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THE USE OF DOSED AND HERBAGE *N*-ALKANES AS MARKERS FOR THE DETERMINATION OF INTAKE, DIGESTIBILITY, MEAN RETENTION TIME AND DIET SELECTION IN GALAPAGOS TORTOISES (*GEOCHELONE NIGRA*)

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Eleven captive Galapagos tortoises (*Geochelone nigra*) were used as study subjects to estimate digesta kinetics, diet composition, intake and apparent digestibility (aD) using *n*-alkanes. The results were compared to observed intakes and digestibility estimated through total faecal collection. Acid-insoluble ash (AIA) and acid-detergent lignin (ADL) were compared to the alkane method for the estimation of aD. Mean retention times (MRT) were estimated in four adult tortoises (ca 40-60 years old) and four juvenile tortoises (4-5 years old) fed a pulse dose of Co-EDTA, Cr-mordanted fibre (particle size <2 mm) and *n*-alkane hexatriacontane (C_{36}). Average MRT for the liquid phase marker Co was nine days in both adult and juvenile tortoises. For the particle phase markers Cr and C_{36}, MRT was 12 days in adult tortoises and eight and nine days, respectively, in juveniles. Digestibility, diet intake and diet composition were estimated in nine Galapagos tortoises fed for 32 days on a standardized diet containing the synthetic *n*-alkanes octacosane (C_{28}), dotriacontane (C_{32}) and C_{36}. In four juvenile tortoises kept individually, total faecal collection was performed and the faecal recovery rates of *n*-alkanes were estimated for pentacosane (C_{25}), heptacosane (C_{27}), C_{28}, nonacosane (C_{29}), hentriacontane (C_{31}), C_{32}, tritriacontane (C_{33}) and C_{36}. Intakes calculated with the alkane-pair C_{31} and C_{32} overestimated intake by a factor 1.5. After correction for the relative recoveries of alkanes there was no significant difference between estimated and observed intakes. Observed aD of organic matter (OM) was 67.5%. Estimated aD of OM with the internal marker C_{36} alkane, ADL and AIA were 48.5%, 38.9% and 18.3%, respectively. Estimates of diet composition using alkanes in individual animals accurately reflected directly-observed composition. Observed selection of a certain feedstuff was recognized with the alkane method. This is the first report of the use of *n*-alkanes as digestive markers in reptiles and it confirms that *n*-alkanes may be used for determining diet intake and the passage through the gut of the particulate digesta phase, and for estimating diet composition. The possibility of estimating different aspects of digestive strategies with the same marker type is a major asset of the alkane technique.

Key words: tortoise, reptile, *n*-alkane, *Geochelone*, digestibility markers, digestion, intake, diet composition

INTRODUCTION

In domesticated animals, digesta markers are used routinely to calculate faecal output and to estimate kinetics within the digestive tract. It may be because no reptile has been domesticated that a pulse or continuous dose marker system has not been studied in depth in any reptile species. The large number of marker systems (see Hatch & Afik (1999) for a summary of passage time markers) used in nutrition studies in reptiles, sometimes without thorough validation, is a disadvantage for the comparison of results obtained in different studies. A concentration on fewer marker systems would therefore be an important achievement. A marker system that could be of special interest under these circumstances uses the *n*-alkanes, which have been used to study different aspects of digestive strategies, such as digestibility, diet intake, food selection, and digesta kinetics in mammals and birds (Dove & Mayes, 1996; Hameleers *et al.*, 1996). These markers, which are found in the epicuticular waxes of plants as mixtures of different carbon-chain lengths, have received considerable attention in the last 15 years. Comprehensive reviews on the alkane method in domestic ruminants have been published (Dove & Mayes, 1991; Dove & Mayes, 1996). A major advantage of the *n*-alkane technique is that it allows the estimation of digestibility and intake with the same marker system and therefore considerably reduces labo-

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ratory work. Whereas digestibility is estimated by the use of an odd-chain *n*-alkane, naturally present in plants, as internal marker, the method for estimating intake is based on the combined use of a natural odd-chain *n*-alkane and a dosed even-chain *n*-alkane. The low, but measurable, concentrations of the even-chain alkane normally present in the diet is taken account of in the intake calculation. In theory, the reliability of intake estimates is not affected by the digestibility of the ration, or the marker recoveries in the faeces, if the recoveries are sufficiently similar between the natural and dosed alkanes (Dove & Mayes, 1991). In domestic ruminants the alkane combinations C_{31}/C_{32} and C_{32}/C_{33} have been the most reliable for this procedure. The *n*-alkanes have also been used as markers to estimate dietary proportions of different plant species or plant components (Dove, 1992; Malossini *et al.*, 1994; Salt *et al.*, 1994; Dove & Moore, 1995). As different plant species tend to have differing mixtures of odd-chain alkanes (chain lengths in the range 21 to 35 carbon atoms) diet composition can be estimated from the patterns of alkanes in the faeces and in the dietary components. Similarly, the dietary proportions of different component feedstuffs can be estimated by having them labelled with separate synthetic *n*-alkanes (usually even-chain) (Hatt *et al.*, in press).

A further application of *n*-alkanes as markers for digesta kinetic studies has been demonstrated in recent studies in ruminants (Mayes *et al.*, 1997; Hatt *et al.*, 1998).

