

Evaluation of urinary resveratrol as a biomarker of dietary resveratrol intake in the European Prospective Investigation into Cancer and Nutrition (EPIC) study

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Abstract

In vitro studies have shown several beneficial properties of resveratrol. Epidemiological evidence is still scarce, probably because of the difficulty in estimating resveratrol exposure accurately. The current study aimed to assess the relationships between acute and habitual dietary resveratrol and wine intake and urinary resveratrol excretion in a European population. A stratified random subsample of 475 men and women from four countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) cross-sectional study, who had provided 24-h urine samples and completed a 24-h dietary recall (24-HDR) on the same day, were included. Acute and habitual dietary data were collected using standardised 24-HDR software and a validated country-specific dietary questionnaire, respectively. Phenol-Explorer was used to estimate the intake of resveratrol and other stilbenes. Urinary resveratrol was analysed using tandem MS. Spearman's correlation coefficients between estimated dietary intakes of resveratrol and other stilbenes and consumption of wine, their main food source, were very high ($r > 0.9$) when measured using dietary questionnaires and were slightly lower with 24-HDR ($r > 0.8$). Partial Spearman's

Abbreviations: 24-HDR, 24-h dietary recall; DQ, dietary questionnaire.

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correlations between urinary resveratrol excretion and intake of resveratrol, total stilbenes or wine were found to be higher when using the 24-HDR (R^2_{partial} approximately 0.6) than when using the dietary questionnaires (R^2_{partial} approximately 0.5). Moderate to high correlations between dietary resveratrol, total stilbenes and wine, and urinary resveratrol concentrations were observed. These support the earlier findings that 24-h urinary resveratrol is an effective biomarker of both resveratrol and wine intakes. These correlations also support the validity of the estimation of resveratrol intake using the dietary questionnaire and Phenol-Explorer.

Key words: Resveratrol: Biomarkers: Urine: Intakes: Wine

Resveratrol (3,5,4'-trihydroxystilbene) is the most well-known stilbene. This polyphenol class also includes piceid (resveratrol glucoside), pallidol and viniferins (resveratrol oligomers), and piceatannol (tetrahydroxystilbene). In the last two decades, resveratrol and other stilbenes have received considerable attention, particularly in *in vitro* and preclinical studies, because of their various biological properties such as antioxidant and anti-inflammatory effects^(1,2). Their capacity to modulate several signalling pathways leads to important cellular processes such as apoptosis, inhibition of angiogenesis and tumour growth as well as lipidic and glycaemic modulation^(1,2). However, evidence from epidemiological studies regarding effects on health is lacking, because of the difficulty in accurately estimating resveratrol exposure in large population-based samples, and because of the low levels of resveratrol observed in humans in non-experimental conditions compared with those in experimental conditions^(2,3).

Resveratrol and its derivatives are found in at least seventy-two plant species, including twelve families and thirty-one genera, but they are found in only a limited number of edible foods (online Supplementary Table S1)^(4,5). The primary sources of stilbenes are grapes and wine, especially red wine^(4,5). They can also be found in minor concentrations in peanuts, pistachios, berries^(4,5), dark chocolate⁽⁶⁾, some beers⁽⁷⁾ and in some varieties of tomatoes⁽⁸⁾ and apples⁽⁹⁾. Dietary intake of stilbenes in Europe is between 2 and 3 mg/d, and resveratrol and piceid represent about 50% of the total stilbene intake⁽¹⁰⁾.

Resveratrol and piceid, after hydrolysis, are absorbed in the small intestine and extensively metabolised into glucuronides and sulphates. Unabsorbed resveratrol reaches the colon and is converted into dihydroresveratrol by the microbiota, which can also be absorbed, accounting for as much as 50% of an oral resveratrol dose⁽¹¹⁾. Experiments with radiolabelled resveratrol have shown a high absorption of resveratrol in the gut, with more than 70% of the ingested radioactivity recovered in urine⁽¹¹⁾. To date, very little information is available on the bioavailability of other stilbenes such as piceatannol⁽¹²⁾ and viniferins⁽¹³⁾; although, to the best of our knowledge, they are not parent compounds of urinary resveratrol⁽¹⁴⁾.

