1 Urinary volatile compounds differed across reproductive phenotypes and were
2 altered following an aggression trial in male Siberian hamsters
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ABSTRACT

Chemical communication plays an integral role in social behavior by facilitating social encounters, allowing for the evaluation of social partners, defining territories and advertising information such as species and sex. Odors provide information about the social environment for rodents and other mammals; however, studies identifying chemical compounds and their functions have thus far focused primarily on a few species. In addition, considerably less attention has been focused on how environmental factors and behavioral context alter these compounds during periods of reproductive quiescence. We examined the effects of photoperiod and social context on chemical communication in the seasonally breeding Siberian hamster which displays modest territorial aggression during long “summer-like” days, but *increased* aggression in short “winter-like” days. We collected urine samples from long- and short-day male hamsters to investigate how photoperiod and subsequent changes in reproductive phenotype alter urinary volatile compound profiles. Next, we identified changes in urinary compounds before and after an aggressive encounter. Male hamsters exhibited a diverse urinary profile across photoperiods; however, long-day reproductive males showed higher levels of individual compounds when compared to short-day non-reproductive males. In addition, individual compounds were altered following to an aggressive encounter; some changed only in long days whereas others changed regardless of photoperiod. Further, aggression and circulating levels of testosterone were positively correlated with urinary compounds in long-, but not short-day males. These findings suggest both photoperiod- and aggression-specific physiological regulation of urinary compounds in this species and contribute to a greater understanding of chemical communication more broadly.

KEY WORDS: olfactory communication, scent marking, mammalian semiochemicals, gas chromatography-mass spectrometry, stir bar sorptive extraction, androgens

1. INTRODUCTION

Chemical communication is a key aspect of social behavior in virtually all animals. These communicative signals are used to facilitate social encounters, evaluate social partners, establish territories and advertise information such as identity and sex [1, 2]. Various types of communication, including visual, auditory, tactile, or chemical signals, can be transmitted between sender and receiver. Such modes of communication have been studied across many vertebrate taxa, however, rodents offer an excellent comparative model to study mammalian chemical communication [2, 3]. Rodents are differentiated from other vertebrate taxa because sense of smell is the most important sensory modality; therefore, olfaction provides a sensory map of the environment [4]. In fact, there are functional consequences for rodents with compromised olfaction. For example, mice with disrupted olfaction display abnormal aggressive behaviors [5-7] and abnormal sexual behaviors [7]. Further, distinct chemical compounds that induce aggression [8], dominance [9] and reproductive partner preference [10] have been examined in mice. Identifying functional behavioral consequences and attributing individual compounds to such behaviors is key to a comprehensive understanding of mammalian chemical communication.

The importance of olfaction is clear from evidence in olfactory deficient mice and behavioral responsiveness to individual chemical compounds [5-10]; however, these findings have not been extended to mammalian chemical communication more broadly. Instead, work in mammals has increasingly focused on a relatively limited number of species, particularly mice (but see [11-15] for some notable exceptions). In addition, much of the work on rodents and the chemical complexity expressed has focused on animals that are reproductively functional or behaviors expressed within a reproductive context. For example, the role of the major

histocompatibility complex (MHC) in the production of chemical compounds, and the functions of these compounds, has predominantly been examined within mate choice paradigms [2-4]. Further, the use of kin recognition and the functions of scent marking have been examined within a reproductive context [2-4, 16, 17]. Despite these advances in rodent chemical communication much less is known about how changes in reproductive phenotype, and subsequent changes in endocrine status, within a non-reproductive social context influence individual chemical compounds. Using ecologically relevant model systems to study how the environment, including both social and non-social factors and endocrine status, influence chemical signaling strongly expands upon the important findings that have emerged from the mouse model system.

Siberian hamsters (*Phodopus sungorus*) breed seasonally and are well established for the study of photoperiodic changes in physiology and behavior [18, 19], making this species an excellent model to examine how season and behavioral context alter individual urinary compounds. First, hamsters in short “winter-like” days exhibit gonadal regression and display *increased* territorial aggression when compared with hamsters in long “summer-like” days [18, 20]. This inverse relationship between gonadal steroids and aggression makes this species amenable to disassociating the individual effects of reproductive phenotype and aggression on chemical signaling. Second, Siberian hamsters employ chemical signaling in a variety of ways, including the use of urine, feces, sacculi and ventral glands in the wild and in the laboratory [21-24].