Most research has been conducted with sheep and cattle (Dove & Mayes, 1996), but the alkane method has also been applied to non-domestic ruminants and non-ruminant species, such as the giraffe (*Giraffa camelopardalis*) (Hatt *et al.*, 1998; Clauss, 1998), pigs and horses (Mayes *et al.*, 1995; Ordakowski *et al.*, 2001), mountain hares (*Lepus timidus*) (Hulbert, 1993) and rabbits (Letso, 1996). Gudmundsson & Halldorsdottír (1995) successfully used synthetic alkanes incorporated into diets to estimate dietary intake and digestibility in farmed fish. In birds, *n*-alkanes have been evaluated in chickens (Hameleers *et al.*, 1996) and pigeons (*Columba livia*) (Hatt *et al.*, in press).

The present experiments were conducted to evaluate the use of *n*-alkanes as markers in a reptilian species, just as they have been applied to study digestive strategies in mammalian and in avian species. The aim was to validate the alkane method in tortoises in two experimental trials. In Trial 1 we compared estimates of digesta mean retention times (MRT) obtained using *n*-alkanes as markers with those derived from the use of the respective liquid-phase and particulate-phase markers, Co-EDTA and Cr-mordanted fibre. The use of alkanes for estimating dietary intake, digestibility and composition was examined in Trial 2. Digestibilities determined using *n*-alkanes were compared with measurements obtained from the internal markers acid-detergent lignin (ADL) and acid-insoluble ash (AIA), and from total faecal collection. Estimates of the dietary proportions of different feed components acquired from dietary and faecal *n*-alkane patterns were compared to directly observed diet composition.

MATERIAL AND METHODS

ANIMALS

Eleven Galapagos tortoises (*Geochelone nigra*) of various body masses and ages from Zurich Zoo (Switzerland), were used in this study. All juvenile animals (between 4 and 8 years) were hatched at Zurich Zoo. Adults were wild-caught animals and their ages could only be guessed, based on the time they had been kept at the zoo. The animals shared the enclosure with five adult Aldabra tortoises (*Geochelone gigantea*).

The heated indoor enclosure measured 65 m^2 and the flooring was concrete except for a 3 m^2 sand pit for egg deposition. The outside enclosure measured 265 m^2 and the floor consisted of grass, gravel and concrete. There was a heated shelter (20-25°C) in the outside enclosure into which all animals could retreat if it was cold outside. Animals were kept in their usual enclosure throughout the study, except that during Trial 2 juvenile tortoises (nos. 11, 15, 28, 29) were separated in individual pens of 1.5 m^2 each for individual feeding and total faecal collection. Juveniles were kept indoors and adults outdoors during Trial 1 (August/September). Throughout Trial 2 (February/March) all animals were housed indoors.

The ambient temperature (aT) and relative humidity (rH) were determined daily during both trials. Juvenile animals nos. 11, 15, 28 and 29 were weighed before and after every trial. For logistical reasons all other animals were only weighed before and two months after the entire study period.

DIETS

During Trial 1 the tortoises were fed their usual diet. Adult animals, which were kept outdoors, had access to fresh and dried rye-grass. Browse (*Ficus* and *Salix*) was offered on a daily basis, and a mix of vegetables (fennel, celery, parsley, carrots), fruit (apples, pears, banana), cottage cheese and a vitamin/mineral supplement was offered twice daily. The diet of juvenile tortoises contained a higher proportion of herbs (basil, parsley, sage) than the diet of adults, and it lacked fruit. Grass was offered only twice a week. The diet of juveniles was cut in small pieces (approximately 1 cm), whilst the diet of adults was chopped into pieces several centimetres. in length. All animals had access to water *ad libitum*.

In Trial 2 all animals received the same diet, which consisted of a total daily amount of 5470 g on a fresh matter (FM) basis for the entire group, and was composed of 75% dried grass, 14% tortoise pellets, 8% apples and 3% lettuce on a dry matter (DM) basis. The DM content of the entire diet was 51%. Pellets were commercial tortoise pellets (Dorswal, Roswal Products,

Zurich, CH) into which *n*-alkanes at a concentration of approximately 1700 ppm per kg DM for each *n*-alkane were mixed before pelleting. The alkanes used were octacosane (C_{28}), dotriacontane (C_{32}) and hexatricontane (C_{36}) (Fluka Chemie GmbH, Buchs, CH). The reason for including three *n*-alkanes was to allow estimation of the faecal recovery factors relative to their carbon-chain lengths.

Digesta Kinetics (Trial 1)

The four adult tortoises and four of the juveniles (nos. 11, 15, 28 and 29) were fed a pulse dose of indigestible markers with their morning food. Juveniles were fed the marker mixed with chopped cabbage (*Brassica oleracea*). Adults were individually offered markers in kiwi fruit and tomato halves.

The preparation of the sodium salt of the monovalent Co-EDTA anion was made according to Udén *et al.* (1980). The salts (15.5 g) were redissolved in 200 ml distilled water and mixed with the tortoise pellets described above. The final Co-concentration was analysed to be approximately 1700 ppm on a DM basis and animals were dosed with 0.8 mg Co per kg body mass (BM). For the identification of the particulate phase, two markers – Cr-mordanted cell wall of dried grass and tortoise pellets labelled with C_{36} alkane – were used. The preparation of Cr-mordanted fibre (1% Cr on a DM basis) with a particle size of <2 mm was performed according to Udén *et al.* (1980). Each animal was dosed with 20 mg of Cr.

