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Seasonal patterns of melatonin alter aggressive phenotypes of female Siberian hamsters

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Abstract

Many animal species exhibit year-round aggression, a behaviour that allows individuals to compete for limited resources in their environment (eg, food and mates). Interestingly, this high degree of territoriality persists during the non-breeding season, despite low levels of circulating gonadal steroids (ie, testosterone [T] and oestradiol [E_2]). Our previous work suggests that the pineal hormone melatonin mediates a 'seasonal switch' from gonadal to adrenal regulation of aggression in Siberian hamsters (*Phodopus sungorus*); solitary, seasonally breeding mammals that display increased aggression during the short, 'winter-like' days (SDs) of the non-breeding season. To test the hypothesis that melatonin elevates non-breeding aggression by increasing circulating and neural steroid metabolism, we housed female hamsters in long days (LDs) or SDs, administered them timed or mis-timed melatonin injections (mimic or do not mimic a SD-like signal, respectively), and measured aggression, circulating hormone profiles and aromatase (ARO) immunoreactivity in brain regions associated with aggressive or reproductive behaviours (paraventricular hypothalamic nucleus [PVN], periaqueductal gray [PAG] and ventral tegmental area [VTA]). Females that were responsive to SD photoperiods (SD-R) and LD females given timed melatonin injections (Mel-T) exhibited gonadal regression and reduced circulating E_2, but increased aggression and circulating dehydroepiandrosterone (DHEA). Furthermore, aggressive challenges differentially altered circulating hormone profiles across seasonal phenotypes; reproductively inactive females (ie, SD-R and Mel-T females) reduced circulating DHEA and T, but increased E_2 after an aggressive interaction, whereas reproductively active females (ie, LD females, SD non-responder females and LD females given mis-timed melatonin injections) solely increased circulating E_2. Although no differences in neural ARO abundance were observed, LD and SD-R females showed distinct associations between ARO cell density and aggressive behaviour in the PVN, PAG and VTA. Taken together, these results suggest that melatonin increases non-breeding aggression by elevating circulating steroid metabolism after an aggressive encounter and by regulating behaviourally relevant neural circuits in a region-specific manner.

KEYWORDS

aggression, aromatase, biological rhythms, environmental cues, pineal, steroidogenesis

1 | INTRODUCTION

Animals inhabiting temperate zones experience marked variations in climate and resource availability across the annual cycle. Such fluctuations in temperature, water and food availability in the environment require individuals to respond to temporal and spatial niches from winter to summer. The unique demands of these extreme conditions result in reproductive inhibition during unfavourable conditions.[1,2] Thus, seasonally breeding animals limit reproduction to the spring and summer, which coincide with relatively warm ambient temperatures and abundant resources, and shift to reproductive quiescence during the winter, when physiological and behavioural adaptations tend to be geared towards survival rather than reproduction.[1-4]

As with most species that live in temperate habitats, Siberian hamsters (*Phodopus sungorus*) use photoperiod (ie, day length) as the primary environmental cue to coordinate seasonally appropriate changes in physiology and behaviour. These animals breed during the summer and undergo gonadal regression, a marked reduction (> 10%) in body mass, and changes in thermoregulation during the winter.[5,6] In the wild, Siberian hamsters are solitary and occur at low population densities (ie, one to six individuals per km^2), and both males and females defend their territories year-round.[7] Thus, unlike other species where reproduction and aggression often co-occur during the breeding season, Siberian hamsters display increased aggression during the short, 'winter-like' days of the non-breeding season, despite gonadal regression. This increased aggression during periods of reproductive quiescence suggests an uncoupling of aggressive behaviour from gonadal hormones in both sexes.

Similarly, robust levels of aggression in the relative absence of gonadal steroids have been shown in other vertebrates, including song sparrows (*Melospiza melodia*),[8-11] spotted antbirds (*Hylophylax naevoides*),[12-14] beach mice (*Peromyscus polionotus*), deer mice (*Peromyscus maniculatus*)[15] and Syrian hamsters (*Mesocricetus auratus*),[16] suggesting that animals have evolved alternative neuroendocrine mechanisms to regulate aggressive behaviour across the seasons. The current working model in birds and mammals posits that non-breeding aggression is facilitated by increased metabolism of non-gonadal prohormones to biologically active androgens (ie, testosterone [T]) and oestrogens (eg, oestradiol [E_2]) via converting enzymes, particularly in brain regions that are associated with aggressive behaviour. Our laboratory and others have shown that adrenal dehydroepiandrosterone (DHEA) is a key hormone for maintaining high levels of aggression during periods of reproductive quiescence.[17-19] DHEA is an androgen and a prohormone that can be rapidly metabolised into biologically active steroids, including T and E_2. Although DHEA is synthesised primarily by the adrenal glands, circulating DHEA is capable of passing through the blood-brain barrier and can be metabolised to biologically active steroids in brain regions that express the appropriate steroidogenic enzymes.[20,21] In addition to a shift in hormonal source, seasonal changes in steroid synthesis and metabolism within target tissues have been reported. For example, heightened steroid metabolism, including increased expression of the enzyme aromatase, which catalyses the conversion of T to E_2, and elevated sensitivity to hormones (eg, E_2), such as increased expression of oestrogen receptors, within discrete brain regions, have been reported as key regulators of aggression in birds,[22,23] mammals,[24-26] and species that display a sex role reversal in aggression.[27,28] Collectively, these mechanisms are consistent with the hypothesis that seasonal changes in aggression map onto changes in neuroendocrine processes; however, they do not describe, in turn, how aggression influences these processes.

Dynamic shifts in neuroendocrine mechanisms that occur on a seasonal timeframe suggest that animals modify how they physiologically respond to social challenges. The physiological effects of social challenges are often studied within the frameworks of the challenge hypothesis or the winner effect.[29,30] Specifically, aggressive encounters can trigger rapid and marked increases in circulating androgens; therefore, elevated T might enable animals to aggressively defend a territory or acquire a mate, and it may prime individuals for future competitions.[31] Support for the challenge hypothesis, however, is mixed in males.[32] Because the majority of studies investigating the challenge hypothesis have used males in breeding condition, it is difficult to disassociate the individual contributions of seasonal elevations in T and socially induced changes in T.[33] Furthermore, social elevation of T in females does not appear to be a common phenomenon.[34,35] To date, most studies that have examined the challenge hypothesis or winner effect have only measured the effects of an aggressive challenge on circulating T. Thus, alternative mechanisms, such as socially mediated changes in steroid synthesis (eg, DHEA to E_2), are largely unexplored, although they likely play a key role in enabling high levels of aggression during the non-breeding season.

Although previous work has carefully demonstrated the neuroendocrine mechanisms underlying seasonal aggression, the role of environmental cues, such as photoperiod, in mediating these mechanisms is relatively understudied. Photoperiod is translated from an environmental cue to a biochemical signal via a multisynaptic pathway, in which environmental light is perceived by retinal ganglion cells in the eye, processed in the hypothalamus of the brain, and transduced from a neural to an endocrine signal through the release of the hormone melatonin by the pineal gland.[36,37] Because melatonin secretion is high at night and low during the day, the pattern and duration of melatonin secretion closely tracks changes in photoperiod across the annual cycle and, thus, plays a critical role in regulating seasonal reproduction. Specifically, a long duration of melatonin secretion, which is indicative of short days (SDs) (ie, < 12.5 hours light per day), results in reproductive quiescence; whereas a short duration of melatonin secretion, which is encoded as long days (LDs), results in reproductive functionality.[36,37] In addition, recent work from our group suggests that melatonin mediates a 'seasonal switch' from gonadal regulation of aggression during LDs to adrenal regulation of aggression during SDs in some seasonally breeding animals, including Siberian hamsters.[17,18] We have previously demonstrated that exogenous melatonin administration elevates aggressive behaviour in LD male hamsters.[16,38] Furthermore, we have shown that timed melatonin injections, which mimic SD patterns of melatonin secretion, increases aggression in LD females[39] and that long-term timed melatonin administration induces

gonadal regression, elevates aggressive behaviour, and produces SD-like changes in circulating androgen profiles in LD males.[40] We have also found that administering melatonin *in vitro* to cultured adrenal glands elevates adrenal DHEA output in SD, but not LD females, whereas treating cultured ovaries with melatonin increases DHEA output in LD, but not SD females.[39] Although our findings suggest that melatonin increases aggression during SDs by altering peripheral steroid synthesis, it is unclear how melatonin acts in the brain to mediate seasonal changes in aggression and whether melatonin modulates aggression via neuroendocrine circuits that are independent of reproduction.

In the present study, we tested the hypothesis that melatonin mediates seasonal aggression by elevating steroid metabolism, both in circulation and in brain regions associated with aggressive behaviours. To delineate the precise manner by which melatonin acts to affect seasonal aggression, we directly manipulated the melatonin signal in circulation by administering a timed melatonin injection, which summates with endogenous melatonin secretion and mimics a SD-like signal, or a mis-timed melatonin injection, which exposes tissues to melatonin but allows animals to remain reproductively functional. Adult female hamsters were housed in LD or SD photoperiods and treated with either timed melatonin, mis-timed melatonin, or control injections, and aggressive behaviour was measured after 10 weeks of treatment. Moreover, to assess whether melatonin regulates seasonal shifts in peripheral and neural steroid metabolism, baseline and aggression-induced levels of circulating DHEA, T and E_2 were measured and aromatase immunoreactivity was quantified in three brain regions: the paraventricular hypothalamic nucleus (PVN) and ventral tegmental area (VTA), which regulate reproduction and its associated behaviours[41,42] and the periaqueductal gray (PAG), which has been implicated in modulating aggressive behaviour.[43,44] We predicted that female hamsters given a SD-like melatonin signal (eg, via SD photoperiods or timed melatonin injections) will exhibit gonadal regression, elevated levels of aggression and aggression-induced increases in steroid metabolism, both in circulation and in brain regions associated with aggressive, but not reproductive behaviours. Conversely, we predicted that females exhibiting a LD-like melatonin signal (eg, via either LD photoperiods or mis-timed melatonin injections) will remain reproductively functional, show relatively lower levels of aggression and exhibit aggression-induced increases in steroid metabolism in brain regions associated with reproductive, but not aggressive behaviours.

