Chapter 24
Chemical Signals in Giant Panda Urine (*Ailuropoda melanoleuca*)

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24.1 Introduction

The rarest member of the bear family, the Giant panda (*Ailuropoda melanoleuca*) lives mainly in rainy bamboo forests high in the mountains of western China at elevations between 1500 and 3000 m, where they almost entirely feed on the leaves, stems, and shoots of various bamboo species. Restricted and degraded habitat, through logging and agricultural operations, is the greatest threat to giant pandas. Since the mid-1970s three range-wide surveys have been conducted and results from the most recent survey in 2004 indicated a total wild population of 1600 individuals ~40 % more than were thought to exist in the 1980s (http://wwf.panda.org). The Giant panda is a conservation reliant endangered species and establishing self-sustaining panda bear populations in breeding stations and zoos are long-term aims to create healthy, self-sustaining wild panda populations through the release of captive pandas to strengthen small isolated populations of wild pandas. However, a slow reproductive rate in captivity (Hu and Wei 1990) limits the reproductive success of pandas in zoos.

According to Zhang et al. (2004) the most common reasons for mating failure were motivation in males and/or the inability to achieve intromission, even if almost all female pandas displayed strong behavioral estrus. A husbandry reducing stress and establishing a more natural environment are among the factors associated with increased reproduction (Zhang et al. 2004). A 2013 report shows a total number of registered pandas in captivity of 332 individuals held in China and at institutions in

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Europe and North America in which only 25 cubs were born in 2011 (International Studbooks for Rare Species of Wild Animals in Captivity 2013).

Unfortunately, panda breeding management is hampered by difficulties inherent in the maintenance and breeding of this species in zoos. This can be attributed, in part, to the unique reproductive life history patterns of Giant pandas. Reproductive biology of pandas is complicated by three main traits, namely experiencing only one single estrus with spontaneous ovulation in the spring (seasonality), delayed implantation and the occurrence of pseudo-pregnancy (Spady et al. 2007).

In the wild, Giant pandas are usually solitary, except during the breeding season between March and May where females may breed with multiple males. Behavioral and non-behavioral cues regulate many aspects of social interactions related to reproduction in mammals. Among the non-behavioral cues, vocal (Charlton et al. 2010) and chemical [pheromones (Karlson and Luscher 1959)] stimuli release specific behavioral or endocrine reactions in the recipient. Mechanisms of pheromone release into the environment are manifold and secretion with urine represents one mechanism. Giant pandas are known to rely heavily on chemical signals released with anogenital gland secretions (AGS) and urine to coordinate their reproductive and social activities (Schaller et al. 1985; Swaisgood et al. 1999; Nie et al. 2012a).

Scent markingin pandas using both sources is higher during the mating season than the non-mating season (Nie et al. 2012a). Male pandas mark their territory by urinating on trees. It has been assumed that they urinate as high as possible so that rival males know how large they are (Swaisgood et al. 2004). Females often mark with urine and AGS, especially around the time of sexual receptivity (Kleiman et al. 1979; Kleiman 1983; Schaller et al. 1985). AGS contain volatile components which consist of a series of short- and long-chain fatty acids (Zhang et al. 2008; Hagey and Macdonald 2003). Female's urine also appears to function as a chemical signal (Swaisgood et al. 2000) and high concentrations of short chain fatty acids have been demonstrated to appear 6–8 days prior to sexual receptivity (Dehnhard et al. 2004). It has been shown that male pandas displayed a significant investigatory preference for estrous over non-estrous female urine, as indicated by sniffing and flehmen (Swaisgood et al. 2002). Thus female scent may play an important role in coordination of mating, e.g., to attract males, to signal estrus, and to act as an aphrodisiac.

Chemical analyses using mass spectrometry identified 951 chemical components from scent glands, urine, and vaginal secretions of pandas collected during the spring breeding season (Hagey and Macdonald 2003). The scent marks of the two genders contained a similar composition of chemicals but varied particularly in the concentration of short chain fatty acids making up more than one half of the gland secretion in males and approximately one eighth in females (Hagey and Macdonald 2003). A study of Zhang et al. (2008) solely found 39 different substances in anogenital gland secretions of pandas collected in December. Short chain fatty acids were not confirmed, however, distinct amounts of long chain fatty acids (C10–C24) were detected. The divergence in compounds among the two studies might be explained by differences in the analytical systems and the season during which samples were collected.

Chemical analyses of urinary compounds are only limited. A study of Yuliang et al. (2012) found 56 substances, 38 of them were putatively identified, whereby

fatty acids were not detected. This contrasts to our previous findings demonstrating an increase in fatty acids during spring when ovarian steroid secretion increases (Dehnhard et al. 2004).

The endocrine events associated with the peri-ovulatory period have been described by measuring the corresponding urinary steroid hormone metabolites (McGeehan et al. 2002). The excretions of estrogens by female giant pandas increased approximately 8 days prior to observed matings, were at maximum during the proceptive period, and decreased to basal levels during the period of receptivity (Bonney et al. 1982; Hodges et al. 1984). In captivity, successful reproduction depends on the ability of animal managers to detect estrus for the accurate timing of mate pairing and artificial insemination. Thus, daily estrogen measurements aiming to detect the estrogen peak as well as behavioral and morphological indices are used for reproductive management.

