Journal of Chemical Ecology, Vol. 31, No. 5, May 2005 (©2005)
DOI: 10.1007/s10886-005-4252-2

COMPARATIVE INVESTIGATION OF THE VOLATILE URINARY PROFILES IN DIFFERENT *Phodopus* HAMSTER SPECIES

HELENA A. SOINI,[1] DONALD WIESLER,[1] RAIMUND APFELBACH,[2] PATRICIA KÖNIG,[2] NINA Y. VASILIEVA,[3] and MILOS V. NOVOTNY[1,*]

[1]*Institute for Pheromone Research and Department of Chemistry, Indiana University, Bloomington, IN 47405, USA*

[2]*Institute of Zoology, Animal Physiology, University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany*

[3]*Institute of Ecology and Evolution, Russian Academy of Sciences, Leninsky pr. 33, 117071 Moscow, Russia*

(Received November 5, 2004; accepted January 5, 2005)

Abstract—Stir bar sorptive extraction method was used for investigation of the urinary volatile profiles in male and female *Phodopus campbelli* and *Phodopus sungorus* hamsters. Additionally, female *Phodopus roborowsky* urinary profiles were characterized. A quantitative analytical approach allowed comparisons of 17 selected compounds in urine. Results showed that *campbelli* and *sungorus* species show similar urinary volatile profiles for males and females. Differences appeared only in concentrations. Several unique compounds, such as pyrazine derivatives, were found to be gender- and age-specific. *P. roborowsky* females exhibited a completely different urinary volatile profile from *campbelli* and *sungorus* females, featuring a unique set of substituted quinoxalines.

Key Words—Pheromone candidates, hamster urine, urinary volatiles, *Phodopus campbelli*, *Phodopus sungorus*, *Phodopus roborowsky*, stir bar, sorptive extraction, gas chromatography–mass spectrometry.

* To whom correspondence should be addressed. E-mail: novotny@indiana.edu

INTRODUCTION

Urinary scent marking is an important means of chemical communication in mammals. Examples include rodents (Ralls, 1971; Novotny et al., 1990), canids (Jorgenson et al., 1978; Raymer et al., 1984), and red deer (*Cervus elaphus*) (Bakke and Figenschou, 1990), among others. Urinary scent marks may convey multiple messages, ranging from the species chemosignals and individual-recognition compound patterns to aggression and dominance signaling to various primer pheromone activities. Chemical identification of various scent constituents has become essential in elucidating the physiological and behavioral responses pertaining to olfaction. Such activities have thus far been most successful with investigations in the house mouse (*Mus domesticus*) where distinct chemical compounds have been linked to endocrinology and behavior (for review, see Schwende et al., 1986; Novotny et al., 1990), whereas their direct action on the vomeronasal neurons has also been verified (Leinders-Zufall et al., 2000; Sam et al., 2001).

Hamsters have been yet another frequently studied rodent in terms of olfaction. Here most studies have centered on investigations of flank gland markings (in both genders) and female vaginal secretions. Gland marking behavior of the Syrian golden hamster (*Mesocricetus auratus*) has been well established (Johnston, 1975, 1985), whereas the dense and viscous urine of this desert animal is generally viewed as less important in terms of chemical communication. Vaginal secretions of the Syrian golden hamster are also involved in a distinct communication function (Johnston, 1974; Singer et al., 1976) containing aphrodisin (a protein of the lipocalin family). However, it is improbable that aphrodisin serves a direct pheromonal function (Singer et al., 1987; Singer and Macrides, 1990; Vincent et al., 2001); it is more likely that it functions as a pheromone carrier (Briand et al., 2000).

Among the additional members of the hamster family, such as *Phodopus sungorus* (*P. sungorus*), *P. campbelli*, and *P. roborowsky*, fewer studies have accumulated to date. Secretions of the ventral gland and supplementary sacculi have recently been characterized chemically (Burger et al., 2001a,b). Additional glands and secretions may also be used in chemical communication. Unlike with the Syrian hamster, the *Phodopus* hamsters seem to involve urine odors (Lai et al., 1996). In a recent study of the effects of cross-fostering between *P. campbelli* and *P. sungorus* (Vasilieva et al., 2001), the young hamster appeared to learn quickly the olfactory cues from family members. Whereas we do not know currently the origin of the scent cues used in the adaptation process, the urinary odor (if composed of chemically distinct molecules) would be suggested. Earlier studies (Laska and Hudson, 1995) demonstrated that—at least in the squirrel monkey (*Saimiri sciureus*)—urine contains a considerable amount of information of a potential signal value. Consequently, the purpose of this study

was to characterize the volatile urinary constituents of *P. campbelli* and *P. sungorus* with respect to gender and age. In addition, due to the availability of a few *P. roborowsky* urinary samples, a comparison was also made with this species.

Whereas species and individual recognition may involve quantitative arrays of different chemosignals, it was deemed necessary to employ a highly quantitative technique for the extractions of urinary organic constituents. Based on the previously described stir bar extraction procedure for aqueous media (Baltussen et al., 1999, 2002), we have recently adapted this methodology to quantitative profiling of volatile and semivolatile compounds in biological media (Soini et al., 2005). Its excellent reproducibility has permitted reliable determinations of differences in volatile urinary profiles of different hamster species in this study. Numerous chemically distinct profile constituents were subsequently identified through a combined capillary gas chromatography–mass spectrometry and quantified with an element-specific detector.

METHODS AND MATERIALS

Animals and Age Groups. All three species of the genus *Phodopus* (*P. campbelli*, *P. roborowsky*, and *P. sungorus*) were available; gender, ages, and number of animals are listed in Table 1. Animals were born and raised in captivity and kept in indoor rooms in solid-bottom, polycarbonate cages (30 × 15 × 15 cm) with wood-chip bedding material. For all animals, the bedding material was identical (autoclaved, natural wood chips from pinewood). Bedding material was not subjected to chemical analyses. Since *Phodopus* hamsters are social

TABLE 1. CODES AND AGES FOR THE *Phodopus* HAMSTER GROUPS

Species	Subject groups	Age (months)	Number of animals
Phodopus campbelli female	cf4	4	3
	cf9	9	7
P. campbelli male	cm4	4	4
	cm9	9	3
	cm14	14	3
Phodopus sungorus female	sf1	1	3
	sf10–11	10–11	7
P. sungorus male	sm1	1	4
	sm10–11	10–11	5
	sm14	14	1
Phodopus roborowsky female	rf12	12	4

animals, they were kept in groups (three to five animals) of the same sex or in family units (a pair and its litter) for several weeks. The colony was maintained on a 14-hr light/10-hr dark light cycle with lights off at 10:00 hr; temperature was 21 ± 1°C. All animals had free access to hamster chew and water. Sunflower seeds, fruits, and lettuce were occasionally provided as a dietary supplement.