2 | MATERIALS AND METHODS

2.1 | Animal housing

Adult female Siberian hamsters (*P. sungorus*, > 60 days of age) were reared and maintained in a breeding colony at Indiana University under long days (16:8 hour light/dark photocycle) and group-housed at weaning (post-natal day 18). Hamsters were given *ad libitum* access to standard laboratory rodent chow (Lab Diet 5001; PMI Nutrition, Brentwood, MO, USA) and tap water. Ambient temperature was maintained at 20 ± 2°C and relative humidity was maintained at 55 ± 5%. All procedures were performed in accordance with the National Institutes of Health (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 #11-006).

2.2 | Photoperiodic manipulations and *in vivo* melatonin administration

Prior to the start of photoperiodic manipulations, experimental (ie, resident) hamsters were individually housed (n = 70) for a one-week acclimation period on a long day (LD) (16:8 hours) photocycle. After the acclimation period, experimental hamsters were either transferred to a room on a short-day (SD) (8:16 hours) photocycle (n = 28) or were relocated to a new room on a LD cycle (n = 42). All hamsters were given daily s.c. injections of melatonin (15 μg day^{-1} dissolved in 1:10 ethanol:saline solution; M5250; Sigma-Aldrich, St Louis, MO, USA) or a control solution (1:10 ethanol:saline solution), as described previously.[39,40,45] LD hamsters were divided into three treatment groups and received either a timed melatonin injection (Mel-T, n = 14) 2 hours prior to lights off to extend the LD pattern of endogenous melatonin secretion to mimic that of SD animals; a mis-timed melatonin injection (Mel-M, n = 13) 8 hours prior to lights off to supplement circulating melatonin, but at a time at which the injection fails to summate with endogenous melatonin secretion; or a control injection (LD, n = 15) (Figure 1). All SD hamsters received control injections (SD responders, SD-R: n = 14; SD non-responders, SD-NR: n = 14). Hamsters remained in their photoperiodic regimens and were administered melatonin or control injections for a period of 10 weeks.

2.3 | Reproductive phenotypes

Seasonal phenotypes were determined based on *a priori* criteria that have been previously established for Siberian hamsters.[46-48] Throughout the study, hamsters were weighed on a weekly basis to track changes in body mass, and coat colour was assessed to document the transition from summer brown/grey to winter white.[47,48] In addition, oestrous cycles were monitored via vaginal cytology for all experimental animals 5 days prior to behavioural trials to determine whether females were cycling, as described previously.[25,47-49] Hamsters were characterised as reproductively functional if they had functional reproductive tissues (ie, ovaries and uterine horns), displayed no significant change in body mass (≤ 10%) and maintained a brown/grey coat colour. By contrast, hamsters were characterised as reproductively inactive if they had regressed reproductive tissues, exhibited a significant decrease in body mass (> 10%) and had a white coat colour. Moreover, the reproductively active phenotype in SDs (SD-NR) was characterised using the same criteria described above for LD animals. Non-responsiveness to SDs, in which hamsters do not undergo gonadal regression or reduce body mass in response to SD photoperiods and generally respond physiologically and

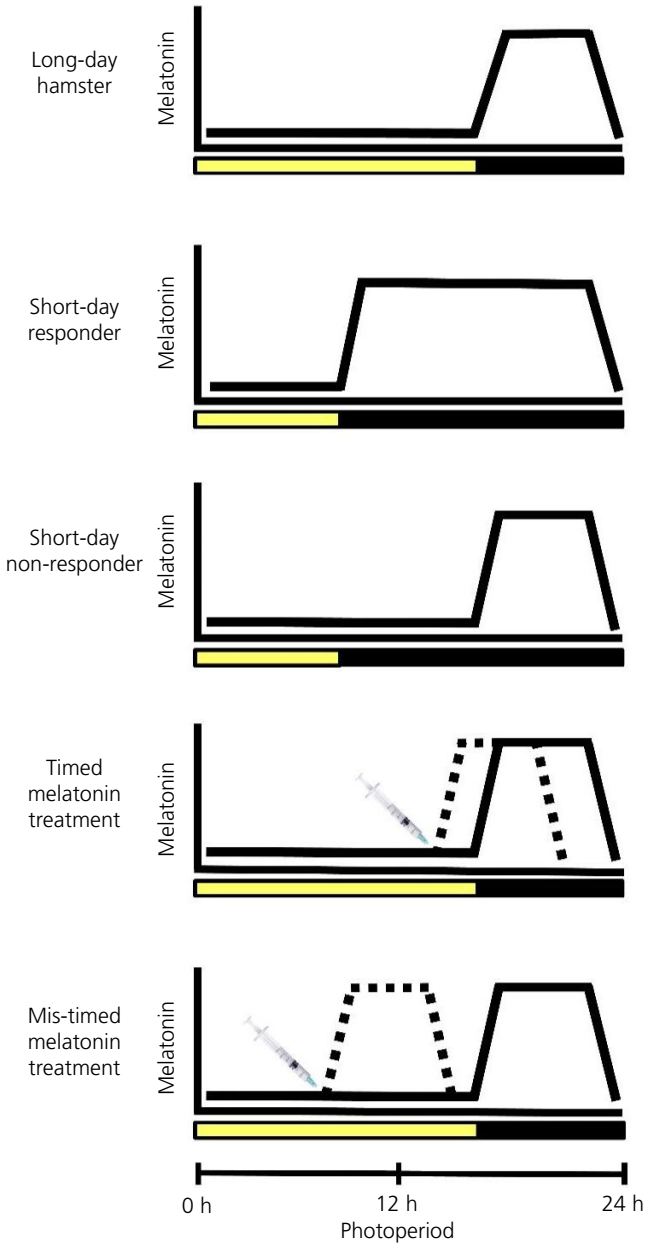

FIGURE 1 Melatonin profiles of female hamsters that received photoperiodic manipulations and/or melatonin injections. Melatonin secretion is shown on the y-axis and photoperiod (ie, day length) is shown on the x-axis (yellow lines: light phase, black lines: dark phase). Long-day hamsters exhibit a relatively short duration of melatonin secretion compared to short-day responders, which have a relatively long duration of melatonin secretion. Although short-day non-responders are housed under short-day photoperiods, their melatonin profiles do not expand in response to treatment. Therefore, these animals have a relatively short duration of melatonin secretion that is indistinguishable from that of long-day hamsters. Timed melatonin treatment summates with endogenous melatonin secretion, expanding the melatonin profile of these animals to mimic that of short-day responders. By contrast, mis-timed melatonin treatment fails to summate with endogenous melatonin secretion, resulting in a melatonin profile that mimics that of long-day hamsters

behaviourally like LD animals, has been documented previously in this species and affects approximately 30% of the population.[36,50,51]

2.4 | Quantification of aggressive behaviour

Same-sex aggressive encounters were conducted within the first 2 hours of the dark phase using methods described previously.[39,47] Aggression was assessed using a same-sex resident-intruder paradigm, in which an unfamiliar stimulus animal (ie, intruder) is placed into the home cage of an experimental animal (ie, resident) and the animals are allowed to interact for 5 minutes. Intruder hamsters were of approximately the same age and body mass (±5%) and their parents were different from those of the experimental animals with which they were paired. Intruders were pair-housed with a sibling (n = 30) and remained in LDs prior to behavioural testing and for the duration of the study. All trials were recorded under low-illumination red lights using a HandyCam Digital Camcorder HDR-SR7 (Sony, Tokyo, Japan). Aggressive behaviours (ie, latency to first attack, number and duration of attacks and chases) were scored for each experimental animal by two trained observers using ODLOG (https://www.macropodsoftware.com). Scores from the two individuals were averaged, and inter-rater reliability was accepted if less than 10% variation occurred. Measures of aggression were defined according to previous studies in male and female Siberian hamsters.[40,47,48]

2.5 | Blood sampling and tissue processing

To compare the effects of an aggressive interaction on circulating hormone profiles across seasonal phenotypes, a pre-aggression and post-aggression blood sample was collected from each experimental animal after 10 weeks of treatment. Pre-aggression samples were drawn 24 hours prior to behavioural trials to control for circadian rhythmicity in hormone concentrations, and post-aggression blood samples were collected immediately (≤ 2 minutes) after behavioural testing. Animals were lightly anaesthetised using isoflurane (Isothesia; Henry Schein Animal Health, Melville, NY, USA) and blood was drawn from the retro-orbital sinus into microcapillary tubes. Samples were allowed to clot for 1 hour, clots were removed and samples were centrifuged at 2410 ***g*** for 30 minutes at 4°C. Serum was aspirated and stored in sealable micro-centrifuge tubes at −20°C until enzyme immunoassay analysis.