In the wild, males deposit their scent marks, composed of both urinary and ventral gland secretions, on the boundaries and throughout their home ranges [24]. The location of these chemical signals suggests that males use urinary and ventral gland signals for territorial defense [24]. In further support of this, sexually experienced males scent mark over conspecific male

urine in a laboratory setting, therefore urine seems to be a signal involved in scent marking duels [21]. In addition to urinary signaling, male and female hamsters actively excrete ventral gland compounds across breeding seasons [25]; for males, they are differentially altered by photoperiod and aggression [25]. Males in short days generally display elevated levels of ventral gland compounds and further show aggression-induced elevations [25]. Moreover, male hamsters presented with ventral gland secretions display increased investigation of the secretion in short days when compared to long days [26]. These data suggest that ventral gland signaling is used more during periods of reproductive quiescence [25], whereas urinary signaling may be used more so during periods of reproductive activity [21]. During the breeding season, hamsters survive with little rainfall therefore produce highly concentrated urine [27, 28]. This corroborates high concentrations of urinary compounds that have previously been reported in laboratory derived male hamsters; females have low levels when compared to males [29]. These data suggest that concentrated urine, an adaptation for surviving in harsh Siberian summers, may be a chemical signal used more during the long days of the reproductive breeding season than in the short days of the non-breeding season [21, 25]. By identifying specific urinary compounds that change with aggression and reproductive phenotype (i.e., breeding condition and levels of the gonadal hormone testosterone), we can begin to connect the complexity of odors in Siberian hamsters to their functional significance.

In this study, we examined photoperiodic changes in the urinary compound composition of male Siberian hamsters, as well as aggression-induced changes of individual urinary compounds. We hypothesized that photoperiod and aggression would induce changes in individual urinary compounds. We also investigated the potential role for photoperiodic variation in the gonadal androgen testosterone (T) to regulate urinary compound composition. It is known

that short-day males with regressed gonads display increased aggression despite low levels of T, whereas males in long days display less aggression concomitant with elevated T levels [18]. Therefore, we predicted that specific urinary compounds would be differentially associated with T concentrations across photoperiods. To test this, we collected urine and identified and quantified urinary volatile organic compounds (VOCs) using gas chromatography-mass spectrometry (GC-MS) [29]. Urine samples were collected from individual hamsters across photoperiods and prior to and following an aggressive encounter. The findings from this study allowed us to identify photoperiod- and behavior-specific changes in urinary compounds and further provide an understanding of the chemical ecology of mammalian chemical communication within an aggressive context.

2. MATERIALS AND METHODS

2.1 Animal housing and photoperiodic treatment

Adult (>60 days of age) hamsters were reared in a breeding colony maintained at Indiana University, Bloomington. Hamsters were bred and subsequently housed under long days (light:dark, 16:8h) and group-housed at weaning (postnatal day 18). Ambient temperature was maintained at 20 ± 2 °C and relative humidity at $55 \pm 5\%$. Hamsters were given *ad libitum* access to laboratory rodent chow (Lab Diet 5001, PMI Nutrition) and water. 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.

Resident hamsters were individually housed ($n = 32$) and intruder hamsters were group housed (4 animals per cage; males, $n = 16$) in the colony room for a one-week acclimation period. Subsequently, a subset of hamsters was transferred to a room on a short-day light cycle

(light:dark: 8:16h), whereas the remaining hamsters were relocated to a new room on the long-day light cycle (light:dark, 16:8h). All hamsters remained in their photoperiodic regime for eight weeks. Reproductive state was determined based on *a priori* criteria previously established for Siberian hamsters [18]. Hamsters were characterized as reproductively competent if they had functional reproductive testes, displayed no significant change in body mass (<10%) and maintained a brown/grey coat color (long-days; $n = 12$). Reproductive incompetence, in contrast, was characterized by regressed testes, a significant decrease in body mass (>10%) and a white coat color (short-days; $n = 16$). A subset of animals did not respond, with respect to reproductive physiology, to the short-day photoperiodic regime based on our *a priori* criteria. This reproductive morph that persists in short days has been previously documented [30, 31]. These animals (short-day non-responders: $n = 4$) were excluded from further analyses due to insufficient numbers.

2.2 Aggressive behavior recording and analysis

Same-sex aggressive encounters were recorded and analyzed using previously outlined methods [18, 20, 32]. Aggression was assessed using the resident-intruder paradigm, which consists of placing an unfamiliar intruder hamster into the home cage of a resident hamster 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). Scores from two individuals were averaged and inter-rater reliability was accepted if less than 10% variation occurred. A principal components analysis (PCA) was used on these aggression variables to

generate a composite score for each hamster. Aggression variables loaded strongly onto the first component and explained 76.16% of the total variance (*Table SI*); therefore we used PC1 as an aggression score for subsequent analyses.

2.3 Identification and quantification of urinary volatile compounds

In order to identify and quantify hamster urinary compounds, male urine was collected one hour before behavioral trials (week eight, pre-aggression samples) and immediately following (< 5 min; week eight, post-aggression samples) behavioral trials. The changes in urinary volatiles from pre- to post-aggression were likely not due to *de novo* synthesis/metabolism, but rather were volatiles stored in the bladder and differentially excreted in the absence (pre-aggression) or presence (post-aggression) of a social encounter (see examples of compounds added to urine upon elimination: [8, 9, 33]; reviewed in: [34, 35]). An additional subset of animals were sampled prior to photoperiodic treatment (week 0, baseline; $n = 14$) and again following photoperiodic treatment (week 8, pre-aggression; $n = 14$), in order to quantify reproducibility of urinary compounds within individuals and to gauge changes in urinary compounds that occur in the absence of a social encounter. Individual male hamsters were placed in a clean cage and urine was collected from the bottom of the cage as soon as the animal urinated. All urine samples were collected during the dark phase to control for circadian fluctuations. Samples were frozen at -20 °C until analyzed and were thawed just prior to the sample preparation. If the sample was contaminated (e.g., feces), the sample did not undergo chemical analysis.