In *P. campbelli* and *P. sungorus*, the males and females represented different age groups. In *Phodopus* species, sexual maturation is reached between postnatal days 30 and 45. During the first 4 mo of life, animals may be regarded as young adults; animals between 9 and 14 mo-old are in their prime. Life span lasts for up to 2 yr.

Sample Collection. Urine was collected from all three hamster species (University of Tübingen, Germany). To collect urine, animals were removed from colonies and kept individually in small metabolic cages until they produced about 1 ml of urine, or up to 4 hr. If an animal did not produce enough urine in one sampling session, the procedure was repeated the following day. Estrous female hamsters were not subjected to urine collection (estrous state was checked regularly). Samples were kept frozen until analyzed.

Sample Preparation. All glassware was washed with distilled water and acetone and dried at 80°C in the oven. Volatile and semivolatile compounds were extracted from 1.0 ml of undiluted urine by sorptive extraction with a Twister PDMS polymer-coated stir bar (10 mm, 0.5-mm film thickness, 24 µl PDMS volume, Gerstel GmbH, Mülheim an der Ruhr, Germany) for 60 min. Stirring speed was 800+ rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnic, Oberschleissheim, Germany). After 60-min extraction time, a stir bar was rinsed with a small amount of distilled water, dried gently on the paper tissue, and was placed in the glass injector liner for mass spectrometry (MS) identification or in the TDSA autosampler tube for a gas-chromatographic (GC) quantification.

Mass Spectrometry. A Finnigan MAT Magnum ion trap gas chromatograph–mass spectrometer (GC-MS) system was used for the compound identification (Finnigan MAT, San Jose, CA, USA). The system was provided with a DB-5 capillary column (30 m × 0.25 mm, i.d., 0.25-µm film thickness, J&W Scientific, Folsom, CA, USA). Helium carrier gas head pressure was 12 psi. At the beginning of the column, a loop of uncoated deactivated silica tubing (30 cm × 0.25 mm, i.d.) was attached by using a universal Press-Tight Connector (Restek Corporation, Bellefonte, PA, USA) as described earlier (Ma et al., 1999). The loop was cooled with liquid nitrogen, while the Twister stir bar was held in the injector liner for 15 min at 250°C for the thermal desorption of the analytes. Subsequently, the desorbed compounds were cryotrapped into the liquid nitrogen cooled loop. After removing liquid nitrogen cooling, the GC temperature was held at 40°C for 5 min and increased to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. The manifold and transfer line temperatures were 220 and

300°C, respectively. The ion trap was operating in the positive electron ionization mode. Spectra were scanned from 40 to 350 msu (1 scan/sec).

Gas Chromatography. Gas-chromatographic (GC) equipment for the quantitative analysis consisted of an Agilent GC Model 6890 with an Atomic Emission Detector (AED) Model G2350A (Agilent Technologies Inc., Wilmington, DE, USA) and a Thermal Desorption Autosampler (TDSA, Gerstel GmbH). The separation capillary was DB-5 (30 m × 0.25 mm, i.d., 0.25-μm film thickness from J&W Scientific Folsom, CA, USA). Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection system CIS-4. TDSA operated in a splitless mode. Temperature program for desorption was 20°C (0.5 min), then 60°C/min to 280°C (10 min). Temperature of the transfer line was set at 280°C. CIS was cooled with liquid nitrogen to −60°C. After desorption and cryotrapping, CIS was heated at 12°C/sec to 280°C with the holding time of 10 min. Temperature program in the GC was 40°C for 5 min, then increasing to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. Carrier gas head pressure was 14 psi (flow rate 1.2 ml/min). The GC unit was operated in the constant flow mode. The emission lines for carbon (193-nm), sulfur (181-nm), and nitrogen (174-nm) were monitored in the atomic plasma emission detection.

Statistical Analysis. Pirouette Lite (Infometrix, Inc., Woodinville, WA, USA) was used for exploratory multivariate analysis to obtain hierarchical cluster patterns for classification of analysis data and for establishment of chemical relations within the subject groups. When appropriate, group comparisons were calculated using the nonparametric Mann–Whitney *U* test.

RESULTS AND DISCUSSION

According to the literature (Sokolov and Vasilieva, 1993; Vasilieva et al., 1990) and the results presented here, *P. campbelli* and *P. sungorus* seem closely related. Yet, they are widely considered as two distinct species. Therefore these two species were compared against each other. *P. roborowsky* will be discussed separately.

I. Campbelli and Sungorus Groups. Campbelli and *sungorus* hamster groups each consisted of 10 males and females (Table 1). Preliminary screening showed that both groups exhibited relatively similar chemical constituents in their profiles. Differences were seen mainly in the levels of compounds within the same age and gender groups. Gender and age inflicted certain profile differences, which will be discussed in more detail below.

The compounds identified by GC-MS in the adult animals are shown in Table 2A. A short list of compounds (numbers 1–17) in Table 2B indicates

TABLE 2A. IDENTIFIED AND PARTIALLY IDENTIFIED COMPOUNDS IN URINE OF MALE *P. sungorus* AND *P. campbelli* HAMSTERS (AGE 10–11 MO)

