

Research



Cite this article: Munley KM, Trinidad JC, Demas GE. 2022 Sex-specific endocrine regulation of seasonal aggression in Siberian hamsters. *Proc. R. Soc. B* **289**: 20220668. <https://doi.org/10.1098/rspb.2022.0668>

Received: 11 April 2022

Accepted: 23 August 2022

Subject Category:

Behaviour

Subject Areas:

behaviour, physiology, neuroscience

Keywords:

androgens, adrenal glands, biological rhythms, melatonin, neurosteroids, territoriality

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.6169755>.

Sex-specific endocrine regulation of seasonal aggression in Siberian hamsters

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Coordinating physiological and behavioural processes across the annual cycle is essential in enabling individuals to maximize fitness. While the mechanisms underlying seasonal reproduction and its associated behaviours are well characterized, fewer studies have examined the hormonal basis of non-reproductive social behaviours (e.g. aggression) on a seasonal time scale. Our previous work suggests that the pineal hormone melatonin facilitates a 'seasonal switch' in neuroendocrine regulation of aggression in male and female Siberian hamsters (*Phodopus sungorus*), specifically by acting on the adrenal glands to increase the production of the androgen dehydroepiandrosterone (DHEA) during the short-day (SD) photoperiods of the non-breeding season. Here, we provide evidence that the activity of 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (3 β -HSD), a key enzyme within the steroidogenic pathway that mediates DHEA synthesis and metabolism, varies in a sex-specific and melatonin-dependent manner. Although both male and female hamsters displayed increased aggression in response to SDs and SD-like melatonin, only males showed an increase in adrenal 3 β -HSD activity. Conversely, SD and melatonin-treated females exhibited reductions in both adrenal and neural 3 β -HSD activity. Collectively, these results suggest a potential role for 3 β -HSD in modulating non-breeding aggression and, more broadly, demonstrate how distinct neuroendocrine mechanisms may underlie the same behavioural phenotype in males and females.

1. Introduction

Many species face marked fluctuations in their environment on a seasonal basis, which alter the accessibility of resources that are critical for maximizing fitness. Consequently, natural selection has favoured the evolution of physiological and behavioural adaptations that allow individuals to prioritize investing in reproduction or survival across the annual cycle [1]. To mediate these energetic trade-offs, animals use environmental cues to predict seasonal conditions and adjust their physiology and behaviour accordingly [2]. Although many factors vary on a seasonal time scale, photoperiod (i.e. day length) is a relatively 'noise-free' cue from which species can coordinate these adaptations with the appropriate time of year. In mammals, the hormone melatonin serves as the biochemical signal from which animals modulate seasonal rhythms. Photoperiod is converted from an environmental cue into a biochemical signal through a complex pathway, which culminates in the release of melatonin by the pineal gland. Because melatonin secretion is high at night and low during the day, the pattern and duration of melatonin production transmits information about day length to the brain and peripheral tissues that are sensitive to melatonin [3]. Although the role of melatonin in regulating seasonal reproduction is well characterized, relatively fewer studies have examined how melatonin controls other seasonal social behaviours, such as aggression.

Aggressive behaviour allows animals to compete with conspecifics for access to limited resources in their habitat [4]. Thus, many species display

high levels of aggression during the breeding season, when obtaining a mate and defending a territory is essential for enhancing an individual's chances of reproductive success. To date, the majority of research investigating the proximate mechanisms regulating aggressive behaviour have focused on the role of gonadal steroids (e.g. testosterone (T) and 17 β -oestradiol (E₂)) in modulating aggression during the breeding season [5]. Some species, however, are equally or more aggressive during the non-breeding season despite gonadal regression, suggesting that they face additional selective pressures that favoured the evolution of alternative, gonadal-independent mechanisms to modulate aggressive behaviour across the annual cycle. There is considerable evidence that these animals use extra-gonadal sources of steroid hormones, including adrenal steroids (e.g. dehydroepiandrosterone (DHEA)) and neurosteroids (i.e. steroids synthesized *de novo* in the brain), to regulate aggressive behaviour [6]. Indeed, seasonal plasticity in steroid concentrations, steroidogenic enzymes and steroid receptors have been documented in the brain and peripheral tissues of several songbirds and rodents that show high levels of non-breeding aggression, including song sparrows (*Melospiza melodia*), spotted antbirds (*Hylophylax n. naevioides*), beach mice (*Peromyscus polionotus*), deer mice (*Peromyscus maniculatus*) and Siberian hamsters (*Phodopus sungorus*) [7–10].

Our group has investigated the role of melatonin in regulating seasonal aggression in Siberian hamsters, a solitary species in which both males and females exhibit increased aggression during the non-breeding season [11,12]. Specifically, our work suggests that melatonin controls aggressive behaviour by coordinating a 'seasonal switch' from gonadal regulation of aggression during the breeding season to adrenal regulation of aggression during the non-breeding season [6]. We have shown that LD male and female hamsters given timed melatonin injections, which mimic an SD-like melatonin signal in circulation, show higher levels of aggression than LD hamsters given control injections and exhibit SD-like variation in baseline and aggression-induced circulating androgen and oestrogen levels [13,14]. We have also demonstrated that timed melatonin administration and exposure to SDs decreases concentrations of DHEA, T and E₂ in brain regions associated with aggression in male hamsters, including the anterior hypothalamus (AH) and periaqueductal gray (PAG) [15]. Recently, we determined that lentiviral-mediated overexpression of the MT₁ melatonin receptor, the primary melatonin receptor in Siberian hamsters, in the adrenal glands causes SD-like increases in aggression in male hamsters, but does not alter circulating DHEA levels [16], which could suggest that adrenal MT₁ receptors regulate seasonal aggression directly via neural substrates. There is also emerging evidence of a role for melatonin in modulating aggressive behaviour in other seasonal and non-seasonal mammals, including California mice (*Peromyscus californicus*), greater long-tailed hamsters (*Tscheskia triton*), house mice (*Mus musculus*) and humans [17–20]. While these findings suggest that melatonin modulates aggression and steroid concentrations in the brain and periphery on a seasonal time scale, the roles of adrenal and neural steroids in contributing to increased aggression are unclear.

