J Chem Ecol (2008) 34:398–407
DOI 10.1007/s10886-008-9441-3

Potential Chemosignals in the Anogenital Gland Secretion of Giant Pandas, *Ailuropoda melanoleuca*, Associated with Sex and Individual Identity

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Received: 13 September 2007 / Accepted: 29 January 2008 / Published online: 22 February 2008
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Abstract With a combination of dichloromethane extraction and analysis by gas chromatography–mass spectrometry (GC-MS), we found 39 compounds (corresponding to 38 GC peaks) in the anogenital gland secretion (AGS) of captive adult giant pandas, *Ailuropoda melanoleuca*, during the non-mating season. In addition to indole, squalene, and some of the straight-chain fatty acids that had been characterized previously from the AGS of giant pandas, we identified several new compounds such as decenal, two isomers of decadienal, phenylacetic acid, 5-methylhydantoin, hydroquinone, phenylpropanoic acid, and erucic acid. Quantitative comparison of the relative abundances of the 20 main GC peaks revealed that 5-methylhydantoin, indole, and erucic acid are putative female pheromones, whereas squalene and hydroquinone are putative male pheromones. In addition to the presence of a few individual-specific compounds, the relative abundances of most of the 21 constituents varied more among individuals than within individuals. This suggests that individual identity might be coded in both digital and analog form. The chemical composition of different AGS samples from the same pandas consistently displayed a minimum cluster distance, much smaller than that between samples from different individuals in a hierarchical linkage cluster (average linkage) dendrogram. Our results indicate that the AGS might contain an "odor fingerprint." Although putative sex pheromones such as squalene and erucic acid should be assessed further by bioassay, our study suggests that synthetic chemosignals might be useful in modulating the behavior and physiology of giant pandas.

Keywords *Ailuropoda melanoleuca* · Anogenital gland secretions (AGS) · Giant panda · Individuality · Pheromone · Sex · Sex specificity

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Introduction

Pheromones play crucial roles in modulating sexual and social behavior in mammals (Brown and MacDonald 1985). However, compared with our knowledge of pheromonal compounds of insects, mammalian chemical signals have seldom been characterized structurally, partially owing to their complex composition. Nonetheless, a large amount of information has been amassed about pheromonal functions of whole odorant secretion/excretion in mammals (Brown and MacDonald 1985; Novotny et al. 1999). For a comprehensive understanding of mammalian chemical communication, it is necessary to combine bioassay and

chemical analysis to identify the pheromonal components involved (Singer et al. 1997; Novotny et al. 1999). Fortunately, a systematic approach to screening for pheromonal compounds from numerous scent sources by gas chromatography (GC) has been established in mice, whereby odorant components that vary with biological characters can be considered as pheromone candidates for further verification by bioassay (Singer et al. 1997; Novotny et al. 1999). By using this approach, we have recently characterized some new pheromone components from the preputial glands of the house mouse, *Mus musculus*, and Brandt's vole, *Lasiopodomys brandtii*, and from the flank glands of the golden hamster, *Mesocricetus auratus* (Zhang et al. 2007a, b; J.X. Zhang, unpublished data). These new pheromone components include high boiling compounds such as hexadecanol, hexadecyl acetate, farnesyl acetate, tetradecanoic acid, and hexadecanoic acid (Zhang et al. 2007a, b; J.X. Zhang, unpublished data). The discovery of these higher boiling compounds has extended the spectrum and concept of mammalian pheromones.

The giant panda, *Ailuropoda melanoleuca*, is an endangered species that inhabits fragmented mountainous areas of Sichuan, Shaanxi, and Gansu provinces in China. Poor in eyesight and hearing ability, pandas mainly use their anogenital gland secretions (AGS) and urine to communicate with each other and mediate their social interactions (Schaller et al. 1985; Swaisgood et al. 1999; Liu et al. 2002). Behavioral tests with giant pandas have revealed that the AGS carries a wealth of information about sex and individuality (Swaisgood et al. 2000; White et al. 2002, 2003; Liu et al. 2005; Tian et al. 2007). Male and female pandas show different behavioral responses to AGS samples from conspecific donors of the same and opposite sex. The chemosensory response in the panda differs between the sexes and is age-dependent (Swaisgood et al. 2000; White et al. 2002, 2003; Liu et al. 2005; Tian et al. 2007). Chemical analysis of the AGS should provide further information about pheromone-based behavioral discrimination in the panda. So far, however, sex-and age-dependent pheromonal components have not been identified due largely to the distractive presence of numerous inactive alkanes (e.g., Yuan et al. 2004; Liu et al. 2006) and to inadequacies in the analytical system, which is often capable of detecting only a small portion in the repertoire of odorant compounds (Hagey and MacDonald 2003).

In the current study, we improved our analytical system and used the relative abundances of the principal GC peaks (Sun and Müller-Schwarze 1998a, b) to compare qualitatively and quantitatively male and female AGS samples and to search for components that differ between the sexes in giant pandas during the non-mating season. With the relative standard deviation (RSD) and hierarchical cluster analysis of the relative abundances of the main GC peaks, we compared AGS samples among individuals. This method has been successfully used in similar studies in several *Mustela* species, the house mouse, the Brandt's vole, and the golden hamster (Zhang et al. 2003, 2005, 2007a, b; J.X. Zhang, unpublished data).

