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Seasonal and reproductive variation in chemical constituents of scent signals in wild giant pandas

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Seasonally reproducing animals show many behavioral and physiological changes during the mating period, including increased signaling for intrasexual competition and mate attraction. We collected 102 anogenital gland secretions (AGS) from marking trees in Foping Nature Reserve, and used gas chromatography mass spectrometry to analyze these chemical composition. Of these marks, all but one were from males, confirmed with DNA analysis. We found that several chemical constituents, especially volatile compounds, were present only during the mating season and that the relative abundance of many compounds changed as a function of breeding season, whereas nonvolatile compounds were lower in the mating season. This seasonal variation in chemical composition of AGS most likely plays an important role in governing giant panda reproduction, including mate location, attraction, and male-male competition. The chemical properties of many of these putative chemosignals—such as volatility and longevity—are suggestive of these roles, and undoubtedly contribute to successful reproduction for this species with a characteristically sophisticated chemical communication system. We also found a number of important differences between the chemical constituents of AGS from wild pandas and those found in previous studies with captive pandas, suggesting that inappropriate chemosignal composition may contribute to poor reproductive success in captive breeding programs.

giant panda, chemical communication, anogenital gland secretions, chemical composition, reproduction

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INTRODUCTION

Olfactory communication is a dominant mode of communication in many mammals (Brown and MacDonald, 1985), the mammalian olfactory system collects the information by detecting a large number of volatile and non-volatile chemicals with vastly diverse molecular structures (Wang and Liu, 2012). Compared with other signaling modalities, olfactory communication has several advantages: chemosignals are long-lasting, can be broadcast over a wide range, and can encode highly specific information closely tied to the animal's physiological status (Wyatt, 2014). Further, chemosignals allow communication among individuals without

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direct visual contact, and can take place over large distances and time spans. Chemical signals originated from urine, feces and specialized scent gland secretions play an important role in intraspecific communication of many mammals (Müller-Schwarze, 2006), especially carnivores, such as Ursidae, Hyaenidae, Canidae, Felidae, Herpestidaeand, and some Mustelidae species (Zhang et al., 2005; Burgener et al., 2009; Martín et al., 2010; Jordan et al., 2010; Rosell et al., 2011; Gilfillan et al., 2017).

Chemosignals often transmit complex information on individual identity, gender, reproductive status, social status, body condition and kin relations in mammals (Brennan and Kendrick, 2006; Johansson and Jones, 2007; Stoffel et al., 2015). For seasonally breeding species, chemical signals play a key role in transmitting reproductive information (Burger, 2005), indicating females' sexual receptiveness and motivating males' sexual arousal (Nielsen et al., 2011), and thus typically show sex differences or temporal changes during the mating season (Scordato et al., 2007; Tobey et al., 2009; Rosell et al., 2011). For example, 3,4-dehydro-*exo*-brevicomin and 2-sec-butyl-4,5-dihydrothiazole in the male house mouse (*Mus domesticus*) urine are responsible for the attraction of females and play a role in estrus synchronization (Jemiolo et al., 1985, Jemiolo et al., 1991). In the mating season, the European mole (*Talpa europaea*) anal secretions contain a large number of chemical compounds, but are dominated by large concentrations of C5–C10 carboxylic acids outside the mating season (Khazanehdari et al., 1996). For several weeks prior to ovulation, female Asian elephants (*Elephas maximus*) release *(Z)*-7-dodecen-1-yl *acetate*, as an essential component of their female-to-male sex pheromone, in their urine to attract males (Rasmussen et al., 1997). The complexity and relative concentrations of the semiochemicals of sternal gland secretions vary seasonally in male koalas (*Phascolarctos cinereus*), with the most odorous and complex mixtures occurring during the mating season (Tobey et al., 2009).

As a solitary species, giant pandas (*Ailuropoda melanoleuca*) only rarely have direct contact with other conspecifics and most social interactions take place in a short window during the mating season (Schaller et al., 1985; Pan et al., 2001; Nie et al., 2012). Consequently, opportunities for other communication modalities are limited and pandas rely heavily on olfactory communication (Swaisgood et al., 1999; Swaisgood et al., 2000; Swaisgood et al., 2002; Wei et al., 2015). Behavioral experiments and semiochemical analysis in captive pandas confirm that both urine and anogenital gland secretions (AGS) are used as chemical signals, conveying individual identity, sex, reproductive condition, age and competitive status (Swaisgood et al., 2000; White et al., 2002; White et al., 2003; Liu et al., 2006; Zhang et al., 2008). Female urine serves as a male attractant, containing chemical cues of estrus status that males extract utilizing the vomeronasal organ, thus aiding in the ability to locate mates dispersed on the landscape (Swaisgood et al., 2000; Swaisgood et al., 2002; White et al., 2004). Male pandas also use odors to advertise age and competitive status as part of their reproductive strategy (Swaisgood et al., 2002; Liu et al., 2006; Zhang et al., 2008). However, no previous investigations have elucidated the use of semiochemicals by wild pandas and examined their potential roles in advertising reproductive status and coordinating reproduction.

Here, we test whether giant panda semiochemicals vary with season and reproductive status, as has been found for scent-marking behavioral patterns (Swaisgood et al., 2000; Nie et al., 2012). We used gas chromatography mass spectrometry (GC-MS) to analyze the chemical composition of anogenital gland secretions of wild giant pandas, combined with behavioral observations, to explore seasonal behavioral and physiological changes in wild giant pandas. Specifically, we predicted that giant pandas should produce more complex and easily transmitted scent signals during the mating season than in the non-mating season.

RESULTS

Sex and individual identification of AGS samples

We collected a total of 27 fecal DNA samples and 102 AGS samples (mating season: n=61; non-mating season: n=41) during this study (Table S1 in Supporting Information). We documented AGS deposition with camera trap photos for 30 (29.41%) of the 102 AGS marks, and were able to identity individuals using individually distinctive pelage marking for 73.33% of photos. In all cases where we had both DNA and photo identification, both methods yielded the same conclusion regarding individual identity. DNA analysis of fecal samples revealed 9 adult individuals (8 males, 1 female) associated with deposition of the 49 AGS samples (19 of them were also confirmed by photos). Photo identification of individuals depositing AGS and DNA analysis of AGS marks confirmed that at least 60 (58.82%) were deposited by the same 9 pandas. Thus, all individuals detected by fecal DNA were also detected by AGS DNA and no additional individuals were detected. Since all but one of the DNA-assayed samples were deposited by males (Table S1 in Supporting Information), we restricted our analyses to male AGS.