Few studies assessing the ability of urinary resveratrol to serve as a biomarker of wine and resveratrol intake have been published. These studies have been conducted in Mediterranean countries where wine intake is high⁽¹⁵⁾. One interventional⁽¹⁶⁾ and two observational studies^(17,18) showed high correlations between habitual intake of wine or resveratrol and piceid and the concentration of resveratrol metabolites in spot urine samples. High correlations were also observed between dihydroresveratrol glucuronide and resveratrol concentrations measured in 24-h urine

samples and acute intake of wine on the day of urine collection in a cross-sectional study in four European countries^(19,20).

In the present study, we aimed to investigate the correlation between acute and habitual dietary intakes of resveratrol and stilbenes, and their main food sources, and concentrations of urinary resveratrol in 24-h urine. First, our study assessed the usefulness of urinary resveratrol as a biomarker of resveratrol and wine consumption. Second, it evaluated the validity of using a dietary questionnaire (DQ) and the Phenol-Explorer food composition database⁽⁵⁾ to estimate the dietary resveratrol intake.

Methods

Study population

The European Prospective Investigation into Cancer and Nutrition (EPIC) study is a cohort study with over half a million participants of both sexes mostly recruited from the general population between 1992 and 2000 in twenty-three centres in ten European countries⁽²¹⁾. In a convenience subsample of 475 men and women, aged 50–61 years, a single 24-h dietary recall (24-HDR) and 24-h urine samples were collected on the same day between the years 1995 and 1999⁽²⁰⁾. Approval for the study was obtained from the ethical review boards of the International Agency for Research on Cancer and from all participating institutions. All participants also provided written informed consent.

Dietary and lifestyle information

Short-term dietary data were collected using a single 24-HDR harmonised across countries (EPIC-Soft)⁽²²⁾. The 24-HDR was administered in a face-to-face interview. Habitual dietary data were assessed at recruitment using a centre-specific quantitative DQ (France, Germany, Greece and Italy, except Naples) or a semi-quantitative FFQ (Naples) developed and validated in each country/centre^(21,23). The mean time interval between the DQ and the 24-HDR interview varied by country, from 1 d to 3 years later⁽²⁴⁾. Dietary resveratrol and resveratrol 3-O-glucoside (piceid) intakes were estimated using Phenol-Explorer⁽⁵⁾, a comprehensive food composition database on polyphenols, as described in detail elsewhere⁽¹⁰⁾. Resveratrol aglycone equivalents were calculated as the sum of resveratrol and piceid contents after conversion of piceid to resveratrol equivalents on the basis of molecular weight. Total energy intake was estimated using the standardised EPIC Nutrient Database⁽²⁵⁾.

Data on education, physical activity and smoking history were collected at baseline using questionnaires^(24,26). Data

on age, body weight and height were self-reported by study participants during the 24-HDR interview.

Samples and analytical method

Urine samples over a 24-h period were collected and stored at -20°C using boric acid as a preservative. Completeness of the collection of 24-h urine samples was monitored using *p*-aminobenzoic acid (PABA). Samples with PABA recovery <70 and >110% were excluded from this study⁽²⁷⁾.

Resveratrol was analysed in 24-h urine samples using ultra-performance liquid chromatography–tandem MS⁽²⁸⁾. In brief, urine samples were treated with a β -glucuronidase/sulfatase enzyme mixture and extracted twice with ethyl acetate; all phenolic groups in resveratrol were quantitatively dansylated using a differential isotope-labelling method and quantified using tandem MS. The limit of quantification (LOQ) for resveratrol was 0.02 μ M. Inter-assay and intra-assay CV were <10%. Urinary excretion of resveratrol was expressed as μ mol/24-h. A small proportion of urinary excretion measurements (*n* 46) did not meet predetermined analytical quality control criteria and were therefore excluded from statistical analysis.

