

RESEARCH ARTICLE

Photoperiod modulates the gut microbiome and aggressive behavior in Siberian hamsters

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ABSTRACT

Seasonally breeding animals undergo shifts in physiology and behavior in response to changes in photoperiod (day length). Interestingly, some species, such as Siberian hamsters (*Phodopus sungorus*), are more aggressive during the short-day photoperiods of the non-breeding season, despite gonadal regression. While our previous data suggest that Siberian hamsters employ a ‘seasonal switch’ from gonadal to adrenal regulation of aggression during short-day photoperiods, there is emerging evidence that the gut microbiome, an environment of symbiotic bacteria within the gastrointestinal tract, may also change seasonally and modulate social behaviors. The goal of this study was to compare seasonal shifts in the gut microbiome, circulating levels of adrenal dehydroepiandrosterone (DHEA) and aggression in male and female Siberian hamsters. Hamsters were housed in either long-day (LD) or short-day (SD) photoperiods for 9 weeks. Fecal samples were collected and behaviors were recorded following 3, 6 and 9 weeks of housing, and circulating DHEA was measured at week 9. SD females that were responsive to changes in photoperiod (SD-R), but not SD-R males, displayed increased aggression following 9 weeks of treatment. SD-R males and females also exhibited distinct changes in the relative abundance of gut bacterial phyla and families, yet showed no change in circulating DHEA. The relative abundance of some bacterial families (e.g. *Anaeroplasmataceae* in females) was associated with aggression in SD-R but not LD or SD non-responder (SD-NR) hamsters after 9 weeks of treatment. Collectively, this study provides insight into the complex role of the microbiome in regulating social behavior in seasonally breeding species.

KEY WORDS: Aggression, Dehydroepiandrosterone, Gut–brain axis, Microbiota, Day length, Seasonality

INTRODUCTION

The gut microbiota is the collection of bacteria, archaea, fungi, viruses and protists that live in the gastrointestinal tract (reviewed in Shreiner et al., 2015). These microbes are not only responsible for metabolic functions but they can also influence the brain and behavior, the immune system and numerous other physiological systems (reviewed in Cani, 2018; Shreiner et al., 2015; see also Sylvia et al., 2017). Disruption of the gut microbiome has been

linked to a variety of diseases, including irritable bowel syndrome, inflammatory bowel disease, obesity, diabetes, multiple sclerosis, autism, allergic disease, depression and anxiety (reviewed in Ghaisas et al., 2016; see also O’Mahony et al., 2009).

The gut microbiome, the genes encoding microbial communities, has been predominately studied in model systems, such as germ-free (GF) mice, but these models do not allow us to fully understand the role of these microbiota in the natural environment. Further, because behavior in GF mice is often observed in isolated tests, there is limited information about the role of the microbiome in modulating social behavior (Clarke et al., 2014; Desbonnet et al., 2014; Nguyen et al., 2015; Sylvia et al., 2017, 2018; reviewed in Collins et al., 2012). The relationship between the gut microbiome and social behavior (e.g. aggression) remains relatively understudied, especially in non-traditional systems, such as Siberian hamsters. Studying the gut microbiome in a non-traditional model organism that naturally changes behavior in response to changes in season allows us to understand the mechanisms underlying the relationships among the microbiome, physiology and behavior.

Siberian hamsters exhibit pronounced shifts in reproductive physiology and its associated behaviors in response to seasonal changes in photoperiod (i.e. day length) (Bartness and Wade, 1985). Photoperiod is the primary cue that Siberian hamsters use to anticipate changes in season, and shifts in day length are physiologically encoded by changes in the pattern and duration of melatonin secretion (Bartness et al., 1993; Goldman, 2001; reviewed in Walton et al., 2011). In response to this signal, hamsters will display specific characteristics during long-day (LD; characteristic of the breeding season) or short-day (SD; characteristic of the non-breeding season) photoperiods. During long, ‘summer-like’ days, hamsters have a brown pelage and functional gonads (Jasnow et al., 2000; Rendon et al., 2016). In contrast, hamsters housed in short, ‘winter-like’ days develop lighter pelage, exhibit gonadal regression, decrease body mass and show a pronounced increase in aggression (Jasnow et al., 2000; Rendon et al., 2016). Unlike these short-day responders (SD-R), a subset of animals housed in SDs does not respond to seasonal changes in photoperiod; these short-day non-responders (SD-NRs) typically exhibit a LD-like behavioral and physiological phenotype (Freeman and Goldman, 1997).

Previous work from our lab has examined the mechanisms underlying SD increases in aggressive behavior in Siberian hamsters. Classic neuroendocrine studies focus on the role of circulating gonadal steroids, such as testosterone and estradiol (E₂), in directly regulating aggression in birds and rodents (reviewed in Soma, 2006; Wingfield, 1984). However, some species utilize alternative neuroendocrine pathways independent of circulating gonadal steroids to maintain or increase aggression during the non-breeding season, despite gonadal regression (reviewed in Soma et al., 2008; Wingfield and Soma, 2002). We have shown that both

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male and female Siberian hamsters increase circulating levels of the adrenal hormone dehydroepiandrosterone (DHEA) during SDs (Rendon et al., 2015; Scotti et al., 2008). DHEA is an androgen that can pass through the blood–brain barrier and be converted to biologically active androgens and estrogens within brain regions that possess the appropriate steroidogenic enzymes (Pradhan et al., 2010; reviewed in Soma et al., 2015). Thus, region-specific metabolism of DHEA to testosterone and/or E_2 likely allows these animals to regulate the neural circuits relevant to aggression during the non-breeding season (reviewed in Munley et al., 2018; Rendon and Demas, 2016). Taken together, these studies suggest that Siberian hamsters employ a ‘seasonal switch’ from gonadal regulation of aggression during LDs to adrenal regulation of aggression during SDs.

Because gut microbes produce numerous byproducts that may be involved in physiological and behavioral changes (Foley et al., 2014; Hanstock et al., 2004; reviewed in MacFabe, 2015; Sylvia and Demas, 2018b), the microbiome may play an important role in mediating this ‘seasonal switch’ in neuroendocrine mechanisms. Previous work suggests that the gut microbiome may change seasonally and is related to aggressive behavior. Specifically, Siberian hamsters treated with broad-spectrum antibiotics show changes in gut microbial communities that are associated with decreased aggression (Sylvia et al., 2017). The gut microbial composition is also different between dogs classified as aggressive and non-aggressive (Kirchoff et al., 2019). In addition, other studies suggest that the gut microbiome changes on a seasonal basis. For example, chickens and mice exhibit seasonal shifts in the gut microbiome (Cui et al., 2016; Hieke et al., 2019; Wang et al., 2018). Further, body mass correlates with the relative abundance of Proteobacteria, *Citrobacter* and Firmicutes in LD male Siberian hamsters (Bailey et al., 2010). While these studies suggest that seasonal shifts in the gut microbiome may modulate social behavior, the specific mechanisms underlying this relationship have yet to be explored.

Moreover, recent work suggests that the gut microbiome can vary substantially between males and females (Sylvia and Demas, 2018a; Sylvia et al., 2017, 2018; Vemuri et al., 2018). For example, castrated male mice, which are incapable of producing gonadal steroids, exhibit a gut microbiome that more closely resembles that of female than male mice (Yurkovetskiy et al., 2013). In Siberian hamsters, antibiotic treatment is associated with more robust changes in the gut microbiome of males than females; however, repeated antibiotic treatment decreases aggression in a single treatment for females rather than two treatments for males, and aggression returns to baseline levels during the recovery period for males, but not for females (Sylvia et al., 2017). These findings suggest that studying both sexes is valuable and necessary when investigating the role of the gut microbiome in mediating social behavior.

The goal of the current study was to examine how seasonal changes in photoperiod affect the gut microbiome, circulating DHEA and social behavior of both male and female Siberian hamsters. We hypothesized that 9 weeks of photoperiodic treatment would be sufficient to elicit significant changes in the gut microbial composition of SD-R hamsters compared with LD and SD-NR hamsters. Furthermore, we predicted that seasonal shifts in the gut microbiome would be correlated with changes in physiology (e.g. body mass and serum DHEA levels) and aggressive behavior in a sex-specific manner. Collectively, this study aimed to enhance our understanding of the links among the often separately researched gut microbiome, circulating hormones, photoperiod and behavior across the sexes in a non-traditional system.