In the current study, we tested the hypothesis that melatonin increases non-breeding aggression by altering the activity of steroidogenic enzymes critical for the conversion of DHEA to biologically active steroids in the adrenal glands and brain of Siberian hamsters. Specifically, we characterized

seasonal and melatonin-dependent changes in the activity of 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (3 β -HSD), a key enzyme within the 'seasonal switch' pathway that converts pregnenolone to progesterone and DHEA to androstenedione and, thus, plays a critical role in mediating DHEA synthesis and metabolism. We housed male and female hamsters in LD or SD photoperiods and administered timed melatonin injections to a subset of LD hamsters, which summate with the endogenous melatonin profile of these animals to mimic an SD-like signal. Following 10 weeks of treatment, we measured aggressive and non-aggressive social behaviours (e.g. investigation, self-grooming). Moreover, we quantified the synthesis of progesterone and androstenedione via 3 β -HSD in the adrenal glands and the AH and PAG, two brain regions associated with aggression which we have previously shown to exhibit seasonal and melatonin-dependent changes in neurosteroid levels in male hamsters [15,21], using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We hypothesized that melatonin mediates increased aggression during the non-breeding season by elevating adrenal DHEA secretion and the conversion of prohormones (i.e. pregnenolone and DHEA) to biologically active steroids (i.e. androstenedione, T and E₂) in the brain. Thus, we predicted that male and female hamsters exhibiting an SD-like melatonin signal, either via timed melatonin administration or exposure to SDs, will display higher levels of aggression and will show increases in adrenal progesterone synthesis and neural androstenedione synthesis.

2. Methods

(a) Experimental animals

Adult male and female Siberian hamsters (*Phodopus sungorus*, greater than 60 days of age) were raised and maintained in a breeding colony under long days (16 L:8 D h), group-housed at weaning, and given *ad libitum* access to standard laboratory rodent chow (Teklad global 18% protein diet; Envigo, Madison, WI, USA) and tap water. Ambient temperature was 20 \pm 2°C, and relative humidity was 55 \pm 5%.

(b) Photoperiodic manipulations and melatonin administration

Hamsters (males: $n = 26$, females: $n = 26$) were individually housed for a two-week acclimation period under LDs. Hamsters were then transferred to short days (SDs; males: $n = 12$, females: $n = 12$; 8 L: 16 D h) or kept in LDs (males: $n = 14$, females: $n = 14$). Melatonin profiles were manipulated in a subset of LD hamsters (LD-M; males: $n = 8$, females: $n = 8$), which were administered timed subcutaneous injections of melatonin (15 μ g day⁻¹ USP Reference Standard; Sigma Aldrich, St. Louis, MO, USA) dissolved in 1:10 ethanol:saline solution [16]. All remaining hamsters (males: $n = 18$, females: $n = 18$) were given daily injections of control (1:10 ethanol:saline) solution. Injections were administered 2 h before lights out, which extended the LD pattern of endogenous melatonin secretion to mimic that of SD hamsters [22]. Hamsters remained in photoperiodic treatments and were given injections for 10 weeks.

(c) Seasonal phenotypes

Following 10 weeks of treatment, seasonal phenotypes were determined based on *a priori* criteria for this species [7] (electronic supplementary material). LD hamsters had functional

reproductive tissues and displayed no significant change in body mass. Conversely, LD-M and SD hamsters (males: $n = 8$; females: $n = 8$) had regressed reproductive tissues and showed a significant reduction in body mass (greater than 4%). A subset of SD hamsters (males: $n = 4$, 33.3%; females: $n = 4$, 33.3%) failed to respond to photoperiodic treatment and were classified as 'non-responders' using the same criteria described above for LD hamsters. These hamsters were excluded from the study due to insufficient numbers for statistical analysis. Non-responsiveness to SDs has been previously documented in this species and typically affects between 10 and 50% of the population [7,15,23].

(d) Behavioural testing

After melatonin and photoperiodic treatment, social behaviour was measured using a same-sex resident-intruder paradigm [13] (electronic supplementary material). Aggressive (i.e. latency to first attack, number and duration of attacks and chases, aggression score (number of 5 s intervals during which attacking and/or chasing behaviour was displayed)) and non-aggressive social behaviours (i.e. frequency and duration of nose-to-nose investigations, anogenital investigations and self-grooming) were scored using BORIS v. 7.10.2 [24]. Principal component analyses were used to generate a composite 'aggression score' (PC_{Agg}) and to generate a composite 'investigation score' (PC_{Inv}) for both sexes. The first principal component of each analysis explained a significant proportion of the total variance (PC_{Agg} : 63.2%, PC_{Inv} : 63.6%), had a large eigenvalue (greater than 2.5) and was strongly loaded by all variables included in each analysis (i.e. loading values < -0.3 or greater than 0.3; electronic supplementary material, table S1).

(e) Tissue collection and processing

Tissues were collected following behavioural testing (electronic supplementary material). Brains were cut into coronal sections (thickness: 250 μm) using a Leica CM 1850 cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on microscope slides. The AH and PAG were microdissected from both hemispheres using Miltex disposable biopsy punches (1 mm diameter; Integra LifeSciences, Plainsboro, NJ, USA). Microdissected punches of the AH (2.208 ± 0.059 mg) and PAG (2.555 ± 0.050 mg) were placed into polypropylene microtubes and stored at -80°C . Brain sections were Nissl stained and imaged using a Motic EasyScan digital slide scanner (Kowloon, Hong Kong) to verify the locations of microdissected punches.

(f) *In vitro* measurement of 3β -HSD activity

The *in vitro* conversions of isotopically labelled pregnenolone (PREG) to progesterone (PROG) and isotopically labelled DHEA to androstenedione (AE) via 3β -HSD were measured in adrenal and brain tissue using a previously published incubation assay with minor modifications [25]. Tissues were homogenized in 200 μl cold sucrose-phosphate buffer using a bead-mill homogenizer (Bead Ruptor 24 Elite; Omni International, Kennesaw, GA, USA) and centrifuged for 30 min at $1442\times g$ at 4°C . Supernatants (adrenals: 16 μl , brain: 160 μl) were incubated with PREG- c_2d_2 and DHEA- d_2 (adrenals: 8 mM, brain: 500 μM) and the 3β -HSD cofactor β -nicotinamide adenine dinucleotide (NAD^+ , 1 mM). In addition, abiraterone acetate and fadrozole hydrochloride (pharmacological inhibitors of cytochrome P450c17 and aromatase, 5 $\mu\text{g ml}^{-1}$ each) were added to protect formed PROG- c_2d_2 and AE- d_2 from further metabolism. Samples were incubated (adrenals: 3 h, brain: 6 h) at 37°C with shaking (speed: 60 rpm). Reactions were terminated by flash freezing in dry ice, and samples were stored at -80°C . Total protein content was determined via the Bradford assay (electronic supplementary material).