Compound	GC-MS (min)	RI[a]	Compound	GC-MS (min)	RI[a]
Acetone	1.00	760	An alkenylpyrazine (C-4)	24.48	1165
Butanone	1.42	766	A terpene	24.88	1173
2-Pentanone	1.87	773	2-Nonanol	25.10	1177
3-Pentanone	2.45	781	Phenylethylamine	25.58	1186
3-Hexanone	5.05	819	Phenylacetone	26.55	1204
4-Heptanone	8.82	868	Phenylacetonitrile	27.20	1217
2,6-Dimethylpyridine	9.27	876	A dimethylpropylpyrazine	30.17	1273
2-Heptanone	10.30	896	An ethylpropylpyrazine	30.53	1280
Heptanal	10.33	897	An alkenylpyrazine (C-5)	30.80	1285
2,5-Dimethylpyrazine	10.85	906	Methylsalicylate (food)	31.33	1295
3-Hepten-2-one	12.80	943	A butylmethylpyrazine	31.58	1300
Benzaldehyde	14.08	968	Decanal	32.62	1319
1-Methylpiperidine	15.03	986	Formanilide	32.80	1323
6-Methyl-1-hepten-2-one	15.68	998	Benzothiazole	33.15	1329
Octen-2-ol	16.30	1010	Quinoline	34.08	1347
6-Methyl-5-hepten-2-one	16.43	1012	An alkylpyrazine (C-6)	35.87	1381
Phenol	16.85	1020	Geraniol	36.33	1390
An alkylpyrazine (C-3)	17.20	1027	An alkenylpyrazine (C-6)	36.43	1392
A trimethylpyrazine	17.37	1030	An alkylpyrazine (C-6)	37.02	1403
A propylpyrazine	17.68	1036	2-Undecanol (branched)	37.92	1420
Acetophenone	21.82	1114	Indole	38.42	1429
Methylaniline	22.38	1125	Undecanal	38.67	1434
4-Nonanone	22.82	1133	2-Undecanol (branched)	39.42	1448
1-Octanol	23.03	1138	An alkylpyrazine (C-8)	47.05	1593
6-Methyl-2-octanol	23.82	1152	Geranylacetone	49.20	1634
A methylpropylpyrazine	24.05	1157	An alkylpyrazine (C-8)	50.38	1656
			Vitamin K (menadione from food)	52.47	1696

[a]Retention index on DB-5 column phase.

substances that have been used for quantitative measurements by TDSA-GC-AED. A typical MS total ion chromatogram (TIC) of the male hamster urine is shown in Figure 1. Peak numbers refer to Table 2B.

Compound identification was made by GC-MS based on retention times, spectra, and known standard compounds. Based on the total ion chromatogram (TIC) profiles (as shown in Figure 1), the compound profiles were quantitatively compared with the GC-AED profiles, whereas the peak identities were assigned for these measurements. Figure 2 shows a typical GC-AED compound profile (carbon 193-nm line) for a male *campbelli* hamster.

TABLE 2B. COMPOUNDS QUANTIFIED BY GC-AED

Compound no.	(min)	Compound name
1	8.82	4-Heptanone
2	10.30	2-Heptanone
3	10.85	2,5-Dimethylpyrazine
4	17.68	A propylpyrazine
5	22.38	Methylaniline
6	22.82	4-Nonanone
7	24.05	A methylpropylpyrazine
8	24.48	An alkenylpyrazine (C-4)
9	25.10	2-Nonanol
10	27.20	Phenylacetonitrile
11	30.17	A dimethylpropylpyrazine
12	30.53	An ethylpropylpyrazine
13	32.80	Formanilide
14	36.33	Geraniol
15	36.43	An alkenylpyrazine (C-6)
16	37.02	An alkylpyrazine (C-6)
17	37.92	2-Undecanol (branched)

Within the complex urinary compound profiles, average levels were calculated for 17 identified compounds (Table 2B). Averages were grouped pertaining to the different species/gender/age groups. Calculations were based on the corresponding integrated peak areas for each compound. Logarithmic ($\log_{10}$) transformations log(peak area + 1) were used to normalize the graphs due to large numerical values of the integrated peak areas (Zar, 1999).

In general, male hamster urine contained higher levels of all compounds in both *campbelli* and *sungorus* species when compared with females. Typically, urinary profiles in *campbelli* and *sungorus* males were closely related. Figure 3 shows average levels of 17 compounds (from Table 2B) for *campbelli* females (9 mo) and males (9 mo) and *sungorus* females (10–12 mo) and males (10–11 mo).

Several quantified compounds were either gender- or age-specific in *campbelli* and *sungorus*. Also, some of the compounds were typical for the particular hamster species. Hamster groups *campbelli* females (cf), *campbelli* males (cm), *sungorus* females (sf), and *sungorus* males (sm) were further divided into subgroups based on their ages. The group codes and number of individuals in each group are shown in Table 1. Summary of the identified species/gender/age-specific compounds is shown in Table 3.

Multivariate hierarchical cluster analysis (HCA) in Figure 4 shows "similarity degrees" of different hamster groups based on the averages of 17 quantified compounds. Clusters connected closest to 1.0 on the *X*-axis mark

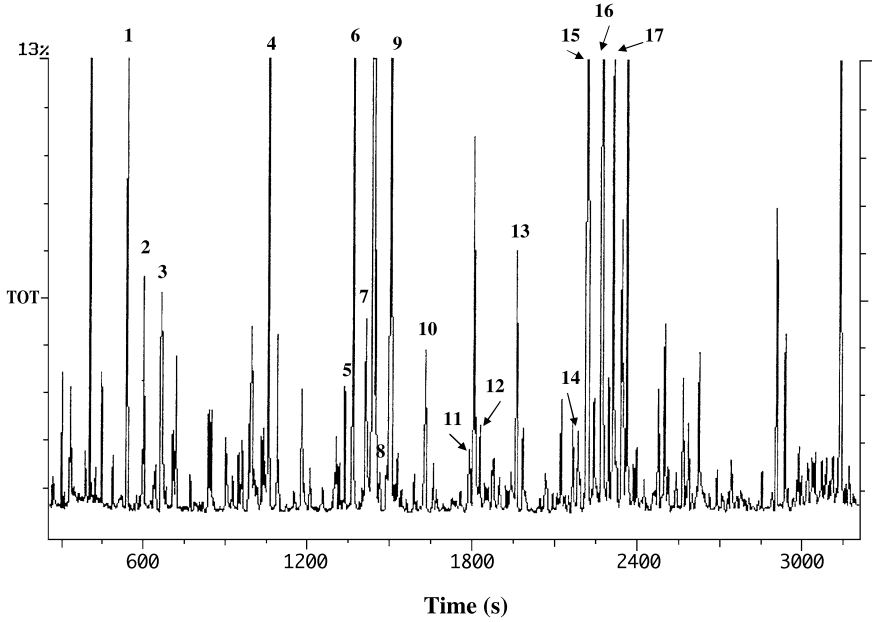

FIG. 1. A GC-MS total ion chromatogram (TIC) of the SBSE-extracted urine of a male *P. sungorus* hamster. Analytical conditions are described in the text. Numbers refer to identified compounds in Table 2B used for quantification by GC-AED.