MATERIALS AND METHODS

Animal housing and photoperiodic treatment

Male and female adult (2–7 months of age) Siberian hamsters, *Phodopus sungorus* (Pallas 1773), were group-housed in polycarbonate cages (28×17×12 cm) and raised in LD conditions (light:dark, 16 h:8 h) in a colony maintained at Indiana University. Experimental animals ($N=26$ males, $N=26$ females) were moved to a new holding room under LD conditions, individually housed and allowed to acclimate for 1 week. Following the 1 week acclimation period, male and female hamsters were randomly assigned to either the LD ($N=9$ males, $N=9$ females; light:dark, 16 h:8 h as above) or SD ($N=17$ males and $N=17$ females; light:dark, 8 h:16 h) group and housed for 9 weeks, as outlined in previous studies for this species (Rendon et al., 2015). For all conditions, relative humidity was $55\pm 5\%$, ambient temperature was $20\pm 2^\circ\text{C}$, and hamsters had *ad libitum* access to purified tap water and standard laboratory rodent chow (Lab Diet 5001, PMI Nutrition). 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 (BIACUC) at Indiana University (protocol no. 16-025).

Seasonal phenotypes

Seasonal phenotypes were determined following 9 weeks of photoperiodic treatment based on *a priori* criteria for male and female Siberian hamsters (Jasnow et al., 2000; Rendon et al., 2015; Scotti et al., 2007). Starting with the acclimation week, all hamsters and total food intake were weighed weekly, and reproductive tissue mass was recorded at the conclusion of the study. LD hamsters ($N=18$; $N=9$ males, $N=9$ females) maintained body and reproductive tissue mass and exhibited brown pelage. Of the hamsters placed in SD conditions ($N=34$; $N=17$ males, $N=17$ females), 53% of both males and females physiologically responded to changes in photoperiod (SD-R; $N=9$ males, $N=9$ females). SD-R hamsters were classified as animals that exhibited $\geq 5\%$ loss of body mass and gonadal regression (compared with the average testes or ovaries mass of LD animals at week 9). A white pelage was used to help confirm each classification. Animals that did not meet these criteria were classified as SD-NR hamsters. Approximately 47% of males and 47% of females failed to respond to changes in photoperiod (SD-NR; $N=8$ males, $N=8$ females). The proportion of SD-NR hamsters in this study is within expectations based on past studies, which found that approximately 30% of Siberian hamsters and other rodents fail to respond to SD conditions (Goldman, 2001; Gorman and Zucker, 1995; Lynch et al., 1989; Rendon et al., 2017).

Fecal sampling and microbiome analysis

Fecal samples were obtained from each animal following 0 (pre-treatment), 3, 6 and 9 weeks of photoperiodic housing. Hamsters were removed from their cages and held over a sterile container to collect fecal samples. Hamsters were then returned to their cages. Fecal samples were stored at -80°C until further processing.

DNA was extracted from fecal samples (males: $N=6$ per treatment group; females: $N=6$ per treatment group) using a commercially available kit (Maxwell RSC Tissue DNA Kit, Promega, Madison, WI, USA) (Sylvia and Demas, 2018a; Sylvia et al., 2017, 2018); 80 μl of TE buffer, 20 μl of RNase A solution and 300 μl of lysis buffer were added to each sample. Samples were then homogenized and centrifuged at 4°C for 5 min at 1200 rpm. The supernatant was used for automated extraction (Maxwell Rapid Sample Concentrator Instrument, Promega). In addition to experimental samples, two negative controls were simultaneously extracted to indicate any

contamination (elution buffer only; and TE buffer, RNase A solution and elution buffer all together). The purity and quality of DNA were verified with the Take3 microvolume plate (BioTek, Winooski, VT, USA) and 4200 TapeStation system (Agilent, Santa Clara, CA, USA).

Following Maxwell processing, samples were sent to the Indiana University Center for Genomics and Bioinformatics (Bloomington, IN, USA), where multiplexed amplicon libraries spanning the V4 hypervariable domain of the microbial 16S ribosomal RNA (rRNA) gene were prepared using NEXTflex 16S V4 Amplicon-Seq Library Prep Kit 2.0 (catalog number: NOVA-4203-01, Bioo Scientific, Austin, TX, USA; Earth Microbiome primers 515F-806R). Agencourt AMPure XP Magnetic Beads were used to clean the samples, PCR primers targeting the V4 domain amplified the samples, and the Illumina MiSeq v3 (600 cycle) platform was used to determine sequence information. Operational taxonomic units (OTUs) were determined through Swarm and matched against the Silva database, as described in previous studies (Armanhi et al., 2016; Mahé et al., 2014; Sylvania et al., 2018).

Behavioral testing and analyses

Behavioral videos were recorded following 0 (pre-treatment), 3, 6 and 9 weeks of photoperiodic treatment and were analyzed for same-sex aggression, investigation, grooming and scent-marking behaviors using previously outlined methods (Jasnow et al., 2000; Rendon et al., 2016). Specifically, within the first 2 h of the dark phase, an unfamiliar same-sex intruder ($N=10$ males, $N=10$ females) was placed into the home cage of the experimental (i.e. resident) hamster ($N=26$ males, $N=26$ females), and the animals were allowed to interact for 5 min. Intruders were housed in LD conditions in groups of 2, and intruders were of approximately the same age and mass ($\pm 10\%$) as the resident animals with which they were paired. All trials were recorded (Sony HandyCam Digital Camcorder HDR-SR7) under low-illumination red lights.

We scored aggression (latency to first attack and frequency and duration of attacks and chases), investigation (frequency and duration of anogenital and nose-to-nose investigation), grooming (frequency and duration of self-grooming) and scent-marking behaviors (frequency and duration of scent depositing) with ODLog (Macropod Software, Eden Prairie, MN, USA) using previously outlined methods (Jasnow et al., 2000; Rendon et al., 2015, 2016; Sylvania et al., 2017).

Tissue collection and blood sampling

All animals were anesthetized with isoflurane vapor following behavioral testing at week 9 to collect a terminal blood sample from the retro-orbital sinus (Sylvania et al., 2018). Hamsters were then euthanized using a lethal intraperitoneal injection of ketamine and xylazine mixture in 0.9% saline. Testes (males), ovaries (females), uterine horns (females), epididymal white adipose tissue (males, EWAT; pads surrounding testes and likely metabolically supporting reproductive capabilities) and parametrial white adipose tissue (females, PWAT; pads surrounding ovaries and likely metabolically supporting reproductive capabilities) were removed and weighed (Bailey et al., 2017; Carlton and Demas, 2015; Jaubert et al., 1995). Blood samples were clotted for 1 h at room temperature, the clots were removed, and the samples were centrifuged at 4°C for 30 min at 2500 rpm. Serum was stored at -20°C until further processing.

Serum DHEA quantification

Serum DHEA concentration was measured using a commercially available enzyme immunoassay kit (DHEA ELISA kit ADI-901-093, Enzo Life Sciences, Farmingdale, NY, USA; assay sensitivity

2.90 pg ml^{-1}). The validity of this assay was determined by comparing male and female Siberian hamster serum samples of varying dilutions with a standard curve generated using reference standards provided by the kit. This assay has some cross-reactivity with sulfated DHEA (30%) and low cross-reactivity with androstenedione (0.73%), androsterone (0.29%), pregnenolone (0.28%) and testosterone (0.10%). All serum samples were run neat or diluted 1:2 or 1:4 with assay buffer to ensure 20–80% binding on a 4-parameter logistic standard curve (Microplate Manager 6 version 6.2, Bio-Rad Laboratories, Hercules, CA, USA). Samples were run in duplicate according to the manufacturer's instructions. Samples from animals of different treatment groups were counterbalanced across two plates from the same kit lot number (05071801). Samples with a coefficient of variability (CV) greater than 20% and a maximum binding less than 20% or greater than 80% were re-analyzed. The inter-assay CV was 12.8% and the average intra-assay CV was 10.9%.

Statistical analyses

All statistical analyses were performed in R v.1.1.383 (<http://www.R-project.org/>), and we attributed statistical significance at $P<0.05$ after controlling for false discovery rate in the case of multiple comparisons (Verhoeven et al., 2005). Females and males displayed significantly different quantities of several behaviors, including duration and frequency of attacks. Therefore, females and males were separated during statistical analysis. For behavioral analyses, mixed model ANOVA were used to compare the duration or frequency of aggression, investigation, grooming and scent-marking behaviors across treatment groups and time points. If a significant result was found between the interaction of treatment and time, pair-wise relationships were explored using Tukey's honest significant difference *post hoc* tests. For organ mass, body mass and serum DHEA analyses, variance and normality were assessed using Levene's tests and Shapiro–Wilk tests, respectively. If data had both equal variances and a normal distribution, one-way ANOVA were used to compare organ and body mass across treatment groups and time points (male and female body mass, male EWAT mass and paired testes mass) and to compare serum DHEA levels across treatment groups at the week 9 time point. Log transformations were used to transform some data to attain equal variances and normality. If data exhibited a normal distribution, but did not have equal variances, Welch's ANOVA were used (female PWAT mass and uterine horns mass).