Methods and Materials

Subjects Sixteen adult giant pandas (M/F = 1:1, age >5yr) housed at the Bifengxia Panda Base ($N = 10$) and Wolong Breeding Center ($N = 6$), China Conservation and Research Center for the Giant Panda, Wolong, China were used as AGS donors. Six subjects were kept in traditional enclosures and ten in semi-natural enclosures (Table 1). Each traditional enclosure contained a night pen (5.8 × 2.3m) and an outdoor yard (5.8 × 13m) with grass, some climbing apparatuses, and a small pond as a water source. Each outdoor yard adjoined two others via a cement wall in which there was a small wire mesh fence door (1 × 10m). Each semi-natural enclosure contained a night pen (4 × 3m)

Table 1 Backgrounds and rearing details of individual giant pandas, *A. melanoleuca*, used for analysis of anogenital gland secretions

Number	Traditional Enclosures				Semi-natural Enclosures			
	Studbook No.	Name	Year of Birth	Sex	Studbook No.	Name	Year of Birth	Sex
1	474	Youyou[a]	1998	Female	357	Zhuangzhuang[a]	1989	Male
2	495	Yeye[a]	1999	Female	413	Didi	1994	Male
3	502	Wugang[a]	1999	Male	479	Qingqing	1999	Male
4	503	Lulu[a]	1999	Male	512	Lesheng	2000	Female
5	511	Ximei[a]	2000	Female	518	Longsheng	2000	Male
6	594	Qiangqiang	1987	Male	525	Longfei	2000	Male
7					547	Meiqing	2002	Female
8					549	Zhuyun	2002	Female
9					581	Caocao	2002	Female
10					656	Zizhu	1999	Female

[a] Indicates pandas housed at Wolong Breeding Center; others were housed at the Bifengxia Base.

and an outdoor yard (25 × 60m) that stretched along a 35 to 40° mountain slope with pine trees, shrubs, herbs, some bamboo, *Fargesia robusta*, and a small pond as a water source. Concrete walls separated these adjoining outdoor yards as well. Each subject could hear and see neighboring animals over the walls in certain areas of the yards. All subjects were provided with a daily routine of food consisting of steamed buns (five to six times per day), as well as apples and carrots (three to four times per day). Bamboo was available *ad libitum*. Additional details of housing, management, and diet are available elsewhere (Liu et al. 1998, 2003).

Scent Sample Collection and Extraction Scent samples were collected in 2006 during the non-mating season. The pandas used in our study were docile after several years in captivity, and hence, it was easy for us to collect their scent marks without any anesthesia. On 23 and 27 December 2006, we collected AGS samples with clean and sterilized cotton swabs (Chengdu Medical Inc., Chengdu, Sichuan, China) by rubbing each swab directly on the surfaces of panda anogenital glands while the subjects were induced with an apple or a carrot to sit by a zookeeper. All swabs used were previously treated with alcohol (99%) overnight and air-dried before use for sample collection. The swabs were only handled with gloved, not bare, hands. Odor samples of some pandas were collected twice (one collection on each of the two collection dates). We sealed all samples individually in clean glass vials with lids lined with Teflon and immediately stored them at −20°C in Bifengxia and Wolong, respectively, for 1wk. Later, the samples were packed on ice and air-shipped to the laboratory in Beijing (about 2-hr flight) and stored at −20°C for less than 1wk until they were extracted for analysis by GC–mass spectrometry (GC-MS).

We used dichloromethane (purity >99.5%, Beijing Fine Chemical Company, Ltd., Beijing, China) to extract the compounds from the AGS samples. To do so, we used a clean pair of scissors to cut away the outer 1mm layer of cotton that contained the AGS secretion and then put the resulting cotton pieces into a vial containing dichloromethane. The cotton was extracted in a volume of dichloromethane that reflected an extract concentration of approximately 1mg/10μl solvent. After 12hr, we removed the cotton pieces and stored the remaining solution at −20°C for less than 2d until they were analyzed by GC-MS. We wore PE disposable plastic gloves (Shanghai Dudeli Plastic Inc., Shanghai) when we handled the materials and never touched the swabs, cotton pieces, or instruments with bare hands.

GC-MS Analysis Analytical GC-MS was performed on an Agilent Technologies Network 6890N GC system coupled with 5973 Mass Selective Detector with the NIST/EPA/NIH Mass Spectral Library (2002 version; Agilent Technologies 2002). Chemstation Software (Windows 2000) was used for data acquisition and processing. The GC was equipped with a 30m HP5-MS glass capillary column (0.25mm i.d. × 0.25-μm film thickness). Helium was used as the carrier gas at the flow rate of 1.0ml/min. The temperature of the injector was set at 280°C. The oven temperature was programmed as follows: 100°C as the initial temperature, which was increased by 5°C/min up to 260°C and held for 10min. Finally, the temperature was increased to 280°C and held for 10min for a post-run cleaning of the column. Unknowns were identified by matching their retention times and mass spectra with authentic analogs after separation with the non-polar column (HP5-MS) and confirmed in some cases by using a polar column (DB-WAX, 30m long, 0.25mm i.d. × 0.25-μm film thickness) for separation and matching of retention times and mass spectra with standards. For the analysis with the polar column, GC injector temperature was set at 270°C; the oven temperature was initially set at 100°C, then increased by 5°C/min to 250°C and held for 20min at the flow rate of 1.0ml/min. Electron impact ionization was used at 70eV. Transfer line temperature was 280°C. Scanning mass ranged from 30 to 450amu. One microliter of sample was injected in the splitless mode.