However, a long-lasting continuous estrogen increase which may also include intermittent declines (Bonney et al. 1982) might complicate a precise estrus prognosis because this would require anticipation of the further course; for example, whether an attained high value is followed by a decrease (and thus reflects preovulatory peak concentrations) or by an additional increase closer to ovulation. Thus, urinary pheromones or volatiles with concentrations that are elevated coincident with the pre-ovulatory LH surge might be excellent indicators of ovulation to detect the relatively brief receptive period in female pandas (McGeehan et al. 2002).

We hypothesized that female panda urine might possess estrous-related signals and that urinary volatiles, which might serve as pheromones in panda communication, could be used to evaluate ovarian function. Pheromones may represent more informative indicators of behavioral and physiological conditions than measurements of circulating or excreted hormones, which do not contain communicator functions. Using a combined approach of hormonal analysis and urinary volatile monitoring we aimed to detect reproductive stage-related substances. We selected three different methods for volatile analyses: (1) solid-phase microextraction [SPME; (Arthur and Pawliszyn 1990)], to reproduce data that had been published earlier based on one female panda (Dehnhard et al. 2004), (2) static headspace (SHS) injecting air saturated with volatiles from above the urine, and (3) stir bar sorptive extraction (SBSE) using a TWISTER stir bar adsorbing volatile as well as nonvolatile urinary compounds. Finally, we aimed to identify estrous-related substances and to assess their reliability for monitoring ovarian function.

24.2 Material and Methods

24.2.1 Animal and Sample Collection

One adult female and one adult male (both 11 years) giant pandas maintained in the zoo of Edinburgh were involved in the study. Urine samples from the female were collected on a daily basis between January 21st and October 15th, 2013 and February 28th

and September 25th, 2014, respectively. Urine samples from the male were collected irregularly between February 13th and May 21st, 2014. All samples were frozen immediately after collection, stored at −20 °C and shipped to Berlin by overnight express, delivered next day, and kept at −20 °C until analyses.

24.2.2   Determination of Female and Male Reproductive Status

Conjugated steroids were hydrolyzed, extracted, and measured with two enzyme immunoassays (EIA) being specific to either total estrogens or pregnanediol. Hormone determinations were carried out as described in a previous paper (Meyer et al. 1997). Testosterone determinations were carried out following hydrolysis (Meyer et al. 1997) as described in a previous paper (Kretzschmar et al. 2004).

In both years artificial inseminations (AI) were scheduled according to the estrogen courses and performed at the beginning of the post peak decrease indicating the period of receptivity (Bonney et al. 1982; Hodges et al. 1984), additionally confirmed by the detection of the ovulatory LH peak in blood applying a semiquantitative LH assay (WITNESS® LH test).

24.2.3   Solid-Phase Microextraction (SPME)

Solid-phase microextractionuses a fiber coated with an adsorbent that can extract organic compounds from the headspace above urine. Extracted compounds are desorbed upon exposure of the SPME fiber in the heated injector port of a gas chromatograph (GC). SPME was carried out with a CTC Combi Pal system autoinjector at 70 °C for 60 min using a fiber coated with a 85 µm Carboxen/PDMS layer. Sampling was done in the headspace above the surface of 5 ml diluted, Acetate-buffered urine (containing 2 ml urine; 2.5 ml water; 0.5 ml 2 M acetate-buffer, pH 4.8; 1.83 g NaCl, and 2 µg undecanoic acid as internal standard) in 20 ml headspace vials (Shimadzu, Duisburg, Germany).

24.2.4   Static Headspace Analyses (SHS)

Headspace vials (20 ml) were filled with 2 ml diluted, acetate-buffered urine (containing 1 ml urine; 1.0 ml 0.5 M acetate-buffer, pH 4.8 and 2 µg camphor as internal standard). SHS was carried out at an incubation temperature of 70 °C for 30 min using a CTC Combi Pal system autoinjector. Thereafter a volume of 2000 µl from the air above the urine surface was injected into the gas chromatograph (GC) using a syringe temperature of 85 °C.

24.2.5 *Stir Bar Sorptive Extraction (SBSE)*

SBSE was done in 20 ml headspace vials (Shimadzu, Duisburg, Germany) containing 1 ml urine, 1 ml 0.5 M acetate-buffer (pH 4.8), and 2 µg undecanoic acid as internal standard. Polydimethylsiloxane (PDMS) coated stir-bars (Twisters) for sorptive extraction (Gerstel GmbH, Mulheim an der Ruhr, Germany) were conditioned according to Jakubowska et al. (2009). They consisted of a 10 mm long glass-encapsulated magnetic stir bar with an external PDMS coating of 0.5 mm thickness. The stir bar was inserted into the headspace vial filled with acetate-buffered urine and stirred at 1000 rpm for 60 min at room temperature. After extraction, the stir bar was removed from the sample using a magnetic tweezer and excess of water was dabbed away with a lint-free tissue.