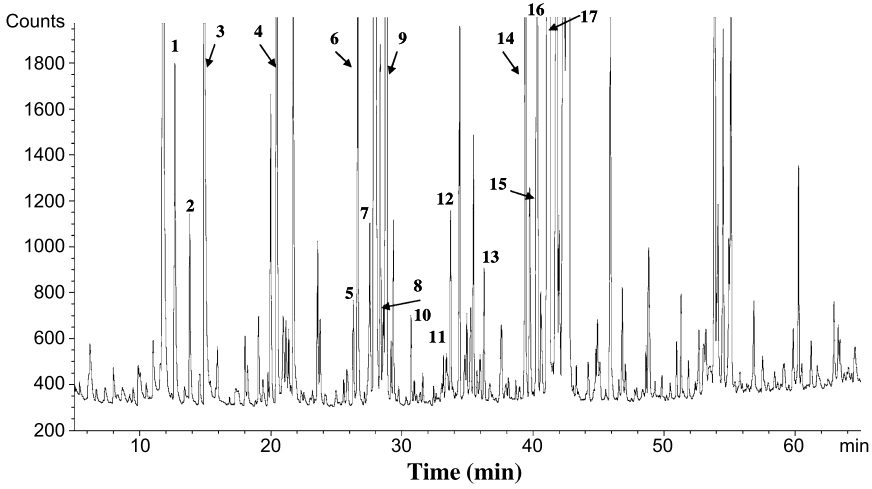

FIG. 2. A urinary volatile profile of a male *P. campbelli* hamster (cm-9) by GC-AED, carbon line 193-nm. Separation conditions are described in the text.

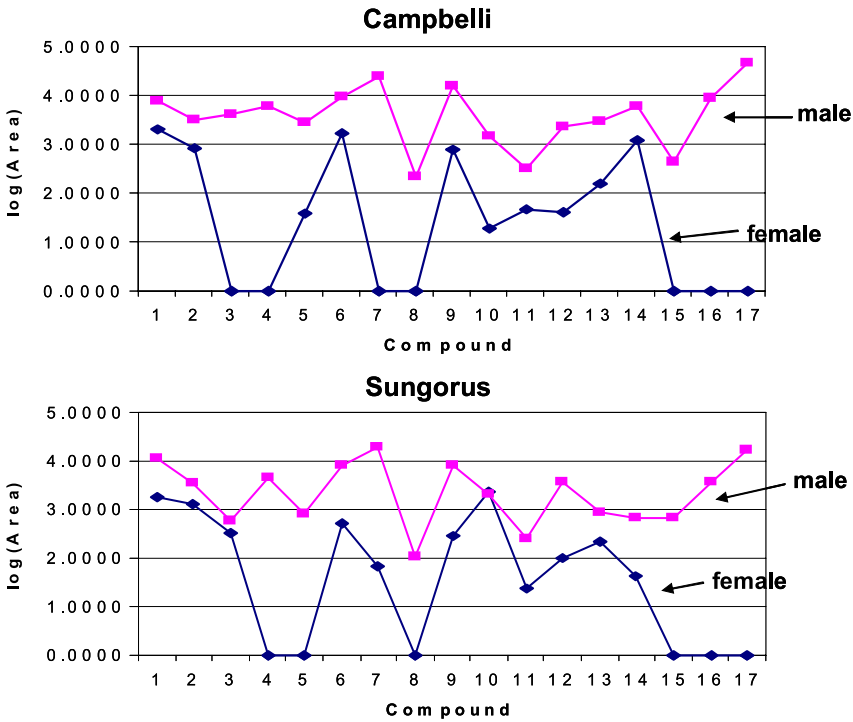

FIG. 3. Averages of levels for compound numbers 1–17 for mature female and male *campbelli* and *sungorus* hamsters. Logarithmic values for peak areas were used [log(peak area + 1)].

the most similar group properties. As shown in Figure 4, volatile profiles of male *campbelli* and *sungorus* hamsters for 17 identified compounds relate closely to each other. Within the corresponding female profiles, relation is not so clear.

Additional comparative qualitative information was obtained from compound profiles by GC-AED sulfur 181-nm line chromatograms (Figure 5). GC-AED facilitated detection of sulfur compound profiles at trace picogram levels (Soini et al., 2005). Characterization by GC-MS was not successful for these compounds due to low levels. As noted for the GC-AED carbon 193-nm line chromatograms (average results in Figure 3), also sulfur-containing, yet unidentified compounds appeared at higher levels in male hamster urine than in female urine.

Method Precision. Reproducibility of extraction and the GC analysis was investigated with the pooled male *campbelli* hamster urine. Four parallel

TABLE 3. SUMMARY OF GENDER- AND AGE-SPECIFIC COMPOUNDS AND THEIR AVERAGE[a] VALUES FOR *P. campbelli* AND *P. sungorus* GROUPS QUANTIFIED BY GC-AED

Compound		*P. campbelli*				*P. sungorus*			
		Adult		Juvenile		Adult		Juvenile	
No.	Name	Male (9)	Female (9)	Male (4)	Female (4)	Male (10–11)	Female (10–11)	Male (1)	Female (1)
3	2,5-Dimethylpyrazine	3.6	0	3.9	0	2.4	2.1	0	0
4	A propylpyrazine	3.8	0	3.9	0	3.6	0	0	0
5	Methylaniline	3.5	0.9	3.5	2.1	2.7	0	0	0
7	A methylpropylpyrazine	4.4	0	4.7	0	4.3	1.3	3.7	0
8	An alkenylpyrazine (C-4)	2.0	0	4.1	0	1.4	0	0	0
10	Phenylacetonitrile	3.2	0.4	0.8	0.3	3.3	3.4	0	0
14	Geraniol	3.8	0.9	1.9	0	2.4	0.9	1.7	0
15	An alkenylpyrazine (C-6)	2.3	0	3.3	0	2.6	0	0	0
16	An alkylpyrazine (C-6)	3.9	0	4.4	0	3.6	0	2.6	0
17	2-Undecanol (branched)	4.7	0	2.9	0	4.2	0	4.6	0

[a]Log (peak area + 1).