Tentative identifications were made by matching the mass spectra of the GC peaks with those in the MS library (Agilent Technologies 2002). Thirteen (corresponding to 13 GC peaks) of the 39 tentatively identified compounds were verified by matching retention times and mass spectra with those of the authentic standards of *E*2-decenal (peak 1), 5-methylhydantoin (peak 2), hydroquinone (latter portion of peak 4), indole (peak 5), *E*2,*E*4-decadienal (peak 6), phenylpropanoic acid (peak 7), tetradecanoic acid (peak 13), hexadecanoic acid (peak 16), heptadecanoic acid (20), *Z*9-octadecenoic acid (peak 22), octadecanoic acid (peak 23), erucic acid (peak 31), and squalene (peak 38; Table 2). Although the chromatographic and spectral data for *Z*9-octadecenoic acid matched that of the authentic standard, we did not analyze this compound by dimethyldisulfide derivatization to unambiguously establish the position of the double bond. Thus, we take the more conservative approach and designate it as "an octadecenoic acid."

Phenylpropanoic acid (97%), tetradecanoic acid (99.5%), hexadecanoic acid (98%), heptadecanoic acid (95%), octadecanoic acid (97%), *Z*9-octadecenoic acid (97%), *E*-2-decenal (95%), *E*2,*E*4-decadienal (95%), indole (99%), and hydroquinone (99%) were purchased from ACROS Organics, Geel, Belgium. 5-Methylhydantoin (97%) and squalene (98%) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Erucic acid (96.5%) was purchased from Tokyo Kasei Kogyo Co. LTD., Tokyo, Japan.

Table 2 GC-MS data and identified compounds in anogenital gland secretion of giant pandas, *A. melanoleuca*

Peak No.	Retention Time (min)	Compounds	Diagnostic Ions [*m/z* (%relative intensity)]
1	4.79	*E*2-decenal[a]	83(100),70(81),43(40),55(40),110(32),69(29),57(27),67(23),*154*[b](0)
		Phenylacetic acid	91(100),*136*(57),92(30),65(21),39(12)
2	5.01	5-methylhydantoin[a]	42(100),*114*(83),43(70),57(7),86(7),70(5)
3	5.52	A decadienal	81(100),41(42),39(29),67(19),53(16),*152*(9)
4	5.59	Hydroquinone[a]	*110*(100),81(43),53(21),55(15),82(13),54(12),39(11),111(8),51(58)
5	5.70	Indole[a]	*117*(100),90(45),89(40),63(14)
6	5.93	*E*2,*E*4-decadienal[a]	81(100),41(19),67(17),67(17),57(15),55(13),55(13),95(12),39(11)
7	6.44	Phenylpropanoic acid[a]	91(100),104(58),*150*(47),77(17),78(16),65(15),51(14),39(9)
8	7.69	A decenoic acid	73(100),43(90),55(65),69(52),86(43),98(42),81(40),60(15),*170*(0)
9	9.50	Pentadecane	57(100),43(81),71(44),85(38),99(10),*212*(5)
10	11.62	Hexadecane	57(100),43(85),71(82),85(47),99(18),115(10),*226*(10)
11	13.01	Tridecanoic acid	73(100),60(85),43(67),55(62),129(57),171(47),57(45),*214*(25)
12	13.72	Heptadecane	57(100),71(75),43 (61),85(48),41(35), 55(23), 99(18)*240*(10)
13	15.16	Tetradecanoic acid[a]	73(100),60(90),43(72),55(71),129(51),185(30),*228*(17)
14	17.17	Pentadecanoic acid	73(100),60(87),43(87),55(85),57(81),129(48),199(40),*242*(40)
15	18.43	Hexadecanoic acid (branched)	73(100),43(98),57(80),60(79),129(52),213(50),*256*(45)
16	19.14	Hexadecanoic acid[a]	73(100),60(80),43(75),57(70),129(60),*256*(60),213(50),227(8)
17	20.33	Heptadecanoic acid (branched)	43(100),73(90),55(85),57(85),60(83),129(65),227(65),*270*(55)
18	20.46	Heptadecanoic acid (branched)	57(100),55(86),43(75),73(61),69(55),60(51),71(49),129(43),*270*(32)
19	20.59	Heptadecenoic acid	55(100),69(75),83(58),97(54),84(46),43(41),98(33),250(24),*268*(6)
20	20.98	Heptadecanoic acid[a]	73(100),43(86),60(84),57(68),*270*(63),55(60),129(34),227(30)
21	22.19	Octadecanoic acid (branched)	43(100),73(80),57(78),60(70),241(62),129(58),*284*(56), 185(34)
22	22.36	An octadecenoic acid[a]	55(100),69(75),83(63),97(58),43(58),264(23),60(15),*282*(4)
23	22.79	Octadecanoic acid[a]	73(100),43(90),*284*(83),55(82),60(80),57(78),129(72),185(40),241(49)
24	23.98	Unknown compound	174(100),328(15),175(4),224(4),343(3)
25	24.1	A nonadecenoic acid	55(100),69(79),83(64),97(54),43(48),67(39),57(36),60(12), *296*(2)
26	24.42	Nonadecanoic acid	73(100),43(94),*298*(94),55(84),57(84),60(80),129(56),71(47)
27	25.73	An eicosenoic acid	55(100),69(75),83(61),97(58),43(49),84(32),292(25),60(16),*310*(3)
28	26.12	Eicosanoic acid	73(100),43(96),55(91),*312*(89),57(87),60(71),69(58),129(57),84(45)
29	27.39	A heneicosenoic acid	55(100),69(95),83(88),97(72),43(52),306(42),98(41),60(16),*324*(1)
30	27.81	Heneicosanoic acid	43(100),57(88),73(84),*326*(84),69(82),60(67),84(61),97(55)
31	28.89	Erucic acid[a]	55(100),69(74),83(65),97(58),43(54),96(30), 320(28), 60(19),*338*(3)
32	29.35	Docosanoic acid	43(100),55(83),57(82),*340*(80),73(75),60(59),69(56),97(46)
33	30.54	A tricosenoic acid	55(100),83(76),69(71),97(54),43(50),57(37),334(32),60(22),*352*(2)
34	30.89	Tricosanoic acid	55(100),57(86),43(85),*354*(83),73(76),60(55),83(48),97(47),69(45)
35	31.87	A tetracosenoic acid	55(100),69(74),83(65),97(62),43(60),96(34),348(26),60(24), *366*(2)
36	32.34	Tetracosanoic acid	55(100),43(96),*368*(90),57(85),73(67),60(60),83(59),71(59)
37	32.67	A cholestatriene	349(100),*364*(66),141(33),350(28),251(20),365(18),237(18)
38	33.01	Squalene[a]	69(100),81(55),41(22),95(15),137(14),136(13),121(13),341(3),*410*(2)