Chemical compounds in AGS of wild male pandas

Using GC-MS analysis, we tentatively identified 104 different compounds from the 102 AGS samples (mating season: n=61; non-mating season: n=41), and were able to verify 19 of them by matching retention times and mass spectra with those of the authentic standards (Table 1, Figure

1). We excluded all chemical compounds identified in tree bark control samples that were found in AGS samples, such as Friedelan-3-one and *D*-Friedoolean-14-en-3-one. The identified compounds were comprised of 27 aldehydes, 19 saturated fatty acids, 12 fatty-acid esters, 10 steroids, 10 ketones, six alkenes, five heterocyclic aromatic organic compounds, four amides, four alcohols, three unsaturated fatty acids, squalene and other compounds (Table 1). The AGS chemical profile for each sample consisted of a subset of the 104 identified compounds ranging from 4 to 54.

Effects of reproductive season on chemical composition

Random forests classification algorithm successfully differentiated reproductive status (OOB estimate of error rate: 4.9%) of the AGS odor samples, with obvious clustering in two distinct groups, the mating and non-mating season (Figure 2). Cholesta-5,7-dien-3-ol, *acetate*, 6,10,14-trimethyl-2-pentadecanone, dodecanal, (*E*)-2-heptenal and (*E*)-7-tetradecene were identified as the most important chemical compounds for classifying the reproductive status of the AGS samples (Figure 3). In addition, AGS from the mating season contained 27 exclusive compounds, including four alcohols (1-octanol; 1-hexadecanol; 1-docosanol and tetracosanol), ten fatty acid esters, and five ketones (2,5-hexanedione; 2-octanone; 2-undecanone; heneicosanone and tricosanone) that were not found in the AGS samples from non-mating season (Table 1). Scent profile complexity, as measured by number of compounds present, increased from the non-mating to the mating season (Welch's t-test: t=−3.6695, df=96.053, P<0.001; mating season: 35.36±1.51, non-mating season: 27.44±1.51).

Both mating season and non-mating season, the number of volatile compounds is significantly higher than that of non-volatile compounds (Paired samples test: mating season: t=14.093, df=64.97, P<0.001; non-mating season: t=10.781, df=43.796, P<0.001). However, the relatively abundance of non-volatile compounds is higher than that of volatile compounds (paired samples test: mating season: t=8.5628, df=118.31, P<0.001; non-mating season: t=10.979, df=66.371, P<0.001) (Figure 4). AGS in the mating and non-mating season also differed with regarding to the relative

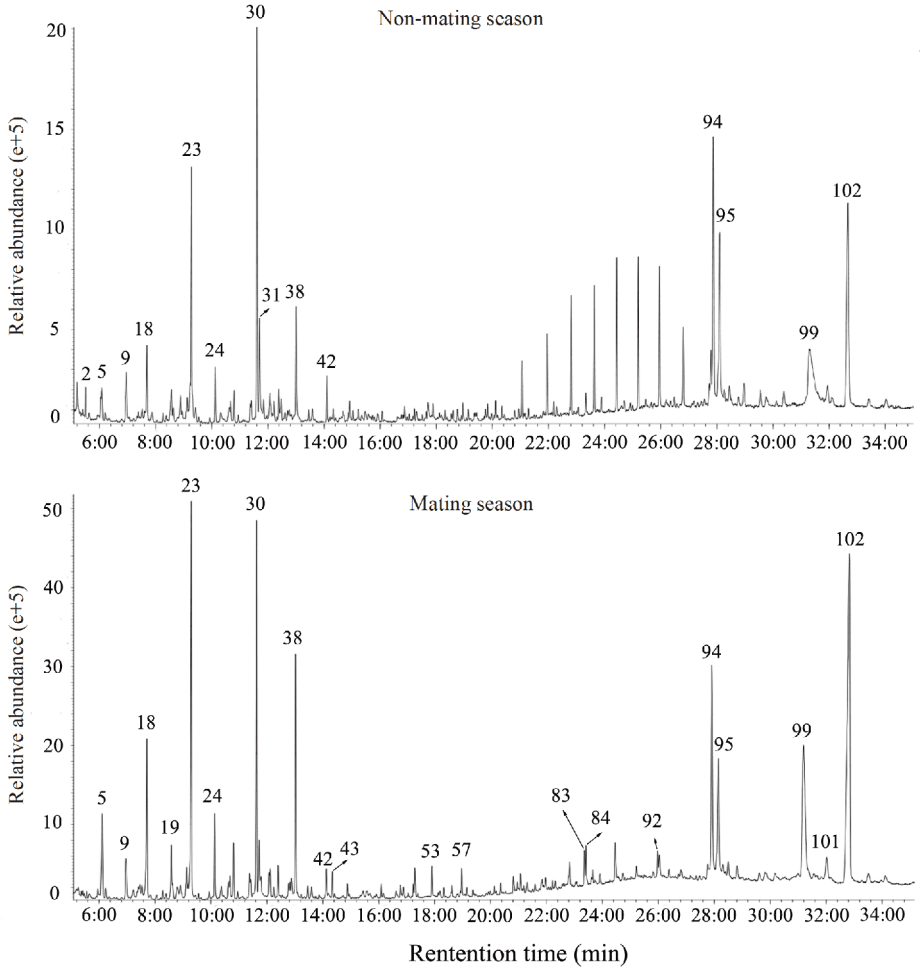

Figure 1 Representative ion chromatograms of giant pandas in mating and non-mating season.

Table 1 Tentatively identified compounds found in anogenital gland secretion (AGS) and seasonal changes in relative abundance in wild giant pandas[a)]