Due to the criteria of higher recoveries of urinary compounds including decanoic acid SBSE was chosen as standard method to continue urine analyses (see Table 24.1). In addition, SBSE also allows enrichment of nonvolatile urinary compounds also adsorbing on the PDMS phase which are undetectable by SPME (Benet et al. 2015). SBSE is also more concerned with ghost peaks or carryover peaks than SPME because it uses 50 µl of sorption media compared to 0.61 µL of sorption medium as typical of 100 µm PDMS fibers (Rodil and Moeder 2008).

Table 24.1 Comparison of extraction efficiency between solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE) based on ten major urinary compounds naturally occurring in panda urine

No	Substance	Retention time	SPME area $\times 10^6$	SBSE area $\times 10^6$
1	Unknown01[a]	9.11	0.54	5.28
2	Methyl salicylate[b]	9.16	1.16	41.97
3	4-vinylphenol[b]	9.36	n. d.	14.93
4	1-(2,4,6-trimethylphenyl)-ethanone[c]	10.63	1.19	15.38
5	Decanoic acid[b]	11.40	0.20	4.93
6	4-(1,1-dimethylethyl)-2-methyl-phenol[c]	12.18	n. d.	41.53
7	Unknown03[a]	13.12	1.74	5.50
8	Unknown04[a]	13.72	0.25	4.44
9	1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butene-1-one[c]	14.60	n. d.	167.10
10	Civetone[c]	21.45	0.15	2.45

n. d. = not detected

[a]Unknown: compounds that did not match >80 % with any substance (NIST MS search)

[b]Compounds verified with authentic standards

[c]Compounds that did match >80 % with substances from library (NIST MS search)

24.2.6 GC-MS Analysis

Sample analysis was performed on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Böblingen, Germany) interfaced with an Agilent 5975C mass selective detector. For SPME we used the rear injector with the splitless injection mode. For SHS as well as SBSE the split/splitless inlet of the front injector was replaced with a Gerstel thermo desorption unit (TDU) which mounts directly onto a Gerstel cooled injection system (CIS). The GC was equipped with an Agilent HP-5MS (5 % phenyl-, 95 % methylsiloxane) fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm). The initial oven temperature was held at 45 °C for 2 min, increased to 105 °C at 15 °C/min, then increased to 175 °C at 10 °C/min, and finally increased to 255 °C at 4 °C/min with a final temperature hold of 2 min. Ultrapure helium at a flow rate of 1.1 ml/min was used as the carrier gas. The TDU was operated in the solvent vent mode.

When conducting SHS the TDU was maintained at 250 °C and volatiles were swept into the CIS. The CIS inlet was operated using an initial temperature of 0 °C cryo-focussing the volatiles and ramped up to 300 °C at 720 °C/min with a final temperature hold of 2 min.

When operating SBSE an initial TDU temperature was held at 20 °C for 30 s, and then increased to 200 °C at 60 °C/min with a final temperature hold of 5 min. The TDU thermally desorbs substances adsorbed on the PDMS layer and sweeps these analytes into the CIS as well. The CIS inlet was also operated in the solvent vent mode using an initial temperature of 25 °C ramped up to 320 °C at 720 °C/min with a final temperature hold of 2 min. The purge flow was set at 3.0 ml/min.

Both, the desorption (TDU to CIS) and the injection (CIS to column) were performed in splitless mode at a helium flow of 70 mL/min. A liner filled with glass wool was installed in the CIS.

The CIS and TDU parameters were set using the Gerstel software installed on the GC-MS computer system, which consisted of a Hewlett-Packard computer with Windows 7 and Agilent MSD Chemstation software.

The MS was operated in the EI mode with the electron voltage set at auto-tune value. MS acquisition was performed in TIC (total ion chromatogram). The chemstation data analysis software was used for peak integration and library searches. Spectra of unknown urinary components were compared with spectra of known components stored in the NIST library containing well over 100,000 spectra. Compounds that match (>80 %: methyl salicylate, 4-vinylphenol, decanoic acid) were verified with authentic standards using both, retention time and spectra. Compounds that did not match (<80 %) with substances from library were regarded as unknowns.

24.2.7 Data Analyses

Urinary steroid metabolite concentrations were standardized to creatinine (Cr) levels to control for water content and are given as ng hormone/mg creatinine (crea). For urinary estrogen profiles, an iterative process was used to calculate basal

concentrations (Herrick et al. 2010). Briefly, the mean of all samples for the female was calculated and samples with concentrations greater than three standard deviations (mean+3SD) above this mean were removed. This iterative process was repeated until there were no samples with concentrations greater than the mean+3SD. The mean of the remaining values was considered as the individual basal concentration. An increase above basal was defined as the day when urinary estrogen concentrations exceeded basal concentrations+3SD for at least three consecutive samples.

All hormone and volatile profiles shown in the figures refer to the period of receptivity when artificial insemination (AI) was carried out (day 0, see Sect. 24.2.2).

