1 **Photoperiod and aggression induce changes in ventral gland compounds exclusively in**
2 **male Siberian hamsters**

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ABSTRACT

Chemical communication is a critical component of social behavior as it facilitates social encounters, allows for evaluation of the social partner, defines territories and resources, and advertises information such as sex and physiological state of an animal. Odors provide a key source of information about the social environment to rodents; however, studies identifying chemical compounds have thus far focused primarily on few species, particularly the house mouse. Moreover, considerably less attention has been focused on how environmental factors, reproductive phenotype, and behavioral context alter these compounds outside of reproduction. We examined the effects of photoperiod, sex, and social context on chemical communication in the seasonally breeding Siberian hamster. We sampled ventral gland secretions in both male and female hamsters before and after an aggressive encounter and identified changes in a range of volatile compounds. Next we investigated how photoperiod, reproductive phenotype, and aggression alter ventral gland volatile compound composition across the sexes. Males exhibited a more diverse chemical composition, more sex-specific volatiles, and showed higher levels of excretion compared to females. Individual volatiles were also differentially excreted across photoperiod and reproductive phenotype, as well as differentially altered in response to an aggressive encounter. Female volatile compound composition, in contrast, did not differ across photoperiods or in response to aggression. Collectively, these data contribute to a greater understanding of context-dependent changes in chemical communication in a seasonally breeding rodent.

KEY WORDS: ventral gland, chemical signaling, olfactory, pheromones, scent marking, sex differences, territoriality, stir bar surface sampling, gas chromatography-mass spectrometry

INTRODUCTION

Chemical communication plays an important role in the social behavior of virtually all animals. Communicative signals are used to facilitate social encounters, evaluate social partners, establish territories, and advertise information such as identity, sex, and physiological state of an animal (Bradbury and Vehrencamp, 2011). Various types of communication can occur through the transmission of visual, auditory, tactile, or chemical signals between sender and receiver. Whereas communication has been studied across many vertebrate taxa, rodents offer an excellent opportunity as a comparative model for mammalian chemical communication (Liberles, 2014; Tirindelli et al., 2009). For most species of rodents, the sense of smell is the most dominant sensory modality; therefore, olfaction provides a critical source of information about the environment for rodents (Johnston, 2003). In fact, rodents with disrupted olfaction display abnormal social behaviors; male mice fail to display territorial aggression and also mount and thrust both males and females (Chamero et al., 2011; Stowers et al., 2002), and female mice fail to display maternal aggression (Chamero et al., 2011; Del Punta et al., 2002). Three areas of research regarding the chemical complexity in rodents has received particular attention: the role of the major histocompatibility complex (MHC) in the production of chemical constituents and the functions of these constituents in mate choice (Johnston, 2003; Liberles, 2014; Tirindelli et al., 2009), the use of odors in kin recognition (Hurst et al., 2001) (Johnston, 2003; Liberles, 2014; Tirindelli et al., 2009), and the functions of scent marking (Arakawa et al., 2008; Johnston, 2003; Liberles, 2014; Tirindelli et al., 2009).

The importance of olfaction for rodents is evident by studies examining consequences of olfaction deficiency (Chamero et al., 2011; Del Punta et al., 2002; Stowers et al., 2002), however, it is clear that mammalian chemical communication has increasingly focused on a

relatively limited number of model species, particularly mice [but see: (Drea, 2015)]. A growing number of studies employing cutting-edge techniques and gene knock-out models have revealed an incredible diversity of chemosensory systems that mediate murine signal detection and transduction and their underlying cellular and molecular mechanisms (Liberles, 2014). For example, the G protein, $G\alpha_o$ is vital for sensory responses to cues including exocrine gland-secreting peptide, and is also essential for the display of male-male territorial aggression and maternal aggression in mice (Chamero et al., 2011). Further, distinct chemical constituents that induce behaviors have been identified in mice [e.g., aggression: (Novotny et al., 1985), dominance: (Novotny et al., 1990); reproductive partner preference: (Jemiolo et al., 1991)]. Despite these and other major advances in mice, much less is known about how changes in reproductive phenotype and social behaviors influence individual chemical constituents in non-murine mammalian models.

The Siberian hamster (*Phodopus sungorus*) is a well-established model for the study of seasonal changes in physiology and behavior (Jasnow et al., 2000; Rendon et al., 2015b; Scherbarth and Steinlechner, 2010; Soma et al., 2015), making this species an excellent model to characterize the interactions of season, sex, and behavioral context on individual chemical constituents. First, both males and females housed in short “winter-like” days exhibit gonadal regression and display *increased* territorial aggression when compared with hamsters in long “summer-like” days (Bedrosian et al., 2012; Jasnow et al., 2000; Rendon et al., 2015b; Scotti et al., 2007). This inverse relationship between gonadal steroids and aggression allows for the disassociation of the individual effects of reproductive phenotype and aggression on chemical signaling. Second, this species exhibits a polymorphism in the reproductive response to short days in that ~30% of the population does not respond to short day lengths by inhibiting

reproductive physiology and behavior. These animals, called reproductive *non-responders*, maintain fully functional reproductive capacity and appear phenotypically indistinguishable from long-day hamsters (Gorman and Zucker, 1995; Lynch et al., 1989) despite maintenance in short days. This distinct polymorphism makes it possible to dissociate the direct effects of photoperiod *per se* from the indirect effects of photoperiod on reproductive phenotype and chemical signaling. Lastly, Siberian hamsters utilize a variety of chemical signaling mechanisms, including deposition of chemical constituents in feces, and urine, sacculi, and ventral glands (Burger et al., 2001a; Burger et al., 2001b; Burger, 2005; Ross, 1998; Soini et al., 2005).

Among the variety of signaling mechanisms in hamsters, the ventral gland chemical trails applied to ground substrate are thought to be of particular importance for territorial marking in this species (Wynne-Edwards, 2003; Wynne-Edwards et al., 1992). Observations of Siberian hamsters in the wild have demonstrated that females use scent-marking to establish boundaries between their home-ranges (Wynne-Edwards, 2003; Wynne-Edwards et al., 1992), which corroborates laboratory studies using naturalistic housing conditions (Wynne-Edwards and Lisk, 1987). Male hamsters presented with conspecific male odors (i.e., urine, bedding material, and saccular secretions) behaviorally responded by increasing ventral gland marking (Feoktistova, 1994), and more specifically, male hamsters presented with ventral gland secretions display increased investigation of the secretion in short-day photoperiods when compared to long-day photoperiods (Sokolov and Feoktistova, 1997). Further, ventral gland secretions, unlike feces and urine, which are partially metabolized, are likely to change during social encounters, providing temporally salient feedback to counterparts within a social interaction. Siberian hamster ventral compounds have previously been analyzed in males, but were undetectable in females (Burger et al., 2001b). Males have larger ventral glands when compared to females,

likely explaining this sex difference in the ability to detect chemical constituents with existing analytical techniques. In the present study, however, we used a stir bar surface sampling method (Soini et al., 2006), and reached a sufficiently low detection limit to quantify female ventral gland chemical constituents, which allowed us to examine sex differences in Siberian hamsters. By identifying and quantifying specific chemical constituents, and coupling such constituents with aggression and reproductive phenotype (i.e., breeding condition and levels of the gonadal hormone testosterone), we can clarify key sex-specific and functionally relevant chemical components contributing to chemical communication in Siberian hamsters.