Peak	R.T. (min)	Tentatively identified compounds	Match quality	Mating season (Mean+SE)	Non-mating season (Mean+SE)	Mating/Non-mating Ratio
1	5.26	2,4-Dimethyl-1-heptene	87	1.161±0.177(23)	0.695±0.332(9)	1.671
2	5.56	4-Methyl-octane	94	0.522±0.080(26)	0.279±0.034(17)	1.874
3	5.66	p-Xylene	83	0.406±0.064(15)	0.222±0.051(7)	1.827
4	5.99	2-Heptanone*	80	0.153±0.012(10)	0.206±0.053(2)	0.745
5	6.07	Heptanal	95	1.159±0.103(53)	0.605±0.066(30)	1.916
6	6.24	3,7-Dimethyl-1-octene	93	0.307±0.013(16)	0.211±0.032(6)	1.453
7	6.37	p-Benzoquinone*	91	1.941±0.909(5)	1.119±0.561(6)	1.735
8	6.64	1R-*.alpha.*-Pinene	97	0.636±0.127(4)	3.078±1.139(6)	0.207
9	6.94	(*E*)-2-Heptenal	90	0.902±0.127(18)	0.751±0.036(32)	1.201
10	6.94	2,5-Hexanedione	85	0.240±0.111(7)	–	–
11	6.96	6-methyl-2-Heptanone*	94	0.989±0.448(31)	0.9212±0.295(5)	1.074
12	7.06	Benzaldehyde	86	0.166±0.047(3)	0.810±0.541(4)	0.205
13	7.4	Hexanoic acid*	87	0.381±0.037(27)	0.325±0.062(7)	1.172
14	7.46	Phenol	95	0.381±0.117(3)	–	–
15	7.53	2-Octanone	86	0.334±0.064(7)	–	–
16	7.55	2-Pentyl-furan	72	0.334±0.049(12)	0.378±0.020(6)	0.885
17	7.61	(*E,E*)-2,4-Heptadienal	90	0.319±0.047(4)	0.274±0.026(9)	1.164
18	7.69	Octanal*	86	1.719±0.142(52)	1.080±0.119(35)	1.591
19	8.58	(*E*)-2-Octenal	96	0.766±0.064(51)	0.540±0.042(27)	1.419
20	8.79	1-Octanol	86	0.315±0.042(15)	–	–
21	8.9	Heptanoic acid*	87	0.552±0.030(41)	0.477±0.074(9)	1.157
22	9.13	2-Nonanone*	80	0.433±0.036(31)	0.473±0.100(7)	0.917
23	9.29	Nonanal*	91	5.390±0.467(56)	3.055±0.284(39)	1.764
24	10.14	(*E*)-2-Nonenal	95	0.788±0.064(53)	0.552±0.043(34)	1.428
25	10.37	Octanoic Acid*	93	0.552±0.048(37)	0.499±0.032(25)	1.106
26	10.62	2-Decanone*	83	0.391±0.029(23)	0.547±0.110(6)	0.715
27	10.81	Decanal*	86	0.673±0.044(51)	0.471±0.029(33)	1.428
28	10.96	(*E,E*)-2,4-Nonadienal	87	0.127±0.012(8)	0.309±0.260(10)	0.4138
29	11.42	2,4-Dimethyl-2-decene	81	0.405±0.070(6)	–	–
30	11.62	(*E*)-2-Decenal*	92	4.203±0.401(58)	3.015±0.313(40)	1.394
31	11.78	Nonanoic acid*	97	1.697±0.348(41)	1.022±0.095(25)	1.661
32	11.83	2-Undecanone	87	1.331±0.000(1)	–	–
33	11.86	Hydroquinone	90	3.230±1.213(9)	1.648±0.298(8)	1.96
34	12.06	Nonanoic acid, ethyl ester	91	0.241±0.003(2)	–	–
35	12.08	A decadienal	–	0.571±0.062(38)	0.502±0.255(34)	1.139
36	12.39	(*E,E*)-2,4-Decadienal	94	0.497±0.058(49)	0.600±0.346(37)	0.829
37	12.77	*trans*-4-Undecenal	60	0.271±0.025(6)	–	–
38	13.02	(*E*)-2-Undecenal*	91	1.921±0.161(55)	1.734±0.160(37)	1.107
39	13.17	Decanoic acid	72	0.206±0.040(7)	0.220±0.087(3)	0.937
40	13.57	Vanillin	95	0.600±0.130(4)	0.208±0.017(2)	2.889
41	13.59	Dodecanal	81	0.288±0.019(45)	0.267±0.037(4)	1.079
42	14.12	(*E*)-7-Tetradecene	64	0.666±0.073(31)	0.670±0.050(36)	0.995
43	14.34	(*E*)-2-Dodecenal	90	0.255±0.021(41)	0.284±0.034(18)	0.899
44	14.87	Tridecanal	91	0.438±0.130(27)	0.205±0.000(1)	2.137
45	15.48	Dodecanoic acid	87	0.405±0.107(11)	0.979±0.188(15)	0.413
46	16.08	Tetradecanal	91	0.262±0.012(46)	0.237±0.019(16)	1.105
47	16.63	Tridecanoic acid	95	0.267±0.056(7)	0.248±0.021(10)	1.076
48	16.78	A tetradecenal	–	0.224±0.017(23)	0.289±0.044(3)	0.775
49	16.82	1-Hexadecanol	91	1.069±0.000(1)	–	–
50	16.89	Ethyl tridecanoate	–	0.201±0.046(4)	–	–
51	17.23	Pentadecanal	90	0.242±0.031(29)	0.362±0.150(3)	0.669
52	17.72	Tetradecanoic acid*	99	0.549±0.114(30)	0.576±0.063(25)	0.952
53	17.90	14-methyl-,(*Z*)-8-Hexadecenal	93	0.331±0.024(38)	0.434±0.030(26)	0.763
54	18.06	Tetradecanoic acid, ethyl ester	94	0.216±0.073(5)	–	–

(*To be continued on the next page*)

(*Continued*)