24.3   Results

24.3.1   Seasonal Profile of Reproductive Hormones

Figure 24.1 shows the course of urinary steroid metabolites in 2013. During January to March basal excretion of estradiol remained essentially constant at approximately 2.6 ng/mg creatinine (crea). On the 8th of April (day −13) estrogens increased above
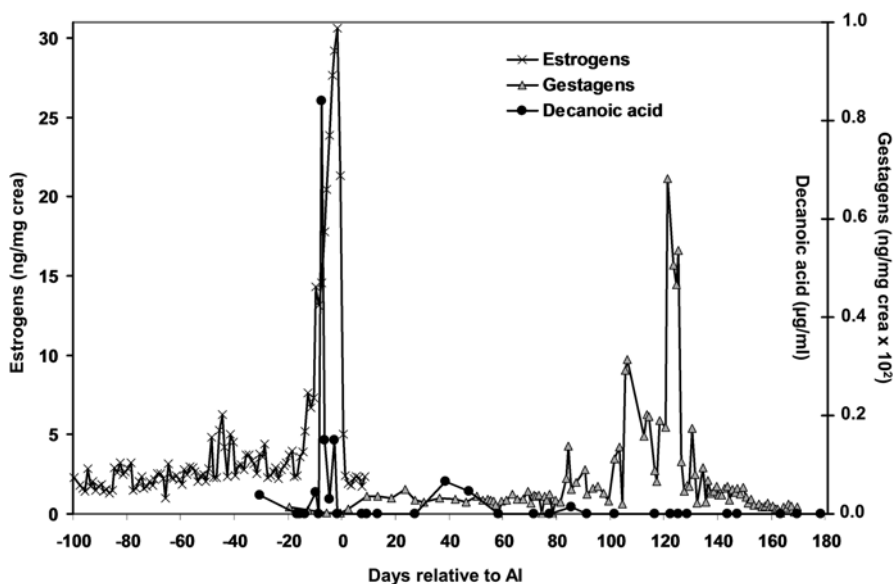
Fig. 24.1 Profiles of urinary estrogens, gestagens and the stir bar sorptive extraction (SBSE) profile of decanoic acid throughout the year 2013. Hormone concentrations were standardized to creatinine (crea) levels to control for water content and are given as ng hormone/mg creatinine. Day 0 is defined as the day of artificial insemination (AI) corresponding to peak receptivity (confirmed by the detection of the ovulatory LH peak in blood)

basal reaching maximal concentrations of 32.8 ng/mg crea 2 days prior to artificial insemination (AI: April 21, equivalent to peak receptivity). Urinary estrogen concentrations fell to baseline levels by 23rd of April and remained low thereafter. Following AI urinary gestagen analyses (pregnanediol) were performed to monitor luteal activity. The slight increase in gestagens mid-July (day 85) might reflect the period of delayed implantation, the distinct increase above basal + 3SD on the 6th of August (282 ng/mg crea, day 105) probably indicates implantation and thus the beginning of pregnancy, which was terminated early September due to an abortion (as evidenced by an ultrasound investigation in the following year).

A similar profile was obtained in 2014 (data not shown) with estrogen concentrations increasing above basal on the 31th of March (day −13) and achieving maximal concentrations between the 9th and 11th of April and dropping to basal levels by the 13th of April, the date when AI was carried out (data not shown). Similar to 2013 luteal activity increased above basal on the 12th of July (day 90) end ended in early September without delivery. Thus, we suspected this period of higher gestagen activity signaled a pseudo-pregnancy.

24.3.2 SPME: Is the Increase in Urinary Fatty Acids Unique Among Individual Pandas?

SPME analyses on the composition of urinary volatiles were carried out throughout the entire year 2013 covering all phases of panda reproduction. The pattern of urinary volatiles remained relatively constant during from January to March. In April urinary fatty acids increased whereby decanoic acid reached maximal concentrations of 0.84 µg/ml 8 days prior to peak receptivity (when AI was carried out, data not shown). This exactly fits with our data obtained one decade earlier where fatty acids likewise peaked 8 days prior to peak receptivity (Dehnhard et al. 2004). In contrast to our 2004 report on pandas, however, this female panda showed maximal decanoic acid concentrations that were only one-tenth as high and octanoic and dodecanoic acid were only barely detectable.

This might be attributed to extreme differences in urinary creatinine (crea) levels increasing from basal concentrations of 1.06 ± 0.75 mg/ml (mean ± SD) during January to March to maximal concentration of 10.7 mg/ml during the period of intense estrogen secretion in April (data not shown). A comparison of area responses in relation to urine concentration by diluting an urine sample from 6.20 mg/g crea down to 0.14 mg/g crea revealed that when crea exceeds concentrations of 2.48 µg/g the recovery of added exogenous decanoic acid decreases whereas the number of endogenous volatiles and their total area stagnated at crea concentrations of 5.0 and 6.2 µg/g. This might indicate an inadequate buffering capacity as well as fiber saturation. Therefore we applied stir bar sorptive extraction (SBSE) using PDMS coated stir-bars (Twister).

