

Surface Polar Lipids Differ in Male and Female *Phlebotomus papatasi* (Diptera: Psychodidae)

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ABSTRACT The polar lipids on the surface of the Old World sand fly, *Phlebotomus papatasi* (Scopoli), were analyzed by high-resolution mass spectrometry. Blood-fed females and nonblood-fed females and males were separately analyzed and compared. The major polar lipids were found to be long-chain diols and fatty acids. Relatively high levels of diacylglycerols were found in blood-fed females and in males. A wide variety of lipids were found at low levels, including esters, sterols, monoacylglycerols, and hydroxy fatty acids. Blood-fed females had several lyso lipids and N-acyl amino acids that were not found on unfed females or males. These substances may be surfactants used in blood feeding. Heneicosenoic acid was found on females at more than twice the level of males, suggesting it could be a component of a female pheromone. Four substances were identified on males at twofold higher levels than on females: tetradienoic acid, methoxyhexadecasphinganine, butyl octadecanoate, and diacylglycerol(14:1/12:0/0:0). These could be short-range pheromones involved in courtship, and they will be further analyzed in future behavioral bioassays.

KEY WORDS high resolution mass spectrometry, blood feeding, courtship pheromone

Phlebotomine sand flies are the vectors of the pathogens that can cause leishmaniasis (Ready 2013), as well as some viral and bacterial pathogens. The World Health Organization estimates that the scope of leishmaniasis includes more than one million new cases and as many as 30,000 deaths each year (WHO 2013). Until vaccines become widely available, improved vector control methods will be needed.

The use of pheromones could be valuable for improving sand fly control strategies. For the New World sand fly *Lutzomyia longipalpis* (Lutz and Neiva, 1912), an oviposition pheromone (Dougherty and Hamilton 1997) and a volatile mating pheromone (Spiegel et al. 2005) have been identified. There is some evidence for mating pheromones in the Old World sand fly *Phlebotomus papatasi* (Chelbi et al. 2011), and a *P. papatasi* female aggregation pheromone has been suggested (Schlein et al. 1984). However, no pheromones have yet been chemically identified in this species. *Phlebotomus* males lack the pheromone-secreting tergal glands found in *Lutzomyia* sand flies (Spiegel et al. 2002). Nevertheless, recent studies of courtship show

antennal contact occurring before mating (Chelbi et al. 2012). For example, the male moves his abdomen up and down, and immediately the female approaches and contacts the male.

Contact pheromones can be more difficult to characterize than volatile pheromones because some types of polar molecules do not become sufficiently volatile upon heating to analyze by gas chromatography unless they are derivatized. However, advances in high resolution mass spectrometry have largely eliminated this problem. Substances separated by reversed-phase high-performance liquid chromatography (HPLC) can be eluted into an electrospray mass spectrometer, and the mass can be measured to a few parts per million accuracy by Fourier-transform methods (Makarov et al. 2006). This is sufficient accuracy to determine the exact molecular formula of each component in a complex mixture. In addition, each component can be broken into fragments that provide molecular structure information.

We report here the analysis of polar lipids extracted from the external exoskeletal surface of *P. papatasi* sand flies. We have identified several potential leads for a male mating pheromone.

Materials and Methods

Sand flies used in this study were from a laboratory colony of *P. papatasi* maintained at USDA, ARS, Knippling-Bushland U.S. Livestock Insects Research Laboratory (Kerrville, TX). The colony was established using pupae from a long-established Israeli strain of *P. papatasi* maintained at the Division of Entomology, Walter Reed Army Institute of Research

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(WRAIR, Silver Spring, MD). Larvae were fed with a sand fly larval diet, a composted mixture of rabbit feces and rabbit food (Young et al. 1981). Male and female flies in the cage were fed daily with 30% sucrose water after emergence. Both larvae and adult flies were maintained at $26 \pm 2^\circ\text{C}$ and a relative humidity (RH) of $85 \pm 2\%$ in an environmental chamber. Female sand flies were blood fed by exposing a population of 3- to 7-d adult male and female sand flies in a cage to blood feeders filled with defibrinated cattle blood for 2 h using an in vitro membrane feeding system.