Urinary VOCs were identified and quantified using gas chromatography-mass spectrometry (GC-MS) using previously established protocols [29]. Volatile compounds were extracted from 0.5 mL of undiluted urine using the stir bar sorptive extraction method

(Twister™, 10mm, 0.5 mm film thickness, 24 µL polydimethylsiloxane (PDMS) volume) for 60 min. Stir bars were obtained from Gerstel GmbH (Mülheim an der Ruhr, Germany) and were conditioned in the TC-2 tube conditioner (Gerstel GmbH) at 290 °C under high helium flow. 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 glass scintillation 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). Prior to extraction, all glassware was washed with acetone and dried in the oven at 80 °C. After extraction, the stir bars were placed into the TDSA desorption tubes in the autosampler.

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 (Gerstel TDSA-CIS-4). 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), followed by injection into the column with a cooled injection assembly (CIS-4). The TDSA operated in a splitless mode. The temperature program for desorption was 20 °C (0.5 min hold time), then 60 °C/min to 280 °C (10 min hold time). Temperature of the transfer line was set at 280 °C. CIS was cooled with liquid nitrogen to -80 °C. After desorption and cryotrapping, CIS was heated at 12 °C/s to 280 °C (10 min hold time).

CIS inlet was operated in the solvent vent mode, a vent pressure of 9 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 psi (flow rate, 1.1 mL/min at the constant flow mode).

2.4 Quantification of urinary volatile compounds

Peak areas of the identified compounds were used for quantitative comparisons between long days and short days. 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 that of the area of the internal standard in the same analytical run. Peak areas with a mean relative proportion of less than 0.1% were identified, but were not included in further statistical analyses.

2.5 Blood sampling and quantification of T

After eight weeks of the photoperiodic regime, 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 was 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 [32]. Samples were diluted (1:20 or 1:40), assayed in duplicate according to the manufacturer's recommended protocol and were balanced across two plates of the same kit lot. Samples with C.V. greater than 10% and maximum binding less than 20% or greater than 80% were re-analyzed. Intra-assay variability was less than 4.5% and inter-assay variability was 2.98%.

2.6 Statistical analyses

All statistical analyses were run in JMP v. 11.0.0 (SAS Institute, Inc., Cary, NC) and statistical significance was attributed at $p < 0.05$. We transformed each variable to attain normality and equal variances. Principal component analyses (PCAs) were conducted on aggression data (*Table S1*) as well as baseline, pre-and post aggression levels of urinary compounds (*Table 2 and Table S2*). PCAs were used to reduce the dimensionality of the data by loading inter-correlated variables into independent components as well as to determine how urinary compounds group together; variables that loaded strongly (≤ -0.5 or ≥ 0.5) within a component confirm statistical relationships between those compounds. PCs with an eigenvalue greater than 1 were retained for analysis. Paired t -tests were used to compare baseline (week 0) and pre-aggression (week 8) levels of urinary compounds within groups to determine reproducibility of compounds. In addition, repeatability of urinary compounds of each individual was calculated as described previously [36]. T -tests were used to compare reproductive physiology, aggression measures, pre-aggression levels of urinary compounds and T between long- and short-day animals. Repeated-measures ANOVAs were used to compare pre- and post-aggression urinary compounds and T levels, followed by paired t -tests if there was a significant photoperiod x aggression interaction. Spearman's rank correlations were used to quantitatively assess relationships between aggression, T levels (both pre- and post-aggression values) and PCs generated for pre-and post-aggression urinary compounds.

3. RESULTS

3.1 Short-day hamsters had non-functional reproductive physiology but were more aggressive than long-day hamsters

Short-day males had significantly decreased body mass (16%, $t_{26} = -6.43$, $p < 0.001$), regressed testes mass ($t_{26} = 20.09$, $p < 0.001$; *Figure 1B*) and decreased circulating levels of T ($t_{26} = 4.34$, $p = 0.001$; *Figure 1C*), when compared to long-day males. Short-day males also displayed more ($t_{26} = 2.19$, $p = 0.03$; *Figure 1A*) and longer ($t_{26} = 3.89$, $p = 0.03$) attacks, more ($t_{26} = 3.11$, $p = 0.04$) and longer ($t_{26} = 3.39$, $p = 0.04$) chases and displayed more overall aggression (aggression score: $t_{26} = 3.80$, $p = 0.04$; *Table S1*). The latency to first attack did not differ across photoperiods ($t_{26} = -1.08$, $p = 0.15$).