(g) LC-MS/MS analysis

Isotopically labelled analogues of PROG and AE (PROG- d_9 and AE- d_7 in 20% acetonitrile with 0.1% formic acid, 50 nM each) were added to each sample and used to quantify PROG- c_2d_2 and AE- d_2 concentrations. Steroids were extracted from tissue homogenates using an acetonitrile-based solid phase extraction protocol (Agilent Technologies, Santa Clara, CA, USA) [15]. Concentrations of PROG- c_2d_2 , AE- d_2 , their isotopically labelled analogues (PROG- d_9 , AE- d_7), their endogenous analogues (PROG, AE; to confirm endogenous PROG and AE production in tissue homogenates), and their isotopically labelled precursors (PREG- c_2d_2 , DHEA- d_2) were measured using an Easy NanoLC 1200 HPLC coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, WA, USA; electronic supplementary material). Data were analysed using the Xcalibur Quan browser v. 2.2 (Thermo Fisher Scientific, Waltham, WA, USA). PROG- c_2d_2 and AE- d_2 concentrations were determined via isotopic internal standard quantification (electronic supplementary material). 3β -HSD enzymatic activity was calculated by dividing the amount of PROG- c_2d_2 and AE- d_2 generated by tissue homogenates during the incubation assay by the total protein content. MS/MS transitions for isotopically labelled and endogenous steroids, accuracy and precision measurements for the LC-MS/MS protocol and 3β -HSD activity assay, and representative extracted ion chromatographs are available in the electronic supplementary material, (tables S2–S4, figures S3–S4).

(h) Statistical analyses

Statistical testing was performed using R v. 4.1.2 [26], and statistical significance was attributed at $p < 0.05$ after controlling for false discovery rate when performing multiple comparisons. A two-way analysis of variance (ANOVA) was used to assess the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on percentage change in body mass, and one-way ANOVAs were used to determine the effects of melatonin and photoperiodic treatment on reproductive tissue mass. Permutational multivariate analyses of variance (PERMANOVAs) with 999 permutations were used to evaluate the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on aggression, investigation and self-grooming based on Euclidean distances. Two-way multivariate analyses of variance (MANOVAs) were used to assess the effects of melatonin and photoperiodic treatment, sex, and the interaction between treatment and sex on PROG synthesis, AE synthesis, and the ratio of AE:PROG synthesis across tissues and brain regions. If a multivariate test reported a significant effect or a trend towards a significant effect ($p < 0.10$) of treatment, sex, and/or the interaction between treatment and sex for one or more of the variables included in an analysis, two-way ANOVAs or generalized linear models (GLMs) and *post-hoc* testing (Tukey's HSD tests for two-way ANOVAs and Dunn's tests for GLMs) were conducted to examine pairwise comparisons (electronic supplementary material) [15,27].

3. Results

(a) Seasonal phenotypes

As expected, timed melatonin administration and exposure to SDs induced characteristic changes in body mass and reproductive physiology in male and female hamsters (electronic supplementary material, table S5). There was a significant effect of treatment, but there was no effect of sex or the interaction between treatment and sex on percent change in body mass ($F_{3,40} = 4.180$, $p = 0.004$, $R^2 = 0.355$; treatment: $p < 0.001$, $\eta^2 = 0.349$; sex: $p = 0.696$, $\eta^2 = 0.004$; interaction: $p = 0.843$, $\eta^2 = 0.009$). Specifically, LD-M and SD hamsters showed a

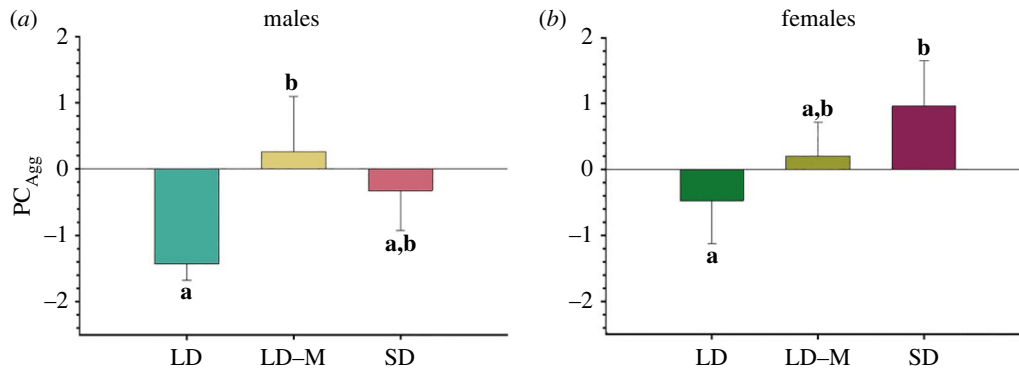


Figure 1. Male and female hamsters given timed melatonin injections and exposed to short days showed increased aggression. (a,b) Composite aggression score (PC_{Agg}). LD, long-day hamsters; LD-M, long-day melatonin hamsters; SD, short-day hamsters. Bar heights represent means \pm SEM (males: $n = 5-8$; females: $n = 6-8$). Different letters indicate a significant difference between treatment groups within each sex ($p < 0.05$). (Online version in colour.)

significant decrease in percent change in body mass relative to LD hamsters ($p \leq 0.035$). In addition, timed melatonin injections and exposure to SDs induced gonadal regression; LD-M and SD males exhibited a significant reduction in paired testes mass ($F_{2,18} = 21.41$, $p < 0.001$, $R^2 = 0.704$, $\eta^2 = 0.653$) and LD-M and SD females showed a significant reduction in paired ovarian mass ($F_{2,16} = 4.288$, $p = 0.032$, $R^2 = 0.349$, $\eta^2 = 0.349$) compared to LD males and females.