Principal coordinates analyses (PCoA) were performed to visualize differences in microbial communities between treatment groups and across time points, and multi-variate non-parametric ANOVA of dissimilarities (PERMANOVA) were run to determine whether microbial communities were affected by treatment, time or an interaction of treatment and time based on Bray–Curtis distance (Sze et al., 2014). The Shannon–Wiener index was calculated to determine alpha diversity, and two-way ANOVA were used to determine statistically significant differences in alpha diversity between treatment groups and across time points (Hill, 1973; Jost, 2006). Bray–Curtis dissimilarity scores were calculated across treatments and were converted to percentage differences between groups for a more clear comparison (Maziarz et al., 2018). Mixed model ANOVA were used to compare the relative abundance of each phylum and family across treatments and time points. If a significant result was found between the interaction of treatment and time, pair-wise relationships were explored using Tukey's honest significant difference *post hoc* tests. In addition, Spearman's rank correlations were used to assess potential relationships between the relative abundance of bacterial phyla and families, aggressive and

non-aggressive social behaviors, serum DHEA levels and body mass for each treatment group and sex at the week 9 time point alone or across all time points.

RESULTS

Reproductive phenotypes differ across photoperiod

After 9 weeks of photoperiodic treatment, SD-R males had significantly lower body mass ($P<0.001$; Fig. 1A), paired testes mass ($P<0.001$; Fig. 1B) and EWAT mass ($P<0.001$; Fig. 1C) compared with both LD and SD-NR males. Similarly, SD-R females also had significantly decreased body mass ($P<0.001$; Fig. 1D), uterine horn mass ($P<0.001$; Fig. 1E) and PWAT mass ($P<0.001$; Fig. 1F) after 9 weeks of treatment compared with both LD and SD-NR females.

Photoperiod significantly increases aggression in females, but not in males

The duration of attacks ($F_{11,52.4}=1.546$, $P=0.144$), number of attacks ($F_{11,52.8}=1.464$, $P=0.1735$) and latency to first attack ($F_{11,51.8}=1.142$, $P=0.097$) in SD-R males were not significantly different from those for SD-NR males and LD males across time (Fig. 2A,B; Table S1).

In comparison, attack duration ($F_{11,50.5}=3.107$, $P=0.003$) and number of attacks ($F_{11,51.1}=2.986$, $P=0.004$) in SD-R females were significantly different from those for SD-NR and LD females across time (Fig. 2D,E; Table S2). Specifically, at week 9, SD-R females exhibited a longer attack duration than LD and SD-NR females ($Z=2.852$, $P=0.041$; Fig. 2E). SD-R females at week 0 also displayed a significantly higher number of attacks than SD-NR

females at week 0 ($P=0.008$) and all other groups at later weeks ($P<0.05$; Fig. 2D). Overall, SD-R females had a significantly shorter latency to first attack than SD-NR females ($F_{2,22.6}=3.997$, $P=0.033$; Table S2).

Photoperiod does not affect non-aggressive behaviors

The frequency of anogenital sniffing was similar across photoperiodic groups and time in males (treatment: $F_{2,23}=0.608$, $P=0.553$; time: $F_{3,75}=0.473$, $P=0.702$; interaction: $F_{11,50.8}=0.676$, $P=0.754$; Fig. 2C) and females (treatment: $F_{2,23}=0.955$, $P=0.399$; time: $F_{4,76.1}=2.223$, $P=0.074$; interaction: $F_{11,50.5}=1.228$, $P=0.293$; Fig. 2F). Other investigative, grooming and scent-marking behaviors were not different across photoperiodic treatments (data not shown). However, there were differences across time and in the interaction between time and treatment. Specifically, in males and females, the duration and frequency of nose-to-nose sniffing were affected by time (data not shown) and the interaction of time and treatment (Tables S1 and S2). The duration and frequency of nose-to-nose sniffing was higher in SD-NR males, SD-R males, LD females and SD-NR females at baseline (data not shown). However, overall, SD-R males and females tended to remain relatively stable in investigative, scent-marking and grooming behaviors.

Photoperiod affects microbial diversity in a sex-dependent manner

Based on PERMANOVA analyses, gut microbial communities were not significantly different between the sexes ($P=0.114$). However, all analyses were completed separately for each sex because behavior was significantly different between the sexes. In

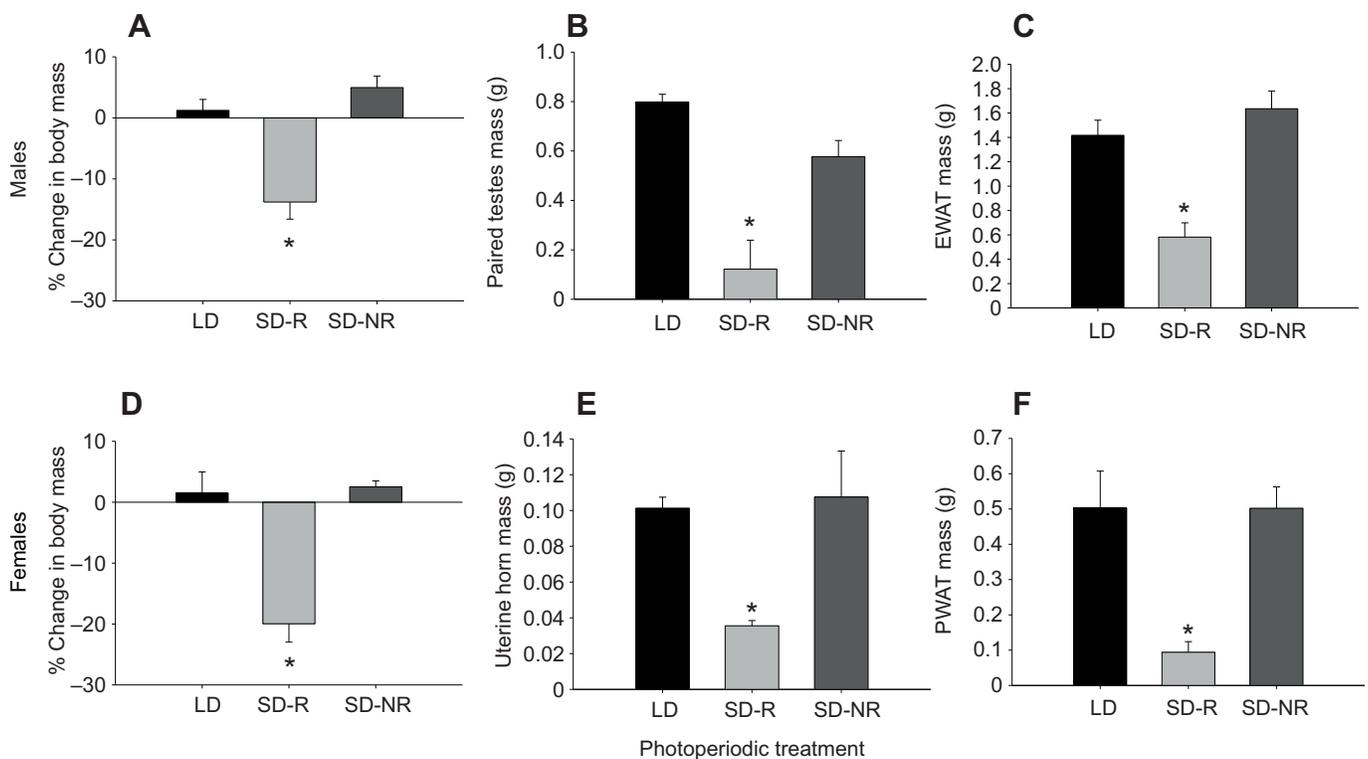


Fig. 1. Percentage change in body mass, reproductive tissue mass, and epididymal and parametrial white adipose tissue (EWAT/PWAT) mass following 9 weeks of photoperiodic treatment. Percentage change in (A,D) body mass; (B,E) reproductive tissue mass; and (C,F) EWAT/PWAT mass in male (A–C) and female (D–F) long-day (LD) hamsters, and short-day responsive (SD-R) and non-responsive (SD-NR) hamsters. Bars represent means \pm s.e.m. (LD: $N=9$, SD-R: $N=9$, SD-NR: $N=8$, for both males and females). An asterisk indicates a statistically significant difference between group means ($P<0.001$; male and female percentage change in body mass, male paired testes mass and EWAT mass: one-way ANOVA; female uterine horns mass and PWAT mass: Welch's ANOVA).