268 *Figure 1. Photoperiodic changes in aggression and reproductive phenotype. (A) number of*
269 *attacks; (B) paired testes mass; (C) baseline testosterone. Bar heights represent means $\pm$ S.E.M. **
270 *statistically significantly different (t -test, $p < 0.05$).*

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3.2 Siberian hamsters displayed a diverse and repeatable composition of urinary compounds

Through analyzing individual GC-MS generated total ion chromatograms (TIC) (*Figure S1*) we identified 39 urinary VOCs from several compound classes: alkenols, aldehydes, amides, amines, esters, heterocyclic and aromatic compounds, ketones and terpenes (*Table 1*). Among the 39 identified compounds, we quantitatively compared 24, which were compounds that had a relative proportion greater than 0.1%.

Urinary VOCs from baseline (week 0) to pre-aggression (week 8) did not statistically differ across time, therefore, were highly reproducible (PC1: $t_{13} = -2.26$, $p = 0.77$; PC2: $t_{13} = 1.44$, $p = 0.72$; *Table S2*) and repeatable (PC1: $F = 1.58$, $r = 0.69$, $p = 0.007$; PC2: $F = 2.10$, $r = 0.52$, $p = 0.02$; *Table S2*). Further, when examining each specific VOC, all 24 were highly reproducible within individuals when maintained in long days (p 's > 0.05 ; *Supplementary Material – Results*). These results indicate that urinary VOCs are reliable within individuals and consistently different among individuals.

Table 1. List of identified urinary compounds from male hamsters.

¹ urinary compounds also identified in a previous study (Soini et al., 2005)

Compounds	Retention time (min)
<u>Alkenols</u>	
trans-2-octen-1-ol	22.64
<u>Aldehydes</u>	
phenylacetaldehyde	19.86
nonanal	24.50
decanal ¹	31.68
<u>Amides</u>	
N-phenylformamide	31.98
hexadecanoamide	83.99
9-octadecanoamide	93.99
<u>Aromatic amines</u>	
o-toluidine	21.63

<u>Acids</u>	
benzoic acid	30.07
hexadecanoic acid	76.42
<u>Esters</u>	
ethyl benzoate	29.02
ethyl salicylate	36.36
propyl benzoate	36.60
<u>Heterocyclic and aromatic</u>	
2,6-dimethylpyridine ¹	9.27
2,5-dimethylpyrazine ¹	11.15
2-acetyl-1-pyrroline	12.29
3-hepten-2-one ¹	12.98
a trimethylpyrazine ¹	16.92
a propylpyrazine ¹	17.37
2-methylvinylpyrazine	18.10
acetophenone ¹	21.30
2-methyl-5-propylpyrazine	23.50
an ethylvinylpyrazine	23.62
a C3-vinylpyrazine	29.91
a C4-vinylpyrazine	35.16
a dipropylpyrazine	35.97
indole ¹	37.20
<u>Ketones and lactones</u>	
4-heptanone ¹	8.97
2-heptanone ¹	9.96
6-methyl-5-hepten-2-one ¹	15.95
3-octen-2-one	20.38
4-nonanone ¹	22.09
γ -6-dodecalactone	60.08
<u>Terpenes</u>	
limonene	18.88
p-cymene	18.90
o-cymene	19.24
m-cymene	19.90
perillene	24.66
geranylacetone	47.40

3.3 Reproductive phenotype altered urinary compound levels

Male urinary compound compositions did not change across photoperiods; all urinary compounds were present in all hamsters. However, long-day males generally had elevated levels of each compound. Ten urinary compounds were elevated in long days when compared to short days, including five pyrazines and two ketones: N-phenylformamide ($t_{26} = -2.67$, $p = 0.001$); 2-acetyl-1-pyrroline ($t_{26} = -1.96$, $p = 0.04$); a propylpyrazine ($t_{26} = -1.82$, $p = 0.04$); 2-methylvinylpyrazine ($t_{26} = -2.55$, $p = 0.002$); 2-methyl-5-propylpyrazine ($t_{26} = -2.69$, $p = 0.02$); an ethylvinylpyrazine ($t_{26} = -2.86$, $p = 0.03$); a C4-vinylpyrazine ($t_{26} = -2.42$, $p = 0.04$); γ -6-dodecalactone ($t_{26} = -2.11$, $p = 0.02$); 4-heptanone ($t_{26} = -3.88$, $p = 0.04$); and 2-heptanone ($t_{26} = -1.82$, $p = 0.03$). In contrast, only one trimethylpyrazine compound was elevated in short days when compared to long days: a trimethylpyrazine ($t_{26} = 1.52$, $p = 0.03$) (Table S3).