Statistical analyses

Urinary resveratrol concentrations that fell below the LOQ were set to values corresponding to half the LOQ. Descriptive statistics (medians and 10th and 90th percentiles) including consumers and non-consumers were used for both urinary excretions and dietary intakes. Kruskal–Wallis tests were used to compare urinary resveratrol levels between groups of subjects with different baseline demographics and lifestyle characteristics. Spearman’s rank correlations were used to assess the association between urinary resveratrol levels and dietary variables (stilbenes and stilbene-rich foods) estimated using DQ and 24-HDR. Partial Spearman’s correlations (R^2_{partial}) were computed to assess the contribution of dietary resveratrol intake (using DQ or 24-HDR) to urinary resveratrol levels while adjusting for other possible confounders: sex, BMI, age at recruitment, smoking status (never smoker, former smoker, current smoker) and total energy intake (measured in the DQ or in the 24-HDR, as appropriate). All statistical tests were two sided and the significance level was $P < 0.05$. All analyses were conducted using the Statistical Analysis Software, release 9.3 (SAS Institute Inc.).

Results

Of the 429 subjects with urinary resveratrol data, fifty-two participants had urinary resveratrol concentrations <LOQ. The median urinary excretions by centre, sex, age, BMI, smoking status and total energy intake are shown in Table 1. Excretion was higher in male subjects, in those aged 50–60 years and in those who were overweight (BMI 25– <30 kg/m²) or had greater energy intake. The highest total urinary resveratrol concentrations were observed in Italy and France (0.09–0.19 μ mol/24 h), whereas the lowest concentrations were observed in Greece (0.04 μ mol/24 h). A similar pattern of

Table 1. Urinary resveratrol excretion (μ mol/24 h) according to socio-demographic and lifestyle factors in the European Prospective Investigation into Cancer and Nutrition study (Medians and 10th and 90th percentiles (P10 and P90))

Characteristics	<i>n</i>	Median	P10	P90	<i>P</i>
Centre					<0.001
Paris (FRA)	67	0.14	0.03	0.48	
Florence (ITA)	45	0.17	0.03	0.84	
Varese (ITA)	51	0.15	0.03	0.95	
Ragusa (ITA)	17	0.19	0.04	1.21	
Turin (ITA)	42	0.15	0.02	0.69	
Naples (ITA)	20	0.09	0.03	0.84	
Greece	56	0.04	0.02	0.10	
Heidelberg (GER)	59	0.09	0.04	0.24	
Potsdam (GER)	118	0.08	0.03	0.54	
Sex					0.010
Men	198	0.10	0.03	0.75	
Women	277	0.09	0.02	0.41	
Age (years)					0.061
<50	151	0.09	0.02	0.51	
50–60	201	0.11	0.03	0.60	
>60	123	0.08	0.02	0.48	
BMI (kg/m²)					0.043
<25	207	0.09	0.02	0.65	
25– <30	186	0.10	0.03	0.53	
≥ 30	82	0.08	0.02	0.34	
Smoking status					0.111
Never smoker	241	0.09	0.02	0.48	
Former smoker	129	0.10	0.03	0.71	
Current smoker	92	0.07	0.02	0.42	
Unknown	13	0.17	0.05	0.67	
Total energy intake (kJ/d)					0.003
<7448	141	0.08	0.03	0.19	
7448–9937	140	0.08	0.05	0.20	
>9937	148	0.11	0.06	0.31	

FRA, France; ITA, Italy; GER, Germany.

habitual wine consumption by country was observed; the median intake ranged from 13 ml/d in Greece to 125 ml/d in Italian centres (data not tabulated).

Medians and 10th and 90th percentiles of the intake of resveratrol and resveratrol 3-*O*-glucoside and their food sources (e.g. wine, grapes, peanuts and red wine) are described in Table 2. Medians of the sum of resveratrol and piceid intake obtained using the 24-HDR were lower (0.10 mg/d) than those obtained using the DQ (0.42 mg/d). As expected, a higher number of subjects with negligible intake of the sum of resveratrol and piceid was found using the 24-HDR (*n* 82) than using the DQ (*n* 0). Similar results were observed for wine, with medians varying between 0 g/d (276 non-wine consumers) and 45 g/d (seventy non-wine consumers) in the 24-HDR and in the DQ, respectively.

Spearman’s correlation coefficients between urinary excretion and intakes of different dietary stilbenes were >0.61 and >0.55 as reported using 24-HDR and DQ (Table 3), respectively. The correlation between the sum of resveratrol and piceid intake and wine consumption was found to be 0.83 using 24-HDR and 0.95 using DQ. DQ data on red wine were collected only in Italy (except in Naples); therefore, data were available for 155 participants only. Correlations between red wine consumption and the sum of resveratrol and piceid intake were found to be lower in the 24-HDR (r 0.71) than in the DQ (r 0.96). No significant correlations were found with either

grape or peanuts. DQ data on peanut intake were collected only in Germany (*n* 177). The consumption of peanuts was negligible (0 g/d in the percentile 90 using both DQ and 24-HDR), and the number of peanut consumers was only

fourteen of 475 subjects using the 24-HDR and thirty-three of 177 individuals using the DQ (Table 2).