Peak	R.T. (min)	Tentatively identified compounds	Match quality	Mating season (Mean+SE)	Non-mating season (Mean+SE)	Mating/Non-mating Ratio
55	18.33	Hexadecanal	91	0.212±0.009(38)	0.198±0.012(6)	1.073
56	18.42	Pentadecanoic acid(branched)			0.169±0.031(2)	–
57	18.62	6,10,14-trimethyl-2-Pentadecanone	95	0.256±0.014(45)	0.203±0.012(6)	1.265
58	18.79	Pentadecanoic acid*	96	0.433±0.087(28)	0.544±0.240(23)	0.795
59	18.96	*Cis*-9-Hexadecenal		0.442±0.029(51)	0.472±0.035(26)	0.936
60	19.08	Pentadecanoic acid, ethyl ester	68	0.326±0.039(4)	–	–
61	19.45	Hexadecanoic acid (branched)		0.476±0.099(12)	0.285±0.039(3)	1.671
62	19.69	9-Hexadecenoic acid	98	0.494±0.000(1)	0.214±0.000(1)	2.308
63	19.81	Hexadecanoic acid*	98	0.752±0.204(20)	0.859±0.099(27)	0.875
64	20.06	Hexadecanoic acid, ethyl ester	98	0.264±0.054(13)	–	–
65	20.36	Heptadecanal	96	0.264±0.019(22)	0.183±0.034(4)	1.445
66	20.43	Heptadecanoic acid(branched)	–	–	0.253±0.000(1)	–
67	20.51	Heptadecanoic acid(branched)	–	0.270±0.000(1)	0.141±0.000(1)	1.915
68	20.74	Heptadecanoic acid*	96	0.379±0.142(5)	0.616±0.088(14)	0.615
69	20.97	(*E*)-15-Heptadecenal	98	0.277±0.134(14)	0.351±0.048(7)	0.789
70	21.01	Heptadecanoic acid, ethyl ester	95	0.216±0.126(3)	–	–
71	21.3	Octadecanal	87	0.296±0.016(32)	0.285±0.000(1)	1.039
72	21.58	An octadecenoic acid	–	0.253±0.036(3)	–	–
73	21.65	Linoleic acid ethyl ester	98	0.425±0.067(20)	–	–
74	21.68	Octadecanoic acid	92	1.589±0.000(1)	0.954±0.399(7)	1.666
75	21.69	Ethyl Oleate	95	0.578±0.176(25)	7.089±0.000(1)	0.082
76	21.7	An octadecadienoic acid	–	1.852±0.000(1)	–	–
77	21.85	Hexadecanamide	96	0.533±0.120(22)	4.383±0.944(3)	0.122
78	21.91	Octadecanoic acid, ethyl ester	91	0.271±0.053(10)	–	–
79	22.6	Nonadecanoic acid	86	0.372±0.165(2)	0.379±0.000(1)	0.98
80	22.92	Heneicosanone	98	1.369±0.640(3)	–	–
81	23.35	4,8,12,16-Tetramethylheptadecan-4-olide	96	0.548±0.029(42)	0.440±0.042(19)	1.247
82	23.41	An eicosenoic acid, ethyl ester	–	1.381±0.000(1)	–	–
83	23.42	(*Z*)-9-Octadecenamide	89	2.412±0.814(33)	19.740±7.310(5)	0.122
84	23.6	Octadecanamide*	86	0.846±0.195(13)	2.051±0.622(5)	0.412
85	23.61	Eicosanoic acid	85	0.369±0.086(2)	–	–
86	23.91	1,21-Docosadiene	98	0.451±0.042(21)	0.380±0.000(1)	1.189
87	24.55	1-Docosanol*	90	3.115±1.518(4)	–	–
88	24.56	Tricosanone	97	0.560±0.135(2)	–	–
89	24.73	Erucic acid	87	0.445±0.054(9)	0.457±0.000(1)	0.973
90	26.14	Tetracosanol	89	4.869±2.459(5)	–	–
91	26.52	Unsaturated wax ester C24	–	0.988±0.000(1)	–	–
92	26.72	(*Z*)-13-Docosenamide	91	1.992±0.614(16)	1.611±0.470(5)	1.236
93	27.18	Squalene	99	10.041±2.849(29)	2.250±0.973(15)	4.462
94	27.92	Cholesta-4,6-dien-3-ol, (*3.beta.*)	96	7.176±0.936(60)	10.152±0.734(40)	0.707
95	28.14	Cholesta-3,5-diene	99	8.426±1.192(57)	16.366±1.754(40)	0.515
96	28.23	Cholesta-8,24-dien-3-ol, 4-methyl-, (*3á,4à*)	87	1.179±0.577(6)	–	–
97	28.48	Unsaturated wax ester C25	–	0.813±0.065(18)	0.930±0.148(7)	0.875
98	28.82	Cholesta-5,7-dien-3-ol, *acetate*	94	0.938±0.093(17)	2.065±0.128(31)	0.454
99	31.17	Cholesterol	97	17.071±1.870(61)	11.662±1.121(40)	1.464
100	31.31	Cholestanol	93	4.651±1.415(10)	2.670±0.619(19)	1.164
101	32.03	Cholest-4-en-6-one	99	1.406±0.085(11)	2.230±0.235(10)	0.611
102	32.78	Cholesta-3,5-dien-7-one	97	10.641±1.036(53)	19.449±1.622(37)	0.547
103	33.51	Cholest-5-en-3-one	92	0.773±0.104(12)	2.228±0.856(2)	0.347
104	33.72	Cholesta-4,6-dien-3-one	99	0.770±0.130(3)	–	–

a) *, Compounds identified using authentic standards, 6-methyl-2-heptanone (98%), *p*-benzoquinone, octanoic acid (99.5%), octanal (99%), nonanal (96%), decanal (97%), pentadecanoic acid (98%) and heptadecanoic acid (98%) were produced by Macklin. Tetradecanoic acid (98%) was produced by Alfa. Hexanoic acid (analytical standard), heptanoic acid (analytical standard), nonanoic acid (99.5%), 2-heptanone (99.8%), 2-nonanone (analytical standard), 2-decanone (99.5%), (*E*)-2-decenal (95%), (*E*)-2-Undecenal (95%), *cis*-11-hexadecenal (95%), 1-docosanol (98%), analytical standard of the (*E*)-2, (*E*)-4-decadienal were purchased from Sigma-Aldrich (Shanghai) trading co. LTD, Shanghai, China.

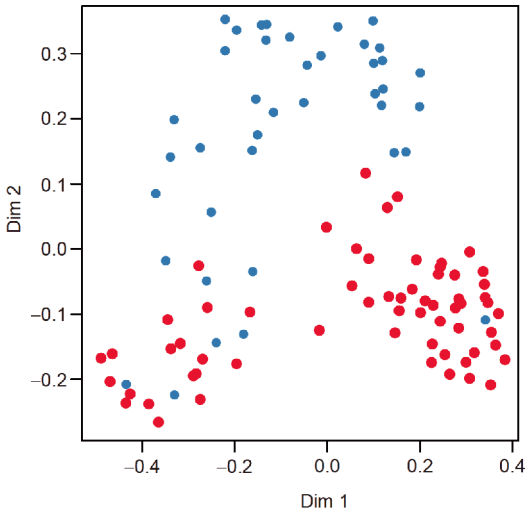

Figure 2 Unsupervised classification to cluster odour samples from mating season or non-mating season (blue: non-mating season; red: mating season).

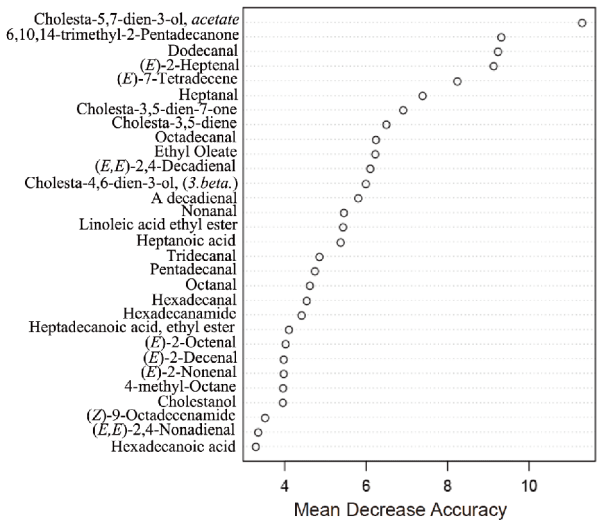

Figure 3 The importance of chemical compounds distinguishing estrous state of adult males emitted from the AGS of the giant panda.

abundance of specific compounds present (Table 1, Figure 4). Alcohols and aldehydes (Wilcoxon signed-rank test, W=1566, P=0.0101), ketones (W=766, P=0.0015) and alkenes (W=998, P=0.0329) were significantly more abundant in the mating than the non-mating season (Figure 5). By contrast, the abundance of steroids (W=513, P<0.001) were lower in the mating than non-mating season (Figure 5). There was no significant difference in amines (W=94, P=0.1875), saturated fatty acids (W=855, P=0.4945), fatty acid esters (W=139, P=0.2845) and heterocyclic aromatic organic compounds (W=134, P=0.3939) between the mating and non-mating season.