Thirteen urinary compound levels did not change with photoperiod, including two 2,5-dimethylpyrazines: phenylacetaldehyde ($t_{26} = -1.60$, $p = 0.56$); nonanal ($t_{26} = 1.06$, $p = 0.47$); decanal ($t_{26} = -1.59$, $p = 0.56$); o-toluidine ($t_{26} = -0.99$, $p = 0.34$); hexadecanoic acid ($t_{26} = -1.51$, $p = 0.62$); ethyl benzoate ($t_{26} = 1.91$, $p = 0.18$); 2,5-dimethylpyrazine ($t_{26} = 0.09$, $p = 0.50$); acetophenone ($t_{26} = -1.60$, $p = 0.56$); a C3-vinylpyrazine ($t_{26} = 1.98$, $p = 0.34$); indole ($t_{26} = 1.76$, $p = 0.23$); 6-methyl-5-hepten-2-one ($t_{26} = 0.23$, $p = 0.82$); 4-nonanone ($t_{26} = -0.68$, $p = 0.51$); and limonene ($t_{26} = -0.43$, $p = 0.68$) (Table S3).

3.4 Aggression altered urinary compound levels

Nine urinary compounds increased following an aggressive encounter in long-day males, but not short-day males: N-phenylformamide ($F_{1,26} = 6.74$, $p = 0.02$); 2-acetyl-1-pyrroline ($F_{1,26} = 5.13$, $p = 0.03$); a trimethylpyrazine ($F_{1,26} = 3.47$, $p = 0.03$); a propylpyrazine ($F_{1,26} = 12.08$, $p = 0.02$); 2-methylvinylpyrazine ($F_{1,26} = 12.21$, $p = 0.002$); 2-methyl-5-propylpyrazine ($F_{1,26} =$

315 2.77, $p = 0.04$); a C3-vinylpyrazine ($F_{1,26} = 3.67$, $p = 0.02$); γ -6-dodecalactone ($F_{1,26} = 3.61$, $p =$
316 0.04); and 6-methyl-5-hepten-2-one ($F_{1,26} = 2.22$, $p = 0.04$) (*Figure 2*). An additional six urinary
317 compounds increased following an aggressive encounter regardless of photoperiod, including
318 one aldehyde, two pyrazines and three ketones: nonanal ($F_{1,26} = 2.57$, $p = 0.02$); 2,5-
319 dimethylpyrazine ($F_{1,26} = 3.21$, $p = 0.04$); an ethylvinylpyrazine ($F_{1,26} = 6.12$, $p = 0.02$); 4-
320 heptanone ($F_{1,26} = 2.73$, $p = 0.01$); 2-heptanone ($F_{1,26} = 2.05$, $p = 0.02$); and 4-nonanone ($F_{1,26} =$
321 3.50, $p = 0.04$) (*Figure 3*).

322 Nine urinary compounds did not change following an aggressive encounter:
323 phenylacetaldehyde ($F_{1,26} = 0.51$, $p = 0.48$); decanal ($F_{1,26} = 0.33$, $p = 0.57$); o-toluidine ($F_{1,26} =$
324 0.76, $p = 0.39$); hexadecanoic acid ($F_{1,26} = 0.02$, $p = 0.89$); ethyl benzoate ($F_{1,26} = 0.42$, $p =$
325 0.53); acetophenone ($F_{1,26} = 2.02$, $p = 0.17$); a C4-vinylpyrazine ($F_{1,26} = 1.46$, $p = 0.24$); indole
326 ($F_{1,26} = 2.02$, $p = 0.17$); and limonene ($F_{1,26} = 1.68$, $p = 0.21$) (*Figure 4*).

Figure 2. Urinary compounds altered after an aggressive encounter in long-day males. Levels of pre-aggression and post-aggression urinary compounds for (A) long days; and (B) short days. * statistically significantly different (repeated-measures ANOVAs followed by paired *t*-tests, $p < 0.05$).

Figure 3. Urinary compounds altered after an aggressive encounter in males across photoperiods. Levels of pre-aggression and post-aggression urinary compounds for (A) long days; and (B) short days. * statistically significantly different (repeated-measures ANOVAs followed by paired *t*-tests, $p < 0.05$).

Figure 4. Urinary compounds not altered after an aggressive encounter in males. Levels of pre-aggression and post-aggression urinary compounds for (A) long days; and (B) short days. * statistically significantly different (repeated-measures ANOVAs followed by paired *t*-tests, $p < 0.05$).

3.5 Urinary compounds were associated with aggression and T

Two PCAs were conducted on pre-aggression and post-aggression urinary compounds to determine how urinary compounds group together; variables that loaded strongly (≤ -0.5 or ≥ 0.5) within a component confirmed statistical relationships between those compounds (*Table 2*). Two pre-aggression urinary compound PCs were extracted (PC1_{PRE-AGG COMPOUNDS} and PC2_{PRE-AGG COMPOUNDS}) which explained 49.45% of the total variance (*Table 2*). For post-aggression urinary compounds, two PCs were extracted (PC1_{POST-AGG COMPOUNDS} and PC2_{POST-AGG COMPOUNDS}) which explained 61.93% of the total variance (*Table 2*). Aggression scores were correlated with PC1_{POST-AGG COMPOUNDS} in long-day males, but were not correlated in short-day males (*Table 3*). Pre-aggression T was correlated with PC1_{PRE-AGG COMPOUNDS} and PC1_{POST-AGG COMPOUNDS} in long-day males, but was not correlated in short-day males (*Table 4*). These correlations between aggression, T and PCs for urinary compounds provide unbiased evidence that individual compounds that loaded strongly onto those components are altered following an aggression trial and may be regulated by T. There were no other significant relationships between aggression, pre- and post-aggression T and urinary compound PCs (*Tables 3 and 4*).