24.3.3   Stir Bar Sorptive Extraction (SBSE)

24.3.3.1   Comparison of the Sensitivity of SBSE and SPME

To comparethe extraction efficiency between SBSE and SPME we used ten endogenous substances some of them identified by computer MS library research and confirmed with those of the authentic standards. Table 24.1 shows a distinct higher recovery when using SBSE. Substances undetectable (n. d.) when applying SPME became clearly detectable following SBSE (no 3, 6, and 9), whereas other substances were extracted with 3- (no 7) up to 25-fold higher recoveries (decanoic acid) when applying SBSE. Therefore SBSE was chosen as standard method to continue urine analyses.

24.3.3.2   Identification of Urinary Volatiles Related to Estrus

Fatty Acids

To increase the buffering capacity and to account for often limited amounts of urine, 1 ml of urine was buffered with an equal amount of 0.5 M acetate buffer adjusting the pH to ~4.8 irrespective diverging urine concentrations. The analysis of the composition of urinary volatiles throughout the samples collected in 2013 reveals dramatic changes during the period of estrogen secretion in April. Again an increase in decanoic acid during that period was confirmed peaking 5 days following the urinary estrogen increase (day −13, see Sect. 24.3.1) and therefore 8 days prior to the day of peak receptivity (Fig. 24.1: day 0). Decanoic acid levels decreased thereafter until the end of the year except a weak intermediate increase at the end of May (day 37). A similar profile was seen in the same set of samples when SPME was used (see Sect. 24.3.2).

The composition of urinary volatiles in samples collected 2014 changed dramatically during the period of estrogen secretion in April. This time the increase in decanoic acid reached peak levels 7 days following the estrogen increase (day −13) and 6 days prior to the day of peak receptivity (data not shown). Decanoic acid levels decreased thereafter until the end of the collection period except two weak intermediate increases on days 34 and 85 (May 17th, July 7th).

Other (Unknown) Estrus Related Substances

Similar to decanoic acid an unknown substance appeared in panda urine during the phase of intense estrogen secretion in 2013 that reached peak levels on day −3 and remained at low levels until the end of the year (Fig. 24.2). The mass spectra of the substance that has yet to be identified is characterized by an intense mass fragment at *m/z* 127 (see Fig. 24.2 insert). A similar seasonal pattern was also obtained in
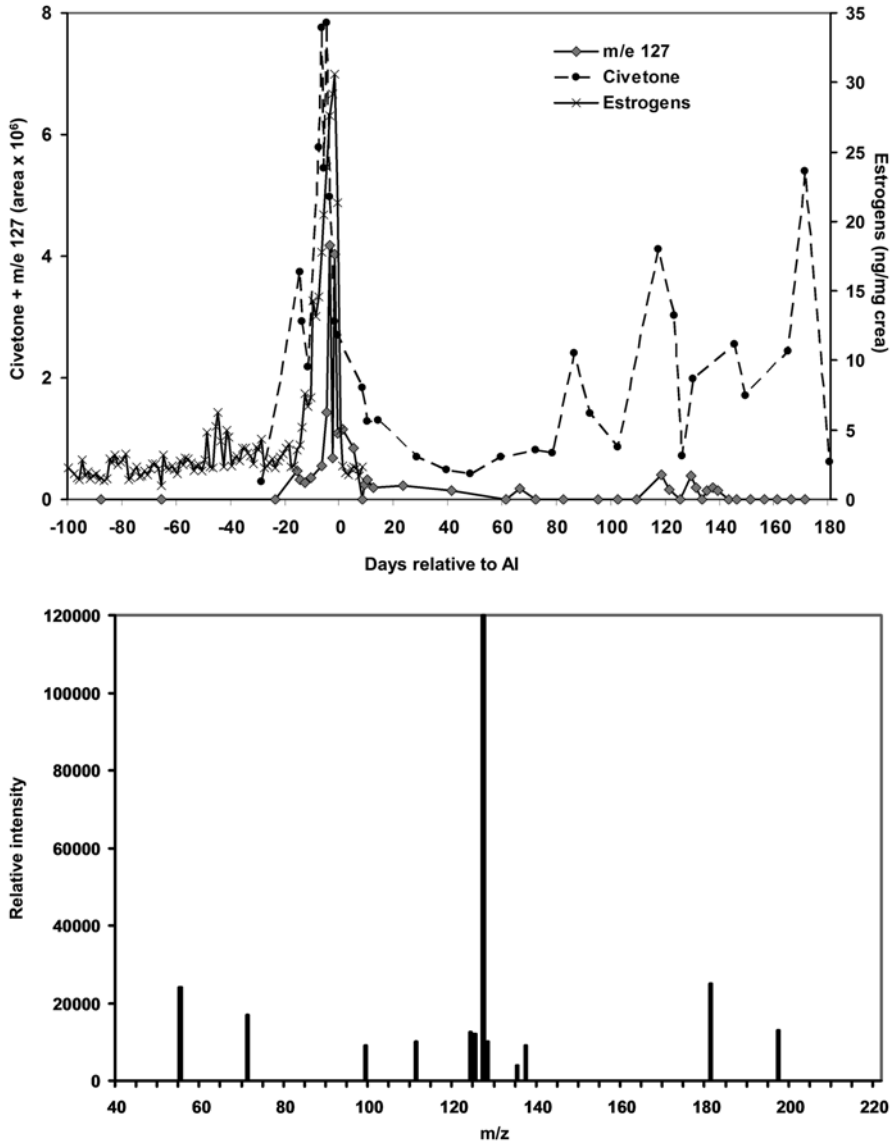
Fig. 24.2 SBSE profiles of an unknown substance with a distinct mass fragment of 127 (see *insert*) and civetone in relation to the urinary estrogen profile during the phase of intensive estrogen secretion and thereafter throughout the year 2013. Day 0 corresponds to the day of AI