Table 2. Dietary intakes of resveratrol and piceid, their food sources and urinary excretion of resveratrol in the European Prospective Investigation into Cancer and Nutrition study (*n* 475) (Medians and 10th and 90th percentiles (P10 and P90))

Variables	LOQ or non-consumers (<i>n</i>)	Median	P10	P90
Urine 24 h				
Urinary resveratrol (μmol/24 h)	52	0.09	0.02	0.54
24-h dietary recall				
Resveratrol (mg/d)	84	0.04	0.00	0.81
Resveratrol 3- <i>O</i> -glucoside (mg/d)	163	0.02	0.00	1.66
Resveratrol + resveratrol 3- <i>O</i> -glucoside (mg/d)	82	0.10	0.00	2.45
Resveratrol aglycone (mg/d)	82	0.07	0	1.74
Wine (g/d)	276	0.0	0.0	350.0
Grapes (g/d)	458	0.0	0.0	0.0
Peanuts (g/d)	461	0.0	0.0	0.0
Red wine (g/d)	354	0.0	0.0	260.0
DQ				
Resveratrol (mg/d)	0	0.17	0.03	0.70
Resveratrol 3- <i>O</i> -glucoside (mg/d)	0	0.24	0.02	1.57
Resveratrol + resveratrol 3- <i>O</i> -glucoside (mg/d)	0	0.42	0.06	2.27
Resveratrol aglycone (mg/d)	0	0.33	0.05	1.61
Wine (g/d)	70	44.5	0.0	283.5
Grapes (g/d)	31	7.1	0.3	33.2
Peanuts (g/d)*	144	0.0	0.0	0.03
Red wine (g/d)†	27	0.0	62.5	333.3

LOQ, limit of quantification; DQ, dietary questionnaire.
 * Peanut data only collected using the DQ in Germany (*n* 177).
 † Red wine data only collected using the DQ in Italy, except Naples (*n* 155).

The results of the multivariate-adjusted partial Spearman's correlations (R^2_{partial}) between the intake of resveratrol and piceid, and their main food sources, and urinary resveratrol are shown in Table 4. R^2_{partial} coefficients were similar (R^2_{partial} approximately 0.6) with resveratrol, piceid and wine using the 24-HDR. R^2_{partial} coefficients were slightly lower (R^2_{partial} approximately 0.5) using data from the DQ. For red wine, the R^2_{partial} coefficients were 0.55 and 0.50 for 24-HDR and DQ, respectively.

Discussion

In the current study, resveratrol concentrations in 24-h urine were correlated with acute (R^2_{partial} approximately 0.6) and habitual (R^2_{partial} approximately 0.5) dietary resveratrol, piceid and wine intake in a multicentre cohort of European adults. Habitual intake of the sum of resveratrol and piceid has previously been reported to correlate with concentrations of resveratrol metabolites (*r* 0.67), glucuronides and sulphates analysed in 24-h urine samples in a subsample of 783 men and women, aged 65 years and older, in the Italian InCHIANTI (Invecchiare in Chianti) study⁽¹⁸⁾. Higher correlations (*r* 0.89) were observed between concentrations of resveratrol metabolites in morning urine samples and the habitual intake of resveratrol and piceid in a subsample of 1000 high-cardiovascular-risk men and women in the Spanish PREDIMED (Prevención con Dieta Mediterránea) study⁽¹⁷⁾. The high correlation in the Spanish study could be due to the large proportion of red wine consumers (77%), whereas in our study the percentage of red wine consumers was much lower (37%). In agreement with this hypothesis, we observed higher Spearman's correlations in countries with high wine consumption, such as Italy (*r* 0.62, 38% non-wine consumers), than in Germany (*r* 0.38, 78% non-wine consumers). Therefore, urinary resveratrol concentration is a more