In the present study, we examined photoperiodic changes in the composition and quantity of volatiles within the ventral glands of both male and female Siberian hamsters during same-sex aggressive encounters, as well as aggression-induced changes in these volatiles. We also investigated the association between the gonadal steroid testosterone (T) and production of volatile compounds within individuals. We predicted that the composition of volatiles would differ photoperiodically such that specific ventral gland compounds would be present in elevated levels in short-day when compared with long-day photoperiods. We also predicted that composition of volatiles would differ with reproductive phenotype such that some ventral gland compounds would be present in greater amounts in reproductively functional hamsters (i.e., long day and short-day non-responders) compared with non-reproductively functional hamsters (i.e., short-day responders). We further predicted that changes in volatile composition would parallel photoperiodic changes in aggression (Jasnow et al., 2000; Rendon et al., 2015b), such that gonadally regressed hamsters would produce elevated levels of specific volatile compounds closely associated with aggression. Previous work in male mice identified volatile compounds that are associated with aggression in gonadally intact males with elevated levels of T (Chamero

et al., 2007; Novotny et al., 1985). Using a hamster model in which functional reproductive physiology and aggression can be dissociated, we expected to identify both T-dependent and T-independent volatile compounds associated with increased aggression. To test these predictions, we sampled secretions of the ventral gland of both male and female Siberian hamsters before and after aggressive interactions, and across photoperiodic conditions that mimicked seasonal phenotypes.

By using a seasonal rodent, Siberian hamsters, to study how social and non-social environmental factors and endocrine status influence chemical signaling in an ecologically relevant context, our findings will complement those that have emerged from studies in laboratory mice. Such insight will also expand our largely-fragmented knowledge of the roles and mechanisms of chemical communication in mammals. Lastly, the results from this study will contribute to our understanding of the chemical ecology of mammalian communication within an aggressive context.

MATERIAL AND METHODS

Animal housing and photoperiodic treatment

Adult (>60 days of age) hamsters were reared, bred and maintained under long days (light:dark, 16:8h), and group-housed at weaning (postnatal day 18). Hamsters were given *ad libitum* access to standard laboratory rodent chow (Lab Diet 5001, PMI Nutrition) and water. Ambient temperature was maintained at 20 ± 2 °C and relative humidity was maintained at $55 \pm 5\%$. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Bloomington Institutional Animal Care and Use Committee at Indiana University.

Prior to the start of the experiment, resident hamsters were individually housed ($n = 76$; male: $n = 51$; female: $n = 20$), whereas intruder hamsters remained group-housed (3-4 animals per cage; males, $n = 25$; females, $n = 9$) in a room on a long-day light (LD) cycle for a one-week acclimation period. Subsequently, a subset of hamsters was transferred to a room on a short-day light (SD) cycle (light:dark: 8:16h), and the remaining hamsters were relocated to a new room on a long-day light cycle (light:dark, 16:8h). All hamsters remained in their photoperiodic regimens for eight weeks. Reproductive phenotype was determined based on *a priori* criteria previously established for Siberian hamsters (Jasnow et al., 2000; Scotti et al., 2007). Briefly, hamsters were characterized as reproductively competent if they had functional reproductive tissues (i.e., testes for males or ovaries, uterine horns, and parametrial white adipose tissue for females), displayed no significant change in body mass ($<10\%$), and maintained a “summer brown/grey” coat color (long days; LD; males: $n = 20$; females: $n = 8$). Estrous cycles were monitored via vaginal cytology (Scotti et al., 2007) to confirm cycling in reproductively competent females. Reproductive inhibition, in contrast, was characterized by regressed reproductive tissues, a significant decrease in body mass ($>10\%$), and a “winter white” coat color (short-day responder; SD-R; males: $n = 21$; females: $n = 8$). The reproductively functional morph in short days was characterized using the same criteria described for long-day females above (short-day non-responder; SD-NR; males: $n = 10$; females: $n = 4$). These non-responders which do not respond reproductively to the short-day photoperiodic regime have been previously documented (Gorman and Zucker, 1995; Lynch et al., 1989). SD-NR males were included in subsequent statistical analyses; however, SD-NR females were excluded due to insufficient numbers.

Quantification of aggressive behavior

Same-sex aggressive encounters were recorded and analyzed using previously outlined methods (Jasnow et al., 2000; Rendon et al., 2015b). Aggression was assessed using the resident-intruder paradigm, which consists of placing an unfamiliar intruder into the home cage of a resident and allowing them to interact for 5 minutes, within the first two hours of the dark phase. Hamsters were approximately the same age and mass ($\pm 10\%$), and from different parents. All trials were recorded under low-illumination red lights using a Sony HandyCam Digital Camcorder HDR-SR7.

Trained observers used ODLog™ (Macropod Software, Eden Prairie, MN) to score aggressive behaviors (i.e., attacks, chases, and latency to first attack), investigative behaviors (i.e., facial and ano-genital investigation) and scent marking behaviors (i.e., self-grooming of ventral gland and depositing of ventral gland secretions on bedding material). Scores from the two individuals were averaged and inter-rater reliability was accepted if less than 10% variation occurred. Two separate principal components analyses (PCA) were used for males and females on aggression (80.30% of the total variance explained), and scent marking variables (61.00% of the total variance explained). Both aggression variables and scent marking variables loaded strongly onto the first component; therefore the composite ‘aggression score’, and composite ‘scent marking score’ were used to examine the effects and associations of volatiles, sex and reproductive phenotype with these behaviors (*Table S1*).

Identification and quantification of ventral gland volatile compounds

Male and female ventral gland secretions were sampled before aggression trials to characterize and quantify volatile compound composition of the ventral gland, and following aggression trials to determine how aggression alters volatile compound compositions. Pre-

aggression samples were taken one hour prior to behavioral trial, and post-aggression samples were taken immediately (< 5 min) following aggressive encounter. Volatile compounds were collected using an *in situ* stir bar sampling method, a technique previously developed for surface skin sampling (Soini et al., 2006). A preconditioned stir bar with the embedded internal standard (see below for details on stir bar preparation) was placed in the collection device and the stir bar was rolled over two separate 5-cm long stretches of the ventral gland (10 cm² skin area). The stir bar was subsequently transferred from the collection device and placed in a capped clean Twister™ glass vial and stored at 4°C for less than 3 days. Previous studies on human skin have shown that the collected samples were chemically stable up to 14 days (Penn et al., 2007; Soini et al., 2006).

Stir bars (Twister™, 10mm, 0.5 mm film thickness, 24 µL polydimethylsiloxane (PDMS) volume) used for ventral gland sample collection were obtained from Gerstel GmbH (Mülheim an der Ruhr, Germany), and conditioned prior to embedding the internal standard in the TC-2 tube container (Gerstel GmbH) at 300 °C under high helium stream. Prior to extraction, all glassware was washed with acetone and dried at 80 °C. An internal standard, 8 ng of 7-tridecanone (Aldrich, Milwaukee, WI), was added in 5 µL of methanol (J.T. Baker, Avantor Materials, Phillipsburg, NJ) to pre-cleaned 20 mL vials containing 2.0 mL high-purity water (OmniSolv, EM Science, Gibbstown, NJ), followed by the addition of a preconditioned stir bar. Stirring speed was 850+ rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnik, Oberschleissheim, Germany). After extraction, the stir bars were stored in the individual vials at 4 °C to sample collection for up to 3 days. The embedded internal standard had been previously found stable at 4 °C for up to 20 days (Soini et al., 2006).

Volatile compounds were characterized and quantified using gas chromatography-mass spectrometry (GC-MS). Quantitative analysis was performed using the Agilent 6890N gas chromatograph connected to a 5973i MSD mass spectrometer (Agilent Technologies, Inc., Wilmington, DE) with the thermal desorption autosampler and cooled injection system (TDSA-CIS 4 from Gerstel). Positive electron ionization mode at 70 eV was used with a scanning rate of 2.47 scans/s over the mass range of 41-350 amu. The mass-spectrometric detector (MSD) transfer line temperature was set at 280 °C. The ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. The separation capillary was DB-5MS (30 m x 0.25 mm, inner diameter [i.d.], 0.25 µm film thickness) from J&W Scientific, Folsom, CA. Samples were thermally desorbed in a Thermal Desorption Autosampler (TDSA automated system), followed by injection into the column with a cooled injection assembly (CIS-4). TDSA operated in a splitless mode, while the temperature program for desorption was 20 °C (0.5 min), then 60 °C/min to 270 °C (10 min hold time). Temperature of the transfer line was set at 280 °C. CIS was cooled with liquid nitrogen to -60 °C. After desorption and cryotrapping, CIS was heated at 12 °C/s to 280 °C (10min hold time). CIS inlet was operated in the solvent vent mode, with a vent pressure of 9.1 psi, a vent flow of 30 mL/min, and a purge flow of 50 mL/min. The temperature program in the GC operation was 40 °C for 5 min, then increasing to 200 °C at the rate of 2 °C/min (10 min hold time). The carrier gas head pressure was 9.1 psi (flow rate, 1.2 mL/min at the constant flow mode).