After post-aggression blood collection, animals were deeply anaesthetised with 0.3 mL of a ketamine (20 mg mL^{-1}) and xylazine (4 mg mL^{-1}) cocktail in 0.9% saline and perfused transcardially with 100 mL of 0.1 mol L^{-1} phosphate-buffered saline (PBS; pH 7.4), followed by 100 mL of 4% paraformaldehyde in PBS (PFA). Necropsies were performed and reproductive organs (ie, ovaries, uterine horns) were dissected, cleaned of fat and connective tissue, and weighed to the nearest mg. Brains were collected, post-fixed for 4 hours in PFA, cryoprotected for 48 hours in 30% sucrose in PBS, transferred to Tissue-Tek® OCT

compound (Sakura, Tokyo, Japan) and stored at −80°C. Coronal sections (40 μm) were cut on a sliding freezing microtome, and free-floating sections were stored in an ethylene glycol-based cryoprotectant solution[52] at −80°C until processing for immunocytochemistry.

2.6 | Quantification of circulating hormones

Pre- and post-aggression levels of serum DHEA, T and E_2 were quantified using commercially available enzyme immunoassay kits that have been previously validated in this species (DHEA: assay sensitivity = 0.108 ng mL^{-1}; ALPCO Diagnostics, Salem, NH, USA; 20-DHEHU-E01[39,53]; T: assay sensitivity = 5.67 pg mL^{-1}; Assay Design, Ann Arbor, MI, USA; 900-065[54,55]; E_2: assay sensitivity = 14.0 pg mL^{-1}; Enzo Life Sciences, Farmingdale, NY, USA; ADI-900-174[25]). Each of these kits is highly specific for the hormone of interest and have negligible or undetectable cross-reactivity with other steroid hormones (DHEA kit: sulfated DHEA = 0.01%, T = 0.01%, E_2 = < 0.01%, cortisol = < 0.01%; T kit: 19-hydroxytestosterone = 14.6%, androstenedione = 7.2%, DHEA = 0.7%, E_2 = 0.4%; E_2 kit: oestrone = 17.8%, oestriol = 0.9%, T = 0.01%, DHEA = < 0.001%). Serum DHEA was analysed neat or diluted 1:2 with assay buffer; serum T was diluted 1:3, 1:4 or 1:8 with assay buffer; and serum E_2 was analysed neat or diluted 1:2 with assay buffer to ensure approximately 50% binding on a four-parameter logistic standard curve (Microplate Manager; Bio-Rad, Hercules, CA, USA). Samples were assayed in duplicate in accordance with the manufacturer's instructions and were counterbalanced across three plates of the same kit lot for each hormone. Samples with a coefficient of variability > 10% and a maximum binding < 20% or > 80% were re-analysed. Intra-assay variability was not greater than 7.95% (DHEA: ≤ 7.60%, T: ≤ 7.95%, E_2: ≤ 3.77%) and inter-assay variability was ≤ 5.89% for each hormone (DHEA: 5.28%, T: 5.89%, E_2: 2.97%).

2.7 | Immunolabelling and quantification of aromatase

Free-floating sections were allowed to equilibrate to room temperature and were washed 5 × 5 minutes in PBS, followed by a 1-hour incubation period in blocking solution, which consisted of PBS + 10% donkey serum (Millipore Sigma, Burlington, MA, USA) and 0.3% Triton X-100 (VWR, Radnor, PA, USA). Sections were then incubated for 48 hours at 4°C in rabbit anti-CYP19 (H-300, raised against the human CYP19 protein; catalogue no. 30086, Lot # H1314; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:200 in PBS + 5% donkey serum and 0.3% Triton X-100 (diluent). After primary antibody incubation, sections were washed 2 × 15 minutes in PBS, followed by a 2 hour incubation period at room temperature in Alexa Fluor® 594 (catalogue no. A-21207; Life Technologies, Grand Island, NY, USA) diluted 1:200 in diluent.[25,56,57] After 3 × 10 minutes PBS washes, sections were mounted onto chrome alum-subbed slides and cover-slipped with Prolong Gold Antifade Reagent with DAPI (4,6-diamidino-2-phenylindole; Life Technologies). In accordance with the manufacturer's instructions, the primary antibody was generated to bind to amino acids 209-503 of the C-terminus of CYP19. Aromatase distribution in the present study is consistent with that reported previously in other rodents.[58-60] Staining was abolished when either the primary or secondary antibodies were omitted (ie, −primary/+secondary or +primary/−secondary, respectively). Samples were counterbalanced across two runs and processed with reagents of the same lot number, and the incubation times for each procedure were strictly controlled.

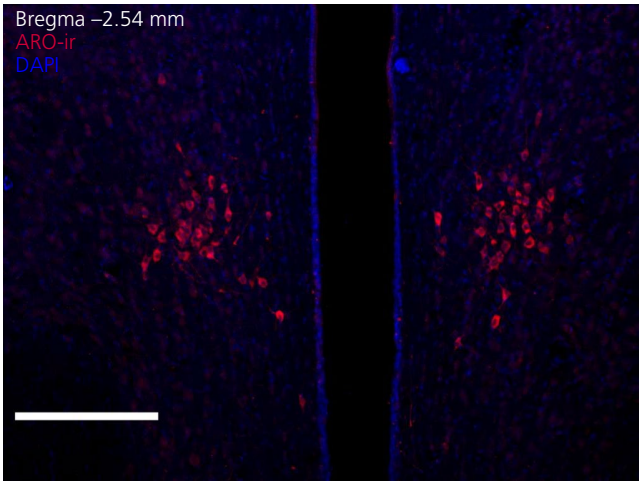

FIGURE 2 Representative photomicrograph of the enzyme aromatase (red) that has been fluorescence labelled and counterstained with DAPI (4′,6-diamidino-2-phenylindole) (blue) in the periaqueductal gray of a short-day responder hamster (scale = 100 μm). ARO-IR, aromatase-immunoreactive

ARO-immunoreactive (-IR) neurones were quantified in the periaqueductal gray (PAG) (Bregma −2.70 mm) (Figure 2) and the ventral tegmental area (VTA) (Bregma −3.28 mm) of the midbrain, as well as the paraventricular nucleus of the hypothalamus (PVN) (Bregma −0.94 mm), which were identified using a mouse brain atlas.[61] Monochrome photomicrographs were shot for each area of interest using an Axio Imager Microscope outfitted with a Z-drive and optical dissector (Apotome; Carl Zeiss, Oberkochen, Germany). Quantification was subsequently conducted from layered monochromone images using PHOTOSHOP CS6 (Adobe Systems Inc., San Jose, CA, USA) and IMAGEJ, version 1.48 (National Institutes of Health, Bethesda, MD, USA). Each region of interest was outlined bilaterally and the cross-sectional area of each region was measured. Fluorescence labelled ARO cells were then counted bilaterally in the largest cross-sectional area for each region. To provide an unbiased estimate of the total number of ARO-IR cells, a sampling area was determined for each region of interest prior to counting (range 200-600 mm^2) and cells were counted in the selected area of predetermined size within the region of interest. Cells were counted when the fluorescence signal was 2 SDs greater than background, and when cells were approximately 7.5 μm in diameter and confirmed to be nuclear-bound. Counts were corrected for size of region for all animals and are expressed as cells per unit area. To account for counting overestimates, an Abercrombie correction was applied before data analysis.[62]

2.8 | Statistical analysis

All statistical analyses were performed in JMP, version 11.0.0 (SAS Institute Inc., Cary, NC, USA) and R, version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria). $P < 0.05$ was considered statistically significant. Normality and homogeneity of variances were assessed using Shapiro-Wilk tests and Levene's tests, respectively. Some data (duration of attacks and chases, baseline serum T and E_2 levels) were log-transformed to satisfy the assumptions of normality and equal variances. A principal component analysis was conducted to reduce aggression variables (ie, latency to first attack, number and duration of attacks and chases) to a composite 'aggression score'. All aggression variables loaded strongly (< −0.30 or > 0.30) onto the first principal component (PC1), which had a high eigenvalue (> 1) and explained 73.8% of the total variance. Because no other PCs from this analysis explained a significant proportion of the variance (< 15%), PC1 (referred to hereafter as 'aggression score') was used to examine the effect of treatment on aggressive behaviour (see Supporting information, Table S1). One-way ANOVA was used to compare aggression variables, reproductive physiology, baseline (ie, pre-aggression) circulating hormone levels, aggression-induced changes in circulating hormone levels and neural ARO-IR abundance between treatment groups. If a statistical test reported a significant effect of treatment, Tukey's honestly significant difference post-hoc analyses were performed to examine pairwise comparisons. Spearman's rank correlations with a Holm-Bonferroni correction for multiple comparisons were conducted on untransformed data to examine potential differences in the relationships between aggressive behaviour, circulating hormone profiles and neural ARO-IR abundance across seasonal phenotypes.

3 | RESULTS

3.1 | Timed melatonin treatment and short-day photoperiods altered reproductive phenotypes and elevated aggressive behaviour

After 10 weeks of treatment, females that were responsive to SD photoperiods (SD-R) and LD females given timed melatonin injections (Mel-T) had regressed reproductive tissues (relative reproductive mass: $F_{4,69} = 27.94$, $P < 0.01$) (Figure 3A) and had a lower body mass (> 10%: $F_{4,69} = 10.92$, $P < 0.01$) than long-day females (LD), females that were non-responsive to SD photoperiods (SD-NR) and LD females given mis-timed melatonin injections (Mel-M). SD-R and Mel-T females also displayed more aggression with respect to number of attacks ($F_{4,69} = 9.72$, $P < 0.01$) (Figure 3B), duration of attacks ($F_{4,69} = 7.70$, $P < 0.01$), number of chases ($F_{4,69} = 6.68$, $P < 0.01$), duration of chases ($F_{4,69} = 6.64$, $P < 0.01$), latency to first attack ($F_{4,69} = 3.69$, $P = 0.02$) and composite aggression score ($F_{4,69} = 10.46$, $P < 0.01$) (Figure 3C) relative to LD, SD-NR and Mel-M females.