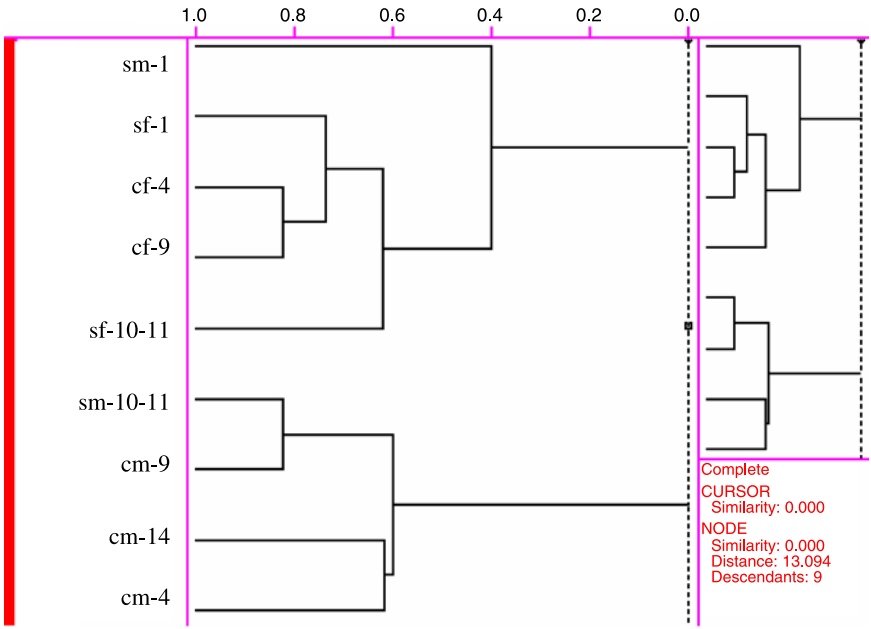

FIG. 4. A hierarchical cluster analysis (HCA) graph for all hamster groups using levels of compounds 1–17 as variables (see Table 2B).

extractions were performed, and the relative standard deviations of peak areas were calculated (RSD). Typical variability for the analytes was between 0.1 and 2.0% (RSD, $N = 4$) as shown in Table 4. A uniform sorptive polymer coating, automated thermal desorption sample introduction, and a constant flow control in the GC-AED system have all contributed to the acceptable reproducibility of the results when no internal standard was deemed necessary.

Role of Pyrazines. In all male *campbelli* and *sungorus* urinary profiles, the presence of pyrazines was a dominating factor, as seen in Table 2A. Compounds with numbers 3, 4, 7, 8, 12, 15, and 16 (Table 2B) appeared to be related to gender and age in both hamster groups. *Campbelli* males showed more *male-specific pyrazines* (2,5-dimethylpyrazine, a propylpyrazine, a methylpropylpyrazine, a C-6 alkylpyrazine, and alkenyl C-4 and C-6 pyrazines). In the *sungorus* group, female urine also contained 2,5-dimethylpyrazine and a C-4 alkylpyrazine, however, with the latter appearing at higher levels in *sungorus* males than in females ($P < 0.02$). Ethylpropylpyrazine (compound 12) levels were significantly higher in both *campbelli* ($P < 0.002$) and *sungorus* males ($P < 0.02$) compared with females. Ethylpropylpyrazine levels were also significantly higher in mature *sungorus* males (10–14 mo) compared with

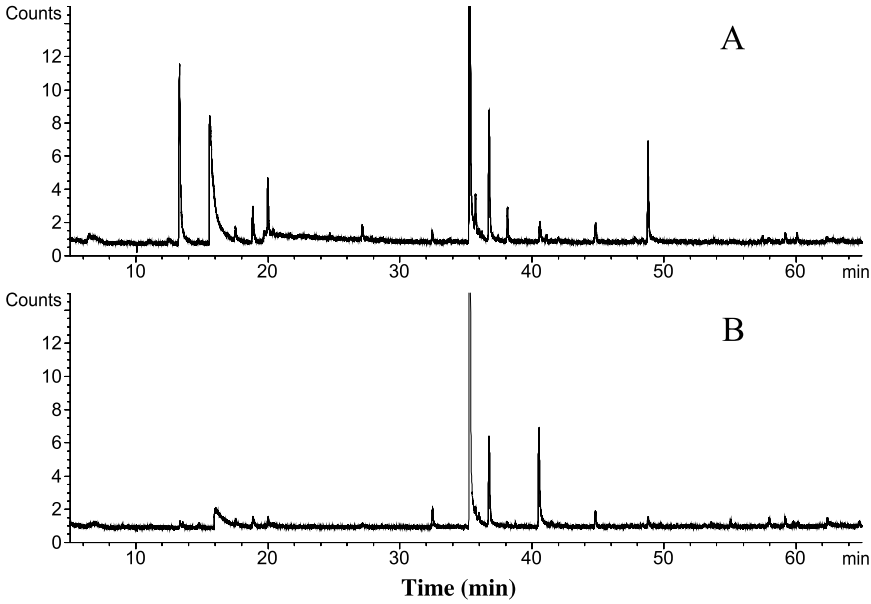

FIG. 5. Comparative urinary volatile profiles for pooled (A) male *sungorus* and (B) female *sungorus* urine by GC-AED sulfur line 181-nm. Compounds were not identified.

juvenile (1 mo) animals ($P < 0.002$). In all *campbelli* and *sungorus* females, the less volatile alkylated C-6 pyrazines (compounds 15 and 16) were absent (below detection limit). Urinary alkenyl C-4 and C-6 pyrazines (compounds 8 and 15) appeared higher in old male hamsters as well as in younger individuals, although the differences were not statistically significant. Figure 6 illustrates average levels of unsaturated alkenyl pyrazines in the *campbelli* male urinary profiles in the different age groups. Large differences in pyrazine levels within gender and age suggest that pyrazines may be under endocrinological control and may thus be an important means of chemical communication for both *campbelli* and *sungorus* hamsters.