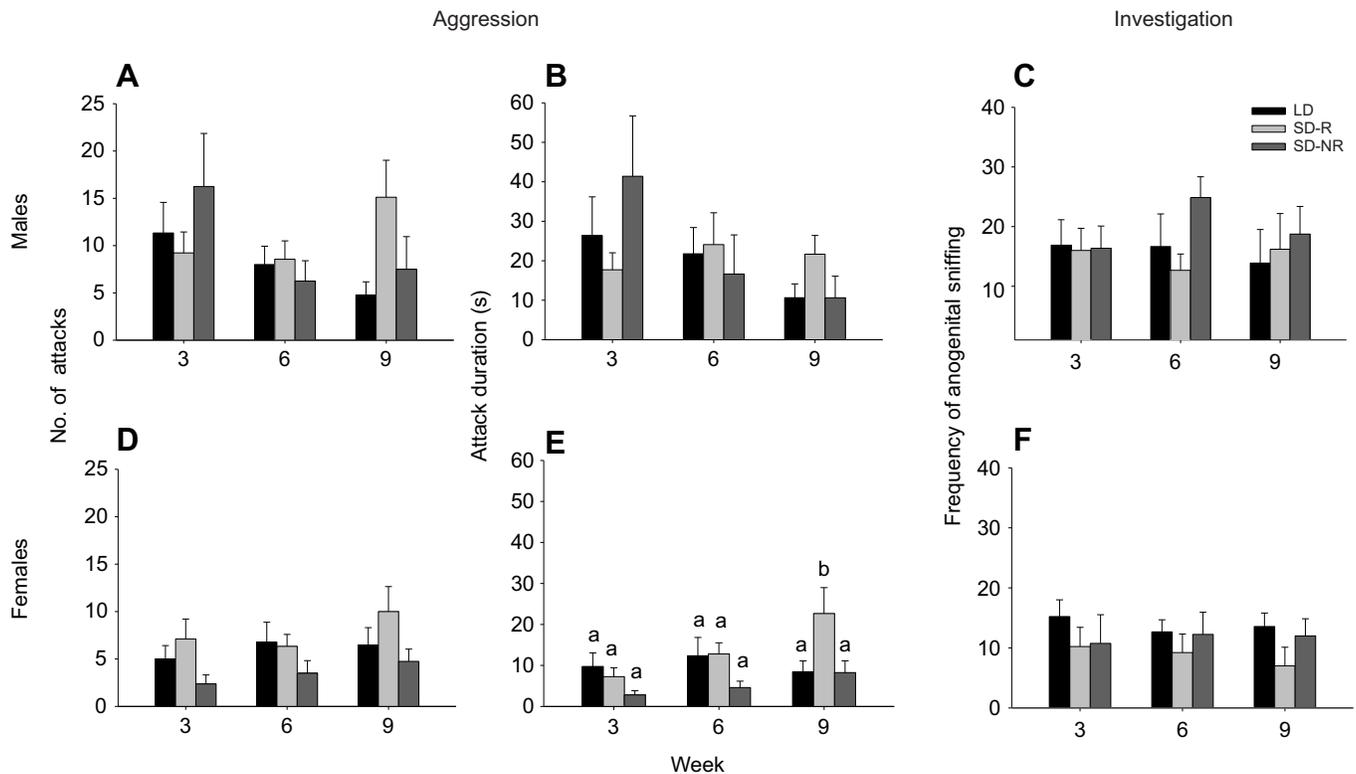


Fig. 2. Aggressive and non-aggressive social behaviors in male and female hamsters following 3, 6 or 9 weeks of photoperiodic treatment. (A,D) Number of attacks; (B,E) attack duration; and (C,F) frequency of anogenital sniffing in male (A–C) and female (D–F) LD hamsters, SD-R hamsters and SD-NR hamsters. Bars represent means \pm s.e.m. (LD: $N=9$, SD-R: $N=9$, SD-NR: $N=8$, for both males and females). Bars with different letters represent statistically different group means ($P < 0.05$; mixed model ANOVA).

males, gut microbial communities were not significantly different across photoperiodic groups ($P=0.182$), time points ($P=0.546$) or the interaction between photoperiodic treatment and time ($P=0.219$; Fig. 3A). Similarly, female gut microbial communities were not different across photoperiodic groups ($P=0.285$), time points ($P=0.340$) or the interaction between photoperiodic treatment and time ($P=0.708$; Fig. 3C).

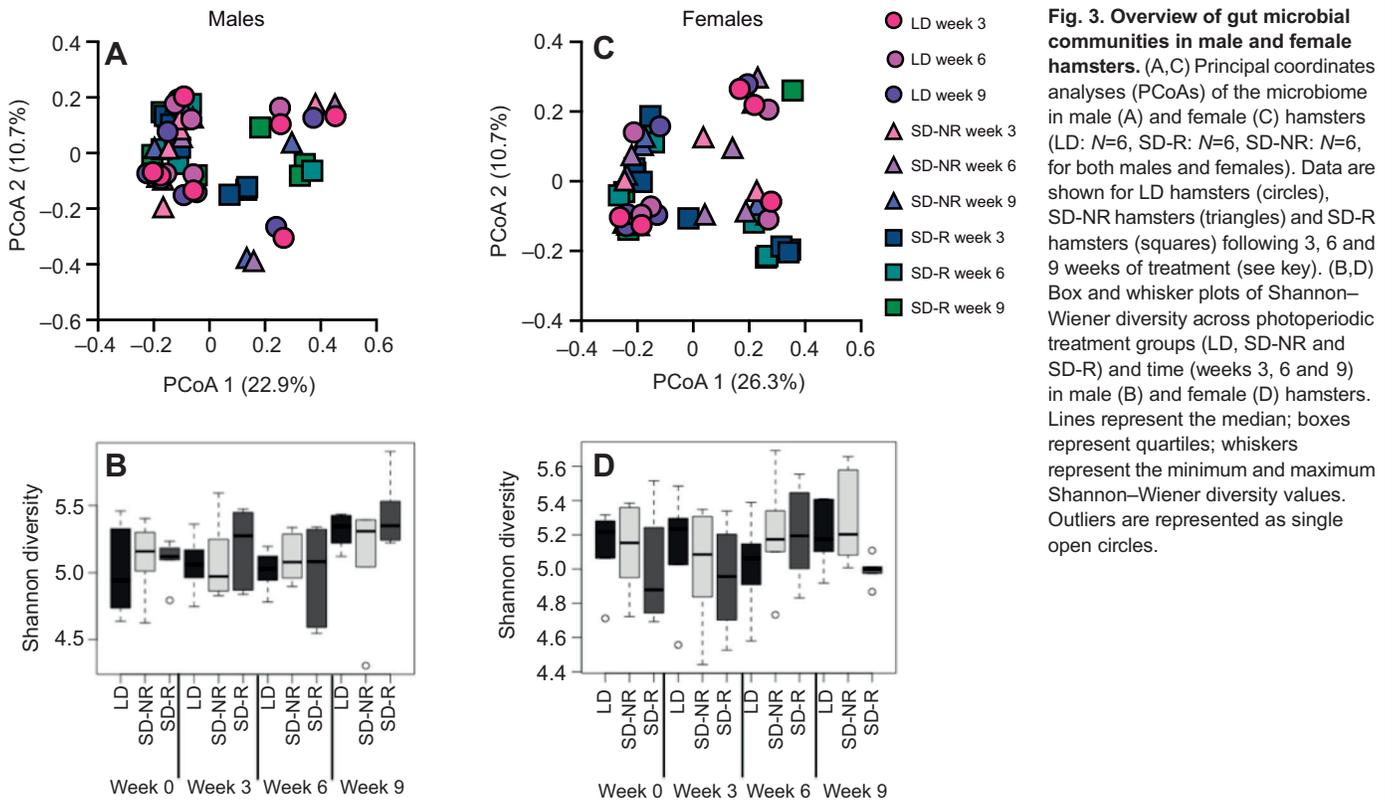
Alpha diversity between males and females was not significantly different ($P=0.623$; Fig. 3B,D). However, time affected alpha diversity in females, but not males. Specifically, all females at week 9 exhibited higher alpha diversity, regardless of photoperiodic treatment ($P=0.043$; Fig. 3D), but there was no effect of time on alpha diversity in males ($P=0.244$; Fig. 3B).