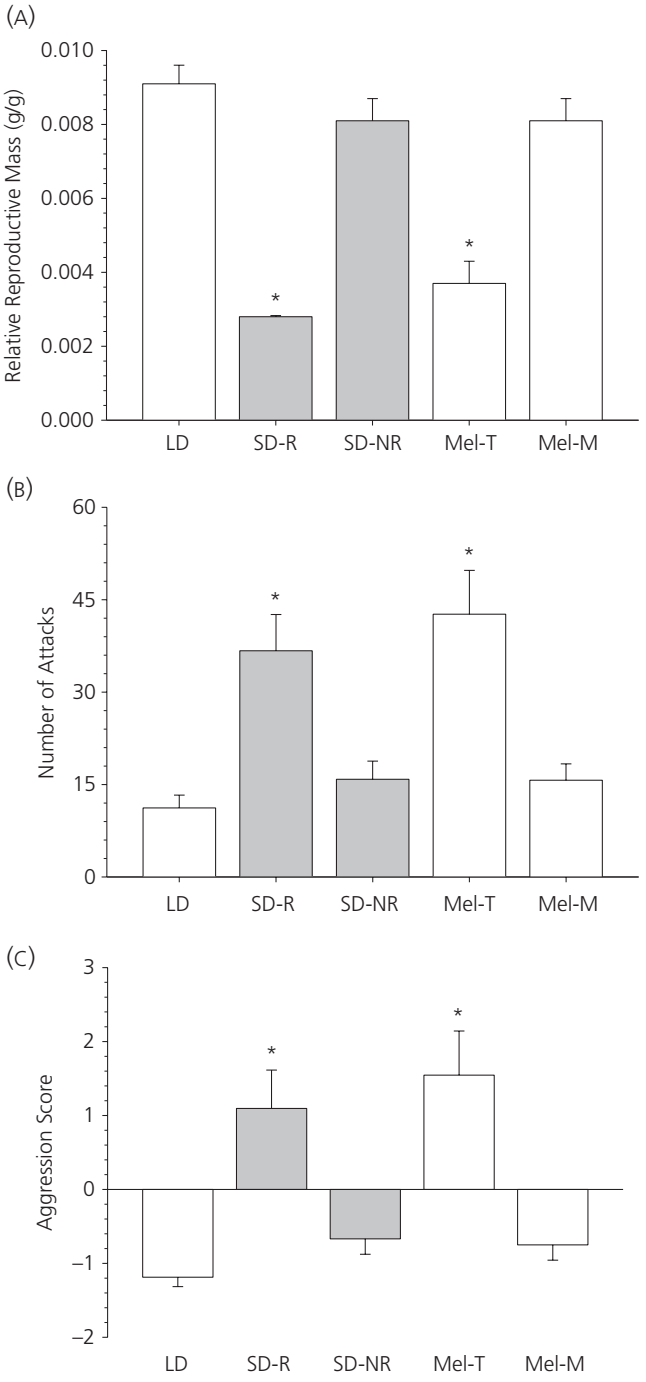

FIGURE 3 Short days and timed melatonin injections altered reproductive physiology and elevated aggressive behaviour. Relative reproductive mass (A), number of attacks (B) and aggression scores (C) of long-day females (LD), females that were responsive to short-day photoperiods (SD-R), females that were non-responsive to short-day photoperiods (SD-NR), LD females given timed melatonin injections (Mel-T) and LD females given mis-timed melatonin injections (Mel-M). Bar heights represent the mean ± SEM (white bars: LD, grey bars: SD; LD: n = 15, SD-R: n = 14, SD-NR: n = 14, Mel-T: n = 14, Mel-M: n = 13). *Significant difference from LD females (one-way ANOVAs with Tukey's honestly significant difference post-hoc analyses, $P < 0.05$)

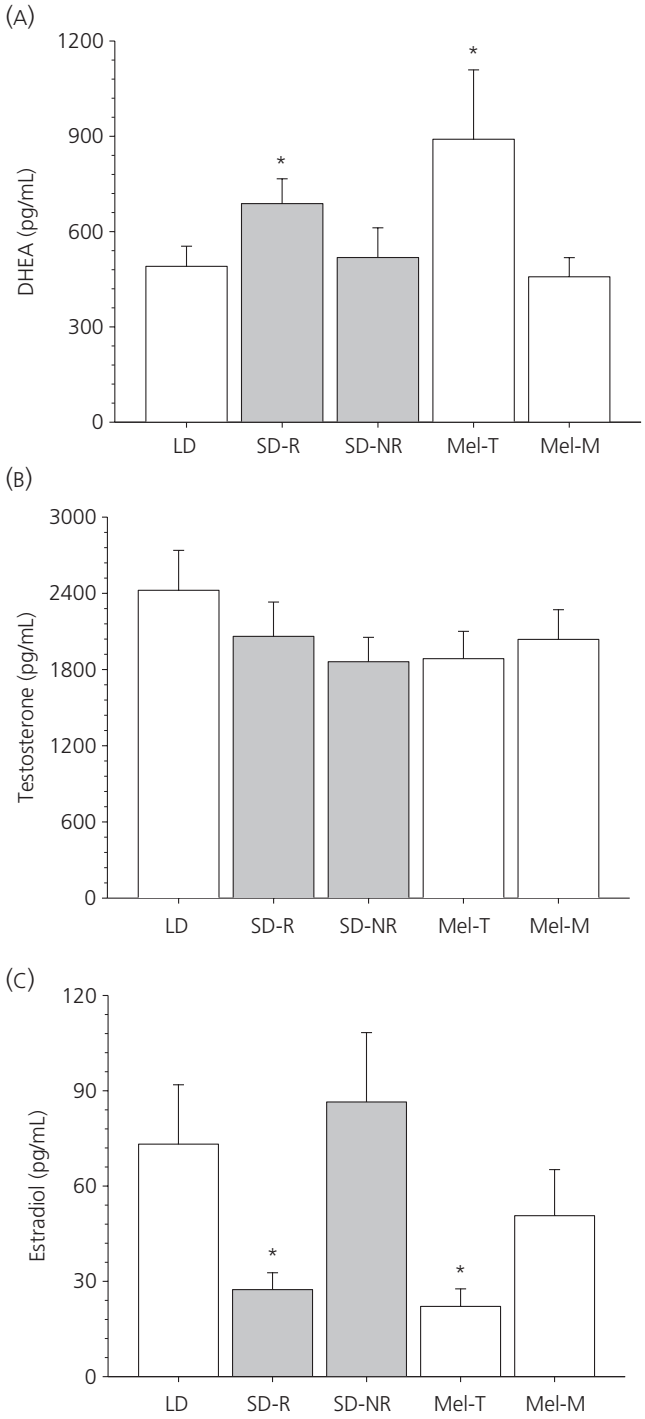

FIGURE 4 Short days and timed melatonin injections increased serum dehydroepiandrosterone (DHEA), but decreased serum oestradiol levels. Baseline (ie, pre-aggression) levels of serum (A) DHEA, (B) testosterone and (C) oestradiol in long-day females (LD), females that were responsive to short-day photoperiods (SD-R), females that were non-responsive to short-day photoperiods (SD-NR), LD females given timed melatonin injections (Mel-T) and LD females given mis-timed melatonin injections (Mel-M). Bar heights represent the mean ± SEM (white bars: LD, grey bars: SD; n = 10 per treatment group). *Significant difference from LD females (one-way ANOVAs with Tukey's honestly significant difference post-hoc analyses, $P < 0.05$)