2014 when the unidentified substance reached maximal concentrations on day −2. In contrast to decanoic acid, which has been demonstrated in the Berlin panda Yan Yan during the two consecutive years 2002 and 2003, we were unable to detect this unknown substance in GCMS files from SPME analyses of 2002 and 2003 based
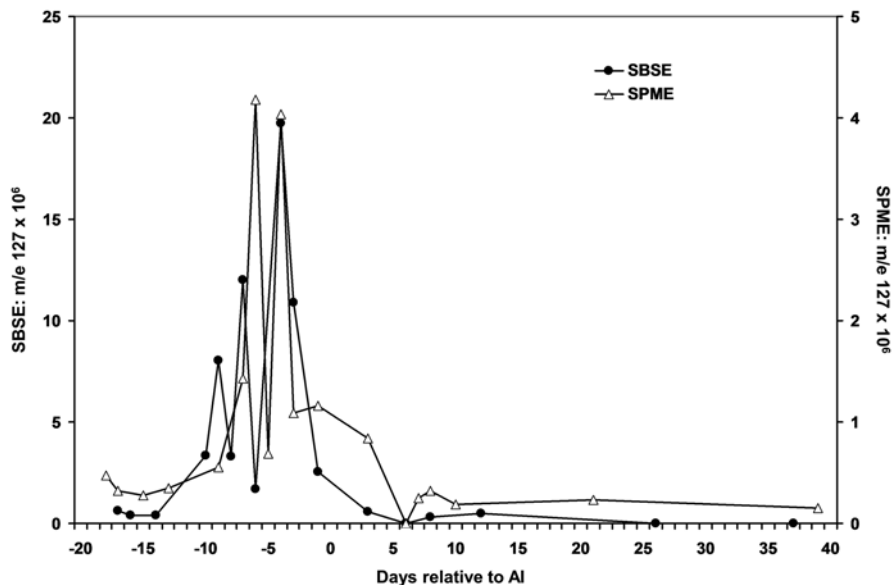
Fig. 24.3 Comparative SBSE and solid phase microextraction (SPME) profiles of urinary decanoic acid throughout the year 2013

on retrospective evaluations. Thus we cannot exclude the *m/z* 127 substance as an individual phenomenon, as it was clearly detectable in the samples from 2013 when SPME was applied (see Fig. 24.3). In addition, *m/z* 127 and its estrogen dependency was also confirmed in the samples collected in 2014 (data not shown) when applying SBSE. Regarding *m/z* 127 SBSE was about five-times more efficient than SPME when comparing samples from 2013 (Fig. 24.3).

In addition to the appearance of decanoic acid and the *m/z* 127 substance several other compounds became detectable during the episode of intensive estrogen secretion in April such as civetone (see Fig. 24.2) and 1-(4-methylphenyl)-ethanone ($C_9H_{10}O$: 93 % match with substance from NIST MS library, not shown). Despite low concentrations in the subsequent months, both substances increased again in mid-July (civetone) and September (methylphenyl-ethanone), respectively, remaining at high levels until the end of the year as shown for civetone (Fig. 24.2) following patterns unrelated to urinary gestagens.