Interestingly, females and males had comparable beta diversity, according to Bray–Curtis dissimilarity scores. In males, SD-R hamsters were 42.1% different from LD hamsters, SD-R hamsters were 45.3% different from SD-NR hamsters, and LD hamsters were 43.1% different from SD-NR hamsters across all time points. In females, SD-R hamsters were 50.3% different from LD hamsters, SD-R hamsters were 49.9% different from SD-NR hamsters, and LD hamsters were 42.8% different from SD-NR hamsters across all time points. However, Bray–Curtis dissimilarity scores increased over time, especially for SD-NR males and SD-R females. Specifically, SD-NR males at week 3 were 41.3% different from baseline; SD-NR males at week 6 were 45.5% different from baseline; and SD-NR males at week 9 were 60.6% different from baseline. Similarly, SD-R females at week 3 were 42.2% different from baseline; SD-R females at week 6 were 44.9% different from baseline; and SD-R females at week 9 were 74.9% different from baseline.

Photoperiod and time affect the relative abundance of gut microbes in males and females

Phyla and families in the male and female gut microbiome were similar across photoperiodic treatment groups at week 0 ($P > 0.10$). However, specific differences in the relative abundance of gut microbes across time were not consistent within each sex. For example, at week 9, males had a significantly greater relative abundance of Patescibacteria ($P \leq 0.028$) than week 0 or week 3 males (Fig. 4A), whereas SD-R females at week 9 had a greater relative abundance of the phylum Firmicutes ($P < 0.05$) than LD females at week 9 and all treatment groups at weeks 0, 3 and 6 (Fig. 4D). Further, SD-NR males at week 9 had a greater relative abundance of Ruminococcaceae than all other treatment groups at weeks 0 and 3 ($P < 0.05$, Fig. 4B). In contrast, SD-R females at week 9 had a higher relative abundance of Ruminococcaceae ($P < 0.05$) than LD females at week 9 and all treatment groups at weeks 0, 3 and 6 (Fig. 4E). SD-R males at week 9 had a higher relative abundance of Marinifilaceae than all treatment groups at week 0 and 3 ($P < 0.05$), and there was a gradual increase in the relative abundance of Marinifilaceae over time in SD-R males compared with LD and SD-NR males (Fig. 4C). The same changes in Marinifilaceae were not seen in females (Table S4).

The bacterial family Anaeroplasmataceae showed the most pronounced response to photoperiodic treatment in females ($P=0.007$; Fig. 4F). Specifically, SD-R females had a higher proportion of Anaeroplasmataceae following 3 weeks of treatment than LD and SD-NR females (SD-R and LD: $P=0.028$, SD-R and SD-NR: $P=0.028$; Fig. 4F). At week 9, the relative abundance of Anaeroplasmataceae in SD-R males also appeared to increase, though not significantly, compared with earlier time points ($P=0.225$; Table S3).



Finally, the following bacterial phyla were significantly different ($P<0.05$) across time points in males, regardless of photoperiodic treatment: Bacteroidetes, Cyanobacteria, Firmicutes, Patescibacteria, Proteobacteria, Spirochaetes and Tenericutes

(Table S3). In addition, the following bacterial phyla were significantly different ($P<0.05$) across time points in females, regardless of photoperiodic treatment: Firmicutes and Spirochaetes (Table S4).

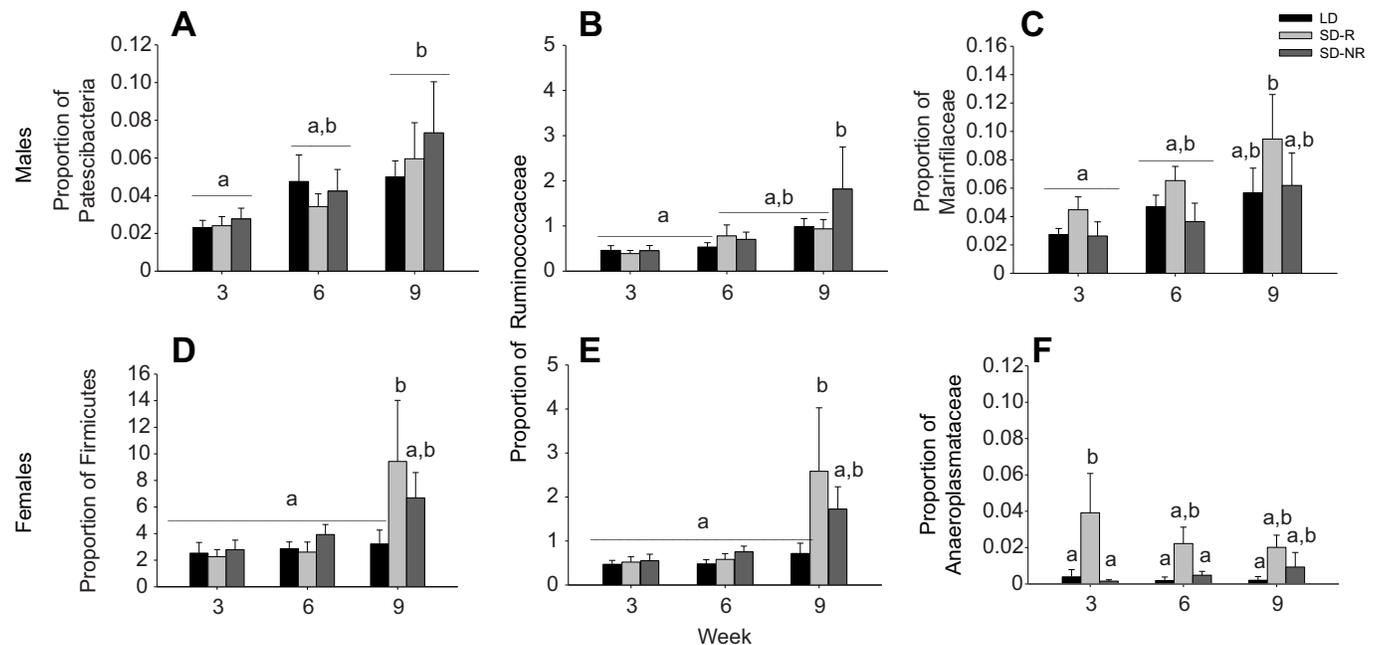


Fig. 4. Relative abundance of bacterial phyla and families that differed in response to photoperiodic treatment and/or time in male and female hamsters. The proportion of bacteria from the phylum Patescibacteria (A), family Ruminococcaceae (B) and family Mariniflacciae (C) showed significant differences across treatments and/or time in male hamsters. The proportion of bacteria from the phylum Firmicutes (D), family Ruminococcaceae (E) and family Anaeroplasmataceae (F) showed significant differences across treatments and/or time in female hamsters. Bars represent means \pm s.e.m. (LD: $N=6$, SD-R: $N=6$, SD-NR: $N=6$ for both males and females). Bars with different letters represent statistically different group means ($P<0.05$; mixed model ANOVA).