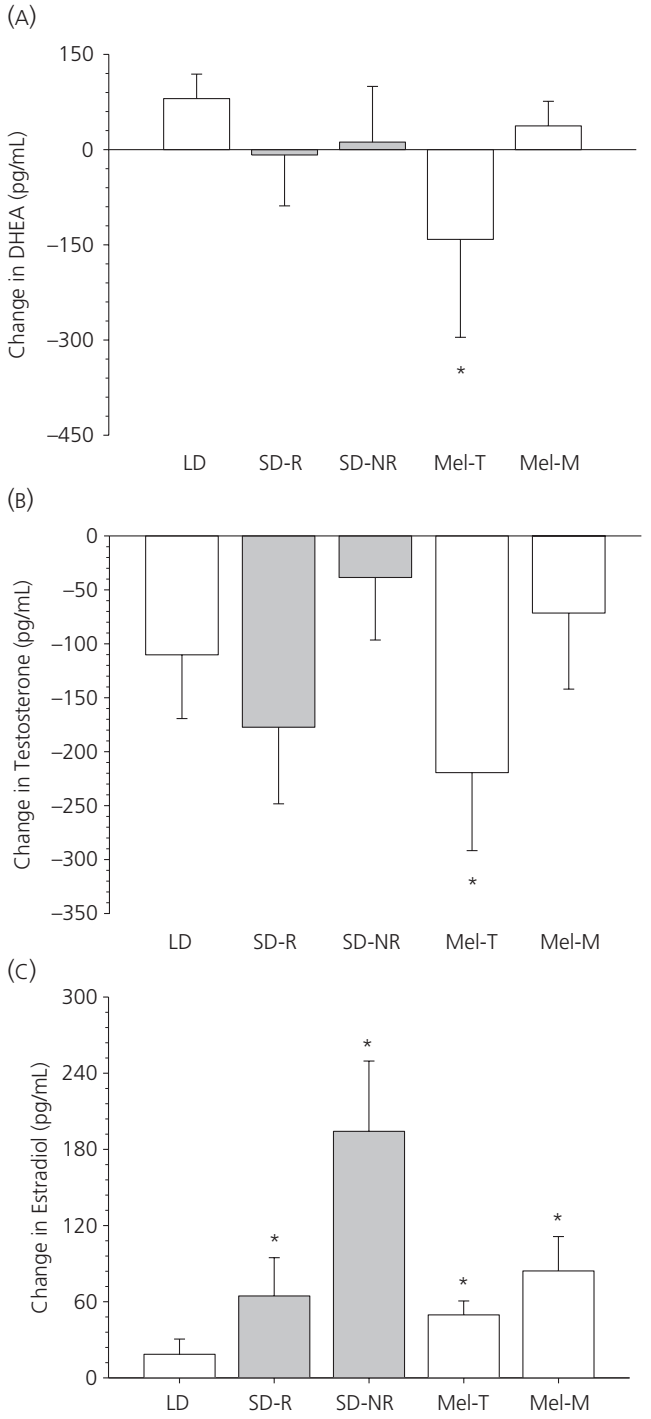

FIGURE 5 Aggressive challenges differentially altered circulating hormone levels across seasonal phenotypes. Aggression-induced changes in serum (A) dehydroepiandrosterone (DHEA), (B) testosterone and (C) oestradiol in long-day females (LD), females that were responsive to short-day photoperiods (SD-R), females that were non-responsive to short-day photoperiods (SD-NR), LD females given timed melatonin injections (Mel-T) and LD females given mis-timed melatonin injections (Mel-M). Bar heights represent the mean ± SEM (white bars: LD, grey bars: SD; n = 10 per treatment group). *Significant difference from LD females (one-way ANOVAs with Tukey's honestly significant difference post-hoc analyses, $P < 0.05$)

3.2 | Baseline levels of circulating hormones differed across photoperiods and in response to melatonin treatment

SD-R and Mel-T females exhibited significant increases in baseline (ie, pre-aggression) circulating DHEA ($F_{4,69} = 2.25$, $P = 0.03$) compared to LD and Mel-M females, whereas SD-NR females exhibited intermediate serum DHEA levels (Figure 4A). Conversely, there was no significant difference in circulating T concentration between the treatment groups ($F_{4,69} = 1.87$, $P = 0.13$) (Figure 4B). SD-R and Mel-T females also displayed significant reductions in circulating E_2 levels ($F_{4,69} = 3.76$, $P < 0.01$) relative to LD and SD-NR females, whereas Mel-M females displayed intermediate serum E_2 levels (Figure 4C).

3.3 | An aggressive challenge differentially induced changes in circulating DHEA, T and E_2 across seasonal phenotypes

Mel-T females exhibited significant reductions in serum DHEA ($F_{4,69} = 3.27$, $P = 0.04$) (Figure 5A) and serum T ($F_{4,69} = 2.58$, $P = 0.02$) (Figure 5B) after an aggressive encounter. None of the other treatment groups, however, showed aggression-induced changes in circulating androgen levels. By contrast, an aggressive interaction caused increases in serum E_2 concentration in SD-R, SD-NR, Mel-T and Mel-M females, but not in LD females ($F_{4,69} = 4.38$, $P = 0.01$) (Figure 5C).

3.4 | Melatonin treatment and short-day photoperiods caused no change in neural aromatase abundance

There was no significant difference in ARO-IR cell density in the PVN ($F_{4,22} = 0.09$, $P = 0.98$) (Figure 6A), PAG ($F_{4,29} = 0.42$, $P = 0.79$) (Figure 6B) or VTA ($F_{4,26} = 0.15$, $P = 0.96$) (Figure 6C) between the treatment groups.

3.5 | Associations between aggression and circulating hormone profiles differed across photoperiods and in response to melatonin treatment

In general, SD-R females showed the strongest associations between aggressive behaviour and baseline (ie, pre-aggression) or aggression-induced changes in circulating hormone profiles. Both aggression score and number of attacks were positively associated with aggression-induced changes in serum DHEA in SD-R females (aggression score: $\rho = 0.96$, n = 7, $P < 0.01$; number of attacks: $\rho = 0.76$, n = 7, $P = 0.05$) and latency to first attack trended towards a significant positive association with pre-aggression

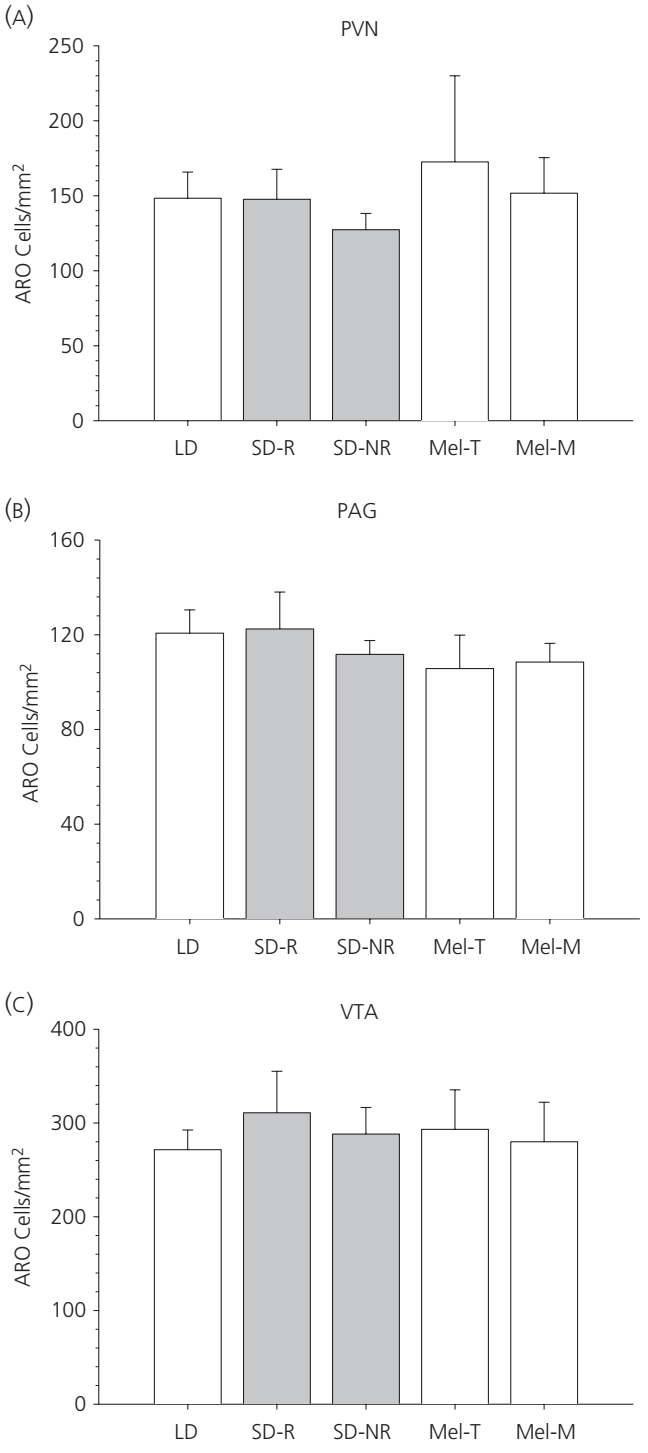

FIGURE 6 Short days and timed melatonin injections did not alter neural aromatase abundance. Aromatase (ARO)-immunoreactive cell density in the (A) paraventricular hypothalamic nucleus (PVN), (B) periaqueductal gray (PAG) and (C) ventral tegmental area (VTA) of long-day females (LD), females that were responsive to short-day photoperiods (SD-R), females that were non-responsive to short-day photoperiods (SD-NR), LD females given timed melatonin injections (Mel-T) and LD females given mis-timed melatonin injections (Mel-M). Bar heights represent the mean ± SEM (white bars: LD, grey bars: SD; LD: n = 6, SD-R: n = 5-7, SD-NR: n = 5-7, Mel-T: n = 5-7, Mel-M: n = 5-7)

TABLE 1 Correlations between aggressive behaviour, circulating hormone profiles and neural aromatase abundance in female Siberian hamsters

		LD		SD-R		SD-NR		Mel-T		Mel-M	
		ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*
Aggression score	Change in DHEA	−0.31	0.54	0.96	**<0.01**	−0.04	0.94	−0.43	0.34	−0.14	0.76
	Change in T	−0.43	0.40	−0.79	**0.04**	0.75	***0.05***	−0.21	0.64	0.25	.59
	Change in E_2	0.09	0.87	0.64	0.12	0.61	0.15	0.25	0.59	0.75	***0.05***
Number of attacks	Pre-aggression E_2	0.06	0.91	−0.58	0.18	0.09	0.85	0.79	**0.04**	−0.07	0.88
	Change in DHEA	−0.06	0.91	0.76	***0.05***	−0.09	0.85	−0.36	0.43	−0.11	0.82
	Change in E_2	0.00	1.00	0.85	**0.02**	0.70	***.08***	0.32	0.48	0.82	**0.02**
Attack duration	Pre-aggression DHEA	−0.09	0.87	−0.18	0.70	−0.86	**0.01**	0.07	0.88	0.50	0.25
	Pre-aggression E_2	−0.09	0.87	−0.29	0.53	−0.14	0.76	0.79	**0.04**	0.54	0.22
	Change in E_2	−0.09	0.87	0.71	***0.07***	0.29	0.53	0.32	0.48	0.71	***0.07***
Latency to first attack	Pre-aggression DHEA	0.09	0.87	0.68	***0.09***	0.14	0.76	0.18	0.70	−0.25	.59
	PAG ARO cell density	0.03	0.96	−0.36	0.43	−0.71	***0.07***	0.47	0.28	0.14	0.76

Note: Spearman's rank correlations between aggressive behaviour, circulating hormone profiles, and neural aromatase abundance in long-day females (LD), females that were responsive to short-day photoperiods (SD-R), females that were non-responsive to short-day photoperiods (SD-NR), LD females given timed melatonin injections (Mel-T) and LD females given mis-timed melatonin injections (Mel-M). Correlations coefficients (ρ) and *P*-values are shown for each analysis, which was performed within treatment groups (LD: n = 6, SD-R: n = 7, SD-NR: n = 7, Mel-T: n = 7, Mel-M: n = 7). Only correlations that are significant ($P < 0.05$, in bold) or trending towards significance ($P < 0.10$, in bold and italics) in at least one treatment group are shown (for a full list of correlation analyses, see Supporting information, Table S2).