Two hours after blood feeders were removed from the sand fly cage, 20 male and 20 blood-fed female sand flies were removed from the cage and placed in glass vials. In addition, 20 nonblood-fed females (hereafter referred to as unfed females) were removed from a separate cage and placed in a glass vial. The flies in each group were anesthetized with CO_2 and then soaked in 5 ml pentane for 5 min. Extracts were transferred to clean vials and dried under a gentle stream of N_2 . The extracts were reconstituted in isopropanol before HPLC-electrospray ionization tandem mass spectrometry. Analyses were conducted on a Q Exactive mass spectrometer (Thermo Fisher, San Jose, CA) with a PicoChip Nanospray Source (New Objective, Woburn, MA) and a PicoChip column (Atlantis dC18; $150\ \mu\text{m}$ by $105\ \text{mm}$; $3\ \mu\text{m}$ particles; Waters, Milford, MA). The on-line separation was performed on a rapid separation nano HPLC system (Thermo Fisher/Dionex, Sunnyvale, CA). The HPLC conditions were—mobile phase A, acetonitrile/water (40:60) containing 10 mM ammonium acetate; mobile phase B, acetonitrile/isopropanol (10:90) containing 10 mM ammonium acetate; flow rate, $1\ \mu\text{l}/\text{min}$; gradient, 10 to 60% B over 5 min, 60 to 99% B over 28 min, and held at 99% B for 15 min. Data-dependent tandem-MS scans were performed using one full MS scan (m/z [mass/charge] 200–2000; 70,000 resolution [m/z 300]) followed by fragmentation in the higher-energy collisional dissociation collision cell of the six most abundant ions in the precursor scan using a normalized collision energy of 35 arbitrary units and mass analysis in the orbitrap at 17,500 resolution. Separate analyses were conducted using electrospray positive and negative ion detections.

Progenesis CoMet (Nonlinear Dynamics Limited, Newcastle, UK; <http://www.nonlinear.com>) software was used to process the Thermo raw data files. Peak alignment and integration were performed, and the relative abundance was generated to obtain fold change statistics between blood-fed females, unfed females, males, and a solvent blank. In addition, total ion current chromatograms were displayed in Xcalibur software (Thermo Fisher, Waltham, MA) and inspected in 10 m/z ranges from 200 to 1000. Peaks with m/z values corresponding to formulas of molecular ions within the expected 5-ppm mass error were tabulated and searched in METLIN (<http://metlin.scripps.edu/index.php>) and lipid maps (<http://www.lipidmaps.org/data/structure/>). The putative lipid identifications were manually verified through examination of the MS/MS spectra. In most cases, many

isomers are possible, and further chemical analysis will be necessary to establish the exact chemical structures.

Results and Discussion

The most abundant polar lipids identified in the surface solvent extracts of *P. papatasi* are shown in Table 1. Molecular formulae were obtained from the high resolution mass spectra molecular ions. In cases where several formulae fit the molecular ion, the table indicates the formula with a mass error closest to the instrument calibration (approximately $-4\ \text{ppm}$ for positive ions and $0\ \text{ppm}$ for negative ions). For some molecules, several different molecular ions were detected (e.g., $[\text{M}+\text{H}]^+$, $[\text{M}+\text{NH}_4]^+$), and in these cases, the table shows data for only one. Tentative molecular identifications are given for those molecules supported by fragment ions in the MS/MS spectra. The main fragments identified are given in Supp Table 1 (online only). Quantification is estimated based on comparisons of the total ion current, which is proportional to both the amount of molecule and also the efficiency of ionization. The total ion chromatograms for the main positive ions are shown in Supp Fig. 1 (online only) and Supp Table 2 (online only). The lipids most likely are present on the cuticle surface, but some substances may be derived from interior cavities, as discussed below.

The major surface polar lipid found in both males and females was heptacosanediol. Additional diols were found in smaller amounts in both males and females: dotriacontanediol, nonacosanediol, pentacosanediol, and octacosanediol. Long-chain diols have been considered rare constituents of insect cuticular lipids (Blomquist and Bagnères 2010). However, these substances may be difficult to detect by GC/MS methods. A recent analysis of *Drosophila melanogaster* cuticular lipids by UV-laser desorption ionization time-of-flight mass spectrometry showed many hydroxyl-modified hydrocarbons, including diols (Yew et al. 2009).