Quantification of the ventral gland volatile compounds

Peak areas of the positively or tentatively identified compounds were used for quantitative comparisons among groups and sexes. Several unidentified compounds were also used for comparisons. Identifications were based on the comparisons of the retention times and

spectra of the known standard compounds. Peak areas of each compound were normalized by dividing each peak area by the peak area of the internal standard in the same analytical run. Peak areas with a mean relative proportion of less than 0.1% within an individual were not included in the further analysis.

Blood sampling and testosterone quantification

After eight weeks of the photoperiodic regimen, blood samples were drawn from the retro-orbital sinus 24 hours prior to behavioral trials (i.e., pre-aggression), and then again within 5 min of completion of behavioral trials (i.e., post-aggression). Serum T levels were quantified using an enzyme immunoassay (EIA; Assay Design 900-065; assay sensitivity = 5.67 pg/mL) that has been validated in this species and has negligible or undetectable cross-reactivity with other steroid hormones (Bedrosian et al., 2012). Samples were diluted (males: 1:20 or 1:40; females: 1:4 or 1:8), assayed in duplicate according to the manufacturer's recommended protocol, and were balanced across five plates of the same kit lot. Samples with coefficients of variation (CVs) greater than 10% or maximum binding less than 20% or greater than 80% were re-analyzed. Intra-assay variability was between 1.47% and 4.48%, and inter-assay variability was 2.27%.

Statistical analyses

All statistical analyses were run in JMP v. 11.0.0 (SAS Institute, Inc., Cary, NC), and statistical significance was attributed at $p < 0.05$. Data were transformed to attain normality and equal variances. Four separate principal component analyses (PCA) were conducted on aggression variables (*Table S1*), scent marking variables (*Table S1*), male ventral gland compounds (*Table 2*), and female ventral gland compounds (*Table 3*) to reduce the number of

variables for analysis, retaining PCs with an eigenvalue greater than 1. Either one-way analyses of variances (ANOVAs) or two-tailed *t*-tests were used to compare reproductive physiology, T, aggression, scent marking, investigation and percentage of chemical class abundance within sexes and across groups. Two-tailed *t*-tests were used to compare levels of compounds between males and females. Repeated-measures ANOVAs were used to compare pre- and post-aggression volatiles and T levels across groups with time as a within-subjects factor, followed by paired *t*-tests. Spearman's rank correlations were used to quantitatively assess relationships between volatiles and T levels. Lastly, effect sizes were calculated; η^2 was calculated for ANOVAs and Cohen's *d* was calculated for pair-wise comparisons.

RESULTS

Photoperiod affected reproductive phenotype: SD hamsters had non-functional reproductive physiology

Male short-day responders had significantly decreased body mass (16%, $F_{2,48} = 33.70$, $\eta^2 = 0.58$, $p < 0.0001$) (*Table S2*), gonadal mass (paired testes mass: $F_{2,48} = 237.34$, $\eta^2 = 0.91$, $p < 0.0001$) (*Figure S1A*), and circulating levels of T ($F_{2,48} = 6.56$, $\eta^2 = 0.21$, $p = 0.003$) (*Figure 1A*), compared with both long-day and short-day non-responder males. Similarly, females had significantly decreased body mass (17%, $t_{12} = -4.90$, $d = -2.45$, $p = 0.0004$) (*Table S2*), gonadal mass (paired ovarian mass: $t_{12} = -3.56$, $d = -1.78$, $p = 0.004$) (*Figure S1B*), and general reproductive masses (i.e., uterine horn mass, and parametrial white adipose tissue mass) compared with both long-day females (*Table S2*). In contrast to males, short-day females did not have decreased circulating levels of T ($t_{12} = -1.06$, $d = -0.53$, $p = 0.32$) (*Figure 1B*), compared with long-day females.

Photoperiod affected aggression: SD hamsters were more aggressive than LD hamsters

Short-day responder males displayed more ($F_{2,48} = 4.91, \eta^2 = 0.52, p = 0.01$) and longer ($F_{2,48} = 4.81, \eta^2 = 0.50, p = 0.01$) attacks, more ($F_{2,48} = 4.50, \eta^2 = 0.36, p = 0.03$) and longer ($F_{2,48} = 1.93, \eta^2 = 0.32, p = 0.04$) chases (*Table S2*), and displayed more overall aggression ($F_{2,48} = 4.19, \eta^2 = 0.49, p = 0.02$) (*Figure 1C*) (see *Table S1* for aggression score details), compared with long-day and short-day non-responder males. Short-day responder males had a shorter latency to first attack, compared with short-day non-responder males; long-day males were intermediate to both groups ($F_{2,48} = 4.19, \eta^2 = 0.25, p = 0.02$). Similarly, short-day females displayed more ($t_{12} = 2.40, d = 1.20, p = 0.03$) and longer ($t_{12} = 2.06, d = 1.03, p = 0.03$) attacks, more ($t_{12} = 2.20, d = 1.10, p = 0.03$) and longer ($t_{12} = 2.08, d = 1.04, p = 0.04$) chases (*Table S2*), and displayed more overall aggression than long-day females ($t_{12} = 2.28, d = 1.14, p = 0.04$) (*Figure 1D*) (see *Table S1* for aggression score details). In contrast to males, the latency to first attack did not differ across photoperiods ($t_{12} = -1.04, d = -0.52, p = 0.33$) (*Table S2*).

Photoperiod affected scent marking in males: SD hamsters scent marked more than LD hamsters

Short-day responder males displayed more scent depositing bouts ($F_{2,48} = 5.04, \eta^2 = 0.37, p = 0.01$) (*Table S2*), and displayed more overall scent marking ($F_{2,48} = 2.14, \eta^2 = 0.31, p = 0.03$) (*Figure 1E*) (see *Table S1* for scent marking score details) compared with either long-day or short-day non-responder males. However, males displayed no group differences in duration of scent depositing bouts ($F_{2,48} = 1.09, \eta^2 = 0.22, p = 0.34$), number ($F_{2,48} = 1.62, \eta^2 = 0.25, p = 0.54$) and duration ($F_{2,48} = 0.61, \eta^2 = 0.25, p = 1.55$) of ventral gland grooming bouts, number ($F_{2,48} = 1.10, \eta^2 = 0.04, p = 0.90$) and duration ($F_{2,48} = 1.06, \eta^2 = 0.02, p = 0.94$) of facial investigations, number ($F_{2,48} = 1.17, \eta^2 = 0.07, p = 0.85$) and duration ($F_{2,48} = 1.15, \eta^2 = 0.06, p$

= 0.86) of ano-genital investigations (*Table S2*). In contrast to males, short-day females displayed longer facial investigations ($t_{12} = 2.02$, $d = 1.01$, $p = 0.03$) compared with long-day females (*Table S2*). Despite this difference, females displayed no photoperiodic difference in number ($t_{12} = 1.27$, $d = 0.64$, $p = 0.59$) and duration ($t_{12} = -1.52$, $d = -0.76$, $p = 0.61$) of scent depositing bouts (*Table S2*), overall scent marking ($t_{12} = 1.55$, $d = 0.78$, $p = 0.32$) (*Figure 1F*) (see *Table S1* for scent marking score details), number ($t_{12} = 1.01$, $d = 0.51$, $p = 0.91$) and duration ($t_{12} = 1.75$, $d = 0.88$, $p = 0.47$) of ventral gland grooming bouts, number of facial investigations ($t_{12} = 1.04$, $d = 0.52$, $p = 0.32$), number ($t_{12} = 1.26$, $d = 0.63$, $p = 0.23$) and duration ($t_{12} = 1.36$, $d = 0.68$, $p = 0.20$) of ano-genital investigations (*Table S2*).