(b) Social behaviour

Hamsters treated with timed melatonin injections or SDs displayed higher levels of aggression than LD hamsters, regardless of sex (figure 1). There was a trend towards an effect of treatment, but there was no effect of sex or the interaction between treatment and sex on PC_{Agg} (aggression (all measures) – treatment: $F_{1,42} = 4.928$, $p = 0.026$, $R^2 = 0.105$; sex: $F_{1,42} = 1.044$, $p = 0.306$, $R^2 = 0.024$; interaction: $F_{2,41} = 3.044$, $p = 0.056$, $R^2 = 0.129$; PC_{Agg} – $F_{3,39} = 1.554$, $p = 0.216$, $R^2 = 0.107$; treatment: $p = 0.092$, $\eta^2 = 0.071$; sex: $p = 0.204$, $\eta^2 = 0.041$; interaction: $p = 0.696$, $\eta^2 = 0.004$; electronic supplementary material, tables S6–S7). LD-M males had a significantly higher PC_{Agg} than LD males ($p = 0.019$), and SD males trended towards an increase in PC_{Agg} relative to LD males ($p = 0.076$; figure 1a). Likewise, SD females exhibited a significant increase in PC_{Agg} compared to LD females ($p = 0.048$), whereas LD-M females had a PC_{Agg} which was intermediate to that of LD and SD females (LD versus LD-M females: $p = 0.203$; LD-M versus SD females: $p = 0.341$; figure 1b). Similar patterns were observed for individual measures of aggressive behaviour, in which LD-M and SD hamsters generally showed increased aggression relative to LD hamsters (e.g. number of attacks and chases, aggression frequency, aggression score; electronic supplementary material, Tables S7–S8). Conversely, there was no effect of treatment, sex, or the interaction between treatment and sex on investigation (treatment: $F_{1,42} = 0.294$, $p = 0.720$, $R^2 = 0.007$; sex: $F_{1,42} = 0.893$, $p = 0.393$, $R^2 = 0.021$; interaction: $F_{2,41} = 0.585$, $p = 0.631$, $R^2 = 0.028$) or self-grooming (treatment: $F_{1,42} = 0.379$, $p = 0.546$, $R^2 = 0.009$; sex: $F_{1,42} = 1.277$, $p = 0.271$, $R^2 = 0.030$; interaction: $F_{2,41} = 0.820$, $p = 0.478$, $R^2 = 0.038$; electronic supplementary material, table S6).

(c) Adrenal 3β -HSD activity

Treatment with melatonin and SDs altered adrenal 3β -HSD enzymatic activity in a sex-specific manner (figure 2). There

was a significant effect of sex, but there was no effect of treatment or the interaction between treatment and sex on PROG synthesis in the adrenal glands (PROG synthesis (all tissues) – treatment: $F_{3,30} = 0.159$, $p = 0.923$, $\eta^2 = 0.032$; sex: $F_{3,30} = 3.355$, $p = 0.032$, $\eta^2 = 0.251$; interaction: $F_{3,30} = 0.573$, $p = 0.637$, $\eta^2 = 0.054$; PROG synthesis in adrenal glands – $F_{3,40} = 3.283$, $p = 0.030$, $R^2 = 0.198$; treatment: $p = 0.776$, $\eta^2 = 0.005$; sex: $p = 0.006$, $\eta^2 = 0.176$; interaction: $p = 0.237$, $\eta^2 = 0.027$; electronic supplementary material, tables S9–S10). LD-M males showed a significant increase in adrenal PROG synthesis relative to LD males ($p = 0.042$), and SD males trended towards an increase in adrenal PROG synthesis relative to LD males ($p = 0.095$). Conversely, LD-M females exhibited a significant reduction in adrenal PROG synthesis compared to LD females ($p = 0.028$), and SD females trended towards a decrease in adrenal PROG synthesis compared to LD females ($p = 0.089$; figure 2a,b). Furthermore, there was a significant effect of sex, but there was no effect of treatment or the interaction between treatment and sex on AE synthesis in the adrenal glands (AE synthesis (all tissues) – treatment: $F_{3,37} = 1.082$, $p = 0.369$, $\eta^2 = 0.080$; sex: $F_{3,37} = 7.832$, $p < 0.001$, $\eta^2 = 0.388$; interaction: $F_{3,37} = 0.080$, $p = 0.971$, $\eta^2 = 0.006$; AE synthesis in adrenal glands – $F_{3,40} = 5.155$, $p = 0.004$, $R^2 = 0.279$; treatment: $p = 0.550$, $\eta^2 = 0.025$; sex: $p = 0.001$, $\eta^2 = 0.243$; interaction: $p = 0.285$, $\eta^2 = 0.038$; electronic supplementary material, tables S9–S10). LD-M and SD males showed elevated adrenal AE synthesis relative to LD males ($p \leq 0.036$), whereas there was no significant difference in adrenal AE synthesis between treatment groups in females ($p \geq 0.458$; figure 2c,d). There was also a trend towards an effect of sex, but there was no effect of treatment or the interaction between treatment and sex on the ratio of AE:PROG synthesis in the adrenal glands (AE:PROG synthesis (all tissues) – treatment: $F_{3,28} = 1.120$, $p = 0.328$, $\eta^2 = 0.128$; sex: $F_{3,28} = 2.945$, $p = 0.050$, $\eta^2 = 0.183$; interaction: $F_{3,28} = 0.798$, $p = 0.506$, $\eta^2 = 0.079$; AE:PROG synthesis in adrenal glands – $F_{3,39} = 0.701$, $p = 0.557$, $R^2 = 0.051$; treatment: $p = 0.470$, $\eta^2 = 0.004$; sex: $p = 0.076$, $\eta^2 = 0.046$; interaction: $p = 0.879$, $\eta^2 = 0.002$; electronic supplementary material, tables S9–S10). LD-M females showed a significant increase in adrenal AE:PROG synthesis ratio compared to LD females ($p = 0.017$), and SD females trended towards an increase in adrenal AE:PROG synthesis ratio compared to LD females ($p = 0.092$). There was no significant difference in adrenal AE:PROG synthesis ratio, however, between treatment groups in males ($p \geq 0.842$; electronic supplementary material, table S11).