Several free fatty acids were also abundant constituents of the *P. papatasi* surface polar lipids: octadecenoic acid, hexadecenoic acid, octadecadienoic acid, and tetradecenoic acid. Previously, dodecanoic acid was identified as the oviposition pheromone of *L. longipalpis* (Dougherty and Hamilton 1997). Among the fatty acids, heneicosenoic acid was observed in blood-fed and unfed females to about the same extent, but the amount was more than twofold lower in males. This suggests the possibility that heneicosenoic acid could be a constituent of a female pheromone.

Aside from the long-chain diols and fatty acids, unfed females had much smaller amounts of surface polar lipids than blood-fed females and males. For example, we observed relatively high levels of some triacyl- and diacylglycerols in blood-fed females and males (DG(12:0/12:0/0:0), DG(14:0/12:0/0:0), DG(16:1/12:0/0:0), DG(16:1/14:0/0:0), DG(18:1/14:0/0:0), and TG(16:1/12:0/12:0)), but these were at much lower levels in unfed females. It is possible that some

Table 1. *P. papatasi* surface polar lipids

m/z measured	m/z Theo.	Mass error (ppm)	Formula	Identification ^a	Relative intensity ^b		
					FF	FU	M
Alcohols							
384.3950 ^c	384.3967	−4.3	C ₂₅ H ₅₂ O ₂	Pentacosanediol	0.1389	0.1347	0.1151
398.4129 ^{c,d}	398.4124	1.3 ^d	C ₂₆ H ₅₄ O ₂	Hexacosanediol	0.0748	0.0340	0.0345
412.4264 ^{c,d}	412.4280	−3.8 ^c	C ₂₇ H ₅₆ O ₂	Heptacosanediol	1.0000	1.1073	0.9579
426.4422 ^{c,d}	426.4437	−3.4 ^c	C ₂₈ H ₅₈ O ₂	Octacosanediol	0.0590	0.0619	0.0454
440.4578 ^{c,d}	440.4593	−3.3 ^c	C ₂₉ H ₆₀ O ₂	Nonacosanediol	0.2102	0.2284	0.1616
482.5046 ^{c,d,e}	482.5063	−3.5 ^c	C ₃₂ H ₆₆ O ₂	Dotriacontanediol	0.6793	0.6883	0.5807
Fatty acids							
200.1777 ^d	200.1776	0.5	C ₁₂ H ₂₄ O ₂	Dodecanoic acid	0.0126	0.0006	0.0020
214.1930 ^f	214.1933	−1.3	C ₁₃ H ₂₆ O ₂	Tridecanoic acid	0.0101	0.0090	0.0083
224.1774 ^f	224.1776	−0.8	C ₁₄ H ₂₈ O ₂	Tetradecadienoic acid	0.0014	0.0010	0.0039
226.1930 ^f	226.1933	−1.2	C ₁₄ H ₂₆ O ₂	Tetradecenoic acid	0.0925	0.0050	0.0113
254.2246 ^{c,e,f}	254.2246	0.1 ^f	C ₁₆ H ₃₀ O ₂	Hexadecenoic acid	0.8616	0.0669	0.1231
280.2403 ^{c,f}	280.2402	0.4 ^f	C ₁₈ H ₃₂ O ₂	Octadecadienoic acid	0.3651	0.0846	0.1266
282.2561 ^f	282.2559	0.8	C ₁₈ H ₃₄ O ₂	Octadecenoic acid	0.9976	0.1636	0.2311
288.2288 ^{c,e}	288.2301	−4.6 ^e	C ₁₆ H ₃₂ O ₄	Dihydroxyhexadecanoic acid	0.0039	0.0000	0.0001
312.2286 ^c	312.2300	−4.4	C ₁₈ H ₃₂ O ₄	Dihydroxyoctadecadienoic acid	0.0013	0.0011	0.0008
314.2458 ^{c,d,g}	314.2457	0.3 ^g	C ₁₈ H ₃₄ O ₄	Dihydroxyoctadecenoic acid	0.0026	0.0016	0.0015
316.2613 ^f	316.2613	0.1	C ₁₈ H ₃₆ O ₄	Dihydroxyoctadecanoic acid	0.0039	0.0003	0.0004
320.2353 ^g	320.2351	0.6	C ₂₀ H ₃₂ O ₃	Hydroxyeicosatetraenoic acid	0.0067	0.0032	0.0029
324.1922 ^c	324.1937	−4.5	C ₁₈ H ₂₈ O ₅	Trihydroxyoctadecatetraenoic acid	0.0034	0.0000	0.0000
324.3012 ^c	324.3028	−4.8	C ₂₁ H ₄₀ O ₂	Heneicosenoic acid	0.0056	0.0058	0.0027
332.2548 ^e	332.2563	−4.6	C ₁₈ H ₃₆ O ₅	Trihydroxyoctadecanoic acid	0.0107	0.0001	0.0001
Esters							
242.2234 ^{c,e}	242.2246	−5.1 ^e	C ₁₅ H ₃₀ O ₂	Propyl dodecanoate	0.0040	0.0002	0.0015