Siberian hamsters displayed a diverse composition of ventral gland compounds

By analyzing individual GC-MS generated total ion chromatograms (TIC) (*Figure S2*) we positively or tentatively identified 75 ventral gland compounds from several compound classes: alcohols, aldehydes, ketones, carboxylic acids, hydrocarbons, amides, terpenes, heterocyclic and aromatic compounds as well as several unidentified compounds were observed. Males showed a more diverse chemical composition compared to females. There were 50 male-specific compounds including 6 unidentified compounds, and 6 female-specific compounds (*Table 1*). We quantitatively compared all compounds that had a mean relative proportion greater than 0.1% within an individual. Among the identified compounds, 23 compounds in the male group and 14 compounds in the female group were quantitatively compared. Additionally, differences within 4 unidentified compounds in the male group were compared.

Males had elevated levels and a more diverse composition of ventral gland compounds than females

Short-day responder and non-responder males displayed a greater proportion of aldehydes ($F_{2,48} = 4.86$, $\eta^2 = 0.17$, $p = 0.01$), and terpenes ($F_{2,48} = 5.52$, $\eta^2 = 0.19$, $p = 0.007$) compared with long-day males. Short-day responder males displayed a greater proportion of carboxylic acids ($F_{2,48} = 5.89$, $\eta^2 = 0.20$, $p = 0.005$), and heterocyclic and aromatic compounds ($F_{2,48} = 6.71$, $\eta^2 = 0.17$, $p = 0.03$) when compared to long-day and short-day non-responder males. In contrast, long-day and short-day non-responder males displayed a greater proportion of ketones ($F_{2,48} = 7.77$, $\eta^2 = 0.24$, $p = 0.001$), and unknown compounds ($F_{2,48} = 8.46$, $\eta^2 = 0.26$, $p = 0.0007$) when compared to short-day responders. Hydrocarbons, however, did not differ across groups ($F_{2,48} = 1.04$, $\eta^2 = 0.04$, $p = 0.36$) (Figure S3A). In contrast to males, females displayed no photoperiodic difference of ventral gland composition within chemical class (aldehydes: $t_{12} = 0.79$, $d = 0.40$, $p = 0.45$; amides: $t_{12} = -0.91$, $d = -0.46$, $p = 0.39$; carboxylic acids: $t_{12} = -0.04$, $d = -0.02$, $p = 0.97$; heterocyclic and aromatic compounds: $t_{12} = -0.03$, $d = -0.02$, $p = 0.99$; and ketones: $t_{12} = -0.15$, $d = -0.08$, $p = 0.88$) (Figure S3B).

In general, males had higher levels of compounds among those shared between the sexes: nonanal (long days: $t_{26} = 3.38$, $d = 1.28$, $p = 0.002$; short-days: $t_{26} = 3.45$, $d = 1.26$, $p = 0.001$), hexadecanoic acid (long days: $t_{26} = 3.89$, $d = 1.47$, $p = 0.008$; short-days: $t_{26} = 3.95$, $d = 1.44$, $p = 0.03$), acetophenone (long days: $t_{26} = -2.70$, $d = -1.02$, $p = 0.01$; short-days: $t_{26} = 2.72$, $d = 0.99$, $p = 0.01$), 2-pentadecanone (long days: $t_{26} = 4.92$, $d = 1.86$, $p < 0.0001$; short-days: $t_{26} = 2.27$, $d = 0.83$, $p = 0.03$), and 6-methyl-5-hepten-2-one (long days: $t_{26} = 2.77$, $d = 1.05$, $p = 0.01$; short-days: $t_{26} = 3.54$, $d = 1.29$, $p = 0.002$). However, there was no sex difference in levels of decanal for long-day animals (long days: $t_{26} = 1.11$, $d = 0.42$, $p = 0.28$; short-days: $t_{26} = 1.56$, $d = 0.57$, p

= 0.13), or in levels of indole for short-day animals (long days: $t_{26} = 4.64$, $d = 1.75$, $p < 0.0001$; short-days: $t_{26} = 1.78$, $d = 0.65$, $p = 0.23$) (Figure 2A,B).

Photoperiod and reproductive phenotype altered ventral gland compounds in males, but not females

In males, photoperiod altered the levels of 8 ventral gland compounds: 3,7-dimethyl-6-octenoic acid ($F_{2,48} = 4.12$, $\eta^2 = 0.27$, $p = 0.02$), 9-octadecenoic acid ($F_{2,48} = 2.72$, $\eta^2 = 0.19$, $p = 0.008$), octadecanoic acid ($F_{2,48} = 4.14$, $\eta^2 = 0.20$, $p = 0.04$), 1-H-pyrrole-2,5-dione ($F_{2,48} = 2.86$, $\eta^2 = 0.30$, $p = 0.02$), squalene ($F_{2,48} = 2.98$, $\eta^2 = 0.19$, $p = 0.04$) (Figure 3A), 2-heptadecanone ($F_{2,48} = 9.92$, $\eta^2 = 0.37$, $p = 0.002$), 2-nonadecanone ($F_{2,48} = 8.37$, $\eta^2 = 0.62$, $p = 0.0008$) (Figure 3B), and o-cymene ($F_{2,48} = 2.34$, $\eta^2 = 0.19$, $p = 0.01$) (Figure 3C). Reproductive phenotype altered the levels of 7 ventral gland compounds such that short-day non-responders had intermediate levels of compounds compared with long-day and short-day responders: 2-pentadecanone ($F_{2,48} = 6.02$, $\eta^2 = 0.33$, $p = 0.005$), 4,8-dimethyl-3,7-nonadiene-2-one ($F_{2,48} = 5.91$, $\eta^2 = 0.21$, $p = 0.005$), 4,8-dimethyl-3,8-nonadiene-2-one ($F_{2,48} = 5.76$, $\eta^2 = 0.26$, $p = 0.006$), 4,8-dimethyl-7-nonen-2-one ($F_{2,48} = 7.67$, $\eta^2 = 0.27$, $p = 0.0001$) (Figure 3B), unidentified compound 2 ($F_{2,48} = 6.36$, $\eta^2 = 0.54$, $p = 0.004$), unidentified compound 3 (m/z 166) ($F_{2,48} = 9.39$, $\eta^2 = 0.24$, $p = 0.0004$), and unidentified compound 4 (m/z 166) ($F_{2,48} = 7.44$, $\eta^2 = 0.36$, $p = 0.002$). Further, short-day responders had intermediate levels of acetophenone ($F_{2,48} = 4.50$, $\eta^2 = 0.31$, $p = 0.02$), and 6-methyl-5-hepten-2-one ($F_{2,48} = 2.74$, $\eta^2 = 0.19$, $p = 0.04$) (Figure 3C) compared with long-day and short-day non-responders. In contrast to males, levels of female ventral gland compounds did not differ across photoperiods (p 's > 0.05 in all cases) (Table S3).