The composition of volatile and non-volatile components of AGS changed as a function of reproductive season (Figure 4). We found a significant difference in the chemical complexity of volatile (t=3.398, df=96.636, P<0.001; mating season: 27.21±1.31, non-mating season: 20.90±1.29) and non-volatile (t=4.1552, df=93.839, P<0.001; mating season: 8.16±0.27, non-mating season: 6.54±0.28) chemical profiles between the mating and non-mating seasons. Further, the relative abundance of these volatile (W=1519, P=0.0188; mating season: 26.29±1.83, non-mating season: 21.10±1.88) and non-volatile (W=3.398, P<0.001; mating season: 50.13 ±2.06, non-mating season: 60.46±3.07) compounds changed significantly across seasons (Figure 4).

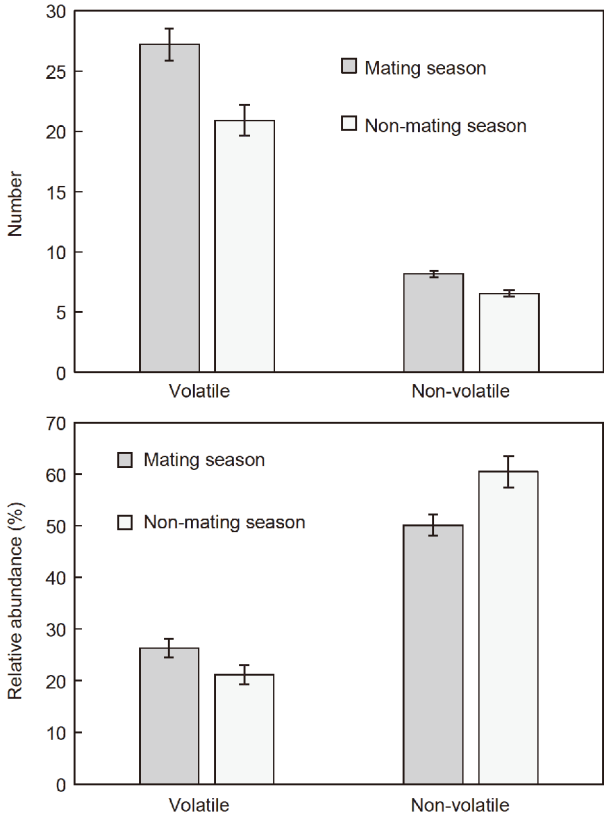

Figure 4 The number and the relative abundance of the volatile and non-volatile chemical compounds in giant panda AGS in mating and non-mating season.

Synergistic effects of the major compounds in wild pandas

To evaluate potential synergistic interactions among inter-correlated suites of chemical constituents of AGS, we performed correlation analysis. We found that several similar compounds (such as six aldehydes, two ketones and three short-chain fatty acids) have significantly positive correlations with each other. Cholesta-4,6-dien-3-ol, (*3.beta.*) correlated positively with Cholesta-3,5-dien-7-one, but Cholesterol had a negative correlation with Cholesta-3,5-diene. Three amides had a significant positive correlations with each other whereas negative correlation with many other compounds except two steroids and all short-chain

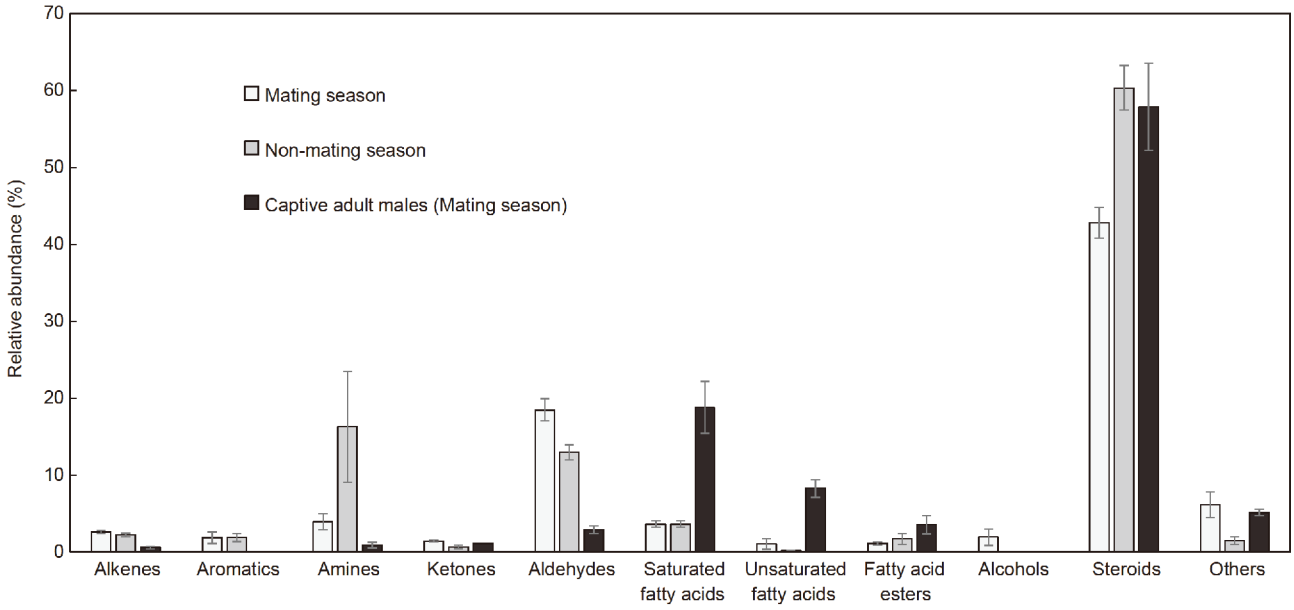

Figure 5 Correlation among twenty-three primary compounds found in giant panda AGS.

aldehydes and ketones. Aldehydes correlated positively with ketones, short-chain fatty acids (C7–C9), but negative correlation with medium- and long-chain fatty acids (C14–C16) (Figure 6).