Table 3. Spearman's correlations between urinary resveratrol (RESV) concentrations and acute intakes of RESV, RESV 3-*O*-glucoside and their main food sources estimated with a single 24-h dietary recall and a dietary questionnaire in the European Prospective Investigation into Cancer and Nutrition study

	Urinary RESV	RESV	RESV 3- <i>O</i> -glucoside	RESV + RESV 3- <i>O</i> -glucoside	RESV aglycone	Wine	Grape	Red wine
24-h dietary recall								
Urinary RESV		0.61	0.63	0.64	0.63	0.61	0.09	0.59
RESV			0.84	0.98	0.98	0.77	0.08	0.69
RESV 3- <i>O</i> -glucoside				0.92	0.90	0.85	0.07	0.73
RESV + RESV 3- <i>O</i> -glucoside					0.99	0.83	0.07	0.71
RESV aglycone						0.92	0.07	0.71
Wine							-0.03	0.71
Grapes								-0.04
Red wine								
Dietary questionnaire								
Urinary RESV		0.57	0.55	0.57	0.58	0.56	-0.01	0.61
RESV			0.92	0.97	0.98	0.90	0.08	0.95
RESV 3- <i>O</i> -glucoside				0.98	0.97	0.96	0.07	0.95
RESV + RESV 3- <i>O</i> -glucoside					0.99	0.95	0.07	0.96
RESV aglycone						0.94	0.07	0.96
Wine							-0.03	0.91
Grapes								-0.03
Red wine*								

* Red wine data only collected using the dietary questionnaire in Italy, except Naples (*n* 155).

Table 4. R^2_{partial} coefficients between dietary resveratrol and resveratrol 3-*O*-glucoside intake and urinary resveratrol concentrations in the European Prospective Investigation into Cancer and Nutrition study (n 429)*

Questionnaires	Dietary variable	Urinary resveratrol ($\mu\text{mol}/24\text{ h}$)
24-h dietary recall	Resveratrol (mg/d)	0.57
	Resveratrol 3- <i>O</i> -glucoside (mg/d)	0.59
	Resveratrol + resveratrol 3- <i>O</i> -glucoside (mg/d)	0.59
	Resveratrol aglycone (mg/d)	0.59
	Wine (g/d)	0.59
	Red wine (g/d)	0.55
Dietary questionnaire	Resveratrol (mg/d)	0.51
	Resveratrol 3- <i>O</i> -glucoside (mg/d)	0.49
	Resveratrol + resveratrol 3- <i>O</i> -glucoside (mg/d)	0.51
	Resveratrol aglycone (mg/d)	0.51
	Wine (g/d)	0.51
	Red wine (g/d)	0.50

* Spearman's correlation model adjusted for centre, sex, age, BMI, smoking status and total energy intake.

suitable biomarker of resveratrol exposure in populations with elevated and regular intakes of resveratrol/wine than in those with low and occasional intakes.

Approximately 70% of dietary resveratrol and piceid consumed is recovered in urine as conjugates of resveratrol and dihydro-resveratrol produced by the microbiota, which account for as much as 50% of an oral resveratrol dose⁽¹¹⁾. Because of extensive metabolism in the gut and the liver, most of the resveratrol found in urine is glucuronidated and/or sulphated⁽²⁹⁾, and only 1% of resveratrol absorbed is found in its free form in urine⁽¹¹⁾. Therefore, in the analysis of urinary resveratrol, it is common practice to measure it after enzymatic hydrolysis to release resveratrol aglycone, as done here⁽²⁸⁾. The current analytical method allowed us to accurately quantify urinary resveratrol using a proper standard, whereas, to date, standards for all conjugated metabolites of resveratrol are not commercially available. In addition, this method simultaneously analyses thirty-four urinary polyphenol aglycones, allowing us to potentially measure several nutritional biomarkers of polyphenol-rich foods at the same time⁽²⁰⁾.