For the preparation of alkane-labelled pellets, C_{36} was dissolved in *n*-hexane (C_6) at 40°C and subsequently mixed with pellets to achieve a concentration of 2300 ppm on a DM basis. Hexane was left to evaporate overnight. Animals were dosed with 46 mg C_{36} per kg BM.

In an earlier investigation with the same juvenile animals dosed with carmine red (66 mg kg^{-1} BM) it was observed that the marker was excreted in faeces for up to 18 days (Liesegang *et al.*, 2000). It was expected that in adults the marker would be excreted over a longer period than in juveniles, so faecal samples were collected daily for 25 d after dosing. As faeces and renal excrements were voided separately, faecal collection was possible without contamination. After collection the samples were frozen at -20°C for at least 48 h and subsequently freeze-dried to constant mass. Before laboratory analysis, the samples were ground to a particle size of approximately 1 mm with a coffee grinder. The analysis for *n*-alkanes was carried out by an adaptation of the method of Mayes *et al.* (1986a). Duplicate dried, ground faecal and food samples of 0.5 g were weighed into 100 x 20 mm borosilicate glass culture tubes fitted with screw caps. All tubes and caps were rinsed prior to use with 2 ml of petroleum spirit to eliminate possible hydrocarbon contamination. A solution of tetratriacontane (C_{34}) (0.8 mg/g) in *n*-hexane was added to the sample by weight (0.2 g) as an internal standard, followed by 7 ml of ethanolic KOH (1M). The tubes were then capped and heated for 16 h at 90°C in a dry-block heater. After cooling to 50 - 60°C, the alkanes were extracted by adding 7 ml of *n*-hexane and 2 ml of doubly distilled water. The supernatant (non-aqueous) layer was separated into new glass culture tubes. The extraction was repeated and subsequently the *n*-hexane was evaporated to approximately 4 ml on a dry block (at 50°C). The extracts were then applied to a column containing silica gel (Kieselgel 60, 70-230 mesh) with a bed volume of 5 ml. Hydrocarbons were eluted into 23 x 54 mm scintillation vials by addition of 2 x 4 ml of *n*-hexane to the column, and subsequently the samples were evaporated to dryness. The purified hydrocarbon extracts were redissolved with 0.5 ml of *n*-hexane and transferred to an autosampler vial for analysis by gas chromatography (for technical details see Appendix A).

For the Co and Cr analysis, 0.35 g of freeze-dried, ground sample was mixed with 5 ml of 72% sulphuric acid and left on a shaker at room temperature overnight. Subsequently, 40 ml of distilled water was added and after vigorous shaking, 20 ml of supernatant was passed through a paper filter. Co and Cr concentrations were measured in the filtrate by atomic absorption spectrometry (for technical details see Appendix B).

The MRT in hours of each marker in the entire gastrointestinal tract was calculated according to Thielemans *et al.* (1978). The following equation was applied:

$$MRT = (\Sigma t \times C \times \delta t) \times (\Sigma C \times \delta t)^{-1}$$

where C is the marker concentration in the faecal sample (mg kg^{-1} DM) at time t (h after administration) and δt is the interval (h) represented by the respective sample.

Diet Intake, Digestibility and Diet Composition (Trial 2)

The experimental period of Trial 2 lasted 32 days, i.e. 25 days of adaptation to the diet to reach a steady state in marker excretion and 7 days of faecal collection. In adults and juveniles (Nos. 3, 5 and 6) individual faecal samples were collected on a daily basis. Total faecal collection was performed for animals nos 11, 15, 28 and 29.

Food was offered at 08.00 h, 10.00 h and 13.00 h. Juvenile tortoises nos. 11, 15, 28 and 29, which were fed and housed individually, were offered the following amounts of food on a FM basis: 45 g, 22 g, 30 g, and 29 g, respectively. There were no refusals during the entire trial. Juveniles defecated once or twice a day. Adults defecated more frequently, up to five times a day. After collection the samples were frozen at -20°C for at least 48 h and subsequently freeze-dried to constant mass. On days where two faecal samples were collected from individual animals, samples were pooled in equal amounts. Samples of diets and pellets were analysed for composition and internal marker concentrations. The procedure for analysis of *n*-alkanes was identical to that used in

TABLE 1. Analysis of diets fed to Galapagos tortoises (*Geochelone nigra*). Values are expressed per unit fresh matter (FM) or dry matter (DM).

		Diet Trial 1 (Juveniles)	Diet Trial 2 (Entire Group)
Dry matter	% FM	16.8	51.0
Crude protein	% DM	20.1	11.3
Crude fibre	% DM	14.9	20.5
Organic matter	% DM	89.9	95.7
Acid-insoluble ash	% DM	-	2.6
Acid-detergent lignin	% DM	-	5.0

Trial 1. In addition, samples were analysed for DM according to Padmore (1990), and crude protein (CP), crude fibre (CF) and organic matter (OM) were determined using standard procedures for Weender analysis. Furthermore, ADL was analysed according to the systems of Van Soest (1994), and AIA was estimated gravimetrically as the filtrate residue after ashing in a muffle furnace at 550°C for 16 h and hydrolysis in 12% hydrochloric acid (4 N).