Aggression altered ventral gland compounds in males, but not females

Nine ventral gland compounds were differentially altered in response to a same-sex aggressive encounter in males, but not in females. Five compounds increased following an aggressive encounter in short-day responder males, but not in long-day and short-day non-responder males: decanal ($F_{1,48} = 1.44$, $\eta^2 = 0.11$, $p = 0.04$), 3,7-dimethyl-6-octenoic acid ($F_{1,48} = 3.95$, $\eta^2 = 0.35$, $p = 0.04$), eicosanoic acid ($F_{1,48} = 1.97$, $\eta^2 = 0.16$, $p = 0.04$), 4,8-dimethyl-3,7-nonadien-2-one ($F_{1,48} = 1.81$, $\eta^2 = 0.14$, $p = 0.02$), and 4,8-dimethyl-3,8-nonadien-2-one ($F_{1,48} = 4.18$, $\eta^2 = 0.42$, $p = 0.02$) (*Figure 4A*). O-cymene increased following an aggressive encounter in long-day males, but not short-day males ($F_{1,48} = 4.06$, $\eta^2 = 0.22$, $p = 0.04$) (*Figure 4A*). 6-methyl-5-hepten-2-one increased following an aggressive encounter in short-day non-responder males, but not in long-days or short-day non-responders ($F_{1,48} = 2.12$, $\eta^2 = 0.18$, $p = 0.03$) (*Figure 4C*). Acetophenone decreased following an aggressive encounter in long-day and short-day responder males, but were not altered in short-day non-responder males ($F_{1,48} = 4.72$, $\eta^2 = 0.17$, $p = 0.04$) (*Figure 4A,B*). In contrast to males, levels of female ventral gland compounds were not altered in response to an aggressive encounter (p 's > 0.05 in all cases) (*Table S3*).

Ventral gland volatile compounds were associated with serum testosterone

Two separate PCAs were conducted on pre-aggression ventral gland compounds for males and females. For males, two ventral gland compound PCs were extracted (PC1_{MALE VG COMPOUNDS} and PC2_{MALE VG COMPOUNDS}), which explained 77.47% of the total variance (*Table 2*). For females, two PCs were extracted (PC1_{FEMALE VG COMPOUNDS} and PC2_{FEMALE VG COMPOUNDS}), which explained 58.29% of the total variance (*Table 3*). Pre-aggression T was correlated with

PC2_{FEMALE} VG COMPOUNDS. There were no other significant relationships between ventral gland compound PCs and T in either sex (*Table 4*).

DISCUSSION

In the present study, we identified ventral gland chemical constituents from individual male and female hamsters using a non-invasive, highly sensitive sampling and analysis method. Although female ventral gland secretions have been examined in pooled samples, previously employed analytical techniques were not sensitive enough to characterize individual female chemical constituents. The present surface sampling method allowed us to characterize both male and female compounds in individual animals, as well as determine the functional effects of photoperiod, reproductive phenotype and aggression on expression of these compounds. Siberian hamster ventral gland secretions included a broad range of chemical compounds, both in terms of the number of chemical classes, as well as structural diversity within each class. Moreover, both photoperiod and aggression directly affected chemical compounds in a sex-dependent manner. Specifically, males had a more diverse representation of chemical classes and generally had higher levels of compounds, and also displayed photoperiodic and aggression-induced changes in individual compounds. Females, in contrast, did not display any significant changes in compound levels, despite also showing similar photoperiod-induced gonadal regression and increased aggression as males did; these findings suggest sex-specific physiological regulation of chemical secretions in this species. Although the precise role of these compounds, either individually or in combination, is not yet known, the present findings suggest an important role for chemical communication in regulating social behavior in this species and likely extend to other seasonally breeding mammals.

Identification of individual hamster ventral gland secretions

We identified 69 male ventral gland secretions representing 10 chemical classes (categorized on the basis of overall structure, and according to functional groups that categorize compounds based on the same or similar modes of metabolism), and 33 female secretions representing 5 chemical classes. For both males and females, the majority of the ventral gland constituents (e.g., carboxylic acids, alcohols, ketones) are similar to those that have been identified in other mammalian species (Burger, 2005). Among the additional compounds, we further found both male- and female-specific compounds that are likely used to identify sex within this species. We report several previously unidentified male-specific unsaturated ketones, including 4,8-dimethyl-7-nonen-2-one, 4,8-dimethyl-3,7-nonadien-2-one, and 4,8-dimethyl-3,8-nonadien-2-one, which may be unique to ventral glands in this species (Burger et al., 2001a; Burger et al., 2001b; Burger, 2005; Soini et al., 2005). Lastly, two female-specific methyl ketones, 2-dodecanone, 2-tetradecanone, as well as a tentatively identified female-specific compound, N-hydroxytetradecanamide (tetradecanehydroxamic acid or myristoyl hydroxamate) were observed. This latter compound could serve some special functions for female hamster physiology as hydroxamic acid functional groups have been found to actively target matrix metalloproteinase enzymes which are involved in tumor development (Saban and Bujak, 2009).

Identification of marked sex differences in ventral gland secretions

Both qualitative and quantitative analyses of our data show marked sex differences in hamster ventral gland chemical profiles. Male volatile compound profiles are predominantly composed of male-specific ketones, carboxylic acids, and a set of unknown male-specific compounds. In contrast, volatile compound profiles in females are predominantly composed of ketones, aldehydes, and heterocyclic and aromatic compounds, at lower levels than in males.

These sex differences in levels and relative proportions of volatile chemical compounds likely reflect observed behavioral differences for male hamsters presented with either male or female ventral gland secretions (Feoktistova, 1994). Specifically, in the presence of female secretions, males engage in anogenital scent marking, but in the presence of male secretions, males mark using their ventral gland (Feoktistova, 1994). This suggests that hamsters can identify sex using olfactory cues, and do so in the absence of other sensory cues.

Aggression-induced changes in ventral gland secretions in males but not females

A key finding in this study is that individual ventral gland compounds were differentially altered in response to a same-sex aggressive encounter, regardless of photoperiodic regimen. Changes in levels of individual compounds before and after aggression paralleled the intensity of aggression. Ventral gland compounds were altered most notably in short-day responders, which are reproductively inactive animals that display elevated aggression compared with reproductive animals (i.e., long-day and short-day non-responders) (Jasnow et al., 2000; Rendon et al., 2015b). We also report aggression-induced increases in five individual compounds, and a decrease in one compound in these short-day responders compared to reductively active animals. These animals also displayed increased scent marking behaviors, namely self-grooming of the ventral gland and deposition of ventral gland secretions on bedding material. These behaviors, in addition to aggression, likely influenced the expression of individual compounds reported here. Both long-day and short-day non-responders also showed aggression-induced changes in individual compounds, but these reproductive morphs showed fewer changes in compounds compared with short-day responders. Long-day animals showed increases in one compound, and decreases in another compound, in response to behavioral interactions. Short-day non-responder animals showed increases in a single compound. It is interesting to note that short-day

responders, but not long-day and short-day non-responders, increased the male-specific methyl ketones 4,8-dimethyl-3,7-nonadien-2-one, and 4,8-dimethyl-3,8-nonadien-2-one. Recent work comparing red-sided garter snakes to other species of *Thamnophis* suggests that change in proportions of methyl ketone expression may signal species specificity, and therefore likely plays a role in species recognition (Uhrig et al., 2014). Therefore, the information content of these methyl ketones likely differs between hamsters and snakes; signaling sex identity for hamsters and signaling species identity in snakes (Uhrig et al., 2014). Although the precise function of these methyl ketones is not known in hamsters or snakes, similar compounds likely convey different information among senders and receivers across vertebrates. Both long- and short-day responders displayed aggression-induced decreases in acetophenone, which is a simple compound that serves as a precursor and is intermediate in the metabolism and biosynthesis of other compounds, including a variety of aromatic compounds (Kanehisa and Goto, 2000). Aggression-induced decreases of acetophenone might suggest that this chemical compound could act as a “pro-pheromone”, being metabolized to other chemical compounds displaying aggression-induced increases. It is interesting to note that we report very little overlap in aggression-induced chemicals across photoperiodic groups. The lack of overlap suggests that there is differential metabolic regulation of these synthetic pathways for long-day hamsters and short-day responders; however, such mechanisms explaining this pattern are currently unknown.

