as the positive factor (positive value shown in Table 4), the longer each koala's foraging time, suggesting koalas preferred these volatile compounds. On the other hand, the higher IS ratio of compound shown as the negative factor (negative value shown in Table 4), the shorter each koala's foraging time. It was revealed that the positive and negative influence of each volatile compound as it relates to Eucalyptus leaf selection were different for every individual koala, and there was no strong causal relation to volatile compounds and palatability across individuals. However, among all positive factors, three components (β-linalool, α-thujenal and geranial) were sensed as having a similar "citrus-like" odour quality by means of GC-O analysis (see Table 3), suggesting that most koalas reared in Higashiyama might prefer Eucalyptus leaves with a stronger citrus-like odour. The negative factor components (o-cymene, 2-acetoxy-1,8-cineole, 2,5-diethylphenol, piperitone and α-terpineol) that were rejected by koalas shared strong minty and camphor odour qualities. Though the positive and/or negative factors were different for each koala, odour qualities preferred or rejected by koalas were similar, suggesting that most koalas reared in Higashiyama might prefer Eucalyptus leaves with a stronger citrus-like odour as one possibility.

Some influential factors such as kinship and feeding experience on diet preference can be predicted. Koala diet preference has been anecdotally suggested to be related to that of their mother (Martin and Handasyde 1999). In addition, several species such as rabbits, goats and cats developed their odour preferences in utero from their mothers' food experience (Coureaud et al. 2002; Becques et al. 2009; Hai et al. 2013). In the subjects, only Artie and Clements shared kinship. Both individuals also had a similar preference for α-thujenal. Kinship or feeding experience of the mother during pregnancy may affect an odour preference in koalas. Feeding preference has been demonstrated to be affected by past diet experience in some mammals such as sheep (Villalba et al. 2004) and human children (Harris 2008). All subjects had a similar feeding experience before the experiment. Thus, the effect of feeding experience was unclear. Further study is needed to investigating the effect of past experience.

From these results, the significant multiple regression equations for all individuals can be obtained in order to predict the duration of foraging for each koala, therefore the Eucalyptus species fed to koalas can be assessed. Although this study focused on the relationship between feeding duration and concentration of the volatile compounds contained with high contents, some compounds contained at a low concentration may affect koala responses. This should be investigated in the future study.

General discussion

This study demonstrated that koala feeding duration can be predicted by using multiple regression equations for each individual who possessed different positive and negative factors. The smell of leaves plays an important role as a cue to koala foraging preference. They might have acquired the morphological characteristics and cognitive skills required to distinguish their better diet which contains fewer toxic components, which can be sensed as a specific odour. Plants have evolved some defences against herbivores, including secondary metabolites (see review by Mithöfer and Boland 2012). Herbivores, including koalas, have employed some strategies to use against the defences of the plants. The compounds suggested in this study (β-linalool, α-thujenal, geranial, o-cymene, 2-acetoxy-1,8-cineole, piperitone and α-terpineol) as cues by which koalas determine their preferred food are classified as terpenes, which are one of the plants' secondary metabolites (Martin and Handasyde 1999). Sniffing these compounds to estimate their concentration in the leaves might be the strategy koalas use against plant defences. The effect on leaf preference varied depending on the type of compound. Some did not affect the koala leaf selection, suggesting that koalas might have evolved some degradation mechanisms specialised to specific terpene metabolites. A similar mechanism was reported in Lawler et al. (1998a) in ringtail possums, who decreased leaf intake depending on the concentration of cineole and macrocarpa G.

The findings of the present study provide some valuable suggestions for caring for captive koalas and their food preferences and how to efficiently provide them with Eucalyptus. Volatile compounds may work for koalas to discriminate preferred food, but are difficult to incorporate into daily care in zoos. Further study focused on finding a cue by which caretakers can easily differentiate between leaves based on smell as well as traditional cues such as eucalypt species is necessary to satisfy individually different preference enabling a good care for koala welfare and efficient feeding. Another experimental study might demonstrate that adding some specific volatile compounds to non-preferred leaves increases palatability. The findings of this study will also help estimate the best habitat to be preserved for the conservation of wild koalas. Identifying key tree species provides a practical foundation for effective conservation plans of koalas (Callaghan et al. 2011). The information acquired from this study will assist in the habitat conservation effort based on the framework of diet selection. For example, the habitats in which various Eucalyptus species are distributed may be suitable to be preserved to meet individually different diet and olfactory preference demonstrated in this study.

Conclusion

The koalas may sniff specific terpenes when choosing preferred Eucalyptus leaves. Koala sniff reactions were frequently observed under the selection situation. The GC, GC-MS and subsequent multiple regression analysis revealed that the amount of specific volatile compounds influenced the feeding duration. The majority of the identified compounds included terpenes, suggesting the koalas sense specific compounds to estimate their concentrations in the leaves as a strategy against plant defences. Based on the findings of the present study, the GC, GC-MS and subsequent multiple regression analysis revealed that the amount of specific volatile compounds might influence the feeding duration.

Acknowledgements

This work was financially supported by the Sasakawa Scientific Research Grant from The Japan Science Society (#27-526 and #28-503) to the first author. We are grateful to A. Ito, N. Murai, M. Nagai, S. Otani, A. Takeishi, R. Tateishi and K. Tokita for their support in data collection. We wish to thank Mr. H. Hashikawa, the director of Higashiyama Zoo and Botanical Gardens, and Ms. K. Hara, the director of Kanazawa Zoo, for their permission to conduct this research.

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