AGS differences between captive and wild pandas

Compared with captive pandas, we detected several new compounds in wild individuals that include ten ketones, four alcohols, four heterocyclic aromatic organic compounds and most of the volatile short-chain aldehydes (C7–C11) (Figure 5). In addition, captive giant pandas had significantly high proportions of fatty acids (saturated and unsaturated fatty acids) and esters in AGS, whereas wild pandas had higher proportions of aldehydes, amines and alkenes (Figure 5).

Effects of AGS age on chemical profile

To examine signal persistence, we evaluated the effects of AGS age on chemical profile across four time periods (one day, three days, one week, two weeks). We found there is no obvious change in AGS composition as a function of age in terms of the number of chemical compounds they contained (Figure 7) or the relative abundance of the 12 main chemical compounds (Figure S1 in Supporting Information), indicating that AGS is very stable over a period of at least two weeks.

DISCUSSION

This is the first study to determine the chemical compounds present in the scent marks of wild giant pandas, and we have documented for the first time seasonal variation in chemical profiles for this species. We also have shown that females rarely mark using AGS (only one detected in this study) and, by contrast, males marked frequently throughout the year (see also Nie et al., 2012), with each male repeatedly visiting scent mark stations along our transects. Further, reproductive season influenced chemosignal complexity, with males depositing AGS marks that contained a greater variety of compounds during the mating than non-mating season. Twenty-seven chemical compounds, including four alcohols, ten fatty acid esters and five ketones, were detected exclusively in AGS marks deposited during the mating season.

Functionally, this portfolio of 104 chemical compounds in panda AGS should facilitate efficient and effective communication, consistent with behavioral observations indicating a prominent role for chemical communication in this species (Swaisgood et al., 2004). Some chemosignals can evoke behavioral responses, such as fighting and mating (Ferrero and Liberles, 2010). Long chain alcohols elicited avoidance and aggressive behavior in male lizards (*Acanthodactylus boskianus*) (Khannoon et al., 2011), hexadecanol signals male dominance status in Iberian rock lizards (*Lacerta monticola*), and alcohols (1-octadecanol, 1-eicosanol and 1-docosanol) induce dispersion of young subsocial spiders to reduce competition (*Coelotes terrestris*) (Trabalon and Assi-Bessekon, 2008). Our study identifies several chemical compounds—including four alcohols (1-octanol, 1-hexadecanol, 1-docosanol and tetracosanol) and five ketones—that may serve as semiochemicals, as evidenced by variation in chemical profiles between the breeding and non-breeding seasons, which suggests a role in mating or com-

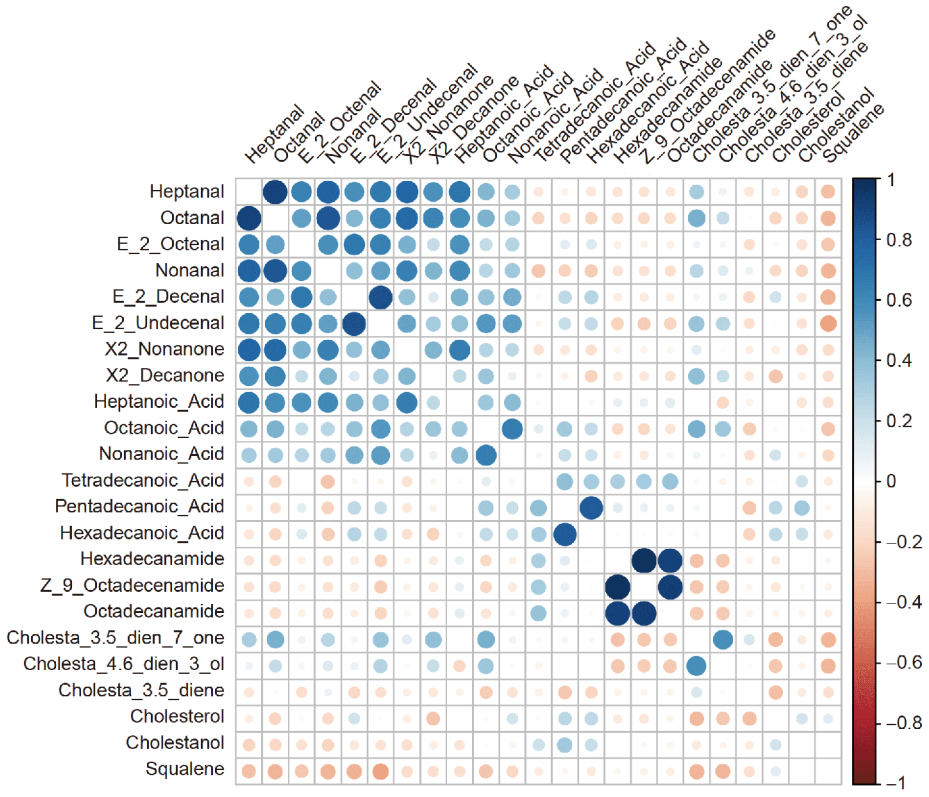

Figure 6 The relative abundance of the different chemical compounds in captive male pandas (Yuan et al, 2004) and mating and non-mating season in wild giant pandas AGS.

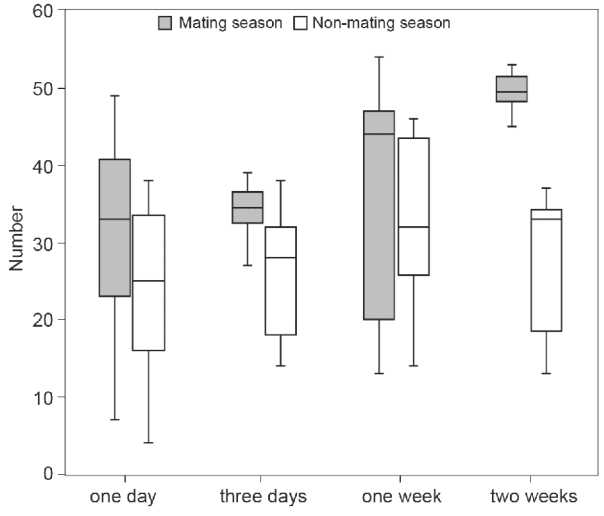

Figure 7 The number of chemical compounds found in male giant panda anogenital gland secretions of various ages collected during the mating and non-mating season.

petition.