Abbreviations: ARO, aromatase; DHEA, dehydroepiandrosterone; E_2, oestradiol; PAG, periaqueductal gray; T, testosterone.

serum DHEA levels in these animals (ρ = 0.68, n = 7, *P* = 0.09). In addition, SD-R and Mel-M females displayed significant positive correlations between number of attacks and aggression-induced changes in circulating E_2 (SD-R: ρ = 0.85, n = 7, *P* = 0.02; Mel-M: ρ = 0.82, n = 7, *P* = 0.02) and both groups trended towards significant positive correlations between attack duration and changes in circulating E_2 (SD-R: ρ = 0.71, n = 7, *P* = 0.07; Mel-M: ρ = 0.71, n = 7, *P* = 0.07). SD-NR females also trended towards a significant positive association between number of attacks and aggression-induced changes in serum E_2 (ρ = 0.70, n = 7, *P* = 0.08) and Mel-M females trended towards a significant positive association between aggression score and changes in serum E_2 levels (ρ = 0.75, n = 7, *P* = 0.05). Conversely, SD-R females showed a significant negative association between aggression score and aggression-induced changes in circulating T (ρ = −0.79, n = 7, *P* = 0.04), whereas SD-NR females trended towards a significant positive association between aggression score and changes in circulating T levels (ρ = 0.75, n = 7, *P* = 0.05). Mel-T females exhibited significant positive correlations between number of attacks and pre-aggression serum E_2 concentration (ρ = 0.79, n = 7, *P* = 0.04) and between attack duration and pre-aggression serum E_2 (ρ = 0.79, n = 7, *P* = 0.04), whereas SD-NR females exhibited a significant negative correlation between attack duration and pre-aggression serum DHEA concentration (ρ = −0.86, n = 7, *P* = 0.01). There were no significant relationships between aggressive behaviour and circulating hormone profiles in LD females (Table 1; see also Supporting information, Table S2).

3.6 | Seasonal phenotypes exhibited distinct relationships between neural aromatase abundance, aggressive behaviour and circulating steroids

LD and SD-R females exhibited distinct associations between aggressive behaviour and neural ARO-IR abundance in the PVN, PAG and VTA. Latency to first attack was significantly positively correlated with ARO cell density in the PVN (ρ = 0.94, n = 6, *P* < 0.01) (Figure 7A) and aggression score trended towards a significant positive correlation with ARO cell density in the PAG in SD-R females (ρ = 0.71, n = 7, *P* = 0.07) (Figure 7C), suggesting that increased aggression (eg, shorter attack latency, higher aggression score) in SD-R females is associated with lower ARO cell density in the PVN and higher ARO cell density in the PAG. Interestingly, aggression score was negatively associated with ARO cell density in the PVN in Mel-T females (ρ = −0.60, n = 5, *P* = 0.28) and was positively associated with ARO cell density in the PAG in Mel-T (ρ = 0.46, n = 7, *P* = 0.29) (Figure 7C) and SD-NR females (ρ = 0.50, n = 7, *P* = 0.25) (Figure 7D), although these relationships were not significant. Conversely, latency to first attack was negatively correlated with ARO abundance in the PVN in LD (ρ = −0.71, n = 6, *P* = 0.11), SD-NR (ρ = −0.50, n = 5, *P* = 0.39), Mel-T (ρ = −0.36, n = 5, *P* = 0.55) and Mel-M females (ρ = −0.80, n = 5, *P* = 0.10) (Figure 7B), although none of these relationships were significant. SD-NR females also trended towards a significant negative correlation between latency to first attack and ARO cell density in the PAG (ρ = −0.71, n = 7, *P* = 0.07) (Table 1), whereas LD females displayed a negative correlation between

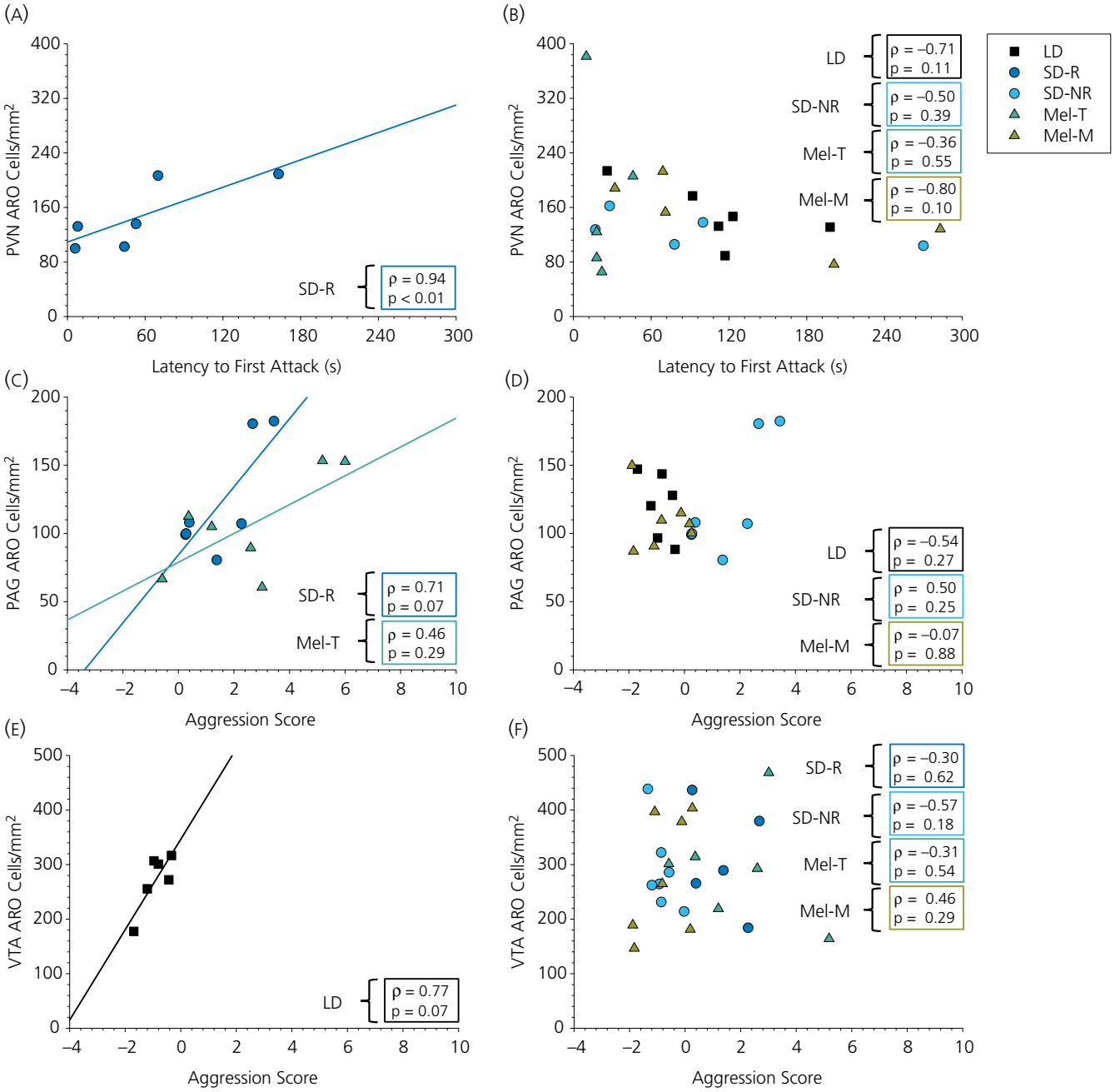

FIGURE 7 Seasonal phenotypes exhibited distinct associations between aggressive behaviour and aromatase (ARO) abundance in the paraventricular hypothalamic nucleus (PVN), periaqueductal gray (PAG) and ventral tegmental area (VTA). (A), Latency to first attack was positively correlated with aromatase abundance in the PVN in females that were responsive to short-day photoperiods (SD-R, dark blue). (B), Latency to first attack or aromatase abundance in the PVN were not correlated in long-day females (LD, black), females that were non-responsive to short-day photoperiods (SD-NR, light blue), LD females given timed melatonin injections (Mel-T, cyan) or LD females given mis-timed melatonin injections (Mel-M, olive). (C), Aggression score was positively correlated with aromatase abundance in the PAG of SD-R and Mel-T females. (D), Aggression score and aromatase abundance in the PAG were not correlated in LD, SD-NR or Mel-M females. E, Aggression score was positively correlated with aromatase abundance in the VTA of LD females. F, Aggression score or aromatase abundance in the VTA were not correlated in SD-R, SD-NR, Mel-T or Mel-M females. Regression lines were generated from Spearman's rank correlations within treatment groups (LD: n = 6, SD-R: n = 5-7, SD-NR: n = 5-7, Mel-T: n = 5-7, Mel-M: n = 5-7)

aggression score and ARO cell density in the PAG ($\rho = -0.54$, n = 6, $P = 0.27$) (Figure 7D), although this relationship did not reach significance. LD animals trended towards a significant positive association between aggression score and ARO abundance in the VTA ($\rho = 0.77$, n = 6, $P = 0.07$) (Figure 7E), whereas SD-R, SD-NR and Mel-T females displayed negative associations between aggression score and ARO abundance in the VTA (SD-R: $\rho = -0.30$, n = 5, $P = 0.62$; SD-NR: $\rho = -0.57$, n = 7, $P = 0.18$; Mel-T: $\rho = -0.31$, n = 6, $P = 0.54$). None of

these relationships, however, were significant (Figure 7F; for a complete list of pairwise comparisons, see also Supporting information, Table S2).