Assessment of resveratrol intake in populations represents a challenge for researchers primarily because of the variability and paucity of data on resveratrol content of some foods, although wine, particularly red wine, has been shown to be the main food source (>92%) for total resveratrol and piceid intakes^(4,10). Resveratrol has been identified only in very few foods, mainly grapes, grape juice and wine. Chocolate, pistachios, peanuts and some varieties of berries^(4,5), beer⁽⁷⁾, tomatoes⁽⁸⁾ and apples⁽⁹⁾ also contain resveratrol, but in very small concentrations, or they are rarely consumed (i.e. berries) (online Supplementary Table S1). Moreover, resveratrol content can vary greatly in the same type of fruit, depending on variety, degree of maturity at harvest, fungal pressure and climate^(4,5). Indeed, resveratrol content in red wines may vary up to 10-fold mainly owing to grape variety, Pinot noir being the variety with the highest content⁽³⁰⁾. The development of Phenol-Explorer in 2009 has facilitated the assessment of resveratrol intake in epidemiological studies⁽⁵⁾. However, to our knowledge, this is the first study correlating the estimation of dietary resveratrol intake using Phenol-Explorer with urinary resveratrol concentrations.

In the current study, similar correlations were observed between urinary resveratrol concentrations and dietary intake of either the sum of or individual resveratrol and piceid, or of

resveratrol and piceid expressed as resveratrol aglycone equivalents. Therefore, urinary resveratrol excretion is a good predictor of dietary resveratrol and piceid. This is because wine, particularly red wine, is the main food source of resveratrol and piceid (>92%)^(4,10). Likewise, in our study, urinary resveratrol level was also significantly correlated with wine consumption, especially with red wine intake, as previously described in other studies^(16,17). Other non-resveratrol-related metabolites such as gallic acid ethyl ester, gallic acid ethyl ester sulphate, methyl-gallic acid sulphate, 4-*O*-methylgallic acid and hydroxytyrosol sulphate were also suggested as adequate biomarkers of red wine^(19,22). Combinations of several metabolites could be better biomarkers of wine intake than individual compounds⁽³¹⁾.

Urinary excretion mainly takes place in the first 4 h after consumption (77% of total excretion), although resveratrol metabolites remain in urine for at least 24 h after intake^(29,32). Therefore, correlations between urinary and dietary resveratrol were found to be higher when using 24-HDR (R^2_{partial} approximately 0.6), a dietary assessment tool for short-term and acute dietary intake, than when using DQ (R^2_{partial} approximately 0.5), a tool for long-term and habitual dietary intake. Similar findings with other polyphenols comparing short- with long-term dietary exposures have been reviewed previously⁽³³⁾. Despite this, partial correlations using DQ were still significant. This is due to the fact that wine was consumed on a regular basis by European adults in the 1990s, especially in wine-producing countries such as France, Italy and Spain. For example, the number of red wine consumers was 121 of 475 and 128 of 155 subjects (data collected only in some Italian centres) on using the 24-HDR and DQ, respectively. Moreover, using urinary resveratrol metabolites as biomarkers of wine consumption, Zamora-Ros *et al.* were able to differentiate non-wine consumers from sporadic consumers (one to three glasses of wine per week) who had consumed their last glasses 3–5 d before urine collection⁽¹⁷⁾. Overall, urinary resveratrol concentrations are adequate biomarkers of short-term and habitual dietary resveratrol intake in countries where wine is regularly consumed.

Our results may be influenced by measurement errors in the 24-HDR and DQ, which may attenuate our findings. However, the 24-HDR was collected using standardised software (EPIC-Soft) and administered in a face-to-face interview⁽²²⁾. Furthermore, DQ were country-specific and previously validated for some resveratrol-rich foods such as wine⁽²³⁾. Second, estimation of dietary resveratrol

intake may be limited by the incompleteness of Phenol-Explorer, although it is currently the most comprehensive database on polyphenols including stilbenes⁽⁵⁾. Finally, the results might also be influenced by laboratory measurement error, although our analytical methodology has fulfilled all criteria to be validated⁽²⁸⁾.

In conclusion, urinary excretion of resveratrol was significantly and positively associated with resveratrol, stilbene and wine intakes estimated by 24-HDR and DQ. The correlation coefficients demonstrate that urinary resveratrol is an adequate biomarker of both acute and habitual resveratrol and wine intake in this population. Moreover, our results support the validity of our methodology, 24-HDR, DQ and the food composition database for estimating resveratrol intake. The findings of this study lay the groundwork for future studies to identify the role of resveratrol in the risk for chronic diseases.

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Supplementary material

For supplementary material/s referred to in this article, please visit <https://doi.org/10.1017/S0007114517001465>

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