24.3.3.3   Substances with Temporal Relationship to Gestagenes

During the episode of increased luteal activity in August 2013 the pattern of urinary volatiles changed again whereby two substances α-terpineol and endo-borneol (match >80 % with substances from NIST MS library) reached peak levels during the episode of highest gestagen production between August 19th and 26th (not shown).
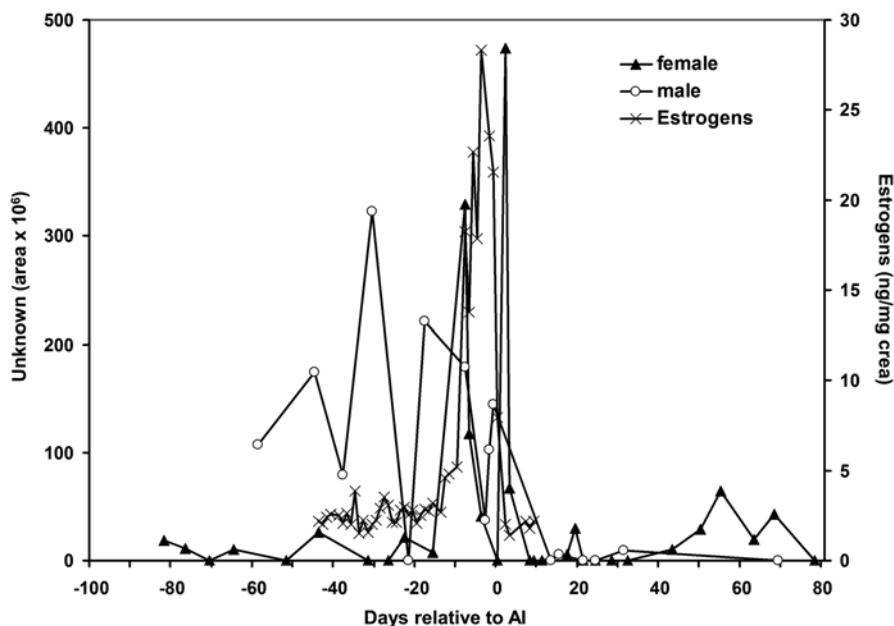
Fig. 24.4 Results of static headspace analyses (SHS) of urine samples of a female and a male panda during 2014 showing the substance at *m/z* 135/192 in comparison. In addition the profile of urinary estrogens is shown

A different result was obtained in samples from the subsequent year when both compounds increased late in the year but were not associated with a high level of luteal activity.

24.3.4 Static Headspace Analyses (SHS)

Samples were analyzed as described in material and methods. Best outcome was reached with incubation at 70 °C for 30 min and a pH-adjustment at 4.8. Adding of salt and enzyme (β-glucuronidase from Helix pomatia) did not improve the analysis (data not shown).

Through SHS only a few compounds were detectable in the urine. One reoccurring substance was an unidentified compound with the *m/z* of 135/192 and a retention time at 11.74 min. The substance reached peak levels on the 15th of April, 2 days following artificial insemination (Fig. 24.4). The substance was found in urine samples from 2013 as well, where its concentration clearly peaked on the 16th of April (here 6 days prior to A.I., data not shown).

In addition, urine samples from the male panda Yang Guang in Edinburgh collected in 2014 were analyzed with SHS. Contrary to our expectations, urine from

the male panda contained this substance as well. Peak levels for Yang Guang were reached on March 13th (Fig. 24.4).

The substance was found in urine samples from both animals until the end of June, when SHS analyses were terminated in favor of SBSE. However, in both animals concentrations dropped to lower levels during April and remained low until the end of June.

24.4 Discussion

The Giant panda (*Ailuropoda melanoleuca*) is an endangered species native to southwestern China living in fragmented habitats and being vulnerable to continued human threats, especially habitat loss. By 2004, there were estimated to be 1600 pandas alive in the wild (IUCN red list). Thus ex situ breeding programs play an important role in the study and recovery of endangered species. One of the many challenges to such programs is establishing a mating protocol that simulates, as closely as possible, what normally occurs under natural conditions. Because females are mono-estrous with a single receptive period of 1–3 days each spring (Kleiman 1983; Schaller et al. 1985) successful reproduction in captivity depends on the ability of animal managers to detect estrus for the accurate management of pairing and artificial insemination. The most useful approach is a skilled combination of monitoring urinary estrogen hormone profiles and careful observations of reproductive behaviors (Zhang et al. 2004).

Giant pandas are a solitary, seasonally mono-estrous species, and as such they certainly rely on chemical signals to coordinate mating efforts (Schaller et al. 1985; Swaisgood et al. 2002). Males displayed a significant investigatory preference for estrous over non-estrous female urine (Swaisgood et al. 2002).

Our systematic SBSE investigations of the composition of urinary volatiles in the female panda throughout two consecutive years revealed a characteristic increase of urinary decanoic acid associated with the seasonal estrogen increase which triggers estrus behavior. The appearance of decanoic acid was also demonstrated in our earlier study where we investigated urine from another female panda during two consecutive years (Dehnhard et al. 2004). Even with a sample size of only two animals, the estrous related appearance of decanoic acid in consecutive years implies that this substance might serve as a pheromone to attract males to females which should be emitted in time before ovulation. In nature, females approaching estrus may attract up to five males that travel long distances and then assemble in the female's home range to compete for copulation opportunities (Schaller et al. 1985; Nie et al. 2012a, b). In captivity it has been shown that 71.0 % of the panda cubs were produced from matings occurring on the day of or the day after the urinary estrogen peak (Huang et al. 2012). In our study AI was performed on the day following the estrogen peak and ovulation was confirmed in 2013 and 2014 by semiquantitative detection of the LH surge in blood plasma when AI was carried out (F. Göritz, personal communication). The occurrence of the decanoic acid peak 6 and 8 days prior

to peak receptivity is similar to our earlier findings (Dehnhard et al. 2004) and might indicate a role of attracting males. This is corroborated with the observation that first scent marking activity usually occurs 6 days prior to the estrogen maximum (McGeehan et al. 2002).