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

Compounds	PC1 _{PRE-AGG} COMPOUNDS	PC2 _{PRE-AGG} COMPOUNDS	PC1 _{POST-AGG} COMPOUNDS	PC2 _{POST-AGG} COMPOUNDS
phenylacetaldehyde	0.33	-0.11	0.45	-0.25
nonanal	0.78	-0.34	0.73	0.54
decanal	0.53	-0.10	0.56	-0.44
N-phenylformamide	0.53	0.61	0.72	-0.28
o-toluidine	0.72	0.26	0.58	-0.45
hexadecanoic acid	0.04	-0.16	0.02	0.31
ethyl benzoate	0.73	-0.33	0.42	0.72
2,5-dimethylpyrazine	0.80	-0.06	0.77	-0.29
2-acetyl-1-pyrroline	0.26	0.57	0.65	-0.29
trimethylpyrazine	0.76	-0.26	0.30	0.16
propylpyrazine	0.46	0.76	0.81	-0.45
2-methylvinylpyrazine	0.69	0.44	0.83	-0.25
acetophenone	0.68	-0.14	0.66	0.70
2-methyl-5-propylpyrazine	0.80	0.46	0.79	-0.31
an ethylvinylpyrazine	0.71	0.42	0.67	-0.36
a C3-vinylpyrazine	-0.05	-0.17	0.79	-0.17
a C4-vinylpyrazine	0.19	0.51	0.70	-0.37
indole	0.65	-0.58	0.60	0.75
γ -6-dodecalactone	-0.03	-0.10	0.33	0.66
4-heptanone	0.22	-0.07	0.80	0.42
2-heptanone	0.60	0.29	0.87	0.07
6-methyl-5-hepten-2-one	0.82	-0.45	0.61	-0.30
4-nonanone	0.78	-0.35	0.78	0.38
limonene	0.56	-0.47	0.71	0.43
Eigenvalue	8.36	3.55	10.49	4.37
% variance explained	34.65	14.80	43.73	18.20

Table 3. Spearman's rank correlations between aggression scores, PC1_{PRE-AGG COMPOUNDS}, PC2_{PRE-AGG COMPOUNDS}, PC1_{POST-AGG COMPOUNDS} and PC2_{POST-AGG COMPOUNDS} in male hamsters. Significant *p*-values are shown in bold.

		Aggression and PC1 _{PRE-AGG COMPOUNDS}		Aggression and PC2 _{PRE-AGG COMPOUNDS}		Aggression and PC1 _{POST-AGG COMPOUNDS}		Aggression and PC2 _{POST-AGG COMPOUNDS}	
Group	<i>n</i>	ρ	<i>p</i>	ρ	<i>p</i>	ρ	<i>p</i>	ρ	<i>p</i>
Long days	12	0.28	0.09	0.13	0.68	0.29	0.02	0.06	0.86
Short days	16	-0.22	0.41	-0.19	0.47	0.20	0.45	0.33	0.21

Table 4. Spearman's rank correlations between pre- and post-aggression T, PC1_{PRE-AGG COMPOUNDS}, PC2_{PRE-AGG COMPOUNDS}, PC1_{POST-AGG COMPOUNDS} and PC2_{POST-AGG COMPOUNDS} in male hamsters. Significant *p*-values are shown in bold.

		Pre-aggression T and PC1 _{PRE-AGG COMPOUNDS}		Post-aggression T and PC1 _{PRE-AGG COMPOUNDS}		Pre-aggression T and PC2 _{PRE-AGG COMPOUNDS}		Post-aggression T and PC2 _{PRE-AGG COMPOUNDS}	
Group	<i>n</i>	ρ	<i>p</i>	ρ	<i>p</i>	ρ	<i>p</i>	ρ	<i>p</i>
Long days	12	-0.59	0.03	-0.04	0.91	0.31	0.32	0.24	0.46
Short days	16	0.23	0.39	0.16	0.56	-0.25	0.35	-0.36	0.17
		Pre-aggression T and PC1 _{POST-AGG COMPOUNDS}		Post-aggression T and PC1 _{POST-AGG COMPOUNDS}		Pre-aggression T and PC2 _{POST-AGG COMPOUNDS}		Post-aggression T and PC2 _{POST-AGG COMPOUNDS}	
Group	<i>n</i>	ρ	<i>p</i>	ρ	<i>p</i>	ρ	<i>p</i>	ρ	<i>p</i>
Long days	12	-0.57	0.04	-0.38	0.22	0.10	0.75	-0.02	0.95
Short days	16	-0.39	0.14	-0.25	0.34	0.12	0.66	0.31	0.24

4. DISCUSSION

In the present study, we identified individual hamster urinary compounds using stir bar sorptive extractions (SBSE), a highly efficient sampling method suitable for trace level analysis of VOCs in a complex biological matrix. Using this method, we measured compounds in individual males, as well as determined the effects of reproductive phenotype and aggression on levels of these compounds. Siberian hamster urine included a broad range of chemical

compounds, both in terms of the number of the chemical classes as well as structural diversity within each class. Moreover, both photoperiod and aggression affected urinary compounds. Long-day males generally had elevated levels and also displayed more photoperiodic- and aggression-induced changes in specific compounds, when compared to short-day males. There were also urinary compounds that increased after an aggressive encounter irrespective of photoperiod. Further, aggression and serum T were correlated with urinary compounds in long but not short days. These findings suggest photoperiod- and aggression-specific physiological regulation of urinary VOC excretion in this species.