Theory suggests that the production and deposition of volatile constituents should be emphasized during the mating season, supporting the need to communicate short-lived messages about breeding readiness and aggressive motivation (Alberts, 1992). Due to their low molecular weight, these compounds are energetically efficient, and diffuse readily, thus increasing signal range and facilitating signal detection by receivers. These volatiles are common constituents of many mammalian secretions (Burger, 2005; Harris et al., 2012). Consistent with predictions, we found an increase in the number of volatile compounds (including short-chain aldehydes, short-chain carboxylic acids and ketones) during the mating season. These compounds likely contribute to the effectiveness of the panda reproductive strategy, which involves several males aggregating around a single estrous female and competing for reproductive access (Schaller et al., 1985; Nie et al., 2012). While field observations indicate a prominent role for male-male competition, experiments with captive pandas have shown that both male and females exercise mate choice, and choice has fitness consequences (Martin-Wintle et al., 2015; Martin-Wintle et al., 2017). As a solitary species, locating mates as well as competitors could be challenging, so these wide-ranging volatiles may aid in the transmission of information pertaining to sexual and aggressive motivation to both male and female recipients (Alberts, 1992; Johansson and Jones, 2007), as well as providing information on mate quality to guide mate-choice decisions.

A significant disadvantage of volatile chemical constituents in scent marks is their relatively short life, as volatiles dissipate more quickly than non-volatiles. Pandas appear to address this shortcoming in several ways. First,

they visit and re-deposit scent frequently (Hurst, 2005). Although we do not know precisely how often pandas re-visit scent stations (our preliminary data suggest an individual panda visits a scent mark station once every two to four weeks), the fact that our data indicate that neither volatile nor non-volatile compounds degrade significantly in two weeks suggests that pandas are capable of maintaining AGS marks fresh enough that chemosignals lose relatively little information content by the time a receiver detects the scent, especially in the mating season when scent station visits are more frequent. Second, chemosignalers can also increase the persistence of volatile constituents by including compounds that act as fixatives (Alberts, 1992; Brennan and Kendrick, 2006). Long-chain squalene, such as we found in panda AGS, retards evaporation of volatiles and extends signal persistence (Greene et al., 2016) by binding and slowly releasing constituents such as carboxylic acid. Cholesterol, one of the primary steroids in panda AGS, provides an apolar matrix that aids in the delivery of semiochemicals in other mammals (Escobar et al., 2003; Harris et al., 2014). These non-volatile high molecular weight compounds typically do not play a direct role in chemical communication, but enhance the functionality of other semiochemicals through these fixative properties. This slow release of volatiles can also provide a "time stamp" that receivers can use to determine signal age.

Many non-volatile high molecular weight compounds do, however, play a role in chemical communication, and are typically found in higher proportions in range marks and recognition signals whose function requires longer signal persistence (Alberts, 1992). Higher molecular weight marks should be selected by environments that encourage semiochemical degradation, such as humid, warm environments (Symonds and Elgar, 2008). Animals living in cool, temperate wet forests tend to produce semiochemicals of medium molecular weight, higher than in dry environments, but lower than in hot, wet tropical forests (Alberts, 1992). Giant pandas live in highly humid but cool environments, where semiochemicals will degrade or be washed away by rainfall (Royer et al., 1993), an effect that if not mitigated may impair mate localization (Wilder et al., 2005). These considerations may explain the abundance and diversity of high molecular weight volatile chemical constituents in panda AGS, with properties that facilitate chemical stability to counteract humidity and extend signal persistence.

A role for semiochemical function in several of the chemical compounds of medium to high molecular weight is suggested by the presence in panda AGS of several semiochemicals with known function in others species. As these are non-volatile, detection may require direct contact with the mark. Notably, pandas are frequently observed licking and closely investigating AGS marks, as well as displaying the flehmen response associated with delivery of chemosignals to the vomeronasal organ (Swaisgood et al., 2000). Squalene and some hormone-like steroids, have chemosignal properties (Pause, 2004; Ferrero and Liberles, 2010) and may encode information, such as sex, identity and physiological status (Alberts, 1992; Escobar et al., 2001; Harris et al., 2012). Squalene has been nominated as a putative male pheromone in giant pandas (Zhang et al., 2008). Two similar androgen derivatives (androstenol and androstenone) that are present at high concentrations in boar saliva can act as classical pheromones and elicit behavioral effects to attract estrous females (Dorries et al., 1997). Martín and López (2006a, 2006b) suggest that cholesta-5,7-dien-3-ol (provitamin D), which is influenced by diet quality, is a reliable semiochemical advertising male quality in Iberian rock lizards. We found that AGS of giant pandas also contains cholesta-5,7-dien-3-ol, *acetate* and cholesta-4,6-dien-3-ol, (*3.beta.*) and a large number of other steroids, and that cholesta-5,7-dien-3-ol, *acetate* was the most important chemical component discriminating reproductive status. Together, the evidence suggests that a number of the steroids and other large molecular weight compounds present in male panda AGS may encode information on individual identity, reproductive status, and competitive status in wild giant pandas. From behavioral experiments, we know that pandas are capable of assessing reproductive status (White et al., 2002), sex and reproductive condition (Swaisgood et al., 2000; Swaisgood et al., 2002), and individual identity (Swaisgood et al., 1999). Future research should evaluate how individual chemical constituents in AGS serve these important communication roles.

Many chemosignals consist of blends of two or more molecules to synergistically form the biologically active signal (Wyatt, 2014), which can elicit a greater response than any individual component (Brennan et al., 2004). Of the 23 most prevalent compounds found in wild pandas, we found six aldehydes that were positively correlated with two ketones and three short chain fatty acids (C7–C9), and many of the aldehydes were correlated with each other. Aldehydes were also the largest class of chemical compounds in male panda AGS, contributing 27 separate compounds. The number and relative abundance of these aldehydes, ketones, and short chain fatty acids also increased during the mating season, suggesting they may interact synergistically to convey important information about reproductive or competitive status. On theoretical grounds, animals are predicted to increase the number of chemical compounds working in concert to maximize information content of chemosignals (Alberts, 1992), and thus our findings are indicative of a complex and sophisticated chemical communication system, consistent with behavioral findings (Swaisgood et al., 2004).