Intakes (I, kg DM d^{-1}) were estimated from the faecal ratios of ingested external and internal *n*-alkanes, the administration rate of the external alkane, and internal alkane concentration in the consumed diet based on the method described by Mayes *et al.* (1986*b*):

$$I = \text{Faeces}/(1 - \text{Digestibility})$$
$$= (D_{32}/F_{32})/(1 - [1 - H_{31}/F_{31}])$$
$$= (D_{32} \times F_{31})/(F_{32} \times H_{31})$$

where F_{31}, F_{32} and H_{31} represent the respective *n*-alkane concentrations (mg per kg DM) in faeces (F) and test diet (H). D_{32} represents the dose (mg) of *n*-alkane ingested per day, which was known from direct observation. The even-chain *n*-alkane C_{32} was used as external (dosed) alkane and hentriacontane (C_{31}) was the internal alkane.

Apparent digestibility coefficients (aD) of organic matter (OM) (% DM) were calculated on the basis of total faecal collection and by using C_{36} *n*-alkane, AIA and ADL as internal markers.

For the estimation of aD of OM (aD_{OM}) with the total faecal collection method, the following standard equation was used:

$$aD_{OM} = 100 \times (I_{OM} - E_{OM})/I_{OM}$$

where I_{OM} represents the amount of OM ingested (g DM) and E_{OM} is the amount of excreted faecal OM (g DM).

For the estimation of aD_{OM} with the markers AIA, ADL, and C_{36}, the following standard equation was used:

$$aD_{OM} = 100\text{-}[(M_d/M_f) \times (N_f/N_d) \times 100]$$

where M_d and M_f are respective percentages of marker in diet and faeces and N_d and N_f represent percentages of nutrient (OM) in diet and faeces, respectively.

The estimation of diet composition with the alkane method was performed with the least-squares optimization procedure described by Newman *et al.* (1995), using the MathCAD 2.52 software (MathSoft Inc.). The method is based on an approximation and uses an iterative algorithm similar to those adopted by Salt *et al.* (1994) and Dove & Moore (1995).

Results are presented throughout as means ± standard error of the mean (SEM) and *n* is the number of individuals or samples. Repeated measures analysis of variance (ANOVA) and the Scheffé *F*-test were used to

TABLE 2. Body masses (BM, kg), age (y) and mean retention times (MRT, h) within the age class (adults and juveniles: Nos. 11,15,28 and 29) of cobalt (Co), chromium (Cr) and n-alkane (C_{36}) in juvenile (J) and adult (A), male (m) and female (f) Galapagos tortoises (*Geochelone nigra*). [a,b] Different superscripts within the same line indicate significant differences ($P<0.05$) by Scheffé *F*-test.

Animal No.	Age (y)	BM (kg) at Trial 1	BM (kg) at Trial 2	Average MRT (h) Co (liquid phase)	Cr (particle phase)	C_{36} (particle phase)
1A f	~60	99.5	98.0	217	270	280
10A m	~40	135.0	-	222	281	267
20A m	~40	139.5	-	189	279	306
30A m	~40	207.0	210.0	260	317	308
Mean±SE				222±30[a]	287±21[b]	290±20[b]
3J f	8	-	36.1	-	-	-
5J m	7	-	28.1	-	-	-
6J f	7	-	37.8	-	-	-
11J m	5	12.3	14.0	217	185	215
15J f	5	6.1	6.8	159	183	206
28J f	4	8.1	8.9	251	195	208
29J f	4	8.1	9.2	227	213	258
Mean±SE				214±39[a]	198±23[a]	222±25[a]

test the difference in MRT between the three markers within the same age group, and difference in intakes and digestibilities between markers and direct observation. For the statistical analysis of the MRT between the age groups an unpaired *t*-test was used. A probability $P<0.05$ was accepted as level of significance.

RESULTS

Environment and Animals

Analyses of diets fed to juveniles in Trial 1 and to all the animals in Trial 2 are summarized in Table 1. Ambient temperatures were relatively stable throughout the study. In the outdoor enclosure aT was 21.3±2.6°C in the afternoon (17.00 h) and rH was 73.5±15.4%. In the inside enclosure aT was 23.2±1.0°C and rH was 88.5±9.7%. All animals were active and appeared healthy throughout the study. No adverse effect of marker feeding was observed.

Body Mass and Mean Retention Times

Values of body mass of individual animals and average MRT within the age class are given in Table 2. Markers were ingested voluntarily by all animals within ten minutes of being offered, with the exception of animal no. 20 which ingested the Cr-mordanted fibre only 24 h later, when this marker was offered in a second attempt. This time difference was taken into account in the MRT calculation.