268.2389 ^{c,e}	268.2402	−4.9 ^e	C ₁₇ H ₃₂ O ₂	Propyl tetradecenoate	0.0096	0.0002	0.0010
270.2546 ^{c,e}	270.2559	−4.9 ^e	C ₁₇ H ₃₄ O ₂	Propyl tetradecanoate	0.0076	0.0009	0.0071
274.2132 ^e	274.2144	−4.5	C ₁₅ H ₃₀ O ₄	MG (12:0/0:0/0:0)	0.0148	0.0009	0.0074
298.2857 ^{c,e}	298.2872	−5.1 ^e	C ₁₉ H ₃₈ O ₂	Propyl hexadecanoate	0.0151	0.0017	0.0108
300.2288 ^{c,d,h}	300.2301	−4.2 ^c	C ₁₇ H ₃₂ O ₄	MG (14:1/0:0/0:0)	0.0040	0.0001	0.0019
302.2442 ^e	302.2457	−5.0	C ₁₇ H ₃₄ O ₄	MG (14:0/0:0/0:0)	0.0056	0.0018	0.0036
320.2702 ^e	320.2715	−4.1	C ₂₁ H ₃₆ O ₂	Propyl octadecatrienoate	0.0030	0.0001	0.0002
322.2857 ^e	322.2872	−4.7	C ₂₁ H ₃₈ O ₂	Propyl octadecadienoate	0.0187	0.0004	0.0015
328.2598 ^{c,e}	328.2614	−4.8 ^c	C ₁₉ H ₃₆ O ₄	MG (16:1/0:0/0:0)	0.0191	0.0017	0.0143
330.2754 ^c	330.2770	−4.8	C ₁₉ H ₃₈ O ₄	MG (16:0/0:0/0:0)	0.0053	0.0043	0.0052
340.3325 ^c	340.3341	−4.8	C ₂₂ H ₄₄ O ₂	Butyl octadecanoate	0.0005	0.0008	0.0022
356.2912 ^{c,h}	356.2927	−4.1 ^c	C ₂₁ H ₄₀ O ₄	MG (18:1/0:0/0:0)	0.0158	0.0063	0.0132
448.4280 ^d	448.4280	0.0	C ₃₀ H ₅₆ O ₂	Tetradecenyl hexadecenoate	0.0533	0.0002	0.0011
456.3798 ^{c,d,h}	456.3815	−3.7 ^c	C ₂₇ H ₅₂ O ₂	DG (12:0/12:0/0:0)	0.5057	0.0063	0.4621
482.3953 ^c	482.3971	−3.7	C ₂₉ H ₅₄ O ₅	DG (14:1/12:0/0:0)	0.0365	0.0001	0.0725
484.4108 ^{c,d,h}	484.4128	−4.0 ^h	C ₂₉ H ₅₆ O ₅	DG (14:0/12:0/0:0)	0.1449	0.0024	0.1419
500.4594 ^d	500.4593	0.2	C ₃₄ H ₆₀ O ₂	Hexadecenyl octadecadienoate	0.0099	0.0003	0.0011
504.4907 ^d	504.4906	0.2	C ₃₄ H ₆₄ O ₂	Hexadecyl octadecenoate	0.0795	0.0017	0.0039
510.4262 ^h	510.4284	−4.2	C ₃₁ H ₅₆ O ₄	DG (12:0/16:1/0:0)	0.0717	0.0000	0.1005
512.4420 ^h	512.4441	−4.0	C ₃₁ H ₆₀ O ₅	DG (12:0/16:0/0:0) ⁱ	0.0493	0.0012	0.0385
538.4577 ^{c,d}	538.4597	−3.7 ^c	C ₃₃ H ₆₂ O ₅	DG (16:1/14:0/0:0) ^j	0.4368	0.0041	0.5978
566.4891 ^{c,d}	566.4910	−3.3 ^c	C ₃₅ H ₆₆ O ₅	DG (16:1/16:0/0:0) ^k	0.3096	0.0093	0.3424
692.5923 ^c	692.5955	−4.6	C ₄₃ H ₈₀ O ₆	TG (16:1/12:0/12:0) ^l	0.4377	0.0417	0.3409
Sterols							
398.3169 ^e	398.3185	−4.0	C ₂₇ H ₄₂ O ₂	Cholestendione	0.0022	0.0018	0.0014
398.3532 ^e	398.3549	−4.3	C ₂₈ H ₄₆ O	Ergostadienol	0.0037	0.0046	0.0028
400.3326 ^c	400.3341	−3.9	C ₂₇ H ₄₄ O ₂	Ketocholesterol	0.0019	0.0017	0.0011
402.3482 ^h	402.3498	−3.9	C ₂₇ H ₄₆ O ₂	Cholestanolone	0.0031	0.0026	0.0023
432.3242 ^g	432.3239	0.6	C ₂₇ H ₄₆ O ₄	Hydroxycholestenol carboxylic acid	0.0004	0.0000	0.0000
Lyso phospholipids							
451.2700 ^f	451.2699	0.3	C ₂₁ H ₄₂ NO ₇ P	LysoPE (16:1/0:0)	0.0047	0.0001	0.0008
477.2856 ^f	477.2855	0.3	C ₂₃ H ₄₄ NO ₇ P	LysoPE (18:2/0:0)	0.0005	0.0001	0.0002
479.3013 ^f	479.3012	0.3	C ₂₃ H ₄₆ NO ₇ P	LysoPE (18:1/0:0)	0.0119	0.0011	0.0032
493.3167 ^d	493.3168	−0.2	C ₂₄ H ₄₈ NO ₇ P	LysoPC (16:1/0:0)	0.0010	0.0001	0.0002
519.3325 ^d	519.3325	0.0	C ₂₆ H ₅₀ NO ₇ P	LysoPC (18:2/0:0)	0.0008	0.0002	0.0003
521.3483 ^d	521.3481	0.4	C ₂₆ H ₅₂ NO ₇ P	LysoPC (18:1/0:0)	0.0036	0.0007	0.0009
523.2911 ^{f,g}	523.2910	0.2 ^f	C ₂₄ H ₄₆ NO ₉ P	LysoPS (18:1/0:0)	0.0006	0.0001	0.0002
596.2964 ^f	596.2962	0.4	C ₂₇ H ₄₈ O ₁₂ P	LysoPI (18:2/0:0)	0.0007	0.0001	0.0003