[a] Compounds verified with authentic standards; other components were identified by comparison with spectra listed in the NIST (Agilent Technologies 2002) mass spectral library and analogous data.
[b] Italicized ions indicate the molecular ion

We converted the peak area of a particular compound into a percentage of the summed peak areas from the 20 main GC peaks (peaks 1–7, 11–14, 16, 20, 22, 23, 26–28, 31, 35, and 38) as a measure of the relative abundance of the relevant compound. If a given GC peak was too small to display the diagnostic MS ions of the corresponding compound, its area was taken as zero.

Statistical Analysis To test the hypothesis that there was sexual dimorphism in the relative abundances of the 20 main GC peaks in the crude extract of the AGS, we first examined the distribution of the raw data by the Kolmogorov–Smirnov test in SPSS for Windows (SPSS Inc. 1999). Then, we used either parametric tests (if the data were normally distributed) or non-parametric tests (if the data were not normally distributed) to analyze the relative abundances of the compounds. We used an independent two-tailed *t* test to analyze for sexual differences in the relative abundances of the compounds represented by peaks 13, 16, 22, 23, 26–28, and 38 (which had normally distributed raw data) and the Mann–Whitney *U* test for the remainder of the 20 main GC peaks (which did not have

normally distributed raw data). All statistical analyses were conducted by using SPSS for Windows (version 10.0; SPSS Inc. 1999). The critical values were set at $\alpha = 0.1$ (used commonly in similar studies, e.g., Gassett et al. 1996) except the intra-individual comparisons where α was set at 0.05.

Hierarchical cluster analysis is a statistical method for finding relatively homogenous clusters of cases based on measured characteristics. We used this analysis (average linkage) with Pearson's correlation coefficients to test the similarity of inter- and intra-individual AGS constituents (based on the 20 main GC peaks in the samples; Zhang et al. 2005).

To determine the variability of the AGS composition between individuals, RSD was used and calculated with the formula:

$$\mathrm{RSD} = (\mathrm{SD}/\mathrm{mean}) \times 100$$

where mean and SD are the average of each volatile peak-area percentage for all same-sex individuals and their standard deviation, respectively (Zhang et al. 2003).

Results

Anogenital Gland Secretion Constituents We tentatively identified 39 compounds from eight male and eight female giant pandas (Fig. 1). Chromatographic and spectral data were used to make these preliminary identifications (Table 2). By matching GC retention times and MS spectra with those of authentic standards, we identified peaks 1, 2 (latter portion of peak 2), 4, 5, 6, 7, 13, 16, 20, 22 (see note in "Methods and Materials"), 23, 31, and 38. The identities of these compounds were verified by a second analysis of the extracts and standards with the DB-WAX polar column.

Compounds in GC peak 2 were not completely resolved when analyzed under our conditions on the HP5-MS capillary column, but the peak included phenylacetic acid [as implied by *m/z* 136 (40 = relative intensity) and *m/z* 91 (100)] and 5-methylhydantoin (5-methyl-2,4-imidazolidinedione) [as indicated by *m/z* 114(94), *m/z* 43(58), and *m/z* 42(100)] (Table 2). GC peak 3 with similar MS data to *E*2, *E*4-decadienal (peak 6) might imply another decadienal (Table 2). Some peaks (e.g., peak 15, 17, 18, and 21) eluted earlier than hexadecanoic acid (peak 16), heptadecanoic acid (peak 20), or octadecanoic acid (peak 23), but showed similar MS data. They may be branched isomers of the corresponding acids. However, their identities were not verified by analysis with authentic standards (Table 2).