The calculated MRT are represented in Table 2. Differences in MRT of the liquid phase marker Co-EDTA between adults and juveniles were not significant (*t*-test, $P=0.74$). The MRT was approximately nine days. Adult tortoises had significantly longer MRT (12 days) for the particle phase markers Cr and C_{36}, compared to the juveniles, where the MRT were eight and nine days, respectively (Cr: $P=0.001$; C_{36}: $P=0.005$). Adult tortoises showed a selective retention factor of 1.3. The selective retention of each of the particulate markers was significant (ANOVA Co-EDTA: Cr – at $P<0.05$ Scheffé-*F*-value 14.3; Co-EDTA: C_{36} – at $P<0.05$ Scheffé-*F*-value 15.9). Juvenile tortoises in contrast showed no selective retention (Co-EDTA: Cr – at $P<0.05$ Scheffé-*F*-value 0.46; Co-EDTA: C_{36} – at $P<0.05$ Scheffé-*F*-value 0.14).

Typical excretion patterns of the adult and juvenile tortoises in this study are exemplified by the animals nos. 10 and 15 respectively (Fig. 1). In juveniles a steep increase or indeed a pulse excretion (for Co) in marker excretion was observed, whereas in adults excretion increased gradually. In adults and juveniles decline of the liquid phase marker Co was more gradual than that of the particle phase markers Cr and C_{36}, which were excreted as a pulse. In both age groups Co reached a maximum of excretion earlier than Cr and C_{36}.

Diet Intake and Digestibility

Although it is common practice to force-feed reptiles during digestibility trials, we let the animals feed voluntarily and accepted the probability that some food would not be eaten. However, this certainly influenced the rate of defecation by some animals within a group, as some individuals ate more food than others. Furthermore, two animals (nos. 10 and 20) refused to eat the diet on a regular basis and had to be excluded from the study.

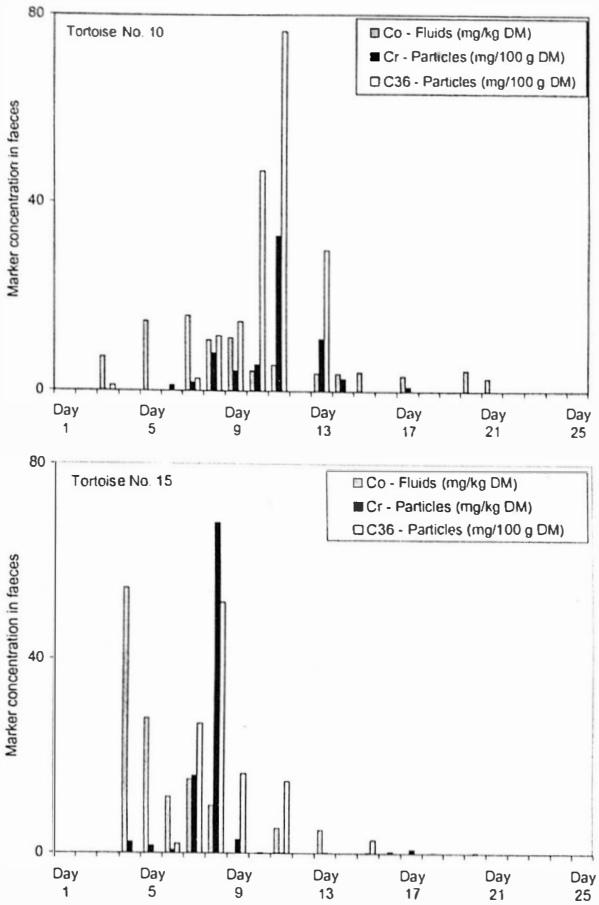

FIG. 1. Faecal concentrations of digesta markers from a single oral dose given to Galapagos tortoises (*Geochelone nigra*) of ~40 y (top) and of 5 y (bottom)

Faecal output of individuals was very variable. On an average daily basis, tortoise no. 11 had an output of 10.2 g DM (number of defaecations, n=6), no. 15 produced 3.3 g DM ($n=4$), no. 28 produced 3.4 g DM ($n=5$) and no. 29 produced 8.2 g DM ($n=5$).

Analyses of feedstuffs offered in Trial 2 are presented in Tables 1 and 3. The preparation of pellets with alkanes produced distinct concentrations of C_{28}, C_{32} and C_{36}. In the four juvenile tortoises kept individually it was possible to calculate *n*-alkane recovery rates based on the average daily intake over the 25 preceding days and faecal samples collected for seven days thereafter (Table 3). Using the *n*-alkane-pair C_{31} and C_{32}, the unadjusted average daily intake was estimated to be 25.0±3.58 g DM. The actual daily mean intake was 16.7±5.14 g DM, so the alkane method overestimated intake significantly (ANOVA at $P<0.05$ Scheffé-*F*-value 15.5), by a factor of 1.5. After taking account of differences in recovery of C_{31} and C_{32} alkanes (C_{31}/C_{32} = 1.36), the estimate was lowered to 18.4±2.64 g DM, which did not differ significantly from the actual daily mean intake (ANOVA at $P<0.05$ Scheffé-*F*-value 0.50).

TABLE 3. Concentration of *n*-alkanes (mg kg^{-1} DM) in feeds offered during Trial 2 and faecal recovery rates calculated for the Galapagos tortoises (*Geochelone nigra*) nos. 11, 15, 28 and 29. * Synthetic alkanes incorporated into tortoise pellet.