Continued on following page

Table 1. Continued

m/z measured	m/z Theo.	Mass error (ppm)	Formula	Identification ^a	Relative intensity ^b		
					FF	FU	M
Miscellaneous							
262.2284 ^c	262.2297	−5.1	C ₁₈ H ₃₀ O	Octadecatrienal/one	0.0014	0.0001	0.0002
303.2761 ^c	303.2773	−4.0	C ₁₇ H ₃₇ NO ₃	Methoxyhexadecaspheganine	0.0021	0.0030	0.0063
345.2502 ^c	345.2515	−3.9	C ₁₈ H ₃₅ NO ₅	N-dihydroxyhexadecanoyl glycine	0.0099	0.0002	0.0002
373.2813 ^c	373.2828	−4.1	C ₂₀ H ₃₉ NO ₅	N-dihydroxyoctadecanoyl glycine	0.0009	0.0001	0.0000

^a Identifications are based on MS/MS fragments; see Supp Table 1 (online only).
^b FF, blood-fed females; FU, nonblood-fed females; M, males; intensity relative to heptacosanediol; components with ≥2:1 intensity ratio shown in bold.
^c Detected as [M+NH₄]⁺.
^d Detected as [M+CH₂CO₂][−].
^e Detected as [M+H]⁺.
^f Detected as [M-H][−].
^g Detected as [M-H₂O-H][−].
^h Detected as [M-H₂O+H]⁺.
ⁱ DG(14:0/14:0/0:0) also detected.
^j DG(18:1/12:0/0:0) also detected.
^k DG(18:1/14:0/0:0) also detected.
^l TG(14:1/14:0/12:0) also detected.

of the identified components were actually contaminants directly transferred to the cuticle from food, or released from the crop or rectum during solvent extraction. However, it seems unlikely that the DGs and TGs found at similar high concentrations on blood-fed females and males could have been directly transferred from food contact, because males and blood-fed females were fed on different food sources.