The origin of decanoic acid is unknown. However, our lab identified decanoic acid in four samples of bladder urine obtained between 1999 and 2003. In addition, decanoic acid has not been found in anogenital gland secretion (AGS) of giant pandas (Zhang et al. 2008) which might rule out the possibility of an extrarenal source from accessory glands of the reproductive tract. However, long chain fatty acids from tridecanoic acid to tetracosanoic (C24) acid had been shown to occur in AGS (Zhang et al. 2008). So far decanoic acid has been found in temporal gland secretions of Asian elephant bulls (*Elephas maximus*) during musth (Rasmussen et al. 1990) and in scents on cheeks and foreheads of the tiger (*Panthera tigris*) emitted during facial marking behavior (Soini et al. 2012). In both species decanoic acid was part of a cocktail of chemical compounds and a pheromonal action of the substance was not investigated.

In the panda comparisons of urinary volatiles can also be complicated by the appearance of a metabolic by-product due to changes in bamboo quality. Profiling of volatile compounds of bamboo from Taiwan has shown changes in urinary constituents and their relative contents when comparing spring and winter bamboo shoots. This particularly concerns the concentrations of methyl salicylate that differed markedly between spring and winter (Chung et al. 2012). This substance has been clearly demonstrated recently in panda urine (Table 24.1) but also in our former analyses. Thus when searching for reproductive state related volatiles that may have semiochemical functions it is important to differentiate between hormone and nutrition caused changes in the profile of substance. Thus, feeding the first spring bamboo might interfere with the appearance of estrus related volatiles. In addition, the increase in urine concentration from approximately 1.06 + 0.75 mg/ml and 0.57 + 0.36 mg/ml creatinine up to 10.7 mg/ml and 9.5 mg/ml, respectively, in 2013 and 2014 during the follicular phase might also impair the recovery of volatiles. Thus an adequate buffering capacity should be chosen to exclude an effect of pH on the recovery of decanoic acid.

When applying static headspace analysis (SHS) we assumed that we would obtain a pattern reflecting similar to what the panda could smell. However, only a few compounds of the urine became detectable. Among them was an unknown substance that reached peak levels in April which were not closely related to estrogen secretion as they occur 6 days before (2013) and 2 days after the day of AI (2014). At approximately the same time, this substance was also detected in urine samples from the male panda coinciding with high urinary testosterone concentrations, but became undetectable when urinary testosterone levels decreased to zero (data not shown). The results from both animals might indicate a relationship to reproductive activities, however, additional analyses and substance identification are needed until potential physiological aspects of the substance can be discussed.

In addition, with SBSE we were able to demonstrate the presence of an unknown substance with a marked ion of 127. The substance shows a clear estrogen dependency increasing during the follicular phase in two consecutive years. Unfortunately our finding is limited to one animal and reanalyses of GCMS data collected 10 years ago did not reveal evidence for this substance in the panda investigated in our previous paper (Dehnhard et al. 2004).

In this paper we also confirmed the presence of civeton in panda urine, a substance which was absent in urines of 20 other mammal species we investigated earlier (Dehnhard et al. 2004). Urinary civetone did not reflect a cycle that can be clearly attributed to reproductive activity. The first distinct increase appears during the estrogenic phase in April but increases were also measured late in September and October. Currently, we have no evidence for a signal function of civetone. Its continuous but variable presence in female panda urine and its absence in other investigated species might imply a biological function. Civetone belongs to the macrocyclic musk compounds. In the wild the powerful musky odor of panda urine had been detected from as far as 5 m (Schaller et al. 1985). The exocrine odor glands of the musk deer (*Moschus moschiferus*) also produce an intensive musk odor which is used by female musk deer for tail marking which attracts male partners over great distances during the rutting season (Meng et al. 2008). There is a distinct possibility that civetone in panda urine might serve as an ingredient for territorial marking in females.

We obtained no evidence for volatile substances that might depend on luteal activity and, thus, may be used as pregnancy indicators as previously described in the Asian elephant (Dehnhard et al. 2003). In pandas, pregnancy diagnosis is complicated by the occurrence of pseudo-pregnancies; which are characterized by a similar duration of luteal gestagen production after infertile matings but also infertile AI. Thus a "true" pregnancy indicator as recently described for felids (Dehnhard et al. 2012) would be of great diagnostic value. However, a comparison between samples from this study between 2013 (when the animal was pregnant but aborted) and 2014 (when the animal was pseudo-pregnant) is inappropriate because of SBSE methodological improvements between the 2 years.

In conclusion, the role of decanoic acid as a pheromone remains to be investigated and studies on their behavioral relevance are required. Is has been shown that environmental enrichment can enhance welfare of captive animals through provisioning of stimuli needed for optimal physiological and psychological well-being which has gained popularity in the last few decades (Rafacz and Santymire 2014). If decanoic acid is of biological relevance for male pandas it could be used as olfactory stimuli to enhance their reproductive behavior and, therefore may have implications for captive-breeding programs.

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