	C-chain length of *n*-alkanes											
	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{34}	C_{36}
Diet Mix	0.0	25.0	0.0	76.1	339.3	221.4	0.0	140.5	342.0	25.4	-	225.3
Pellet	0.0	0.0	0.0	0.0	1805.6*	0.0	0.0	0.0	1723.7*	0.0	-	1613.2*
Apple	0.0	19.2	0.0	204.2	19.5	701.0	0.0	0.0	0.0	0.0	-	0.0
Hay	0.0	24.1	0.0	42.5	0.0	135.5	0.0	191.5	0.0	42.5	-	0.0
Lettuce	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	0.0
Recovery rates	-	0.67	-	0.48	0.86*	0.71	-	0.91	0.67*	1.07	-	0.70*

Digestibility of organic matter, determined from total faecal collection, was 67.5±12.77%. Estimates of OM digestibility derived from alkane C_{36} (48.5±13.35%), AIA (18.3±15.83%) and ADL (38.9±13.67%) were smaller than the observed digestibility, but the alkane method yielded results closest to those obtained by total faecal collection. A significant difference resulted only between total faecal collection and AIA (ANOVA at $P<0.05$ Scheffé-F-value 7.8578).

DIET COMPOSITION

In Trial 2 diet composition was calculated on the basis of the average faecal *n*-alkane concentrations over seven days and was compared with the observed average composition of the consumed diet during the entire study period. The results (Fig. 2) have been grouped for the adults, juveniles that were fed individually (nos. 11, 15, 28, 29) and remaining juveniles (nos. 3, 5, 6) that were kept together with the adults. Lettuce was not included in the diet composition calculation as its *n*-alkane levels were below detection level (Table 3). The diet composition was thus compared with the actual diet composition having omitted the lettuce (pellet 15%, hay 77% and apple 8% on a DM basis). The correspondence between actual and estimated diet composition was very good, particularly for adult tortoises (Fig. 2).

DISCUSSION

To our knowledge this is the first study to investigate the use of *n*-alkanes as digestive markers in reptiles. In Trial 1 we evaluated the C_{36} *n*-alkane as a marker for measuring the rate of passage of the particulate phase of

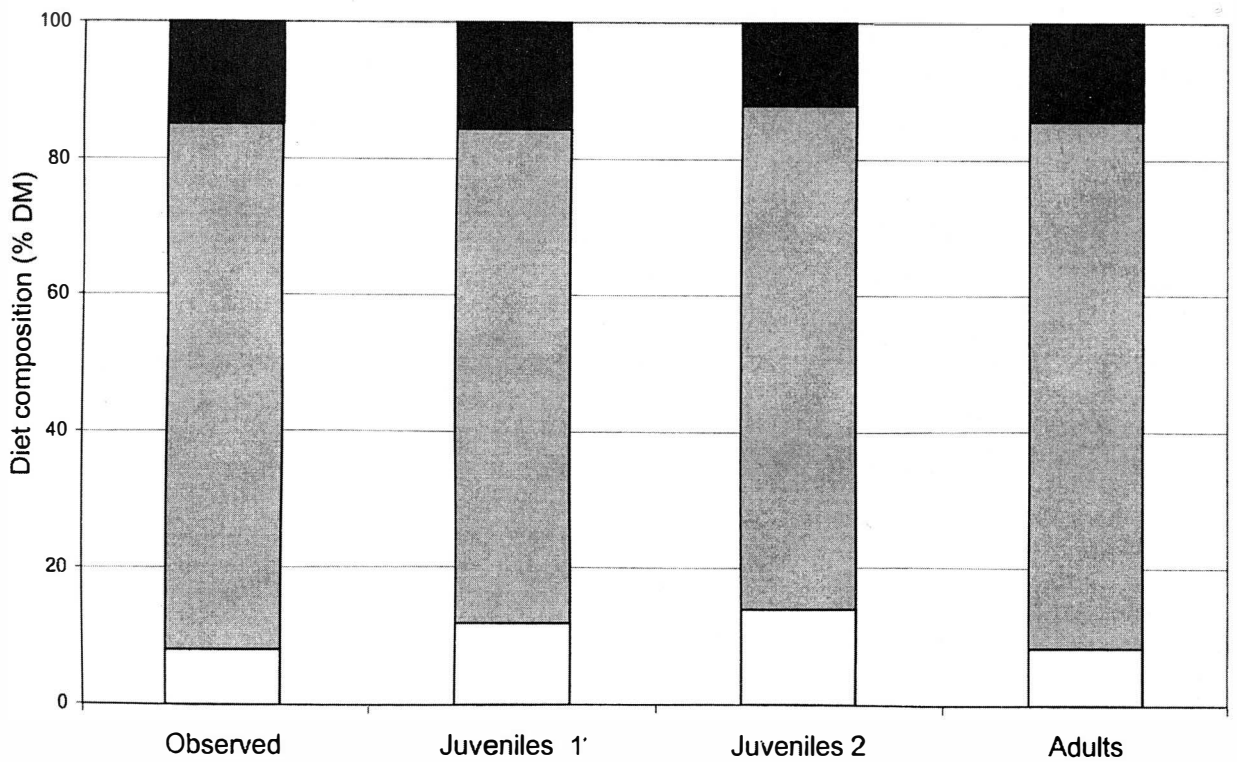

FIG 2. Directly observed diet composition (% of dietary DM) in Galapagos tortoises (*Geochelone nigra*) in Trial 2 compared to mean estimates obtained using *n*-alkanes as faecal markers. Juveniles 1 (n=4) were fed individually. Juveniles 2 (n=3) and Adults (n=2) were fed as a group. Black bars, pellet; shaded bars, hay; open bars, apple.

digesta and compared it with the kinetic marker Cr-mordanted fibre (particle size <2 mm). In our study it appears that C_{36} had a MRT which was not significantly longer than that of the mordanted Cr. The average MRT of the digesta particulate phase in juvenile and adult tortoises was approximately 10 days and this was significantly longer than that of the liquid phase marker, Co-EDTA. This observation agrees with previous results that compared passage-rates of different digesta phases in reptiles (Barboza, 1995; Hatch & Afik, 1999).