In noted contrast to males, we did not detect aggression-induced changes in female ventral gland compounds or changes in scent marking behaviors. Although it is known that females of this species use scent marking to establish boundaries between their home ranges (Wynne-Edwards, 2003; Wynne-Edwards and Lisk, 1987; Wynne-Edwards et al., 1992), these females might shift use of their communicative signals during an aggressive encounter from

chemical to other sensory modalities. In fact, they may use broadband vocalizations, which we have recently shown to be associated with individual levels of aggression in this species (Keesom et al., 2015; Rendon et al., 2015a). An alternative explanation is that there might be a sex difference in detection of individual chemical compounds by the receiver (Baum and Bakker, 2013; Petrulis, 2013); we did not investigate aspects of the receiver's sensory capabilities in the present study.

Photoperiod and reproductive phenotype induced changes in ventral gland secretions

Another key finding is that both photoperiod and reproductive phenotype contributed to substantial changes in levels of individual compounds. There was a significant effect of photoperiod on eight ventral gland compounds; long-day chemical compound levels were generally elevated when compared to short-day levels. Reproductive phenotype also influenced levels of individual chemical compounds in short-day animals. Short-day responders and non-responders displayed differences in photoperiodic-induced changes in levels of chemical compounds, despite experiencing identical photoperiodic treatments. Specifically, short-day non-responders displayed intermediate responses to long-day (i.e., reproductive) and short-day responder (i.e., non-reproductive) hamsters with respect to levels of seven chemical compounds. Short-day responders displayed intermediate responses to long-day and short-day non-responder hamsters with respect to levels of two chemical compounds. These intermediate responses in short-day animals suggests that photoperiod *per se*, as well as reproductive response to photoperiod, contribute to changes in levels of ventral gland volatiles. These data suggest a likely role for both direct and indirect actions of photoperiod on levels of individual chemical compounds in this species.

We show changes in levels of ventral gland compounds that reflect known patterns in photoperiodic-induced changes in reproductive phenotype; however, the proximate mechanisms underlying these responses remain unknown. It is likely that there are both gonadal steroid-dependent and independent effects on individual chemical compounds. Comparing short-day responders and short-day non-responders serves as a means to test T-dependence versus T-independence of gonadal steroid regulation in ventral gland compounds in this species. However, in our comparisons we report no association between serum T and the two principal components extracted for ventral gland compounds. The lack of this association does not rule out a role for gonadal hormones or their receptors for the regulation of ventral gland compounds. For example, modest changes in T, site-specific metabolic conversion, or steroid receptor expression would likely not be captured in quantification of circulating levels of serum T. There is also the potential for other pathways for biosynthesis of ventral gland compounds that are T-independent. Follow-up studies should explore the relationship between ventral gland compound production and gonadal steroids by combining experimental manipulations of reproductive phenotype (i.e., gonadectomies and gonadal hormone replacement), with natural fluctuations of reproductive phenotype (i.e., “functional gonadectomy” induced by maintenance in short days).

Conclusions

In the present study we show that male and female Siberian hamsters have a broad range of chemical compounds secreted by the ventral gland, a gland used for territory marking. These hamsters share broad compound composition with other rodents and mammals, including shared similarity with human skin compounds. Further, sex differences in aggression-induced and photoperiod-induced changes in Siberian hamster ventral gland compounds make this an excellent species in which to explore both the functional significance and physiological

regulation of these identified compounds. Collectively, our data illustrate the important role the ventral gland and its compounds play in regulating hamster chemical communication, and likely extend to other seasonally breeding mammals. These findings advance the understanding of chemical ecology and social behavior broadly.

ACKNOWLEDGEMENTS

We thank M.R. Proffitt (NSF REU) for assistance in behavioral filming, scoring videos for inter-rater reliability, collection of samples for volatile analysis, necropsies, and general animal procedures. Thanks to L.R. Wright for vaginal cytology analysis. We also thank members of the Demas laboratory for valuable discussion and for providing keen feedback on analysis on this project. This work was supported by National Science Foundation Grant IOB-0543798 (to G.E.D.), METACyt (to M.V.N.), Indiana University Lilly Chemistry Alumni Chair (to M.V.N.), Indiana University James Holland Fellowship (to N.M.R.), and Indiana University Graduate School Fellowship (to N.M.R.).

FIGURE CAPTIONS

Figure 1. Males display photoperiodic changes in testosterone, aggressive behaviors, and scent marking, whereas females only display photoperiodic changes in aggressive behaviors. (A) Male baseline testosterone; (B) Female baseline testosterone; (C) Male aggression score; (D) Female aggression score; (E) Male scent marking score; (F) Female scent marking score. LD, long-day animals; SD-R, short-day responder animals; SD-NR, short-day non-responder animals. Bar heights represent means $\pm$ S.E.M. * statistically significantly different (males: one-way ANOVA; females: *t*-test, $p < 0.05$).

Figure 2. Sex differences in abundance of ventral gland compounds; males have more elevated levels than females. (A) Long-day males and females; (B) Short-day responder males and females. Bar heights represent means $\pm$ S.E.M. * statistically significantly different; *t*-test, $p < 0.05$.

Figure 3. Photoperiod altered eight ventral gland compounds and reproductive phenotype altered seven ventral gland compounds in males. (A) carboxylic acids (3 altered by photoperiod), heterocyclic/aromatic (1 altered by photoperiod and 1 altered by reproductive phenotype), and a hydrocarbon (altered by photoperiod); (B) Ketones (2 altered by photoperiod and 5 altered by reproductive phenotype); (C) Terpene (altered by photoperiod) and unclassifiable compounds (altered by reproductive phenotype). LD, long-day males; SD-R, short-day responder males; SD-NR, short-day non-responder males. Bar heights represent means $\pm$ S.E.M. Means with different letters are statistically significantly different (one-way ANOVA, $p < 0.05$).

590 **Figure 4.** Aggression-induced changes in nine ventral gland chemical compounds in males. (A)
591 Long-day males (2 compounds changed); (B) Short-day responder males (6 compounds
592 changed); (C) Short-day non-responder males (1 compound changed). Bar heights represent
593 means $\pm$ S.E.M. * statistically significantly different; repeated-measures ANOVA, followed by
594 paired *t*-tests, $p < 0.05$.

596 **Table 1.** Positively identified and tentatively identified volatile compounds in hamster ventral
597 glands
598

Compounds	Retention time (min)
<u>Alcohols</u>	
2-heptadecanol ^{B,M#}	73.74
1,2-hexadecanediol ^{B,M}	81.01
1,2-heptadecanediol ^{B,M}	83.97
<u>Aldehydes</u>	
2-methyl-2-heptenal ^M	19.69
nonanal [#]	24.00
decanal [#]	31.15
2,4-dimethyl-2,4-nonadienal ^M	43.20
<u>Ketones</u>	
4-methyl-2-pentanone ^M	4.15
2-hexanone ^{M#}	4.47
2-heptanone ^{M#}	10.42
7-octen-4-one ^M	15.05
6-methyl-5-hepten-2-one ^M	15.90
6-methyl-3,5-heptadien-2-one ^M	20.51
2-nonanone [#]	24.20
4-methyl-3-nonanone ^M	26.55
4-decen-3-one ^M	32.01
4,8-dimethyl-7-nonen-2-one ^M	32.80
4,8-dimethyl-3,7-nonadien-2-one ^M	33.21
4,8-dimethyl-3,8-nonadien-2-one ^M	35.83
2-undecanone [#]	38.39
4-methyl-3-undecanone	42.16
2-dodecanone ^{F#}	43.62
geranylacetone [#]	48.27
2-tridecanone ^{F*#}	49.86
4-tridecanone	51.23
5-methyl-4-tridecanone	55.06
3-amino-1-phenyl-propan-1-one	55.15
2-tetradecanone ^{F*#}	57.20
4-benzylidene-2-methyl-2-oxazolin-5-one	59.96
2-pentadecanone ^{B#}	61.84
2-hexadecanone ^{B#}	66.77
2-heptadecanone ^{B#}	72.36
2-octadecanone ^{B, F*#}	76.75
2-nonadecanone ^{B,M}	82.18
2-eicosanone ^{B,M}	85.70
2-heneicosanone ^{B,M}	91.70