GC peaks 9, 10, and 12 were likely alkanes (Table 2). GC peaks 8, 15, 17–19, 21, 24, 25, 29, 30, 32–34, and 36 were tentatively identified as fatty acids and were seldom present in all samples. GC peak 37 might be cholestatriene. These trace GC peaks were excluded from the quantitative analysis (Fig. 1, Table 2).

Sex Differences Quantitative analyses revealed that the relative amount of the compound in peak 38 (squalene) was significantly higher in males and three compounds (peaks 2, 5, and 31) were higher in females (Table 3). Other compounds did not show differences between the sexes. GC peak 2 was only detected in one (Qiangqiang) of the eight males. Mass spectral data indicated that this peak from Qiangqiang only contained phenylacetic acid. However, in five of the eight female subjects, GC peak 2 was composed of 17.82 ± 27.37% ($N = 5$) phenylacetic acid and 82.18 ± 27.37% ($N = 5$) 5-methylhydantoin, which were estimated by each GC area percentage in peak 2 calculated after manually splitting GC peak 2. The latter was more abundant than the former ($t = 2.629$, $df = 4$, $P = 0.058$, paired t test). In other words, 5-methylhydantoin in GC peak 2 was not only female-specific in quality, but it was also more characteristic of chemical signals of females in quantity than phenylacetic acid. Further analysis indicated that phenylacetic acid showed no difference between the sexes (0.133 ± 0.236 vs. 0.022 ± 0.063 for females and males, respectively, both $N = 8$, $Z = 1.532$, $P = 0.125$). GC peak 4 (hydroquinone) was detected in two males (Didi and Longfei) and peak 5 (indole) in three females (Caocao, Zhuyun, and Zizhu). These compounds seem to be sex-specific in the AGS of the panda.

Intra-individual Similarity and Inter-individual Dissimilarity For males, cluster analysis showed that AGS composition in the samples remained relatively constant within individuals (i.e., Qiangqiang, Longsheng, Didi, and Longfei, cluster distance <1; Fig. 2). For females, two scent samples of Zizhu also showed a cluster distance less than one, but Caocao did not exhibit such a similarity in the dendrogram. Instead, the components of her AGS were closer to those of Ximei or Youyou (both females). This indicated that individual female AGS composition might fluctuate to a certain degree. However, further examination of each peak revealed that peaks 2 and 7 were present only in the extract from Caocao, whereas peaks 1 and 3 were present only in extracts from Ximei and Youyou. As a result, these compounds may be the keys for distinguishing Caocao from the other two whose individual attributes were not reflected as expected by the average linkage dendrogram. Likewise, the hierarchical cluster analysis failed to separate males from females despite the presence of sexual differences in several key compounds. In addition, peak 2 was present only in five females (Caocao, Meiqing, Zhuyun, Lesheng, and Zizhu) and peak 1 only in three other females (Yeye, Ximei, and Youyou).