Based on the current knowledge of digesta kinetics, two observations in Trial 1 deserve further discussion. Firstly, although the average body mass of adults differed from the juveniles' by a factor of 17 there was no significant difference in the MRT of the liquid phase. This would suggest that body mass does not have an influence on MRT. Secondly, MRT of particulate and liquid phases were significantly different in adults but not in juveniles. This suggests that juveniles did not show any selective food retention of particles <2 mm, whereas in adults there was significant selective retention.

As for the first observation, it is reasonable to assume that length of gastrointestinal tract increases with body-size. Bjorndal (1979) compared the length of the gastrointestinal tract of two green turtles weighing 50 kg and 82 kg, and the larger animal had a longer gastrointestinal tract. Accordingly, we would expect that adult tortoises should have longer MRT of the liquid phase than juveniles. This was not the case in our study. Indeed, there is other evidence that body size may not have a major influence on MRT in tortoises. In the study of Meienberger *et al.* (1993), where the body masses of tortoises differed by a factor of 12 (0.25 – 3.1 kg), no differences in transit times were noted in relation to body mass. Bjorndal & Bolten (1992) compared 12 g hatchlings with 3000 g adults of *Pseudemys nelsoni*, a herbivorous freshwater turtle, and found MRT of 56 h and 81 h respectively; i.e. body mass differed by a factor of 250, but MRT differed only by a factor of 1.4. If the equation of Illius and Gordon (1992) for the estimation of MRT in mammalian hindgut fermenters is applied in the latter case, one might have expected MRT to differ by a factor of at least 4.1.

In our study, a possible explanation for the adults having the same MRT of the liquid phase as the juveniles could be that MRT in the adults was relatively low owing to the higher amount of fibre in their diet, as a result of the inclusion of larger amounts of browse and dried and fresh grass.

Our observation that juvenile tortoises, in contrast to adults, did not appear to have a selective retention of particles may have been due to different particle sizes of diets. Juveniles' food was cut to sizes of approximately 1 cm, whereas adults' vegetables and fruits where chopped to pieces of several centimetres. As reduction of food particle size in reptiles is limited by poor mastication (Throckmorton, 1976; Norman & Weishampel, 1985) it is reasonable to assume that adults ingested larger food particles than juveniles. Large particles probably do experience some degree of selective retention compared to the fluid phase and travel as boli through the digestive tract of tortoises. The particulate markers used in this study may have been associated with boli and as a result underwent selective retention. In juvenile tortoises, which had a more homogenous diet, this separation of particles and fluids did not occur at a significant level, as shown in Fig. 1. This could explain the steeper increase of marker excretion, or even pulsed excretion (for Co), observed in juveniles, compared to adult tortoises, where selective retention resulted in a more gradual increase and decrease of marker excretion. To test this hypothesis, gut content at different sites would have to be examined, which was not feasible in this study.

We encourage further testing of our observation that body mass appeared not to have a significant influence on MRT of the liquid phase, by comparing MRT of the same diet offered in different particle sizes to tortoises of various sizes.

Concerning the use of *n*-alkanes as a marker system for the particulate phase in digesta kinetic studies, this class of marker can be recommended for further studies in reptiles. Compared to Cr-mordanted fibre at a particle size <2 mm, no significant difference was observed with C_{36}-labelled pellets. An advantage of the alkane method over Cr-mordanted fibre is that different chain lengths of this marker may be combined to study the MRT of particles with different sizes.

One aim of Trial 2 was to estimate *n*-alkane recovery rates. Whereas in domestic ruminants and birds faecal recovery increases with chain length (Dove & Mayes, 1996; Hameleers *et al.*, 1996; Hatt *et al.*, in press), no such pattern could be recognized in the Galapagos tortoises. Our results are in general agreement with observations made in mammalian hindgut fermenters, such as the horse and the pig, where recoveries did not vary significantly with chain length (Mayes *et al.*, 1995; Ordakowski *et al.*, 2001). In our study, however, the recoveries were more erratic than in pigs and horses. An explanation for this may be the MRT and the distribution of alkanes between the solid and liquid phases of digesta. Synthetic alkanes have higher, but still low, proportions in liquid phase than natural alkanes (Mayes *et al.*, 1988). The long MRT of tortoises compared to mammals may have increased differences in recoveries due to different rates of passage. Emulsifier systems in the gut and processes of lipid absorption from the gut may also be important. Further studies on the recovery of alkanes in tortoises and other reptilian species will be needed to clarify this point.