<u>Carboxylic acids</u>	
3-methylbutanoic acid ^M	9.37
2-methylbutanoic acid ^M	9.76
hexanoic acid ^{M#}	17.71
nonanoic acid ^{M#}	37.45
3,7-dimethyl-6-octenoic acid ^M	39.90
dodecanoic acid ^{F#}	54.44
tetradecanoic acid [#]	65.11
a methyltetradecanoic acid ^M	69.67
pentadecanoic acid ^{M#}	71.52
a methyl pentadecanoic acid ^M	74.64
9-hexadecenoic acid ^{M#}	75.23
hexadecanoic acid ^{B#}	76.58
heptadecanoic acid ^{M#}	81.22
9-octadecenoic acid ^{B, M#}	84.01
octadecanoic acid ^{M#}	85.77
nonadecanoic acid ^{M#}	91.33
18-methyl nonadecanoic acid ^M	92.81
eicosanoic acid ^{B, M}	94.11
<u>Hydrocarbons</u>	
2,6-dimethyl-1,5-heptadiene ^M	10.08
1-dodecene [#]	31.50
dodecane [#]	32.11
tridecane [#]	38.90
squalene ^M	92.48
<u>Amides</u>	
heptanamide	25.96
N-hydroxytetradecanamide ^F	71.04
<u>Terpenes</u>	
menthane	15.96
p-cymene [#]	18.86
o-cymene ^{M#}	19.18
limonene [#]	19.51
<u>Heterocyclic and aromatic</u>	
pyridine ^M	4.47
furfuryl alcohol ^M	8.70
2H-furan-5-one (crotonolactone) ^{M#}	11.58
phenol ^{M#}	16.62
1H-pyrrole-2,5-dione ^M	17.34
acetophenone [#]	21.18
4-aminopyrimidine	23.56
phenylacetonitrile ^{M#}	27.29
1-methyl-2-pyridone ^M	29.17
indole [#]	39.90
<u>Other</u>	
Unidentified compound 1	17.72

Unidentified compound 2	28.09
Unidentified compound 3 (m/z 166) ^{B,M}	33.89
Unidentified compound 4 (m/z 166) ^{B,M}	34.95
Unidentified compound 5 (m/z 182) ^M	39.85
Unidentified compound 6 (m/z 196) ^M	45.60
Unidentified compound 7 (m/z 207) ^M	47.67
Unidentified compound 8 (m/z 154) ^M	72.90

599
600 ^BReported in a previous study for male hamsters (Burger et al., 2001); ^M male specific in this
601 study; ^F female specific in this study; ^{F*} more abundant in females in this study,
602 [≠]positively identified with the standard compound.

Table 2. Principal components loading and eigenvalues for analysis of male ventral gland compounds (pre-aggression). Bold values indicate that all variables loaded strongly within the component (≤ -0.5 or ≥ 0.5).

Male compounds	PC1 _{MALE VG} COMPOUNDS	PC2 _{MALE VG} COMPOUNDS
nonanal	0.14	0.83
decanal	-0.02	0.87
3,7-dimethyl-6-octenoic acid	0.89	-0.06
hexadecanoic acid	0.53	-0.08
9-octadecenoic acid	0.93	-0.05
octadecanoic acid	0.95	-0.06
nonadecanoic acid	0.93	-0.05
eicosanoic acid	0.75	-0.09
1H-pyrrole-2,5-dione	0.74	-0.10
acetophenone	0.33	0.75
indole	0.52	-0.16
squalene	0.69	0.08
6-methyl-5-hepten-2-one	0.65	0.09
4,8-dimethyl-7-nonen-2-one	0.96	-0.01
4,8-dimethyl-3,7-nonadien-2-one	0.95	0.01
4,8-dimethyl-3,8-nonadien-2-one	0.89	-0.05
2-pentadecanone	0.86	-0.08
2-heptadecanone	0.82	-0.07
2-nonadecanone	0.86	-0.05
o-cymene	0.07	0.53
unidentified compound 2	0.91	0.08
unidentified compound 3 (m/z 166)	0.94	0.07
unidentified compound 4 (m/z 166)	0.90	0.07
Eigenvalue	13.40	2.12
% variance explained	58.24	19.23

Table 3. Principal components loading and eigenvalues for analysis of female ventral gland compounds (pre-aggression). Bold values indicate that all variables loaded strongly within the component (≤ -0.5 or ≥ 0.5).

Female compounds	PC1 _{FEMALE} VG COMPOUNDS	PC2 _{FEMALE} VG COMPOUNDS
nonanal	0.77	0.02
decanal	0.46	0.40
N-hydroxytetradecanamide	0.17	0.53
tetradecanoic acid	0.36	0.64
hexadecanoic acid	0.62	-0.11
acetophenone	0.89	-0.03
indole	0.40	0.50
6-methyl-5-hepten-2-one	0.00	0.00
2-pentadecanone	0.53	0.68
2-dodecanone	-0.66	0.59
2-tridecanone	0.51	0.70
2-tetradecanone	-0.69	0.61
2-hexadecanone	-0.59	0.66
2-octadecanone	-0.91	0.16
Eigenvalue	4.94	3.22
% variance explained	35.26	23.03

Table 4. Associations of ventral gland compounds (pre-aggression TIC x 10⁶), and serum T levels (pre-aggression levels). Bold values indicate statistical significance using Spearman's rank correlations within photoperiodic groups and sex, $p < 0.05$.

Group	T and PC1 _{MALE} VG COMPOUNDS		T and PC2 _{MALE} VG COMPOUNDS		T and PC1 _{FEMALE} VG COMPOUNDS		T and PC2 _{FEMALE} VG COMPOUNDS	
	ρ	p	ρ	p	ρ	p	ρ	p
Long-day	0.24	0.31	0.02	0.94	-0.11	0.80	0.24	0.46
Short-day responder	-0.37	0.33	0.23	0.55	0.21	0.61	0.74	0.03
Short-day non-responder	-0.06	0.80	0.11	0.64				

REFERENCES

- Arakawa, H., Blanchard, D.C., Arakawa, K., Dunlap, C., Blanchard, R.J., 2008. Scent marking behavior as an odorant communication in mice. *Neuroscience & Biobehavioral Reviews* 32, 1236-1248.
- Baum, M.J., Bakker, J., 2013. Roles of sex and gonadal steroids in mammalian pheromonal communication. *Frontiers in Neuroendocrinology* 34, 268-284.
- Bedrosian, T.A., Fonken, L.K., Demas, G.E., Nelson, R.J., 2012. Photoperiod-dependent effects of neuronal nitric oxide synthase inhibition on aggression in Siberian hamsters. *Hormones and Behavior* 61, 176-180.
- Bradbury, J.W., Vehrencamp, S.L., 2011. *Principles of Animal Communication*, 2 ed. Sinauer Associates, Inc.
- Burger, B., Smit, D., Spies, H., Schmidt, C., Schmidt, U., Telitsina, A., 2001a. Mammalian exocrine secretions XVI. Constituents of secretion of supplementary sacculi of dwarf hamster, *Phodopus sungorus sungorus*. *Journal of Chemical Ecology* 27, 1277-1288.
- Burger, B., Smit, D., Spies, H., Schmidt, C., Schmidt, U., Telitsina, A., Grierson, G., 2001b. Mammalian exocrine secretions XV. Constituents of secretion of ventral gland of male dwarf hamster, *Phodopus sungorus sungorus*. *Journal of Chemical Ecology* 27, 1259-1276.
- Burger, B.V., 2005. Mammalian semiochemicals, *The Chemistry of Pheromones and Other Semiochemicals II*. Springer, pp. 231-278.
- Chamero, P., Katsoulidou, V., Hendrix, P., Bufe, B., Roberts, R., Matsunami, H., Abramowitz, J., Birnbaumer, L., Zufall, F., Leinders-Zufall, T., 2011. G protein Gao is essential for vomeronasal function and aggressive behavior in mice. *Proceedings of the National Academy of Sciences* 108, 12898-12903.
- Chamero, P., Marton, T.F., Logan, D.W., Flanagan, K., Cruz, J.R., Saghatelian, A., Cravatt, B.F., Stowers, L., 2007. Identification of protein pheromones that promote aggressive behaviour. *Nature* 450, 899-902.
- Del Punta, K., Leinders-Zufall, T., Rodriguez, I., Jukam, D., Wysocki, C.J., Ogawa, S., Zufall, F., Mombaerts, P., 2002. Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. *Nature* 419, 70-74.
- Drea, C.M., 2015. D'scent of man: a comparative survey of primate chemosignaling in relation to sex. *Hormones and Behavior* 68, 117-133.
- Feoktistova, N.Y., 1994. Sex discrimination by different odors in the dwarf hamsters: *Phodopus roborovskii* and *Phodopus sungorus*. *Advances in the Biosciences* 93, 333-338.