The minor constituents of the surface polar lipids included various esters containing short-chain alcohols (propyl dodecanoate, propyl tetradecanoate, propyl hexadecanoate, propyl octadecatrienoate, propyl octadecadienoate, and butyl octadecanoate). Most of these esters were at higher levels in blood-fed females than males, and in many cases the amounts in unfed females were very low. In addition, three wax esters were identified (tetradecenyl hexadecanoate, hexadecenyl octadecadienoate, and hexadecyl octadecanoate). These were also more abundant in blood-fed females than in males, and levels were very low in unfed females. Several monoacylglycerols were detected (12-, 14-, 16-, and 18-carbon chains), with MG(12:0:0:0) and MG(14:1:0:0) more abundant in blood-fed females.

Many hydroxy fatty acids were identified at relatively low levels (dihydroxyhexadecanoic acid, dihydroxyoctadecadienoic acid, dihydroxyoctadecenoic acid, dihydroxyoctadecanoic acid, hydroxyeicosatetraenoic acid, trihydroxyoctadecatetraenoic acid, and trihydroxyoctadecanoic acid). All were at higher levels in blood-fed females than in males or unfed females, and the two trihydroxy acids were nearly undetectable in males and unfed females. Hydroxy fatty acids previously have been found at high levels in honey bee royal jelly (Isidorov et al. 2012), and also as O-acetylated fatty acyl chains of glycerides in the oviposition pheromone of *Culex* mosquitoes (Starratt and Osgood 1972, 1973).

Several sterols were identified at low levels that were similar in females and males (cholestendione,

ergostadienol, ketocholesterol, and cholestanolone). A very low level of hydroxycholestenol carboxylic acid was found in blood-fed females, but not in unfed females or males. Because insect sterols are entirely derived from food sources, the low amounts of sterols in our analysis adds further support to the inference that direct contamination of the cuticle by food contact is minimal.

Lysolipids (Supp Fig. 2 (online only)) were found in blood-fed females at higher levels than in unfed females or males. Phospholipase A2 is known to be expressed in salivary glands of Phlebotomine flies (Ribeiro et al. 2010), and thus the lysolipids may reflect the recent feeding activity of the females. A residual amount of lysolipids may remain on the mouthparts after feeding. Alternatively, it is possible that the lysolipids are part of a secretion intended to be used to protect the eggs: previously, lysoPE was identified in house fly larvae and found to have antibiotic activity (Meylaers et al. 2004).

N-acyl glycine was found on blood-fed females (dihydroxyhexadecanoyl, Supp Fig. 3 (online only), and dihydroxyoctadecanoyl), with only traces on unfed females or males. N-acyl glutamine is known as a constituent of the saliva of phytophagous caterpillars (Alborn et al. 1997). It is possible that female sand flies use N-acyl glycine in a function involved with feeding, as N-acyl amino acids are likely to be surfactants.

Four substances were detected on males at approximately twofold or higher levels than on females: tetradecenoic acid, methoxyhexadecaspheganine (Supp Fig. 4 (online only)), butyl octadecanoate, and DG(14:1/12:0/0:0). In future experiments, these compounds will be tested in behavioral bioassays for activity as contact or short range male mating pheromones. The sphinganine derivative is particularly intriguing, as it was recently shown that serine palmitoyltransferase is required for male meiotic cytokinesis in *D. melanogaster* (Guan et al. 2013). Serine palmitoyltransferase catalyzes the first step in sphingolipid biosynthesis, so the sphingolipid pathway is probably highly active during spermatogenesis.

genesis. An interesting possibility is that methoxy-hexadecaspinganine is produced in proportion to the spermatogenesis activity of a particular male. Thus, it could signal to females information about a male's sperm content.

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