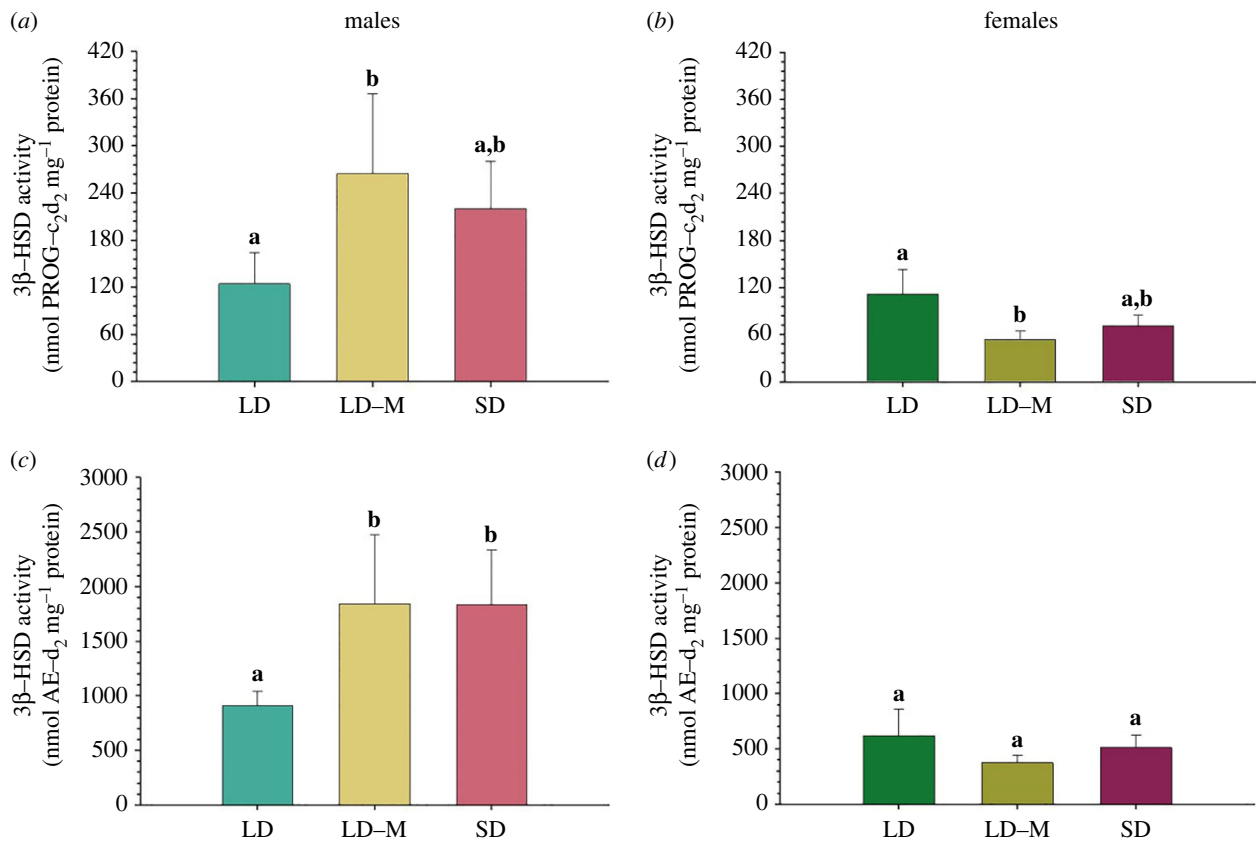


Figure 2. Timed melatonin injections and short days elevated adrenal 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) activity in male hamsters, but reduced progesterone synthesis in female hamsters. (a,b) Progesterone (PROG) synthesis and (c,d) androstenedione (AE) synthesis via 3β-HSD in the adrenal glands. LD, long-day hamsters; LD-M, long-day melatonin hamsters; SD, short-day hamsters. Bar heights represent means ± SEM (males: $n = 6-8$; females: $n = 6-8$). Different letters indicate a significant difference between treatment groups within each sex ($p < 0.05$). (Online version in colour.)

(d) Neural 3β-HSD activity

As observed for the adrenal glands, seasonal and melatonin-dependent changes in neural 3β-HSD activity varied between the sexes (figure 3). There were trends towards an effect of treatment and the interaction between treatment and sex, but there was no effect of sex on PROG synthesis in the AH ($F_{3,38} = 3.103$, $p = 0.038$, $R^2 = 0.197$; treatment: $p = 0.064$, $\eta^2 = 0.102$; sex: $p = 0.236$, $\eta^2 = 0.036$; interaction: $p = 0.072$, $\eta^2 = 0.089$). There was also a trend towards an effect of treatment, but there was no effect of sex or the interaction between treatment and sex on AE synthesis in AH ($F_{3,40} = 1.657$, $p = 0.192$, $R^2 = 0.111$; treatment: $p = 0.053$, $\eta^2 = 0.087$; sex: $p = 0.429$, $\eta^2 = 0.015$; interaction: $p = 0.572$, $\eta^2 = 0.010$; electronic supplementary material, table S10). LD-M and SD females displayed significant reductions in PROG and AE synthesis in the AH relative to LD females ($p \leq 0.047$), but there were no significant differences in PROG or AE synthesis between treatment groups in males ($p \geq 0.497$; figure 3a-d). Moreover, there was a significant effect of sex, but there was no effect of treatment or the interaction between treatment and sex on the ratio of AE:PROG synthesis in the AH ($F_{3,37} = 2.050$, $p = 0.124$, $R^2 = 0.143$; treatment: $p = 0.884$, $\eta^2 = 0.004$; sex: $p = 0.031$, $\eta^2 = 0.125$; interaction: $p = 0.600$, $\eta^2 = 0.022$; electronic supplementary material, table S10). SD females had a significantly higher AE:PROG synthesis ratio in the AH than LD females ($p = 0.041$), and LD-M females trended towards an increase in AE:PROG synthesis ratio compared to LD females ($p = 0.074$). There was no significant difference in AE:PROG synthesis ratio in the AH, however, between treatment groups in males ($p \geq 0.832$; electronic supplementary material, table S11).