652 Gorman, M.R., Zucker, I., 1995. Testicular regression and recrudescence without subsequent
653 photorefractoriness in Siberian hamsters. *American Journal of Physiology-Regulatory Integrative*
654 *and Comparative Physiology* 38, R800-R806.

655 Hurst, J.L., Payne, C.E., Nevison, C.M., Marie, A.D., Humphries, R.E., Robertson, D.H.,
656 Cavaggioni, A., Beynon, R.J., 2001. Individual recognition in mice mediated by major urinary
657 proteins. *Nature* 414, 631-634.

658 Jasnow, A.M., Huhman, K.L., Bartness, T.J., Demas, G.E., 2000. Short-day increases in
659 aggression are inversely related to circulating testosterone concentrations in male Siberian
660 hamsters (*Phodopus sungorus*). *Hormones and Behavior* 38, 102-110.

661 Jemiolo, B., Xie, T.-M., Novotny, M., 1991. Socio-sexual olfactory preference in female mice:
662 attractiveness of synthetic chemosignals. *Physiology & Behavior* 50, 1119-1122.

663 Johnston, R.E., 2003. Chemical communication in rodents: from pheromones to individual
664 recognition. *Journal of Mammalogy* 84, 1141-1162.

665 Kanehisa, M., Goto, S., 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids*
666 *Research* 28, 27-30.

667 Keesom, S.M., Rendon, N.M., Demas, G.E., Hurley, L.M., 2015. Vocal behaviour during
668 aggressive encounters between Siberian hamsters, *Phodopus sungorus*. *Animal Behaviour*, 85-
669 93.

670 Liberles, S.D., 2014. Mammalian pheromones. *Annual Review of Physiology* 76, 151-175.

671 Lynch, G.R., Lynch, C.B., Kliman, R.M., 1989. Genetic analyses of photoresponsiveness in the
672 Djungarian hamster, *Phodopus sungorus*. *Journal of Comparative Physiology A* 164, 475-481.

673 Novotny, M., Harvey, S., Jemiolo, B., 1990. Chemistry of male dominance in the house mouse,
674 *Mus domesticus*. *Experientia* 46, 109-113.

675 Novotny, M., Harvey, S., Jemiolo, B., Alberts, J., 1985. Synthetic pheromones that promote
676 inter-male aggression in mice. *Proceedings of the National Academy of Sciences* 82, 2059-2061.

677 Penn, D.J., Oberzaucher, E., Grammer, K., Fischer, G., Soini, H.A., Wiesler, D., Novotny, M.V.,
678 Dixon, S.J., Xu, Y., Brereton, R.G., 2007. Individual and gender fingerprints in human body
679 odour. *Journal of the Royal Society Interface* 4, 331-340.

680 Petrulis, A., 2013. Chemosignals and hormones in the neural control of mammalian sexual
681 behavior. *Frontiers in Neuroendocrinology* 34, 255-267.

682 Rendon, N.M., Keesom, S.M., Amadi, C., Hurley, L.M., Demas, G.E., 2015a. Vocalizations
683 convey sex, seasonal phenotype, and aggression in a seasonal mammal. *Physiology & Behavior*
684 152, 143-150.

685 Rendon, N.M., Rudolph, L.M., Sengelaub, D.R., Demas, G.E., 2015b. The agonistic adrenal:
686 melatonin elicits female aggression via regulation of adrenal androgens. *Proceedings of the*
687 *Royal Society B: Biological Sciences* 282, 20152080.

688 Ross, P.D., 1998. *Phodopus sungorus*. *Mammalian species*, 1-9.

689 Saban, N., Bujak, M., 2009. Hydroxyurea and hydroxamic acid derivatives as antitumor drugs.
690 *Cancer Chemotherapy and Pharmacology* 64, 213-221.

691 Scherbarth, F., Steinlechner, S., 2010. Endocrine mechanisms of seasonal adaptation in small
692 mammals: from early results to present understanding. *Journal of Comparative Physiology B*
693 180, 935-952.

694 Scotti, M.-A.L., Place, N.J., Demas, G.E., 2007. Short-day increases in aggression are
695 independent of circulating gonadal steroids in female Siberian hamsters (*Phodopus sungorus*).
696 *Hormones and Behavior* 52, 183-190.

697 Soini, H.A., Bruce, K.E., Klouckova, I., Brereton, R.G., Penn, D.J., Novotny, M.V., 2006. *In situ*
698 surface sampling of biological objects and preconcentration of their volatiles for
699 chromatographic analysis. *Analytical Chemistry* 78, 7161-7168.

700 Soini, H.A., Wiesler, D., Apfelbach, R., König, P., Vasilieva, N.Y., Novotny, M.V., 2005.
701 Comparative investigation of the volatile urinary profiles in different *Phodopus* hamster species.
702 *Journal of Chemical Ecology* 31, 1125-1143.

703 Sokolov, V., Feoktistova, N., 1997. Response of adult djungarian hamsters (*Phodopus sungorus*)
704 to odor signals of species of their own and opposite sex during different seasons with the
705 reference to reproduction process. *Izvestiia Akademii nauk. Serii biologicheskaiia/Rossiiskaia*
706 *akademii nauk*, 369-373.

707 Soma, K.K., Rendon, N.M., Boonstra, R., Albers, H.E., Demas, G.E., 2015. DHEA effects on
708 brain and behavior: Insights from comparative studies of aggression. *The Journal of Steroid*
709 *Biochemistry and Molecular Biology* 145, 261-272.

710 Stowers, L., Holy, T.E., Meister, M., Dulac, C., Koentges, G., 2002. Loss of sex discrimination
711 and male-male aggression in mice deficient for TRP2. *Science* 295, 1493-1500.

712 Tirindelli, R., Dibattista, M., Pifferi, S., Menini, A., 2009. From pheromones to behavior.
713 *Physiological Reviews* 89, 921-956.

714 Uhrig, E.J., LeMaster, M.P., Mason, R.T., 2014. Species specificity of methyl ketone profiles in
715 the skin lipids of female garter snakes, genus *Thamnophis*. *Biochemical Systematics and*
716 *Ecology* 53, 51-58.

717 Wynne-Edwards, K.E., 2003. From dwarf hamster to daddy: the intersection of ecology,
718 evolution, and physiology that produces paternal behavior. *Advances in the Study of Behavior*
719 32, 207-262.

- 720 Wynne-Edwards, K.E., Lisk, R.D., 1987. Behavioral interactions differentiate Djungarian
721 (*Phodopus campbelli*) and Siberian (*Phodopus sungorus*) hamsters. Canadian Journal of Zoology
722 65, 2229-2235.
- 723 Wynne-Edwards, K.E., Surov, A.V., Telitzina, A.Y., 1992. Field studies of chemical signalling:
724 direct observations of dwarf hamsters (*Phodopus*) in Soviet Asia, Chemical Signals in
725 Vertebrates 6. Springer, pp. 485-491.
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