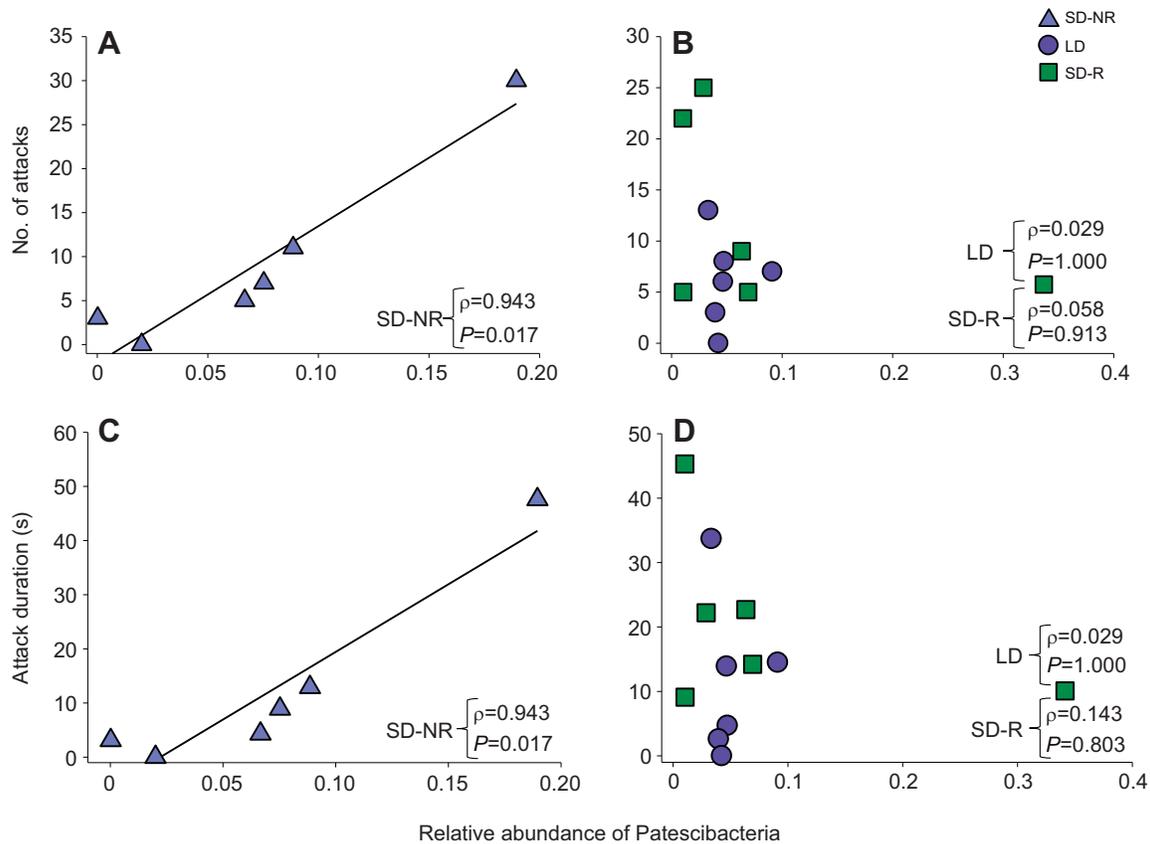


Fig. 5. Correlation between relative abundance of the bacterial phylum Patescibacteria and aggressive behavior in male hamsters after 9 weeks of photoperiodic treatment. (A,B) The relative abundance of Patescibacteria was significantly correlated with the number of attacks in SD-NR males (A), but not in LD or SD-R males (B). (C,D) The relative abundance of Patescibacteria was significantly correlated with attack duration in SD-NR males (C), but not in LD or SD-R males (D). For each treatment group (LD: $N=6$, SD-R: $N=6$, SD-NR: $N=6$), correlation coefficients (ρ) and P -values are shown (Spearman's rank correlations).

The gut microbiome is correlated with behavior and physiology in a sex-specific manner

In SD-NR males, but not LD or SD-R males at week 9, the relative abundance of Patescibacteria was positively associated with number of attacks ($\rho=0.943$, $N=6$, $P=0.017$; Fig. 5A,B; Table 1) and attack duration ($\rho=0.943$, $N=6$, $P=0.017$; Fig. 5C,D). There was no association between number of attacks and the relative

abundance of Patescibacteria at week 9 in females ($\rho=0.350$, $N=18$, $P=0.142$).

In SD-R females, but not in LD and SD-NR females at week 9, the relative abundance of Anaeroplasmataceae was positively correlated with number of attacks ($\rho=0.812$, $N=6$, $P=0.050$; Fig. 6A,B; Table 2) and attack duration ($\rho=0.421$, $N=6$, $P=0.041$; Fig. 6C,D; Table 2). At week 9, males showed no statistically significant correlation between

Table 1. Correlation between the gut microbiome and behavior of male hamsters

Phylum or family	Behavior	ρ	N	P
All time points				
Bacteroidetes	Duration of head neck sniffing	0.655	72	0.034
	Frequency of scent marking	-0.231	72	0.049
	Frequency of grooming	0.339	72	0.036
Muribaculaceae	Frequency of scent marking	-0.263	72	0.023
	Frequency of grooming	0.348	72	0.009
Week 9				
Spirochaetes	Frequency of attack	0.426	18	0.014
	Duration of attack	0.412	18	0.017
Patescibacteria	Frequency of attack	0.387	18	0.037
Firmicutes	Frequency of attack	0.266	18	0.017
	Duration of attack	0.256	18	0.033
Ruminococcaceae	Frequency of attack	0.146	18	0.029
Saccharimonadaceae	Frequency of attack	0.387	18	0.037
Spirochaetaceae	Frequency of attack	0.464	18	0.016
	Duration of attack	0.409	18	0.018

Correlation coefficients (ρ), number of animals (N) and P -values (P) for correlations between the relative abundance of bacterial phyla and families and behavior of male hamsters across all time points and following 9 weeks of treatment. Only significant correlations ($P<0.05$, Spearman's rank correlations) are shown.

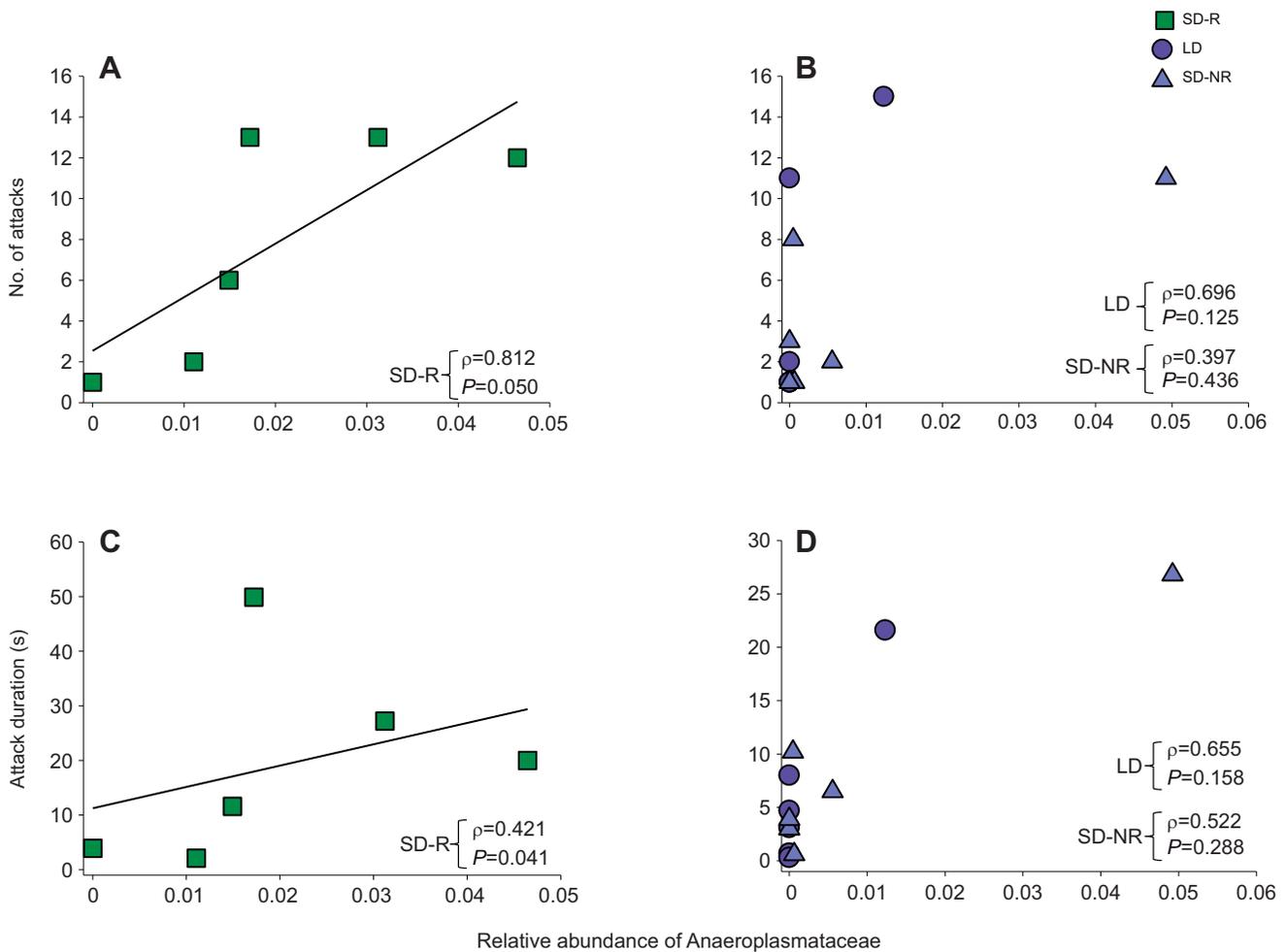


Fig. 6. Correlation between relative abundance of the bacterial family Anaeroplasmataceae and aggressive behavior in female hamsters after 9 weeks of photoperiodic treatment. (A,B) The relative abundance of Anaeroplasmataceae was significantly correlated with the number of attacks in SD-R females (A), but not in LD or SD-NR females (B). (C,D) The relative abundance of Anaeroplasmataceae was significantly correlated with attack duration in SD-R females (C), but not in LD or SD-NR females (D). For each treatment group (LD: $N=6$, SD-R: $N=6$, SD-NR: $N=6$), correlation coefficients (ρ) and P -values are shown (Spearman's rank correlations).

the relative abundance of Anaeroplasmataceae and number of attacks ($\rho=-0.048$, $N=18$, $P=0.510$) or duration of attacks ($\rho=0.126$, $N=18$, $P=0.776$). Additionally, the relative abundance of Anaeroplasmataceae was negatively associated with body mass across all time points in females ($\rho=-0.307$, $N=18$, $P=0.007$; Table 3).