Ketones. Relatively small differences of the levels of 4- and 2-heptanone over the age, gender, and species groups were observed. Figure 7 shows that 2-heptanone levels, as an example (compound 2), in *campbelli* (9 mo) and *sungorus* (10–11 mo) males were relatively close to each other. The same applied to *campbelli* (9 mo) and *sungorus* (10–12 mo) females (data not shown). Statistically significant higher levels of 4-nonanone were found in male urine compared with females in both *campbelli* ($P < 0.02$) and *sungorus* groups ($P < 0.002$). These findings suggest that urinary ketones may not carry specific signaling properties as pyrazines do for *campbelli* and *sungorus* hamsters,

TABLE 4. REPRODUCIBILITY OF 17 QUANTIFIED COMPOUNDS BY GC-AED EXEMPLIFIED BY DETERMINATION OF SAMPLES OF POOLED MALE *P. campbelli* URINE ($N = 4$)

Compound no.	Compound	Average log (peak area)	Standard deviation (SD) of log (peak area)	Relative standard deviation of log (peak area) (RSD %, $N = 4$)
1	4-Heptanone	3.573	0.077	2.2
2	2-Heptanone	3.123	0.040	1.3
3	2,5-Dimethylpyrazine	3.559	0.071	2.0
4	A propylpyrazine	3.548	0.035	1.0
5	Methylaniline	3.014	0.025	0.8
6	4-Nonanone	3.543	0.003	0.1
7	A methylpropylpyrazine	4.610	0.034	0.7
8	An alkenylpyrazine (C-4)	3.564	0.028	0.8
9	2-Nonanol	3.577	0.013	0.4
10	Phenylacetonitrile	Below detection limit		
11	A dimethylpropylpyrazine	3.040	0.033	1.1
12	An ethylpropylpyrazine	3.064	0.021	0.7
13	Formanilide	3.455	0.054	1.6
14	Geraniol	3.226	0.021	0.6
15	An alkenylpyrazine (C-6)	3.873	0.011	0.3
16	An alkylpyrazine (C-6)	4.523	0.014	0.3
17	2-Undecanol (branched)	4.024	0.013	0.3

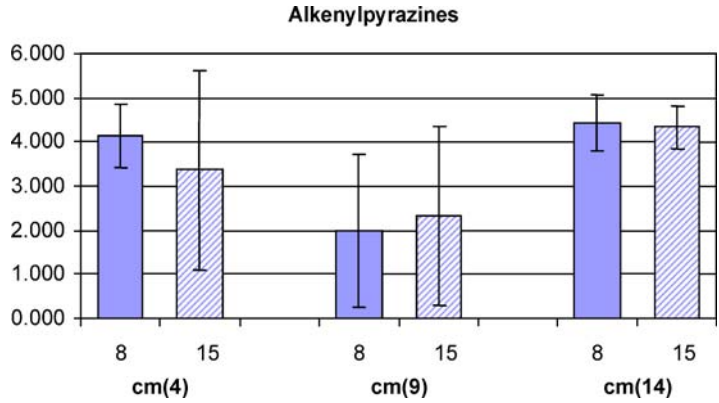

FIG. 6. Averages of alkenyl pyrazine compounds 8 and 15 (see Table 2B) in different male *campbelli* age groups (ages 4, 9, and 14 mo).

although they may be involved in the creation of the baseline scent for the species.

Alcohols. Among the identified alcohols, branched 2-undecanol (compound 17) appeared male-specific in both *campbelli* and *sungorus* species (Figure 3). Surprisingly, branched 2-undecanol levels were already high in the urinary profiles of young *sungorus* males. Levels were declining with age. Geraniol (a terpene alcohol, compound 14) levels appeared somewhat higher in male and female *campbelli* mature hamsters (not a statistically significant

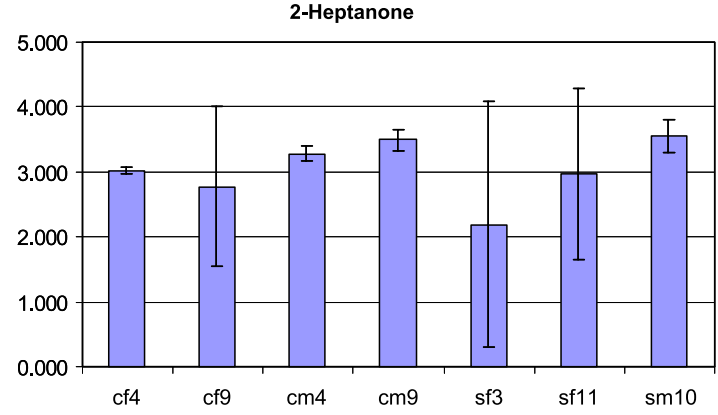

FIG. 7. Average levels of 2-heptanone (compound 2) in different hamster groups.

difference). Only in the *campbelli* group, were geraniol levels higher in males than in females ($P < 0.02$). Young females in both *campbelli* and *sungorus* groups lacked geraniol in their profiles. A branched alcohol, 2-nonanol (compound 9), was found in all age, gender, and species groups. *Sungorus* females showed the lowest levels of 2-nonanol (data not shown) from all groups.

Selected Nitrogen Compounds. Methylaniline (compound 5), phenylacetonitrile (compound 10), and formanilide (compound 13) were independent variables related to each other based on a hierarchical cluster analysis (HCA, data not shown). Within the male profiles, methylaniline was specific for the *sungorus* males only (Figure 3). In the male profiles, methylaniline was shown only in the age group of 9–11 mo (not seen in juvenile and 14-mo-old males). Phenylacetonitrile was not seen in the urine of immature female or male *sungorus* hamsters (1 mo). Among mature *sungorus* males and females, there were no statistically significant differences, whereas the levels in male mature *campbelli* were higher than in females ($P < 0.02$).

Figure 8 shows comparisons of the averages of selected compounds in *campbelli* (9 mo) and *sungorus* (10 mo) male urinary profiles. *Campbelli* average levels tended to be slightly higher than *sungorus* levels. However, the individual variation in their concentrations was clearly larger in the *sungorus* group.

II. P. roborowsky. Within the *roborowsky* group (four female subjects), a completely different urinary volatile pattern was seen. A list of identified or tentatively identified *roborowsky* female urinary compounds is shown in Table 5.