In the present study C_{36} had a recovery rate of 70%. As a result apparent OM digestibility in the four juvenile tortoises (nos. 11, 15, 28 and 29) was underestimated without correction for the recovery rate. Nevertheless the *n*-alkane produced a more accurate digestibility co-

efficient than the internal markers, AIA and ADL. In situations where total faecal collection is not possible the alkane method may be applicable especially for comparative purposes. Further work is necessary to evaluate the variability in faecal recovery of C_{36} alkane under a range of conditions; if such variability were low, reliable estimates of digestibility could be obtained using a recovery correction factor.

A possible reason for ADL not producing satisfactory results is that lignin content in our diet was too low. Van Soest (1994) states that lignin is a better marker in diets with high ADL content, especially above 5% on a DM basis. In our diet the content was 5% (Table 1). The internal marker AIA in the present study had the lowest recovery, as based on the degree to which it caused an underestimation of digestibility. In our case it appears reasonable to assume that AIA was absorbed and excreted in the urine, as this has been described in ruminants (Kotb & Luckey, 1972; Owens & Hanson, 1992). Besides absorption, which causes an underestimation of digestibility, a further disadvantage of the AIA method is the possibility of contamination of faeces, resulting in an overestimation of digestibility. Minerals in the diet that are insoluble in acid may arise from two sources: biogenic mineral fractions in the forage, and contamination from soil and dust (Van Soest, 1994). The latter has already been of concern in Aldabra tortoises studied in the field (Hamilton & Coe, 1982) but contamination may also result through the uptake of sand (geophagy) or stones (lithophagy) which has been described in several species of tortoises and terrapins (Gans & Gans, 1978; Zwart, 2000).

The above mentioned problems with ADL and AIA show that a major disadvantage of these markers, in contrast to the *n*-alkane C_{36}, is that they are not discrete chemical entities; the ADL and AIA in faeces may not be the same material as that determined in the diet.

For the intake estimations the comparatively high recovery of C_{31} in relation to C_{32} resulted in an overestimation of digestibility relative to faecal output, hence an overestimation of intake. This difference in recoveries of synthetic and natural alkanes had to be included for accurate intake estimates, but the results show the utility of the alkane method.

In the present study the intake of dose of C_{32} was known from direct observation. However, in future studies without direct observation it will be important to develop a technique for dosing tortoises reliably. Based on the findings of this study, applying the dosed marker three times daily for 25 days will result in a marker excretion that allows intake calculations.

Estimation of diet composition produced satisfactory results in all animals (Fig. 2). Apples were less precisely estimated than hay and pellets, probably because of their respective alkane concentrations. Whereas hay and pellets could easily be identified by their individual *n*-alkane profiles (C_{31} in hay; C_{28} and C_{32} in pellets), the alkane profile of apples was less characteristic. Nevertheless, individual selection is suspected inasmuch as the estimations suggest that juveniles selected apples more strongly than adults in the mixed-age group. This conclusion is supported by observations during the experiment and goes along with the conclusion of Bjorndal & Bolten (1992) that young tortoises improve the nutritional value of their diet by more effective selection, compared to adult tortoises. The alkane method proved to be valuable for estimating diet composition and it might be of special interest in field trials with respect to seasonal changes in diet and effects on digestion.

In conclusion, this study has clearly demonstrated that alkanes have good potential as dietary markers in herbivorous tortoises. Alkanes were successfully used as markers for the particle digesta phase and for diet composition estimates. Further studies are encouraged to improve recovery of alkanes, and as a result, estimation of diet intake and apparent digestibility coefficients. The possibility of estimating different aspects of digestive strategies with the same marker type is a major asset of this technique and might allow considerable progress towards understanding digestive strategies in tortoises.

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APPENDIX A

Gas chromatograph type: Hewlett Packard 6890 GC Series.
Mode (packed or capillary column): Capillary.
Autosampler type: Hewlett Packard 6890 GC Series.
Injection system: Hewlett Packard 6890 GC Series Injector system.
Injection volume: 1.0 ml.
Detector: FID.
Column: Supelco SPB 1™ Fused Silica Capillary Column 15 m x 0.53 mm ID, film thickness 1.5 mm.
Column temperatures - Programmed: 1 min at 230°C; 7°C/min to 280°C; 10 min at 300°C.
Injector temperature: 300°C.
Detector temperature: 320°C.
Carrier gas: H_2, 4.8 ml/min.
Make-up gas: Nitrogen, 15 ml/min.
Time for analysis: Temperature programmed - 25 min.
Time between run: 5 min.
Replicate injections: none.
Data collection system: Hewlett Packard Chemstation 4.01 linked to Pentium 5/133 Hewlett Packard Vectra Series 4.
Other information: Standard mixture (C_{24} - C_{36}) injected after every 15 sample vials. Response factors calculated after all samples have been run on chromatograph.

APPENDIX B

Atomic absorption spectrometer type: Perkin Elmer Model 3300.
Light: Cobalt WL 240.7 nm Chrom WL 357.9 nm.
Slit Width: 0.2 nm.
Flame gas: air acetylene (C_2H_2).
Gas flows: Oxidant 8.0 l/min Fuel 1.6 l/min.
Calibration: linear plot with 3 standard solutions (0.5 mg/l, 1 mg/l, and 2 mg/l).
Read time: 0.5 s Sample replicates: 3.
Read delay: 0.5 s Standard replicates: 3.
Software: AA Lab Reporter.