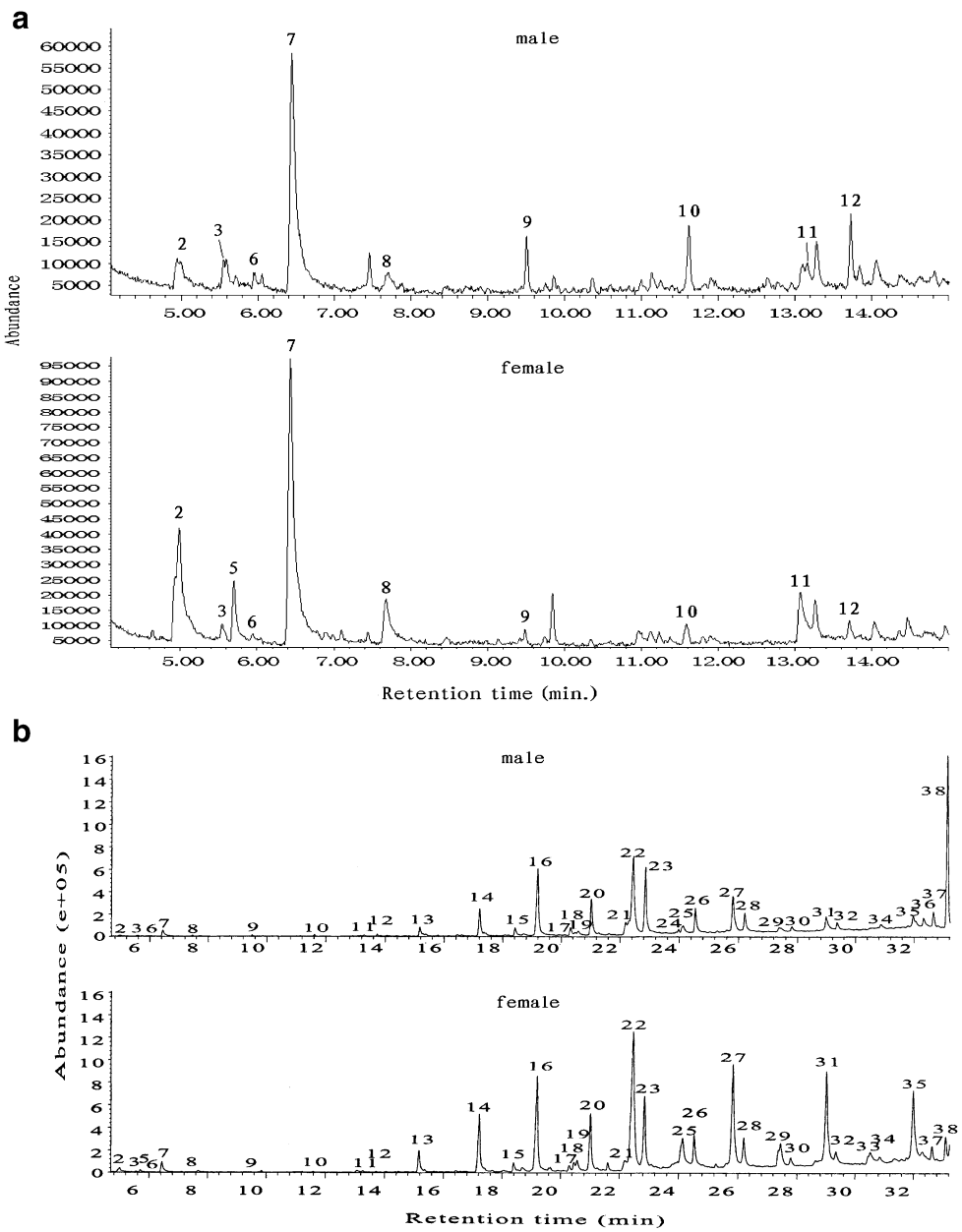

Fig. 1 Representative gas chromatogram of separation of the crude extract of the anogenital gland secretion of male (*top panels* of **a** and **b**, Qiangqiang) and female (*bottom panels* of **a** and **b**, Caocao) giant pandas, *A. melanoleuca*, on a 30-m HP5-MS capillary column. **a** Gas chromatogram (enlarged view) representing 4 to 15 min of retention time; **b** Gas chromatogram (normal view) representing 6–32 min of retention time. The numbers that label the GC peaks correspond to peak numbers in Table 2. Compounds 1 and 4 were undetectable in these two samples

Furthermore, the relative abundances of most compounds in inter-individual scent mark samples of either males or females exhibited much higher RSDs than those of samples drawn five times from the same individual (Ximei; Table 4). This further suggested that many AGS constituents may differ quantitatively among individuals.

Discussion

Our data show that many of the detectable 39 compounds (corresponding to 38 numbered GC peaks) from the AGS of giant pandas are straight-chain fatty acids. This, and the identification of squalene, in general, agrees with the previous findings of Yuan et al. (2004) and Liu et al. (2006), but shows a marked difference from the work of Hagey and MacDonald (2003) who found 111 volatiles smaller than indole. Moreover, we add several new compounds to the AGS of giant pandas that include decenal, decadienal, phenylacetic acid, 5-methylhydantoin, hydroquinone, phenylpropanoic acid, and erucic acid. The divergence in AGS compounds among studies might be explained by differences in the analytical systems, the concentration of samples analyzed, the season during which samples were collected, or the duration of sample storage. We analyzed our samples by injecting aliquots and concentrations that would not overload the column or cause overlap in retention times of the extract constituents. We

Table 3 Sexual differences in relative abundances of major compounds extracted from the anogenital gland secretion of giant pandas, *A. melanoleuca*

Peak No.	Relative Abundance		Statistical Significance	
	Males (N=8)	Females (N=8)	t or Z[a]	P
1[b]	0.479±0.686 (4)[c]	1.628±2.713 (3)	Z=0.116	0.908
2[b]	0.022±0.063 (1)	2.667±4.188 (5)	Z=2.233	0.026
3	0.164±0.201 (6)	0.382±0.479 (7)	Z=1.053	0.292
4[b]	1.112±2.444 (2)	0.000±0.000 (0)	Z=1.461	0.144
5[b]	0.000±0.000 (0)	0.026±0.039 (3)	Z=1.849	0.064
6[b]	0.164±0.245 (5)	0.336±0.492 (3)	Z=1.111	0.267
7[b]	0.157±0.443 (2)	0.316±0.636 (3)	Z=0.745	0.457
11	0.371±1.020 (2)	0.037±0.093 (4)	Z=0.138	0.890
13[b]	2.636±2.119 (7)	2.052±1.344 (7)	t=0.659	0.521
14	6.578±7.885 (8)	5.447±2.030 (8)	Z=1.365	0.172
16[b]	14.90±5.69 (8)	15.95±3.537 (8)	t=0.443	0.664
20[b]	8.911±6.993 (8)	5.932±2.377 (8)	Z=1.155	0.248
22[b]	20.76±7.938 (8)	26.33±7.956 (8)	t=1.402	0.183
23[b]	10.55±2.350 (8)	10.31±2.246 (8)	t=0.208	0.838
26	3.660±1.553 (8)	2.424±1.393 (7)	t=1.676	0.116
27	5.530±2.010 (8)	6.860±6.094 (8)	t=0.586	0.567
28	3.382±1.662 (8)	2.218±1.474 (8)	t=1.481	0.161
31[b]	1.586±1.102 (8)	6.237±6.115 (8)	Z=1.890	0.059
35	3.321±4.802 (8)	3.183±3.418 (7)	Z=0.000	1.000
38[b]	15.73±11.74 (8)	7.343±5.016 (8)	t=1.858	0.084