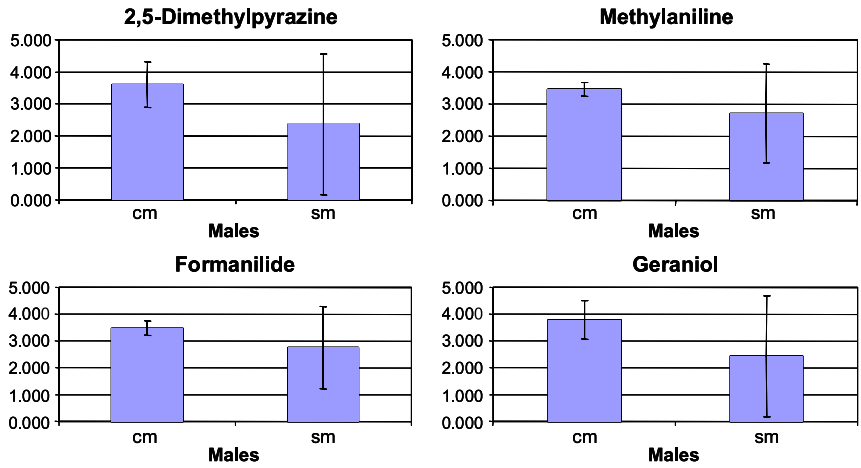

FIG. 8. Comparison of averages and standard deviations of selected compound levels in *campbelli* (cm) and *sungorus* (sm) urinary volatile profiles.

TABLE 5. IDENTIFIED AND PARTIALLY IDENTIFIED COMPOUNDS IN THE URINE OF FEMALE *P. roborowsky* (AGE 12 MO)

Compound	Rt (sec)	Rt (min)	RI[a]
4-Heptanone	544	9.07	879
2-Heptanone	607	10.12	895
3-Hepten-2-one	779	12.98	937
6-Methyl-3-heptanone	847	14.12	954
Benzaldehyde	857	14.28	956
Phenol	996	16.60	990
Acetophenone	1310	21.83	1068
1-Octenol	1356	22.60	1079
1-Octanol	1369	22.82	1082
A cresol	1393	23.22	1088
An unknown pyrazine	1422	23.70	1095
2-Nonanone	1454	24.23	1103
A ketone	1502	25.03	1115
Phenylacetonitrile	1629	27.15	1146
3-Nonen-2-one	1660	27.67	1154
An ester	1730	28.83	1172
An ethylphenol	1790	29.83	1186
1-Nonanol	1820	30.33	1193
Methylsalicylate	1875	31.25	1207
Benzothiazole	1988	33.13	1234
An ester	2020	33.67	1242
An ester	2090	34.83	1260
Geraniol	2163	36.05	1277
A branched ketone	2191	36.52	1284
4-Undecanone	2245	37.42	1298
Indole	2300	38.33	1311
4-Phenyl-3-buten-2-one	2559	42.65	1375
Eugenol	2569	42.82	1377
An alkylquinoxaline (C-3)	2853	47.55	1447
Geranylacetone	2947	49.12	1470
Ethylcinnamate	2981	49.68	1479
A tridecanone	3023	50.38	1489
Menadione	3138	52.30	1517
An alkylquinoxaline (C-4)	3186	53.10	1529
An alkylquinoxaline (C-4)	3220	53.67	1538

[a]Retention index.

Screening of urinary profiles of *P. roborowsky* revealed that few compounds were in common with *campbelli* and *sungorus* (Table 2A). A dominant array of alkyl- and alkenylpyrazines was not present in these samples. Instead, the *roborowsky* urine featured a group of higher-boiling alkyl quinoxalines. The presence of ketones, alcohols, and esters was characteristic for the group of 60

identified compounds. The origin of ketones is likely to be β-and ω-oxidation of fatty acids. This suggests that metabolic pathways in *P. roborowsky* hamsters differ markedly from *P. sungorus* and *P. campbelli* hamsters. Also, based on the large difference on the chemical constituent types in urine, the baseline scent properties of the *roborowsky* female hamster are expected to differentiate from those of *sungorus* and *campbelli* females.

In summary, quantitative data were proven highly reproducible using the stir bar sorptive extraction (SBSE) methodology. Typically, relative standard deviations (RSD, $N = 4$) were 0.1–2.0% for normalized peak areas. This analytical feature provided reliability for the urinary profile comparisons. Ultrahigh sensitivity of the atomic emission detection for sulfur-containing compounds provided extra information about comparative urinary volatile profiles.

The chemical characterization data on *P. campbelli*, *P. sungorus*, and *P. roborowsky* verified relatively similar compound profiles in *campbelli* and *sungorus* (males and females), which differed substantially from *P. roborowsky* (females only, males were not available in the screening study). This suggests that metabolic pathways in *campbelli* and *sungorus* hamsters resemble each other but differ substantially from the *roborowsky* species. In *campbelli* and *sungorus*, different substituted pyrazines dominated the urinary profiles. Several pyrazines and branched 2-undecanol were male-specific in both species. Methylaniline and phenylacetonitrile were age-specific in both male hamster species. The individual compound level variability within the *P. sungorus* was clearly larger than in the *P. campbelli* species.

The urinary profiles of *roborowsky* female hamsters were dominated by ketones, alcohols, and esters. Similar pyrazine arrays, as seen in *campbelli* and *sungorus*, were clearly not observed. Instead, low-volatility alkyl quinoxalines were detected.

One could hypothesize that urinary compound classes such as pyrazines, within a certain volatility range (early eluting pyrazines vs. later eluting pyrazines), may classify the overall perception of the urine odor so that a closely related species may learn the scent codes, as reported (Vasilieva et al., 2001) under cross-fostering conditions. The question remains which urinary compounds carry most crucial information and whether concentration level differences play a role in scent recognition between *campbelli* and *sungorus*. At this time, it is not known whether there is any effect of a seasonal variation on the urinary profiles.

Acknowledgments—We thank Mr. Kevin E. Bruce for his help in preparation of the manuscript. This work was supported by a grant no. DC 24 180 from National Institute of Deafness and Other Communication Disorders, National Institute of Health, U.S. Department of Health and Human Resources, and partially by a grant no. RFBR 04-04-49190a to Nina Y. Vasilieva from the Russian Academy of Sciences.

REFERENCES

BAKKE, J. M. and FIGENSCHOU, E. 1990. Volatile compounds from the red deer (*Cervus elaphus*). Substances secreted via the urine. *Comp. Biochem. Physiol.* 97A:427–431.

BALTUSSEN, E., SANDRA, P., DAVID, F., and CRAMERS, C. 1999. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: Theory and principles. *J. Microcolumn Sep.* 11:737–747.