By contrast, there was no effect of treatment, sex, or the interaction between treatment and sex on PROG and AE synthesis in the PAG (PROG synthesis – $F_{3,32} = 1.639$, $p = 0.200$, $R^2 = 0.133$; treatment: $p = 0.384$, $\eta^2 = 0.027$; sex: $p = 0.326$, $\eta^2 = 0.031$; interaction: $p = 0.470$, $\eta^2 = 0.020$; AE synthesis – $F_{3,39} = 0.879$, $p = 0.460$, $R^2 = 0.063$; treatment: $p = 0.758$, $\eta^2 = 0.005$; sex: $p = 0.151$, $\eta^2 = 0.056$; interaction: $p = 0.893$, $\eta^2 = 0.004$; figures 3a-d; electronic supplementary material, table S10). Interestingly, although there were no differences in PROG or AE synthesis in the PAG in either sex, there was a trend towards an effect of sex, but there was no effect of treatment or the interaction between treatment and sex on the ratio of AE:PROG synthesis in the PAG ($F_{3,30} = 0.333$, $p = 0.802$, $R^2 = 0.032$; treatment: $p = 0.801$, $\eta^2 = 0.004$; sex: $p = 0.091$, $\eta^2 = 0.072$; interaction: $p = 0.248$, $\eta^2 = 0.029$; electronic supplementary material, table S10). SD females had a significantly higher AE:PROG synthesis ratio in the PAG than LD females ($p = 0.045$), but a similar trend was not observed in LD-M females ($p = 0.296$). Conversely, there was no significant difference in AE:PROG synthesis ratio in the PAG between treatment groups in males ($p \geq 0.482$; electronic supplementary material, table S11).

4. Discussion

In the present study, we investigated the role of 3β-HSD, a critical steroidogenic enzyme within the ‘seasonal switch’ pathway that converts DHEA to biologically active steroids, in regulating seasonal aggression in Siberian hamsters. Consistent with previous studies, we showed that male and

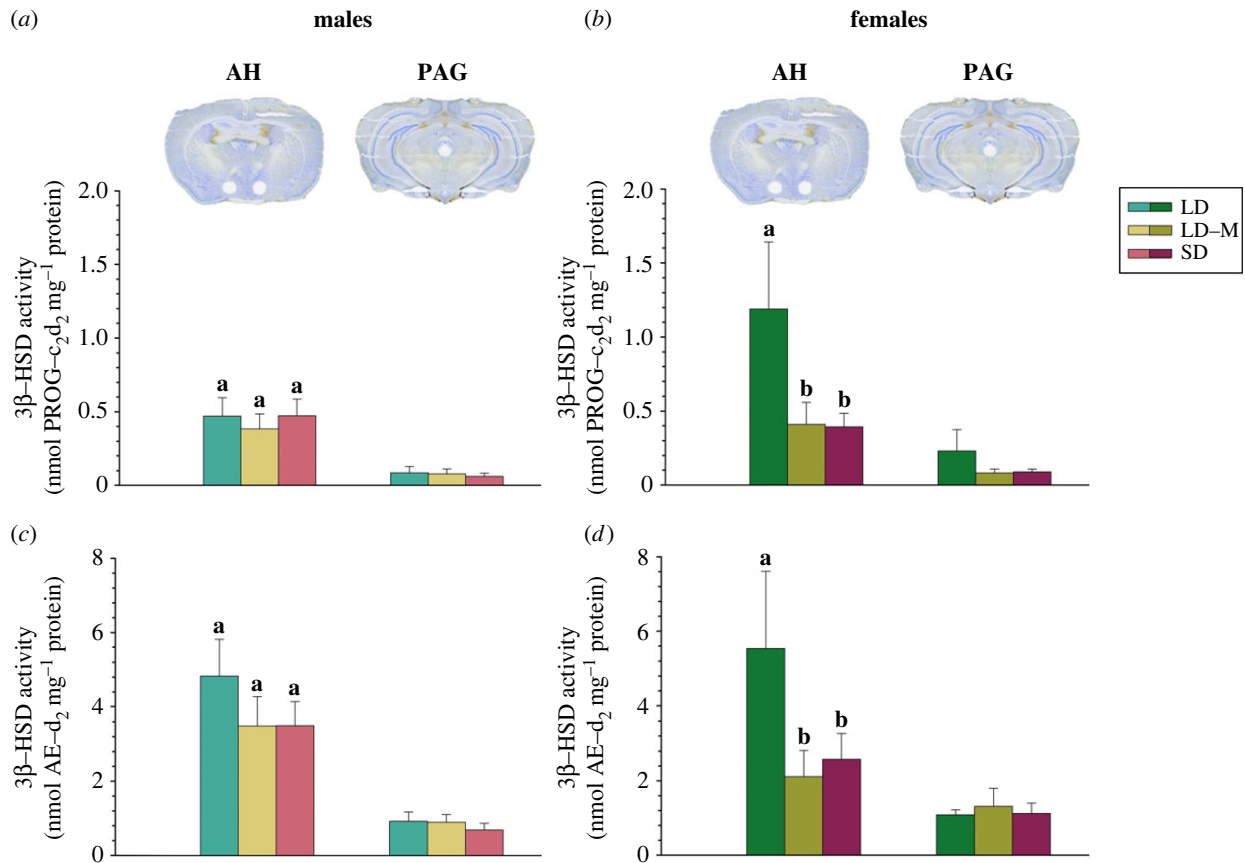


Figure 3. Treatment with melatonin and short days caused sex-specific changes in neural 3β-hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3β-HSD) activity. (a,b) Progesterone (PROG) synthesis and (c,d) androstenedione (AE) synthesis via 3β-HSD in the anterior hypothalamus (AH) and periaqueductal gray (PAG). LD, long-day hamsters; LD-M, long-day melatonin hamsters; SD, short-day hamsters. Bar heights represent means \pm SEM (males: $n = 5$ – 8 ; females: $n = 4$ – 8). Different letters indicate a significant difference between treatment groups within each sex ($p < 0.05$). (Online version in colour.)

female hamsters exhibiting a SD-like melatonin signal, either via timed melatonin administration or exposure to SDs, showed characteristic changes in reproductive physiology and behaviour, including gonadal regression, a reduction in body mass and increased aggression. Moreover, we demonstrated profound sex-specific effects of melatonin treatment and SDs on adrenal and neural 3β-HSD activity, in which LD-M and SD males exhibited an increase in adrenal 3β-HSD activity, whereas LD-M and SD females displayed reductions in adrenal and neural 3β-HSD activity. Collectively, these results demonstrate that male and female hamsters exhibit different seasonal and melatonin-dependent changes in 3β-HSD activity and highlight how distinct neuroendocrine responses may underlie the same behavioural phenotype (i.e. increased aggression) in males and females. To our knowledge, our study is the first to provide evidence that seasonal and sex differences in steroidogenic enzyme activity may underlie the same behavioural phenotype and show that these mechanisms are mediated by melatonin.