Female hamsters also exhibited different associations between neural ARO-IR cell density and circulating steroid profiles across seasonal phenotypes. ARO cell density in the PVN and pre-aggression serum DHEA levels were significantly positively correlated in SD-R females ($\rho = 0.83$, n = 6, $P = 0.04$) and were negatively correlated in LD, SD-NR and Mel-T females (LD: $\rho = -0.60$, n = 6, $P = 0.21$; SD-NR: $\rho = -0.60$, n = 5, $P = 0.28$; Mel-T: $\rho = -0.70$, n = 5, $P = 0.19$), although none of these relationships reached significance. ARO abundance in the PVN was also significantly positively associated with aggression-induced changes in serum DHEA in SD-NR females ($\rho = 0.90$, n = 5, $P = 0.04$), although similar relationships were not observed in any of the other treatment groups. In the PAG, ARO cell density trended towards a significant positive correlation with aggression-induced changes in circulating DHEA and E_2 in SD-R females (change in DHEA: $\rho = 0.68$, n = 7, $P = 0.09$; change in E_2: $\rho = 0.71$, n = 7, $P = 0.07$) and ARO abundance in this region was also positively correlated with aggression-induced changes in circulating E_2 in SD-NR females ($\rho = 0.89$, n = 7, $P = 0.01$). Although Mel-T females showed evidence of a positive correlation between ARO abundance in the PAG and aggression-induced changes in serum E_2 ($\rho = 0.64$, n = 7, $P = 0.12$), these animals trended towards a significant negative correlation between ARO abundance in this region and changes in serum DHEA ($\rho = -0.71$, n = 7, $P = 0.07$). In addition, Mel-T females trended towards a significant positive association between ARO cell density in the PAG and pre-aggression serum DHEA levels ($\rho = 0.68$, n = 7, $P = 0.09$). LD females showed a significant positive correlation between ARO abundance in the PAG and pre-aggression serum T concentration ($\rho = 0.89$, n = 6, $P = 0.02$), whereas Mel-M females exhibited a significant positive correlation between ARO abundance in this region and pre-aggression serum E_2 concentration ($\rho = 0.79$, n = 7, $P = 0.04$). In the VTA, ARO cell density was significantly positively associated with pre-aggression circulating T levels in SD-R females ($\rho = 0.90$, n = 5, $P = 0.04$), whereas ARO cell density in this region tended towards a significant negative association with pre-aggression circulating T in SD-NR females ($\rho = -0.68$, n = 7, $P = 0.09$). LD females trended towards a significant negative correlation between ARO abundance in the VTA and aggression-induced changes in serum T ($\rho = -0.77$, n = 6, $P = 0.07$), whereas Mel-T and Mel-M females showed positive correlations between ARO abundance in this region and changes in serum T (Mel-T: $\rho = 0.66$, n = 6, $P = 0.16$; Mel-M: $\rho = 0.57$, n = 7, $P = 0.18$), although neither of these relationships was significant. Mel-T females also trended towards a significant negative correlation between ARO abundance in the VTA and pre-aggression serum DHEA levels ($\rho = -0.77$, n = 6, $P = 0.07$) (Table 2; for a complete list of pairwise comparisons, see also Supporting information, Table S3).

4 | DISCUSSION

In the present study, we uncoupled the primary environmental cue (ie, ambient day length) from its biochemical signal (ie, melatonin) to test the independent effects of these factors on aggressive behaviour in female Siberian hamsters. We showed that administering a

TABLE 2 Correlations between neural aromatase abundance and circulating hormone profiles in female Siberian hamsters

		LD		SD-R		SD-NR		Mel-T		Mel-M	
		ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*	ρ	*P*
PVN ARO cell density	Pre-aggression DHEA	−0.60	0.21	0.83	**0.04**	−0.60	0.28	−0.70	0.19	0.50	0.39
	Pre-aggression T	−0.20	0.70	0.03	0.96	−0.80	0.10	−0.90	**0.04**	−0.30	0.62
	Change in DHEA	0.14	0.79	−0.49	0.33	0.90	**0.04**	0.60	0.28	0.10	0.87
PAG ARO cell density	Pre-aggression DHEA	−0.20	0.70	0.07	0.88	0.04	0.94	0.68	***0.09***	0.07	0.88
	Pre-aggression T	0.89	**0.02**	0.39	0.38	−0.29	0.53	−0.14	0.76	0.50	0.25
	Pre-aggression E_2	−0.60	0.21	−0.57	0.18	0.61	0.15	−0.11	0.82	0.79	**0.04**
	Change in DHEA	0.54	0.27	0.68	***0.09***	0.00	1.00	−0.71	***0.07***	−0.36	0.43
	Change in E_2	−0.09	0.87	0.71	***0.07***	0.89	**0.01**	0.64	0.12	−0.04	0.94
VTA ARO cell density	Pre-aggression DHEA	−0.26	0.62	0.10	0.87	0.04	0.94	−0.77	***0.07***	0.18	0.70
	Pre-aggression T	−0.66	0.16	0.90	**0.04**	−0.68	***0.09***	−0.20	0.70	−0.07	0.88
	Change in DHEA	−0.09	0.87	−0.30	0.62	0.39	0.38	0.43	0.40	0.04	0.94
	Change in T	−0.77	***0.07***	0.00	1.00	−0.54	0.22	0.66	0.16	0.57	0.18

Note: Spearman's rank correlations between neural aromatase abundance and circulating hormone profiles in long-day females (LD), females that were responsive to short-day photoperiods (SD-R), females that were non-responsive to short-day photoperiods (SD-NR), LD females given timed melatonin injections (Mel-T) and LD females given mis-timed melatonin injections (Mel-M). Correlations coefficients (ρ) and *P*-values are shown for each analysis, which was performed within treatment groups (LD: n = 6, SD-R: n = 5-7, SD-NR: n = 5-7, Mel-T: n = 5-7, Mel-M: n = 5-7). Only correlations that are significant ($P < 0.05$, in bold) or trending towards significance ($P < 0.10$, in bold and italics) in at least one treatment group are shown (for a full list of correlation analyses, see Supporting information, Table S3).

Abbreviations: ARO, aromatase; DHEA, dehydroepiandrosterone; E_2, oestradiol; PAG, periaqueductal gray; PVN, paraventricular hypothalamic nucleus; T, testosterone; VTA, ventral tegmental area.

physiologically relevant melatonin signal in the absence of SD photoperiods is sufficient to increase aggression and produce SD-like changes in circulating hormone profiles, including: (i) elevated serum DHEA levels; (ii) reduced serum E_2 levels; and (iii) aggression-induced increases in circulating steroid metabolism. Furthermore, we demonstrated that the timing of melatonin administration differentially affected LD females, such that timed, but not mis-timed melatonin injections induced gonadal regression and produced SD-like changes in aggression and circulating hormone profiles. Finally, we found that, although timed melatonin injections and SDs did not alter neural ARO abundance, SD-R females showed distinct associations between aggressive behaviour and ARO cell density in the PVN and PAG, and Mel-T females showed evidence of similar relationships between aggression and ARO cell density in these regions. Collectively, our findings suggest that the hormone melatonin regulates increased aggression during the non-breeding season, specifically by elevating circulating hormone metabolism after an aggressive challenge and by modulating the neuroendocrine circuits underlying SD aggression in a region-specific manner.

4.1 | Melatonin facilitates seasonally appropriate mechanisms of aggression

As expected, we found that LD females given timed melatonin injections, which summate with endogenous melatonin secretion to mimic a SD-like pattern, exhibited gonadal regression, decreases in circulating E_2 levels and increased aggression. By contrast, LD females given mis-timed melatonin remained reproductively functional, displayed elevated levels of circulating E_2, and showed decreased levels of aggression. These data illustrate that, in the absence of an environmental cue (ie, SD photoperiods), a SD-like melatonin signal is sufficient to induce seasonal shifts in physiology and behaviour. More importantly, it is not the presence of melatonin per se, but the precise timing of melatonin that is critical for allowing individuals to integrate aspects of their environment and make seasonally appropriate adjustments in these neuroendocrine processes. These findings are in agreement with our previous work in male Siberian hamsters, in which we showed that administering timed melatonin to LD males induces gonadal regression, elevates aggressive behaviour and produces SD-like changes in circulating androgen profiles.[40] It is also likely that this mode of integration for melatonin is involved in the regulation of aggression in other vertebrate species.17,18·63-67 Together, our findings suggest that melatonin mediates increased aggression via an alternative, extra-gonadal source of steroids during the non-breeding season.