BALTUSSEN, E., CRAMERS, C. A., and SANDRA, P. J. F. 2002. Sorptive sample preparation—A review. *Anal. Bioanal. Chem.* 373:3–22.

BRIAND, L., HUET, J.-C., PEREZ, V., LENOIR, G., NESPOULOUS, C., BOUCHER, Y., TROTIER, D., and PERNOLLET, J. C. 2000. Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein. *FEBS Lett.* 476:179–185.

BURGER, B. V., SMIT, D., SPIES, H. S. C., SCHMIDT, C., SCHMIDT, U., TELITSINA, A. Y., and GRIERSON, G. R. 2001a. Mammalian exocrine secretions XV. Constituents of secretion of ventral gland of male dwarf hamster, *Phodopus sungorus sungorus*. *J. Chem. Ecol.* 27:1259–1276.

BURGER, B. V., SMIT, D., SPIES, H. S. C., SCHMIDT, C., SCHMIDT, U., and TELITSINA, A. Y. 2001b. Mammalian exocrine secretions XVI. Constituents of secretion of supplementary sacculi of dwarf hamster, *Phodopus sungorus sungorus*. *J. Chem. Ecol.* 27:1277–1288.

JOHNSTON, R. E. 1974. Sexual attraction function of golden hamster vaginal secretion. *Behav. Biol.* 12:111–117.

JOHNSTON, R. E. 1975. Scent marking by male golden hamsters (*Mesocricetus auratus*). I. Effects of odors and social encounters. *Z. Tierpsychol.* 37:75–98.

JOHNSTON, R. E. 1985. Communication, pp. 121–154, *in* H. I. Siegel (ed.). The Hamster, Reproduction and Behavior. Plenum Publishers, New York.

JORGENSON, J. W., NOVOTNY, M., CARMACK, M., COPLAND, G. B., WILSON, S. R., WHITTEN, W. K., and KATONA, S. 1978. Chemical scent constituents in the urine of the Red Fox (*Vulpes vulpes* L.) during the winter season. *Science* 199:796–798.

LAI, S.-C., VASILIEVA, N. Y., and JOHNSTON, R. E. 1996. Odors providing sexual information in Djungarian hamsters: Evidence for an across-odor code. *Horm. Behav.* 30:26–36.

LASKA, M. and HUDSON, R. 1995. Ability of female squirrel monkey (*Saimiri sciureus*) to discriminate between conspecific urine odours. *Ethology* 99:39–52.

LEINDERS-ZUFALL, T., LANE, A. P., PUCHE, A. C., MA, W., NOVOTNY, M. V., SHIPLEY, M. T., and ZUFALL, F. 2000. Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* 405:792–796.

MA, W., WIESLER, D., and NOVOTNY, M. V. 1999. Urinary volatile profiles of the deermouse (*Peromyscus maniculatus*) pertaining to gender and age. *J. Chem. Ecol.* 25:417–431.

NOVOTNY, M., JEMIOLO, B., and HARVEY, S. 1990. Chemistry of rodent pheromones: Molecular insights into chemical signaling in mammals, *in* D. W. MacDonald, D. Müller-Schwarze, and S. E. Natynczuk (eds.). Chemical Signals in Vertebrates 5. Oxford University Press, New York.

RALLS, K. 1971. Mammalian scent marking. *Science* 171:443–449.

RAYMER, J., WIESLER, D., NOVOTNY, M., ASA, C., SEAL, U. S., and MECH, L. D. 1984. Volatile constituents of wolf (*Canis lupus*) urine as related to gender and season. *Experientia* 40:707–709.

SAM, M., VORA, S., MALNIC, B., MA, W., NOVOTNY, M. V, and BUCK, L. B. 2001. Odorants may arouse instinctive behaviors. *Nature* 412:142–709.

SINGER, A. G. and MACRIDES, F. 1990. Aphrodisin: Pheromone or transducer? *Chem. Senses* 15:199–203.

SINGER, A. G., AGOSTA, W. C., O'CONNELL, R. J., PFAFFMANN, C., BOWEN, D. V., and FIELD, F. H.

1976. Dimethyl disulfide: An attractant pheromone in hamster vaginal secretion. *Science* 191:948–950.

SINGER, A. G., AGOSTA, W. C., CLANCY, A. N., and MACRIDES, F. 1987. The chemistry of vomeronasally detected pheromones: Characterization of an aphrodisiac protein. *Ann. N.Y. Acad. Sci.* 519:287–298.

SCHWENDE, F. J., WIESLER, D., JORGENSON, J. W., CARMACK, M., and NOVOTNY, M. 1986. Urinary volatile constituents of the house mouse, *Mus musculus*, and their endocrine dependency. *J. Chem. Ecol.* 12:277–296.

SOINI, H. A., BRUCE, K. E., WIESLER, D., DAVID, F., SANDRA, P., and NOVOTNY, M. V. 2005. Stir bar sorptive extraction: A new quantitative and comprehensive sampling technique for determination of chemical signal profiles from biological media. *J. Chem. Ecol.* 31:377–392.

SOKOLOV, V. E. and VASILIEVA, N. Y. 1993. Confirmation of the species specificity of *Phodopus sungorus* (Pallas, 1773) and *Phodopus campbelli* (Thomas, 1905) by hybridization. *Dokl. Acad. Naul. SSSR.* 332:122–125 (in Russian).

VASILIEVA, N. Y., TELITZINA, A. Y., and SUROV, A. V. 1990. Confirmation of species identity *Phodopus sungorus* (Pallas, 1773) and *Phodopus campbelli* (Thomas, 1903) by hybridization. All-Union. Meeting Theriol. Soc. Acad. Sci. USSR., Moscow 1:48–49 (in Russian).

VASILIEVA, N. Y., LAI, S.-C., PETROVA, E. V., and JOHNSTON, R. E. 2001. Development of species preferences in two hamsters, *Phodopus campbelli* and *Phodopus sungorus*: Effects of cross-fostering. *Ethology* 107:217–236.

VINCENT, F., LÖBEL, D., BROWN, K., SPINELLI, S., GROTE, P., BREER, H., CAMBILLAU, C., and TEGONI, M. 2001. Crystal structure of aphrodisin, a sex pheromone from female hamster. *J. Mol. Biol.* 305:459–469.

ZAR, J. H. 1999. Biostatistical Analysis 4th edn. Prentice Hall, Upper Saddle River, NJ.