4.1 Hamster urinary compounds have a distinct chemical signature

We identified 39 urinary compounds representing eight chemical classes (categorized on the basis of overall structure and according to functional groups with the same or similar modes of metabolism). The chemical constituents of urine and the ventral gland are strikingly different [25, 37]. Male hamster urinary compounds are notably composed of a group of pyrazines, which are among the most odoriferous substances. The dominating presence of pyrazines has previously been reported in male Siberian hamster urine and is known to be most lacking in females [29]. Further, the three most elevated compounds under the different conditions in this study were 2-methyl-5-propylpyrazine, 2,5-dimethylpyrazine and an ethylvinylpyrazine, which are likely to be male-specific compounds [29]. Ketones were also found as a predominate chemical signature in urine consistent with previous findings [29].

In contrast to urine, the majority of ventral gland constituents were carboxylic acids, alcohols and ketones. In addition, there were several male-specific unsaturated ketones, including 4,8-dimethyl-3,8-nonadien-2-one, which may be unique to this species [25, 37, 38].

There were also two female-specific methyl ketones, 2-dodecanone and 2-tetradecanone [25, 37, 38]. Siberian hamsters have been reported to use urine and ventral gland secretions as scent marks to delineate their respective territorial boundaries, therefore the lack of chemical overlap suggests that these scent profiles convey different information among senders and receivers, which are likely dependent on glandular source of origin [22, 24]. Ventral gland compounds have also been shown to be involved in aggressive encounters, change with reproductive phenotype and are associated with short-day increases in aggression [25]. Our results here nicely parallel these previous findings and suggest that urine is used more during periods of reproductive activity, consistent with previous observations in hamsters in the wild and laboratory [21, 24, 29]. In addition to the ventral gland and urine, both male and female hamsters produce secretions from the supplementary sacculi glands, which are at the opening of their cheek pouches. These secretions are chemically distinct from other glandular secretions, and do not seem to be involved in territorial defense [38]. Instead, buccal secretions are associated with parent-pup interactions and seem critical for successful development in early life [39].

4.2 Hamster urinary compounds and other rodent and mammalian urinary metabolites

In a previous study that compared pooled urine samples of three species in the *Phodopus* genus Siberian hamsters and Campbell's hamsters (*P. campbelli*) were very similar with regards to pyrazine and ketone profiles [29]. Specifically, both species expressed similar levels of each compound, however, Campbell's hamsters displayed more male-specific pyrazines. Although very closely related, these species differ in the degree of paternal care given; Campbell's hamsters display paternal care whereas Siberian hamsters do not. One possibility is that male-specific pyrazines function to advertise male quality, but the information content may differ due to their respective mating systems. The precise information conveyed by these VOCs, however,

is not yet known. Roborovski hamsters (*P. roborovskii*) have a urinary profile composed of substituted quinoxalines, which is completely different than that observed in Siberian hamsters and Campbell's hamsters, suggesting that the metabolic processes likewise drastically differ among these species [29].

When examining other rodents, male and female pine voles (*Microtus pinetorum*) and deer mice (*Peromyscus maniculatus*) share some common compounds, such as pyrazines and ketones, in the urinary profiles with both Siberian hamsters and Campbell's hamsters [29, 40, 41]. However, in contrast to hamsters, female deer mice display elevated levels of pyrazines when compared to male deer mice. These data suggest an important role of pyrazines in rodent chemical communication and demonstrate their multi-faceted roles among species and sexes. Pyrazines have diverse roles among different chemical signaling functions in insects, vertebrates, fungi and bacteria [42]. Both hamsters and deer mice, however, differ markedly with respect to the urinary profiles of house mice, *Mus domesticus*. Siberian hamsters have a different chemical makeup and considerably fewer chemical constituents than the house mouse, which contains a repertoire of over 100 chemicals [2, 43]. Only female mouse urine contains 2,5-methylpyrazine with the distinct biological activity to delay puberty in both female and male juvenile mice [44, 45]. In addition to investigating qualitative differences among Siberian hamster urinary profiles, we examined whether compound levels play a role within a seasonal and aggressive context.