[a] Independent t test was used to analyze compounds 13, 16, 22, 23, 26–28, 38; Mann–Whitney U test was used to analyze the rest of the compounds. The level of significance for each test was set at α=0.1.
[b] Compounds identified using authentic standards
[c] Figures in the parentheses refer to the numbers of individuals for which the compound was detectable.

might have detected more compounds in our extracts by analyzing higher sample concentrations. Nonetheless, the 111 compounds reported by Hagey and MacDonald (2003) included compounds detected from both urine and vaginal secretions in addition to the AGS.

To further screen for pheromone candidates from the identified compounds, we conducted a quantitative analysis

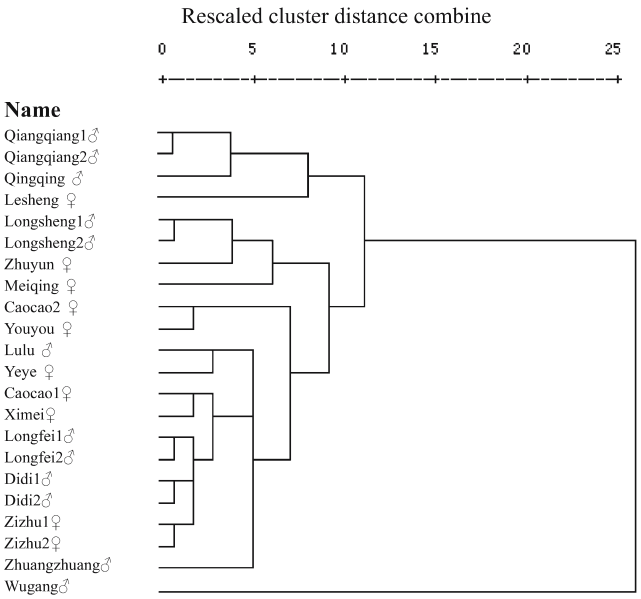

Fig. 2 Hierarchical linkage cluster (average linkage) dendrogram of the anogenital gland secretion of giant pandas, *A. melanoleuca*. The *numbers after the panda names* indicate the date [23 (=1) or 27 (=2) December 2006] on which AGS samples were collected. If no number is present, the sample was collected on 23 December 2006

to determine which compounds covary in relative abundance with sex or individual. For this analysis, all alkanes, which are pheromonally inactive, and high molecular weight fatty acids (present in trace amounts) were excluded (Singer et al. 1997; Novotny et al. 1999; Zhang et al. 2003, 2005, 2007a, b). We selected GC peaks for quantitative analysis by checking each GC profile of all samples; the GC profile in Fig. 1 is representative, but not inclusive of all data from all samples.

Some of the compounds that we detected (e.g., decenal and decadienal) might be products of oxidative degradation of fatty acids in the AGS, either while the secretion was associated with the animal or during our collection, storage, or extraction of the sample. In comparison, comparative analyses of fresh and 1-mo frozen extracts of flank gland samples from golden hamsters in our laboratory revealed no appreciable differences in chemical content (J.X. Zhang, unpublished data). Nonetheless, the relative roles of stable or degradative components in the semiochemical system of giant pandas bears further study. Compounds derived from decompositional processes may have ecological value in giant panda communication.

Our results suggest that the panda AGS contains a wealth of information that codes for sexes and individuality, which is consistent with previous behavioral tests (Swaisgood et al. 1999, 2000; White et al. 2002, 2003, 2004; Liu et al. 2005; Tian et al. 2007). The information concerning sex and individuality might be coded by the components in analog (quantitative differences of common compounds) and/or digital (unique compounds) forms proposed by Sun

Table 4 Variation in relative abundance of AGS constituents from male and female giant pandas, *A. melanoleuca*

GC Peak	RSD[a] of Inter-individuals		Intra-individuals (five replicates of Ximei's AGS sample, ♀)	
	Male (N=8)	Female (N=8)	RSD	Mean±SD
1	142.8	166.7	25.55	5.323±1.359
2	286.4	157.0	–	–
3	122.6	125.4	25.45	1.207±0.308
4	219.4	–	–	–
5	–	150.0	–	–
6	149.4	146.4	19.61	0.969±0.190
7	283.4	201.3	–	–
11	274.9	251.4	–	–
13	80.39	65.5	41.0	2.287±0.939
14	120.0	37.27	17.84	7.527±1.343
16	38.19	22.18	16.02	21.02±3.367
20	78.48	40.07	37.52	6.058±2.274
22	38.24	30.22	20.63	23.22±4.791
23	22.27	21.78	26.98	13.89±3.747
26	42.43	57.47	48.33	2.580±1.247
27	36.35	88.83	57.85	7.201±4.166
28	49.14	66.46	36.26	2.275±0.8237
31	69.48	98.04	68.46	2.973±2.033
35	144.5	107.4	97.14	1.268±1.234
38	74.63	68.31	46.36	2.204±1.020
Mean±SD	113.7±89.66	95.08±67.10	29.25±25.81	
t values[b]	3.662	3.789		
P values	0.002	0.001		