As expected, we found that timed melatonin administration caused SD-like reductions in body and reproductive tissue mass in male and female hamsters. Furthermore, these treatments induced an increase in aggressive behaviour, but did not affect non-aggressive social behaviours (i.e. investigation and self-grooming). Notably, the effects of melatonin treatment and SDs on body mass, reproductive tissue mass, and social behaviour were of a similar magnitude in both sexes, suggesting that seasonal plasticity in reproductive

physiology and behaviour are melatonin-dependent and produce comparable effects in males and females. These data are consistent with our prior work, which showed that timed melatonin injections induce gonadal regression and cause a reduction in body mass in male and female Siberian hamsters [13,14]. These results are also in agreement with previous studies in seasonally breeding rodents, which demonstrated that timed melatonin administration elevates territorial aggression in LD male California mice, male Syrian hamsters (*Mesocricetus auratus*), and male and female Siberian hamsters [7,15,17,28] and that melatonin implants increase social dominance in LD male greater long-tailed hamsters [18]. The importance of melatonin in regulating aggression, but not other non-aggressive social behaviours, underscores the importance of this behaviour in enhancing fitness in males and females of this species. Although the function of SD increases in aggression is still being investigated, we recently showed that food restriction elevates aggressive behaviour in males and females housed in an intermediate photoperiod, which mimics the transition period between the breeding and non-breeding seasons [29], suggesting that increased non-breeding aggression in this species is driven, at least in part, by food availability. Thus, high levels of non-breeding aggression may confer an evolutionary advantage to males and females during a time of the year when food availability is relatively low and competition for limited resources (e.g. territories) is high.

Importantly, we found that male and female hamsters show distinct seasonal and melatonin-dependent

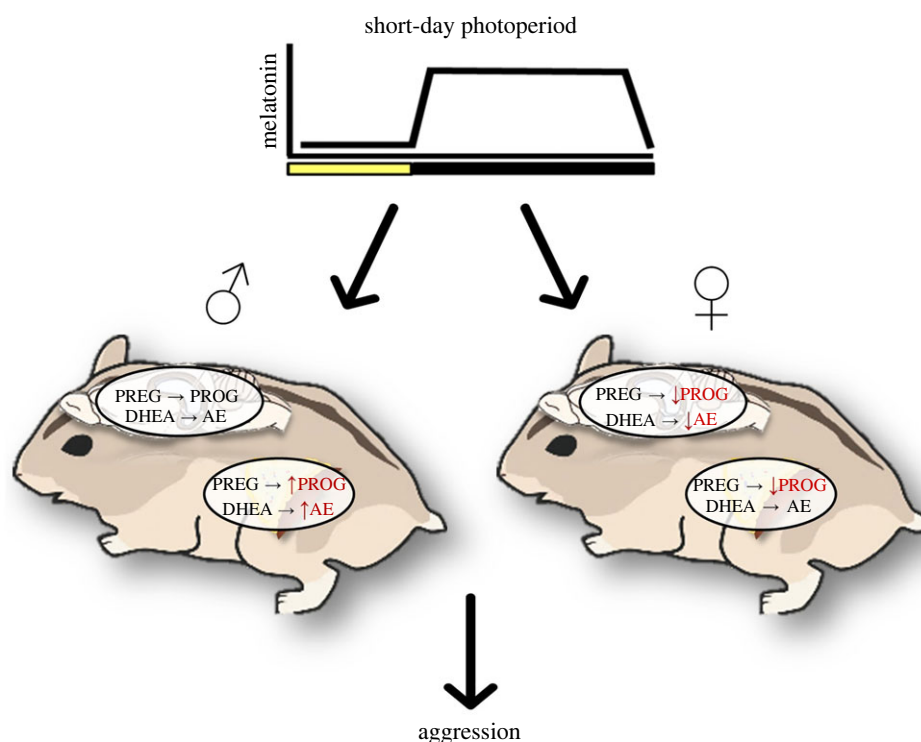


Figure 4. Conceptual model of the neuroendocrine regulation of seasonal aggression in male and female Siberian hamsters via melatonin and 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (3 β -HSD). AE, androstenedione; DHEA, dehydroepiandrosterone; PREG, pregnenolone; PROG, progesterone. (Online version in colour.)

neuroendocrine responses in the adrenal glands and brain regions associated with aggressive behaviour, despite displaying a remarkably similar behavioural phenotype (figure 4). Specifically, LD-M and SD males exhibited *increases* in PROG and AE synthesis in the adrenal glands, but showed no changes in 3 β -HSD activity in the AH or PAG. Furthermore, there was no difference in the ratio of PROG:AE synthesis in the adrenal glands or brain, suggesting that males increase PROG and AE production via 3 β -HSD to a similar degree in the adrenal glands. Conversely, LD-M and SD females showed a *reduction* in PROG synthesis in the adrenal glands and *decreases* in PROG and AE synthesis in the AH. Although reductions in PROG and AE production via 3 β -HSD in females may seem paradoxical to the changes in 3 β -HSD activity observed in males, these neuroendocrine responses culminated in an increase in the ratio of PROG:AE synthesis in the adrenal glands, AH and PAG. Thus, these results suggest that melatonin-treated and SD females selectively decrease PROG production to a greater extent than AE production in these tissues and brain regions, which culminates in increased DHEA metabolism to biologically active androgens and oestrogens (e.g. AE, T and E₂). While our findings suggest that male and female hamsters use alternative strategies for increasing DHEA metabolism, we propose that these mechanisms may contribute to the same behavioural phenotype: elevated territorial aggression during the non-breeding season.

Our study is the first to examine seasonal and sex differences in 3 β -HSD and aggressive behaviour; previous research, however, has documented seasonal plasticity in steroidogenic enzymes in birds and rodents that are highly territorial year-round. Non-breeding male song sparrows exhibit higher 3 β -HSD activity in the caudal telencephalon and ventromedial telencephalon, two brain regions that are associated with aggression, compared with breeding male sparrows

[9]. By contrast, the activity and/or mRNA expression of aromatase (ARO), an enzyme that catalyses the conversion of T to E₂, is higher in diencephalic nuclei that are associated with reproduction in breeding males relative to non-breeding males [30,31]. Interestingly, we recently determined that there are no differences in the density of ARO-immunoreactive cells in the PAG and two brain regions that regulate reproductive behaviours (the paraventricular nucleus of the hypothalamus and ventral tegmental area) between LD and SD female hamsters [14], suggesting that the role of steroid-synthesizing and steroid-metabolizing enzymes in regulating seasonal aggression may be species-specific. The results of the current study enhance our understanding of how the adrenal glands, AH and PAG may modulate seasonal aggression and suggest that the hormonal mechanisms underlying this behaviour may vary between males and females. Collectively, the neuroendocrine responses observed in our study support the hypothesis that male and female hamsters utilize extra-gonadal neuroendocrine mechanisms (i.e. adrenal steroids and neurosteroids) to increase non-breeding aggression. These processes likely allow this species to decouple aggressive behaviour from the hormonal processes underlying reproduction, an adaptive response that enables males and females to maintain high levels of aggression despite gonadal regression.