Serum DHEA levels in males and females were similar across groups after 9 weeks of photoperiodic treatment (males, $P=0.692$; females, $P=0.463$; Table S5). However, serum DHEA levels were significantly correlated with number of attacks ($\rho=0.796$, $N=14$, $P<0.001$; Fig. 7A), duration of attacks ($\rho=0.733$, $N=14$, $P=0.003$;

Table 2. Correlation between the gut microbiome and behavior of female hamsters

Phylum or family	Behavior	ρ	N	P
All time points				
Anaeroplasmataceae	Duration of attack	0.448	72	0.036
	Frequency of attack	0.435	72	0.028
	Latency to first attack	-0.312	72	0.027
Elusimicrobiaceae	Duration of head/neck sniffing	-0.189	72	0.020
	Frequency of head/neck sniffing	-0.184	72	0.025
Week 9				
Anaeroplasmataceae	Frequency of attack	0.707	18	0.003
	Duration of attack	0.716	18	0.004
	Latency to first attack	-0.551	18	0.032
Elusimicrobiaceae	Duration of head/neck sniffing	-0.398	18	0.043
	Frequency of head/neck sniffing	-0.369	18	0.028
Euryarchaeota	Duration of attack	0.331	18	0.015

Correlation coefficients (ρ), number of animals (N) and P -values (P) for correlations between the relative abundance of bacterial phyla and families and behavior of female hamsters across all time points and following 9 weeks of treatment. Only significant correlations ($P<0.05$, Spearman's rank correlations) are shown.

Table 3. Correlations between phyla and families of bacteria and body mass in females and males

Phylum or family	Females			Males		
	ρ	<i>N</i>	<i>P</i>	ρ	<i>N</i>	<i>P</i>
Anaeroplasmataceae	-0.307	18	0.007*	-0.127	18	0.902
Bacteroidetes	0.040	18	0.032*	-0.251	18	0.334
Cyanobacteria	-0.253	18	0.017*	-0.304	18	0.058
Muribaculaceae	0.038	18	0.030*	-0.280	18	0.092
Patiscibacteria	-0.098	18	0.024*	0.043	18	0.321
Proteobacteria	-0.201	18	0.017*	0.034	18	0.887
Saccharimonadaceae	-0.098	18	0.024*	0.043	18	0.321
Tannerellaceae	-0.015	18	0.024*	-0.185	18	0.896
Tenericutes	-0.089	18	0.026*	-0.155	18	0.280

Correlation coefficients (ρ), number of animals (*N*) and *P*-values (*P*) for correlations between the relative abundance of bacterial phyla and families and body mass of female and male hamsters following 9 weeks of treatment. An asterisk indicates a significant correlation ($P < 0.05$, Spearman's rank correlations). Only significant correlations are shown for females. The same correlations are shown for males as a comparison, but none of these associations were significant.

Fig. 7B) and relative abundance of Patiscibacteria ($\rho = 0.506$, $N = 14$, $P < 0.001$; Fig. 7C) in males at week 9. Similar associations were not found in females at week 9 (Table S6).

DISCUSSION

Previous work in our laboratory has demonstrated that seasonal changes in photoperiod cause a reduction in body mass, gonadal regression and increased aggression in SD-R Siberian hamsters. The 'seasonal switch' hypothesis, which suggests that Siberian hamsters switch from gonadal to adrenal regulation of aggression during SD conditions, helps to explain how SD-R hamsters are physiologically capable of increasing aggression during the non-breeding season. While it is unclear whether the gut microbiome plays a role in regulating this pathway, seasonal changes in the gut microbiome have been linked to decreases in body mass in SD-R male hamsters (Bailey et al., 2010). Several studies have provided evidence that the gut microbiome is sexually dimorphic, but the effect of photoperiod on the gut microbiome has yet to be studied in female hamsters. Here, we tested the hypothesis that photoperiodic changes in the gut microbiome affect circulating hormones and aggressive behavior in sex-specific ways.

We found that aggression increased in SD-R females, but not SD-R males at week 9. While photoperiod did not have an effect on overall gut microbial communities or beta diversity in either sex, it altered the relative abundance of specific bacterial phyla and

families in the gut microbiome in a sex-specific manner. In males, the relative abundance of the phylum Patiscibacteria increased over time, independent of treatment group; the relative abundance of the family Ruminococcaceae increased in SD-NR hamsters over time; and the relative abundance of the family Marinifilaceae increased in SD-R hamsters over time. In females, the relative abundance of the phylum Firmicutes and the family Ruminococcaceae increased in SD-R hamsters over time, and the relative abundance of the family Anaeroplasmataceae increased in SD-R hamsters following 3 weeks of treatment. Interestingly, the relative abundance of Patiscibacteria was correlated with aggression at week 9 in SD-NR males, but not in LD males, SD-R males or females. Additionally, the relative abundance of Anaeroplasmataceae was correlated with aggression at week 9 in SD-R females, but not in LD females, SD-NR females or males. Finally, while SD-R males and females showed no changes in serum DHEA levels, DHEA levels at week 9 were correlated with aggression in males but not females. Collectively, these findings suggest that the gut microbiome interacts with the brain and/or periphery to modulate increased non-breeding aggression in a sex-specific manner. Further studies are necessary, however, to investigate the potential mechanisms underlying seasonal changes in the gut microbiome, circulating hormones and aggressive behavior.

Effects of photoperiod on reproductive physiology and behavior

Consistent with previous studies, we found that exposure to SD photoperiods resulted in reductions in body mass and reproductive tissue mass in male and female SD-R hamsters relative to SD-NR and LD hamsters (Bailey et al., 2010; Jasnow et al., 2000; Navara et al., 2007; Rendon et al., 2015). In addition, we found that SD-R males and females exhibited increased aggression in response to photoperiodic treatment, though some of these changes were not statistically significant (Rendon et al., 2015; Scotti et al., 2007). It is possible we did not observe differences in some measures of aggression between treatment groups because of our smaller sample sizes compared with past studies (Bedrosian et al., 2012; Rendon et al., 2016).

Moreover, we did not find any effect of photoperiod on post-behavior serum DHEA levels following 9 weeks of treatment. While the 'seasonal switch' hypothesis proposes that SDs upregulate the hypothalamic-pituitary-adrenal (HPA) axis and increase serum DHEA, we have also shown that aggressive behavior alone can decrease DHEA levels in SD animals (presumably due to conversion to other biologically active steroids; Rendon and Demas,

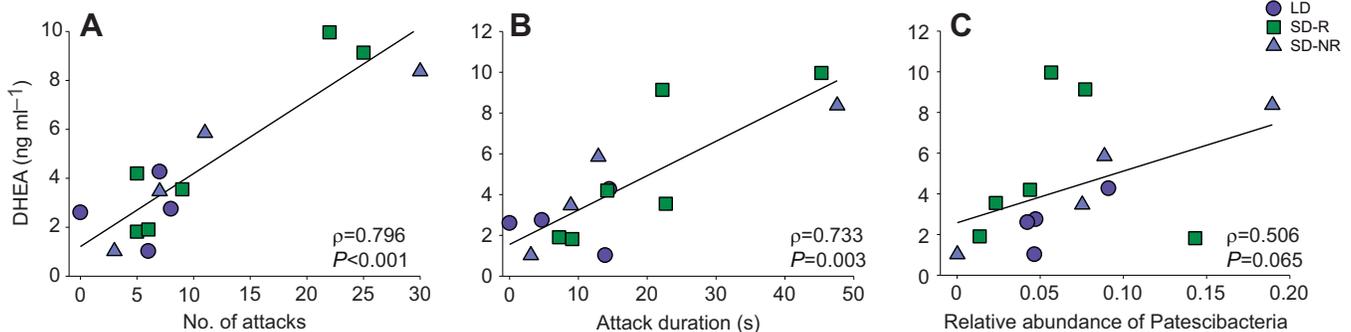


Fig. 7. Correlation between serum DHEA concentration and the gut microbiome and aggressive behavior in male hamsters following 9 weeks of photoperiodic treatment. (A,B) Serum DHEA concentration was significantly correlated with the number of attacks (A) and attack duration (B) in males. (C) The correlation between serum DHEA concentration and the relative abundance of Patiscibacteria in males tended towards significance. For each treatment group (LD: $N = 4$, SD-R: $N = 6$, SD-NR: $N = 4$), correlation coefficients (ρ) and *P*-values are shown (Spearman's rank correlations).