[a] RSD refers to relative standard deviation, which was calculated using the formula RSD = (SD/mean)×100, where mean and SD are the average of each compound peak area (in percentage) and their standard deviation, respectively

[b] An independent t test was used to compare RSDs from the same compounds between the male group and replicated data from Ximei and between the female group and replicated data from Ximei. The level of significance was set at α=0.05

and Müller-Schwarze (1998a, b) for the beaver and exemplified further by three *Mustela* species (Zhang et al. 2003, 2005). Other studies have documented that the odorant compounds that covary in quality or quantity with biological characters can be considered putative pheromones (Singer et al. 1997; Novotny et al. 1999). Based on qualitative and quantitative differences in the AGS extracts from the giant panda, we hypothesize that indole (female-specific), 5-methylhydydantoin, and erucic acid are potential female pheromones, whereas hydroquinone (male-specific) and squalene are potential male pheromones. This concurs with previous results in the elevation of the squalene level with sexual maturation in the panda (Liu et al. 2006). Squalene also has been shown to be active in tamarin, *Saguinus fuscicollis* (Epple et al. 1979) and male Canadian red-sided garter snakes, *Thamnophis sirtalis* (Mason et al. 1989). Indole and long-chain fatty acids pheromones are found in a variety of vertebrates including male ferret, *Mustela furo* (Clapperton et al. 1988), leopard geckos, *Eublapharis macularius* (Mason and Gutzke 1990), and golden hamsters (J.X. Zhang, unpublished data). Some straight-chain fatty acids and various isomers of decadienal are common components of insect pheromone blends (El-Sayed 2005). Such convergent uses of the same compounds in different species show that these compounds may possess some typical chemical properties of pheromones, one of which appears to be their volatility, which allows them to convey airborne cues over a distance.

Hierarchical cluster analysis and RSD of the relative abundances of 20 of the GC peaks in combination with individually unique compounds suggest that individual information may be borne in the giant panda by both the kind and degree of the scent constituents. Such coding patterns for individual identity have been found in three *Mustela* species (Zhang et al. 2003, 2005). In ferrets, for instance, Clapperton et al. (1988) identified inter-individual variation in the combination of five compounds in anal gland secretions. In female ferrets, the intra-individual similarity of urinary volatiles is not always elucidated by hierarchical cluster analysis; instead, dramatic fluctuations of a few compounds reveal an inter-individual dissimilarity (Zhang et al. 2005). Significant differences in RSD within and between individuals have also been used successfully to show the possibility of coding for information about individuality with the anal gland secretion in the Siberian weasel and steppe polecat and preputial gland secretion in the house mouse and Brandt's vole (Zhang et al. 2003, 2007a, b). The preputial glands in mice and voles seldom contain compounds that are unique to a specific individual or sex despite their sexual dimorphism in quantity, whereas the anal glands of carnivores always have noticeable individual-specific compounds (Zhang et al. 2003, 2007a, b). For

example, no two individuals share an identical compound composition in the urine of the lion, *Panthera leo* (Andersen and Vulpius 1999). In the giant panda, a fingerprint-like individual scent composition might be an alternative for the "genetic fingerprint," which provides a new powerful tool in the accurate census of wild giant pandas (Zhan et al. 2006). Preliminary comparisons of the data suggest that there is likely no correlation between the similarity of the AGS composition and genetic distance in the panda. For example, although Qingqing (♂), Longfei (♂), Longsheng (♂), Meiqing (♀), Zhuyun (♀), and Ximei (♀) all are sired by Dadi (♂), and Dadi (♂) and Didi (♂) are sired by Panpan (♂; Xie and Gipps 2005), their AGS compositions failed to reflect such genetic relationships. To conduct a thorough analysis of the extent of the role of genetic distance on AGS composition, one would need to include a chemical analysis of the AGS composition from all parents and exclude experimentally the effects of non-genetic factors, such as microbial community, housing condition, reproductive status, and food (Gosden and Ware 1976; Ferkin et al. 1997; Zhang et al. 2003).

In summary, according to the established methods used to screen for pheromones from numerous scent compounds detected by GC-MS, we propose several putative sex pheromones for the giant panda, especially male-produced squalene and female-produced erucic acid, which were present in all pandas in this study. Our results also show an individual "odor fingerprint" from the AGS of the giant panda. Verification of their pheromonal activity via bioassays and application of synthetic chemosignals to conserve this species will be subjects of our future studies.

Acknowledgments We are grateful to Dr. Steven J Seybold and the anonymous reviewers who gave us many helpful suggestions and comments. This work was supported by grants from NSFC (Nos. 30470233 and 30670268); International Cooperative Giant Panda Projects of State Forestry Administration, China (nos. WH0306 and WH0309) and International Partnership Project of Innovative Research, Chinese Academy of Science (CXTDS2005-4).

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