Although few studies have characterized how the neuroendocrine regulation of seasonal social behaviours may differ between males and females, prior studies have demonstrated sex-specific variation in circulating steroid levels, neural steroid sensitivity, and aggressive behaviour in breeding songbirds [32,33]. Moreover, sex differences in the neural and hormonal mechanisms modulating social behaviour have been documented in non-seasonal mammals, including laboratory rodents and humans [34]. For example, sex-specific effects of stress on neuroanatomy and the

hypothalamic-pituitary-adrenal (HPA) axis, a system of neuroendocrine pathways that function in maintaining physiological homeostasis, have been documented in laboratory rats (*Rattus norvegicus*) [35,36]. Chronic stress impairs temporal object recognition, fear extinction and attentional set-shifting in male rats, but similar behavioural deficits are not observed in females [37,38]. Female laboratory rodents, however, typically display heightened responses in HPA axis activity relative to males [35]. Conversely, chronic stress causes dendritic retraction in the medial prefrontal cortex, a brain region that is important in regulating the HPA axis and executive functioning, in male rats, whereas females either show no changes or exhibit dendritic hypertrophy in this brain region [39,40]. While the focus of our study was on aggressive behaviour, it is intriguing that we observed sex-specific effects of melatonin and SDs on β -HSD activity in the adrenal glands and brain regions associated with aggression, as these tissues (i.e. adrenals, hypothalamus) have been shown to exhibit sex differences in physiological responses to stress. Additional studies are needed to determine the extent of these seasonal, sex-specific effects on steroid synthesis and metabolism and to assess the functional implications of these neuroendocrine responses on behaviour.

Although our findings suggest that seasonal variation in β -HSD activity and aggression are melatonin-dependent, further investigation is necessary to determine the mechanisms by which melatonin acts to influence steroidogenic enzymes and aggressive behaviour. Because MT_1 melatonin receptors (also known as Mel_{1a} receptors in non-mammalian vertebrates), which are believed to be primarily responsible for modulating seasonal changes in physiology and behaviour, are present in all tissues comprising the HPA and hypothalamic-pituitary-gonadal (HPG) axes (i.e. the 'reproductive' axis [41]), melatonin may regulate changes in steroids and social behaviour on a seasonal time scale via peripheral and/or central signalling mechanisms. We recently showed that lentiviral-mediated overexpression of MT_1 receptors in the adrenal glands causes SD-like increases in aggression in male Siberian hamsters. There is no effect of adrenal MT_1 overexpression, however, on serum DHEA levels [16], which may suggest that adrenal MT_1 receptors mediate seasonal behaviours via signalling mechanisms with neural substrates. Moreover, additional studies are necessary to determine if other brain regions that are associated with aggression, in addition to those investigated herein (e.g. lateral septum, medial amygdala, ventromedial hypothalamus [21]), may be important in facilitating the actions of melatonin on aggressive behaviour. Our future research will examine how seasonal plasticity in steroidogenic enzymes, both in the periphery and in the brain, may be regulated by MT_1 receptors and how these local changes in steroidogenesis influence territorial aggression.

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5. Conclusion

In the present study, we demonstrated that Siberian hamsters show pronounced seasonal variation in the activity of the enzyme β -HSD, which is dependent on the pineal hormone melatonin. Furthermore, we observed profound sex-specific effects of timed melatonin administration and SDs on β -HSD activity in the adrenal glands and brain regions associated with aggression; males exhibited increased adrenal β -HSD activity in response to a SD-like melatonin signal, whereas females showed reductions in adrenal and neural β -HSD activity. Additional studies are needed to assess the functional implications of these sex differences in steroidogenic enzyme activity and to determine how melatonin acts via its receptors to control seasonal plasticity in steroidogenesis and aggression. Collectively, these findings enhance our understanding of how melatonin acts indirectly via steroid hormones to regulate aggressive behaviour. More broadly, our study highlights how natural selection has acted, and will continue to act, on the proximate mechanisms underlying seasonal social behaviours in a sex-specific manner.

Ethics. All animal procedures were performed in accordance with the NIH 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. 17-001).

Data accessibility. Data for this study are available from the Dryad Digital Repository (<https://doi.org/10.5061/dryad.70rxwdc0p>) and are provided in electronic supplementary material [42].

Authors' contributions. K.M.M.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing—original draft; J.C.T.: formal analysis, investigation, methodology, resources, validation, visualization, writing—review and editing; G.E.D.: conceptualization, funding acquisition, project administration, resources, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This research was funded by National Institutes of Health Grant R21MH109942 (to G.E.D.), National Science Foundation Grant IOS-1656414 (to G.E.D.), an Indiana Clinical and Translational Sciences Institute Pilot Grant for Research Use of Core Facilities (to K.M.M. and G.E.D.), National Institutes of Health Training Grant T32HD049336 ('Common Themes in Reproductive Diversity,' to K.M.M.), an IU Center for the Integrative Study of Animal Behaviour Predoctoral Fellowship (to K.M.M.), an IU College of Arts and Sciences Dissertation Research Fellowship (to K.M.M.), and Indiana University.

Acknowledgements. We thank members of the Demas lab for assistance with animal procedures, behavioural filming and necropsies; Dr Randalyn Shepard, Dr Karen Rogers and the staff of Laboratory Animal Resources for caring for the animals used in this study; and the Center for the Integrative Study of Animal Behavior's Mechanisms of Behavior Lab, the Light Microscopy Imaging Center and Dr Jason Tennessen for providing access to equipment. We are also grateful to Dr Kim Rosvall, Dr Troy Smith, Dr Cara Wellman and two anonymous reviewers for their feedback on a previous version of this manuscript.

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