2016). Therefore, it is possible that SD-R hamsters had elevated DHEA concentrations prior to behavioral testing, and that engaging in aggressive behaviors decreased circulating DHEA in these animals relative to the other treatment groups. This physiological response may explain why serum DHEA levels appear unchanged after behavioral testing. Although basal DHEA levels were not measured in this study, the observed changes in post-behavior serum DHEA match those of previous studies (Rendon and Demas, 2016; Scotti et al., 2009). Collectively, these results suggest that DHEA acts as a precursor to increase circulating testosterone and E_2 in SD-R hamsters following an aggressive interaction. Furthermore, changes in serum DHEA concentration before and after behavioral testing suggest that there are rapid effects of steroid hormones on aggression (reviewed in Heimovics et al., 2015; Navara et al., 2007).

Seasonal changes and sexual dimorphism in the gut microbiome

Past studies suggest that there may be sex differences in the gut microbiome's response to photoperiod, though to varying degrees (Bailey et al., 2010; Davenport et al., 2014; Hieke et al., 2019; Wang et al., 2018). In the current study, photoperiod differentially affected bacterial diversity and the relative abundance of bacteria in the male and female gut, further suggesting that the gut microbiome may play a role in sex-specific seasonal changes. Specifically, at week 9, female but not male hamsters exhibited increased gut microbial diversity compared with earlier time points; and at week 3, the relative abundance of Anaeroplasmataceae was significantly higher in SD-R females than in LD and SD-NR females, but no differences were found in males. Interestingly, young female and male mice have similar gut microbial compositions, with the exception of the bacterial family Anaeroplasmataceae (Leclercq et al., 2017), suggesting the potential for a conserved sex-specific importance of Anaeroplasmataceae in the gut microbiome. Previous work suggests that bacteria from the family Anaeroplasmataceae aid in carbohydrate, purine and pyrimidine metabolism in some mammals (Petzel et al., 1989).

Moreover, the observed sex-specific response to photoperiodic treatment may be, in part, due to differences in sensitivity to environmental changes and stress. In CF-1 mice subjected to stress from restraint and forced swim sessions, *Ruminococcus gnavus* increased in females, but the opposite effects were found in males (Tsilimigras et al., 2018), suggesting that even short-term stressors can alter the profile of gut microbial communities in sex-specific ways (Bharwani et al., 2016; Desbonnet et al., 2015; Foster et al., 2017; Galley et al., 2014). In the current study, we found differences in the gut microbiome that were unrelated to changes in photoperiod (e.g. differences in the microbiome over time). Because differences in the gut microbiome over time were observed in all photoperiodic treatment groups, these results suggest that stress may have impacted the gut microbiome. Though previous work in our lab has shown that general handling and manipulation does not significantly impact the gut microbiome (Sylvia et al., 2017), future work should further investigate how the potential stress of photoperiodic treatment and repeated handling may affect the gut microbiome.

Not only does the microbiome change in response to photoperiod in SD-R males but also SD-NR males exhibit differences in the microbiome at week 9 (e.g. higher relative abundance of Ruminococcaeae). This finding suggests that SD-R and SD-NR males likely have distinct microbiota, and future work should investigate the mechanisms by which the microbiome of SD responders and non-responders differ over time.

Potential mechanisms mediating relationships among seasonal changes in photoperiod, the gut microbiome and aggression

In the current study, we found that the relative abundance of bacteria (e.g. Anaeroplasmataceae, Ruminococcaeae and Patescibacteria) was positively associated with aggression in a sex-specific manner. Specifically, we found that in females, the relative abundance of Anaeroplasmataceae, a family in the phylum Tenericutes, was positively associated with aggression in SD-R hamsters and negatively associated with body mass, but this same relationship was not found in males. Further, the relative abundance of Ruminococcaeae and Patescibacteria were positively associated with aggression in male but not female hamsters. These data suggest that Anaeroplasmataceae, Ruminococcaeae and Patescibacteria may play sex-specific roles in regulating seasonal changes in body mass and behavior.

Little is known, however, about the precise role of many bacteria in regulating behavior. For example, Patescibacteria are presumed to be either symbiotic or parasitic, suggesting the need for further investigation into the function of these specific microbes (Cui et al., 2019; Frey et al., 2016; Lopez-Fernandez et al., 2018; Sánchez-Osuna et al., 2017). We have previously shown that the relative abundance of Tenericutes is associated with increased aggression in male and female Siberian hamsters treated with a broad-spectrum antibiotic (Sylvia et al., 2017), suggesting that shifts in the relative abundance of these families and phyla (induced by either photoperiodic changes or antibiotics) are likely linked to aggression. Other Tenericutes (e.g. *Anaeroplasmata*) have been associated with increased levels of immunoglobulin A (Beller et al., 2019), suggesting that the immune system may also play a role in the mechanisms regulating aggression. Interestingly, Ruminococcaeae, a family in the phylum Firmicutes, has also been associated with immunoglobulin A production and anti-inflammatory properties (Dowhaniuk et al., 2019; Ingham et al., 2019). However, Ruminococcaeae is best known for its role in producing butyrate, a short-chain fatty acid (SCFA) that serves as the main energy source of colonocytes, reduces inflammation and regulates gene expression (Koh et al., 2016; Zhuang et al., 2019). Increased SCFA levels from a high carbohydrate diet increase aggressive and anxiety-like behaviors in rats, suggesting that microbial metabolites may also contribute to aggressive behavior (Hanstock et al., 2004; reviewed in MacFabe, 2015). The link between microbial metabolites, immune mediators and behavior is further supported by *Bacteroides fragilis*, a species within the phylum Bacteroidetes (associated with investigation and grooming in males in the current study) that alters gut metabolites, targets intestinal junctions through cytokines and modulates anxiety-like, locomotor and social behavior (Desbonnet et al., 2014; Hsiao et al., 2013; Sharon et al., 2014). Finally, because melatonin plays an important role in modulating seasonal changes in physiology and behavior (Jasnow et al., 2000; Rendon et al., 2015) and melatonin has been shown to influence the gut lining (Sommansson et al., 2013), it too may mediate seasonal shifts in the gut microbiome. Collectively, these findings suggest that seasonal changes in the gut microbiome may modulate aggression via peripheral and/or central neuroendocrine mechanisms, and further investigation into the function of specific microbes is needed.

Conclusions

The current study provides evidence that photoperiod is related to sex-specific changes in the gut microbiome and aggression and that the gut microbiome may be one component of the 'seasonal switch' hypothesis. In addition, our data support the idea that several

interconnected systems contribute to seasonal changes in aggression, including the brain, immune mediators (e.g. cytokines), gut hormones and microbial metabolites. We are just beginning to understand the potential benefits of microbiome-targeted human therapies, and continued study of the mechanisms underlying aggressive behavior will likely improve such therapeutic treatments. Taken together, our research sheds important light on an area of research that is critical for understanding the basic mechanisms regulating seasonal shifts in physiology and behavior.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.C.R., K.E.S., J.E.D., G.E.D.; Methodology: C.C.R., K.E.S., J.E.D., G.E.D.; Software: C.C.R., K.E.S., K.M.M.; Validation: C.C.R., K.E.S., K.M.M.; Formal analysis: C.C.R., K.E.S., K.M.M.; Investigation: C.C.R., K.E.S., K.M.M., J.E.D., S.G.H., M.P.V.; Resources: K.E.S., K.M.M., J.E.D., G.E.D.; Data curation: C.C.R.; Writing - original draft: C.C.R.; Writing - review & editing: C.C.R., K.E.S., K.M.M., J.E.D., S.G.H., M.P.V., G.E.D.; Visualization: C.C.R., K.E.S., K.M.M.; Supervision: K.E.S., K.M.M., J.E.D., G.E.D.; Project administration: C.C.R., K.E.S., J.E.D., G.E.D.; Funding acquisition: C.C.R., K.E.S., K.M.M., G.E.D.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.212548.supplemental>

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