4.3 Aggression and urinary compounds

A key finding in this study is that individual urinary compounds were *increased* following a same-sex aggressive encounter and further, overall changes in urinary compounds following an aggression trial were associated with individual levels of aggressiveness. Six urinary compounds were altered including two pyrazines and three ketones (e.g., 2,5-

455 dimethylpyrazine and 4-nonanone). Although we cannot completely rule out that changes in
456 urinary compounds were influenced by other factors besides the aggressive interaction (e.g.,
457 handling), we believe this is unlikely because of the high reproducibility within individuals and
458 across time in non-social encounters. We also report photoperiod-specific increases in select
459 compounds in response to aggression; nine urinary compounds were increased in long days but
460 not short days. Interestingly, these compounds were predominately pyrazines (e.g., 2-methyl-5-
461 propylpyrazine) that have previously been described as mostly male-specific compounds present
462 in adults, but absent or present at low levels in juvenile males [29]. Although males across both
463 photoperiods displayed changes in urinary compounds in response to aggression, long-day males
464 showed a much more robust magnitude of change compared with short-day males. Further, the
465 magnitude of change in the levels of individual urinary compounds before and after aggression
466 did not parallel the intensity of aggression; urinary compounds were altered most notably in
467 long-day hamsters, which display decreased aggression compared with short-day hamsters [18,
468 20]. At a mechanistic level, these distinct changes in urinary compounds in response to
469 aggression suggest that differential metabolic regulation may be responsible for changes to the
470 urinary profile across photoperiods; however, such mechanisms explaining this pattern are
471 currently unknown. While we did not explicitly examine urinary compounds at a functional
472 level, these results suggest that urinary compounds may play a greater role within a long-day
473 context when animals are reproductively competent, and less so in short days when reproduction
474 is inhibited. In contrast, short-day animals appear to shift to the use of ventral gland signaling
475 during aggressive encounters [25]. Taken together, these findings support the idea of an
476 adjustment in signaling across photoperiods, however, neither study directly tested function.
477 Therefore, additional studies will be needed to conduct functional analyses of candidate urinary

volatile compounds identified here in order to gain a greater understanding of their role in the chemical communication of this species.

4.4 Reproductive phenotype and urinary compounds

Another finding of note in the present study is that photoperiod-induced changes in reproductive phenotype contributed to substantial changes in levels of individual compounds. In general, elevated levels of compounds were observed in long days. Baseline levels of pyrazines and ketones were secreted in very high concentrations in long-day hamsters, compared to the moderate levels observed in short-day hamsters. The metabolic processes explaining elevated levels of urinary compounds and also a larger number of urinary compounds that change in response to behavior for long-day animals are likely dependent on elevated levels of gonadal steroids reported here and previously [18, 32]. We show that baseline levels of T are associated with baseline levels of urinary compounds that loaded onto the first principal component. Most urinary compounds that loaded strongly onto the first component also show photoperiod-dependent elevations in these compounds (e.g., 2-methyl-5-propylpyrazine and an ethylvinylpyrazine). Such a strong association suggests a role for T in the regulation of these urinary compounds. Because most, but not all, urinary compounds are associated with T, this suggests that these other compounds may be regulated by other gonadal steroids or by a T-independent mechanism [46].

There were also many urinary compounds that loaded strongly onto the first component that did not show photoperiodic variation (e.g., 2-acetyl-1-pyrroline and γ -6-dodecalactone). Despite the lack of photoperiodic variation, these urinary compounds showed aggression-induced increases that co-occurred with aggression-induced decreases in T in long-day males. This marked decrease in T concomitant with marked increases in urinary compounds suggests that T

is involved in the onset of metabolic conversion of these compounds. In contrast, the aggression-induced changes, irrespective of photoperiod, suggest that there are T- or gonadal steroid-independent effects on these chemical compounds. Modest changes in T, site-specific metabolic conversion, or androgen receptor expression and kinetics were not captured by our quantification of baseline T [46]. In addition, we did not examine non-gonadal mechanisms of aggression by which these urinary compounds could also be regulated [46]. To further explore the relationship between urinary compound production, gonadal steroids and T, future studies should combine the natural fluctuations of reproductive phenotype with experimental manipulations of reproductive phenotype such as gonadectomies and hormone replacement. In addition, future studies should test the likely non-gonadal mechanisms by which both aggression and a subset of urinary compounds are regulated. Such studies are the critical next step needed to gain a greater understanding of the underlying mechanisms regulating chemical communication of this species.

5. CONCLUSIONS

We show that the different photoperiod and aggressive encounter conditions influence variation in levels of specific compounds across the broad range of Siberian hamster urinary compounds. These chemical signals, used for territory marking, also share some of the urinary compound composition with other rodents, although the function of the same chemical compound may differ between species. Further, differences across photoperiods in relative levels of urinary compounds as well as photoperiod-dependent and photoperiod-independent changes in urinary compounds following behavioral trials make Siberian hamsters an excellent species in which to explore both the functional significance and physiological regulation of these compounds in future studies. Our data illustrate the important role of urinary compounds in regulating hamster chemical communication [25, 29, 37, 47] and communication in general [48,

49]. Collectively, these findings advance our understanding of mammalian chemical communication and social behavior more comprehensively.

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