The 104 chemical compounds we identified in the AGS of wild male pandas is higher than that found in captive pandas (Hagey and MacDonald, 2003; Yuan et al., 2004; Zhang et

al., 2008). We detected ten ketones, four alcohols, five heterocyclic aromatic organic compounds and a number of volatile short-chain aldehydes that have not been detected in captive pandas. Wild panda AGS also had higher proportions of aldehydes and lower proportions of fatty acids and esters than captive pandas. These differences between wild and captive giant pandas can be ascribed to the different environmental conditions, stress, genetic differences, hormones, diet and symbiotic bacteria (Lizé et al., 2013; Ezenwa and Williams, 2014; Wyatt, 2014). Inadequate chemical communication has been implicated in reproductive failure of captive pandas (Swaisgood et al., 2004), and it is possible that the conditions in captivity that give rise to these differences in AGS odor profiles may hinder effective communication and be a cause of failed mating in some cases. Future research should address these scent profile differences to determine if there are management actions that can be taken to improve effective communication for mating.

MATERIALS AND METHODS

Sample collection and extraction

From March 2016 to April 2018, we conducted our study in the 293 km^2 Foping National Nature Reserve, Shanxi province, China. Giant pandas deposit scent marks primarily on trails and ridges (Schaller et al., 1985; Nie et al., 2012), so we placed transects on ridges and trails where pandas frequently deposit scent. We visited each transect every two weeks and examined trees for evidence of marking. Scent marks are evident by a discoloring and darkening of the bark caused by AGS deposition, as well as a detectable odor (Nie et al., 2012). Upon identifying a scent mark, we used a sterilized capillary tube to collect fresh AGS samples from the tree surface. For each scent mark, we collected 2–3 capillary tubes, and immediately sealed them in 2 mL glass headspace vials and stored them at −20°C. Camera-traps were used to monitor transects, allowing us to determine how many pandas had visited the site in the interval between semichemical collection, determine the time the scent was deposited (and therefore age of the scent mark when collected) and aiding in individual identification of the panda depositing the scent (Zheng et al., 2016). This information was further supplemented by the collection of fresh fecal samples which were analyzed for DNA to determine sex and individual identification. Following semiochemical collection, we lightly scraped away the dark bark made by AGS to expose the lighter bark underneath to ensure that future scent marks we collected at the same site were deposited by giant panda during the immediately preceding interval and were not contaminated with older semiochemicals. We categorized these samples according to the reproductive period (mating season: February-April; non-mating season: other month) of giant panda (Pan et al., 2001; Nie et al., 2012). All samples were packed on ice and shipped to the laboratory in Beijing and stored at −20°C until analysis. Representative samples of unmarked tree bark were collected for each tree species so that results of chemical analysis of AGS samples could be corrected for contributions of the underlying bark. Using a sterile needle, we extracted AGS samples, added 600 μL dichloromethane (purity>99.8%, Macklin) and vortexed for 15 s. After storing the samples for 12 h at −4°C, we centrifuged the sample for 3 min at 3500 r/min. We then transferred the resulting supernatant to a glass vial and froze it at −20°C until it was analyzed by GC-MS. During all stages of collection and handling of AGS samples, we used latex gloves to avoid contamination with human scent.

Sex and individual identification

We were able to identify most individuals through individually distinctive markings (Zheng et al., 2016), but confirmed and supplemented these data with DNA analysis of feces collected near the scent site. DNA was extracted from feces according to Zhang et al. (2006) using standard controls. Twelve microsatellite primers Ame-μ10, μ11, μ13, μ15, μ22, μ24, μ26, μ27, AY79, AY95, AY161213, AY217 (Lu et al., 2001; Shen et al., 2005; Wu et al., 2009) were used to amplify DNA extracts from fecal samples. We amplified each extract three times; if the genotype could not be determined, we performed two additional amplifications. Two species-specific sexing primer pairs SRY and ZF were designed for sex determination (Zhan et al., 2007), conducted three times for each DNA extract. Individual and sex identification PCR amplification procedures are described in detail in Hu et al. (2010).

Chemical analysis and compound identification

The analyzes were performed in an Agilent Technologies Network 6890N gas chromatograph system equipped with a 30 m HP5-MS glass capillary column (0.25 mm i.d.× 0.25 μm film thickness) coupled with 5973 Mass Selective Detector. Helium gas was set to constant flow (1.0 mL/min) and the spiltless technique was applied. The injector port temperature was set at 280°C. Initial oven temperature was set to 35°C, held for 1 min, and increased by 10°C/min to 280°C and held for 10 min. We increased the temperature to 300°C for 5 min for a post-run cleaning. The entire run lasted 40 min and tests revealed that no compounds eluted after 35 min. Electron impact ionization was used at 70 eV. Transfer line temperature was 280°C. A 2 μL sample was injected using the splitless mode. Scanning mass ranged from 30 to 350 amu.

Compounds were tentatively identified by matching their

retention time and mass spectra with structures available in the NIST 2002 library (Agilent Technologies 2002, USA). All chemical compounds were characterized as "volatile" (molecular weight <300) or "non-volatile" (molecular weight>300) components (Wilson, 1963; Yuan et al., 2004).

Statistical analysis

Because absolute concentration is subject to variability across samples, we used relative abundance of each chemical compound within a sample (i.e., proportion) for statistical analyzes. In order to determine the characteristic odor profiles of the estrous status of adult males, we analysed the proportion of chemical compounds emitted from giant pandas AGS using a random forests classification algorithm (Breiman, 2001) within the *R* package randomForest (Liaw and Wiener., 2002). AGS of male giant pandas contain more chemical compounds than samples; for example, AGS samples from this study contained 104 compounds and each of them acts as a single variable. Therefore, we used random forests, a algorithm which has been well applied in metagenomics for classification and regression (Zhang et al., 2018), and it's also suited for analysis of the chemical compound datasets. For example, it allows for more variables than samples, it rarely overfits the data, it has high classification efficiency and it can create a minimal set of variables which can be used as group predictors.

We used unsupervised classification to cluster odor samples from the mating or non-mating season and calculated a classification accuracy for each random forest using out-of-bag error rates. In order to provide an interpretation of the best predictors for reproductive status from the random forest, we calculated a measure of variable importance using the importance function of the random Forest package and the metric mean decrease accuracy (MDA) (Strobl et al., 2007). MDA refers to the degree to which the classification accuracy decreases without this feature. Therefore, a higher MDA means less misclassification and thus greater accuracy, and ultimately indicates higher variable importance to the classification of a characteristic.

We selected 45 major compounds commonly shared by more than 30 samples for Wilcoxon rank sum test to evaluate the effects of mating season. All tests were two-tailed tests with $P<0.05$ considered as significant. We selected 23 compounds which occurred frequently and in sufficient abundance to run correlation analysis. All statistical analyzes were conducted using R version 3.4.0 (https://www.r-project.org/).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Twelve main chemical compounds in male giant panda AGS of various ages collected during the mating and non-mating season.

Table S1 Backgrounds of anogenital gland secretions samples used for analysis and DNA sex and individual identification of giant pandas, *A. melanoleuca*

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