In addition, we report that reproductively quiescent females (eg, females that have low circulating E_2) displayed elevated levels of DHEA in circulation, whereas reproductively functional females exhibited relatively low levels of circulating DHEA. These data suggest that reproductively quiescent animals rely on an extra-gonadal hormone, the adrenal androgen DHEA, to modulate non-breeding aggression. Furthermore, these findings support our hypothesis that some animals undergo a 'seasonal switch' from gonadal regulation of aggression during the breeding season to adrenal regulation of aggression during the non-breeding season, a mechanism that appears to be conserved, at least in part, across birds and mammals.17-19 Previously, we have shown that short-term exogenous melatonin treatment induces SD-like increases in aggression in male and female hamsters, yet does not affect reproductive physiology or circulating gonadal steroid levels.[16,38,39] Conversely, long-term administration of melatonin induces gonadal regression, elevates aggressive behaviour, and produces SD-like changes in circulating androgen profiles in male hamsters[40]; suggesting that melatonin may be involved in the early onset of responding to changes in ambient day length (eg, the transition from 'spring' to 'fall'). Moreover, we have shown that melatonin acts directly on the adrenal glands to increase the release of DHEA in SD females, whereas melatonin acts directly on the ovaries to increase the release of DHEA in LD females.[39] The present findings build on this hypothesis by suggesting that a long-term, SD-like melatonin signal is critical in allowing female hamsters to utilise an adrenal source of circulating steroids after an initial transition to a reproductively quiescent phenotype, when gonadal steroids are at a seasonal nadir. Our data also implicate melatonin as a key mediator of this 'seasonal switch' from gonadal to adrenal regulation of aggression.

4.2 | Aggression-induced changes in circulating hormone metabolism: Support for the challenge hypothesis?

In the present study, we showed that an aggressive interaction resulted in distinct changes in serum DHEA, T, and E_2 levels across seasonal phenotypes. Mel-T females exhibited decreases in serum DHEA and T, and both Mel-T and SD-R females displayed aggression-induced increases in serum E_2. By contrast, SD-NR and Mel-M females exhibited no significant changes in circulating DHEA and T, but showed aggression-induced increases in circulating E_2. These findings support our previous work, in which we found that SD hamsters display aggression-induced decreases in circulating DHEA and T[40,53,68] and that timed melatonin injections cause decreases in circulating DHEA and T in LD males after an aggressive encounter.[40] Other studies have shown that administering E_2 to male song sparrows (*M. melodia*), white-throated sparrows (*Zonotrichia albicollis*), beach mice (*P. polionotus*) and California mice (*Peromyscus californicus*) increases aggressive behaviour during the non-breeding season,[9,24,69-71] whereas blocking aromatase in circulation decreases aggression in non-breeding male song sparrows.[72] Taken together, these data support the hypothesis that DHEA, via conversion to biologically active T and/or E_2, can maintain or increase aggressive behaviour during periods of reproductive quiescence by compensating for low levels of circulating gonadal steroids. The seasonal uncoupling of these neuroendocrine processes suggests that natural selection has altered the mechanisms regulating non-breeding aggression. Therefore, these compensatory mechanisms are likely broadly applicable in the regulation of aggressive behaviour, particularly in contexts where T does not appear to be the primary player,11,22,27,28,34,73-75 as has been reported for males.[33,76]

Dynamic increases in circulating E_2 after aggressive challenges do not provide support for the challenge hypothesis, as originally described with respect to T.[30] However, because E_2 is the primary gonadal steroid in female hamsters, it provides support for an extension of this hypothesis,35,77 as do reports in female African black coucals (*Centropus grillii*) and Galápagos marine iguanas (*Amblyrhynchus cristatus*).[27,74] These data, along with emerging evidence regarding socially mediated changes in circulating T in females, suggest that this phenomenon is not as generally applicable as previously assumed.34,35 For example, there is no association between territorial aggression and T in female California mice,[73] dark-eyed juncos (*Junco hyemalis*),[75] spiny damselfish (*Acanthochromis polyacanthus*)[78] and white-browed sparrow weavers (*Plocepasser mahali*).[79] Moreover, consistent with observed decreases in circulating T for Mel-T females, female African black coucals,[27] eastern bluebirds (*Sialia sialis*),[80] Galápagos marine iguanas[74] and song sparrows[11] all exhibit decreases in circulating T after aggressive encounters. The diversity of hormonal responses to social challenges reported here and elsewhere illustrate that the complex effects of hormones on behaviour may be paralleled by reciprocal impacts of behaviour on hormones. In addition to circulating T, the challenge hypothesis should be tested through inclusion of relevant hormones for the species, sex and context in which aggression is being observed. Seasonally breeding species that are territorial year-round provide an ecologically relevant framework to test the confound of elevated levels of circulating gonadal steroids during the breeding season because high levels of aggression occur independently of reproduction (eg, when the gonads are regressed and circulating levels of T and E_2 are low).

4.3 | Seasonal differences in the role of neural aromatase in modulating aggressive behaviour

Interestingly, although we observed no differences in ARO-IR abundance in the PVN, PAG or VTA, we found that female hamsters showed distinct associations between neural ARO cell density, aggression and circulating steroid profiles across seasonal phenotypes. Although LD females exhibited a positive correlation between aggression and ARO cell density in the VTA, SD-R females showed opposing relationships between aggressive behaviour and ARO cell density in the PVN and PAG, in which aggression was negatively associated with ARO cell density in the PVN, but positively associated with ARO cell density in the PAG. Mel-T females also showed evidence of a negative correlation between aggression and ARO cell density in the PVN and a positive correlation between aggression and ARO cell density in the PAG, although these relationships were not significant. Similar correlations between aggressive behaviour and ARO cell density in the PVN, PAG and VTA, however, were not observed in SD-NR or Mel-M females. Both the VTA and PVN have been implicated in modulating reproductive behaviours. The VTA contains dopaminergic neurones that project to several forebrain regions and have been shown to regulate motivation, reproduction and parental behaviours.42,81,82 The PVN has a critical role in regulating seasonal reproduction, particularly by serving as a control center for the hypothalamic-pituitary-gonadal (HPG) axis.1-3 Furthermore, the PVN contains neurones that synthesise the neuropeptides arginine vasopressin (AVP) and oxytocin (OXT),83,84 and changes in AVP and OXT levels within the PVN have been associated with reproductive and sexual behaviours.41 Conversely, the PAG serves as a relay between several hypothalamic nuclei associated with aggressive behaviour, including the medial preoptic area and the ventromedial hypothalamus.43 Thus, the PAG has mostly been implicated in modulating aggression.43,44 We also showed that SD-R females exhibit different relationships between neural ARO abundance in these regions and circulating steroids, in which aggression-induced changes in circulating DHEA and E_2 were positively correlated with ARO abundance in the PAG, whereas pre-aggression DHEA and E_2 levels were positively correlated with ARO abundance in the PVN and VTA, respectively. Taken together, these findings suggest that the neuroendocrine circuits mediating reproduction and aggression are uncoupled during the non-breeding season and that aromatase abundance in the PVN and PAG may have opposing actions on aggressive behaviour. Our results also suggest that these circuits are mediated, at least in part, by melatonin.

To date, seasonal differences in neural aromatase and aggressive behaviour have mostly been studied in birds. Male song sparrows have elevated aromatase activity in the ventromedial telencephalon (including the nucleus taeniae, a region that is homologous to the mammalian amygdala), but decreased aromatase activity in the diencephalon (includes the preoptic area) during the non-breeding season.[85] In male Japanese quail (*Coturnix japonica*), aggression is positively associated with aromatase activity in the anterior hypothalamus/preoptic area during the breeding season,[86] whereas male breeding white-crowned sparrows (*Zonotrichia leucophrys*) show no association between neural aromatase activity and aggressive behaviour.[87] Taken together, these results suggest that the relationship between neural aromatase and aggression varies in a seasonal and region-specific manner. In addition, because hormonal signalling can be influenced by several mechanisms, including steroidogenesis, steroid metabolism, and receptor abundance and binding affinity, it is likely that neural oestrogen receptor abundance and/or binding affinity may also mediate seasonal changes in aggressive behaviour. We have shown that SD-R females exhibit increases in oestrogen receptor alpha (ERα) abundance in brain regions associated with aggression, including the PAG, lateral septum and bed nucleus of the stria terminalis (BnST), although not in regions associated with reproduction, including the preoptic area, arcuate nucleus and anteroventral periventricular nucleus of the hypothalamus.[25] SD-R males also display elevated ERα expression in brain nuclei associated with aggression, including the BnST and medial amygdala.[26] Similarly, in male beach mice and deer mice (*Peromyscus maniculatus*), ERα abundance and expression in the BnST are positively correlated with aggressive behaviour during SDs.[88] Further studies are needed to examine how seasonal changes in neural oestrogenic signalling

modulate aggressive behaviour and whether melatonin may regulate these mechanisms.

5 | CONCLUSIONS

Collectively, our findings suggest that melatonin, the biochemical cue for photoperiod in mammals, signals the induction of a suite of physiological modifications that drive seasonal changes in aggressive behaviour. We provide support for the hypothesis that melatonin mediates a 'seasonal switch' from gonadal to adrenal regulation of aggression, specifically by elevating the metabolism of adrenal prohormones into biologically active steroids in circulation following an aggressive interaction and by regulating the neuroendocrine circuits mediating non-breeding aggression in a region-specific manner. These results enhance our understanding of how environmental cues mediate neuroendocrine processes to regulate seasonal shifts in social behaviour. More broadly, these findings provide insight into the patterns and processes by which natural selection can, and has, altered organismal responses on evolutionary, seasonal and immediate timeframes.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

NMR and GED designed the experiments. NMR performed photoperiodic treatments, administered melatonin and control injections, staged behavioural interactions, collected vaginal cytology samples, performed blood and tissue collection, determined reproductive phenotypes, analysed video files, and ran hormone assays. CLP and MAK validated, optimised and performed procedures for neural aromatase histology. ACA and DLB processed tissue prior to histological analysis, and KMM, ACA and DLB quantified aromatase. NMR and KMM conducted statistical analyses, and all authors interpreted the results. NMR, KMM and GED wrote the manuscript, with editorial contributions from CLP, ACA, DLB and MAK.

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DATA AVAILABILITY

Data from this study are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.j0zpc86c6.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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