

Aggressive behaviours track transitions in seasonal phenotypes of female Siberian hamsters

Nikki M. Rendon* , Andrea C. Amez, Melissa R. Proffitt, Elizabeth R. Bauserman and Gregory E. Demas

Department of Biology, Center for the Integrative Study of Animal Behaviour, Program in Neuroscience, Indiana University, Bloomington, IN 47405, USA

Summary

1. Seasonally breeding animals exhibit profound physiological and behavioural responses to changes in ambient day length (photoperiod), including changes in reproductive function and territorial aggression.
2. Species where aggression persists when gonads are regressed and circulating levels of gonadal hormones are low, such as Siberian hamsters (*Phodopus sungorus*) and song sparrows (*Melospiza melodia*), challenge the well-established framework that gonadal hormones are important mediators of aggression.
3. A solution to this apparent paradox is that a season-specific increase in sensitivity to hormones in brain areas associated with aggression offsets low gonadal hormone levels during periods of reproductive quiescence.
4. To test this hypothesis, we manipulated photoperiod to induce natural fluctuations in seasonal phenotype across multiple stages of the annual reproductive cycle in female Siberian hamsters that display increased aggression during short-day reproductive quiescence, suggesting that behaviour persists independent of gonadal steroids.
5. Females were housed in long 'summer' days or short 'winter' days for 10, 24 or 30 weeks to capture gonadal regression, a transition back to a reproductively functional state and full gonadal recrudescence, respectively.
6. Long-day animals maintained reproductive functionality and displayed low aggression across all time points. By week 10, short-day reproductively responsive females underwent gonadal regression and displayed increased aggression; non-responsive animals showed no such changes. At week 24, animals were in a transitional period and displayed an intermediate phenotype with respect to reproduction and aggression. By week 30, short-day females were fully recrudescenced and returned to long-day-like levels of aggression.
7. Consistent with our hypothesis, gonadally regressed females displayed decreases in 17 β -oestradiol (oestradiol) levels, but site-specific increases in the abundance of brain oestrogen receptor-alpha (ER α) in regions associated with aggression, but not reproduction. Increased site-specific ER α may function as a compensatory mechanism to allow increased responsiveness to oestradiol in regulating aggression *in lieu* of high circulating concentrations of hormones.
8. Collectively, these results broaden our understanding of how breeding phenology maps onto social behaviour and the mechanisms that have evolved to coordinate behaviours that occur in non-breeding contexts.

Key-words: agonism, biological rhythms, breeding phenology, competitive phenotypes, estrogens, fertility, melatonin, refractory

Introduction

Seasonally breeding mammals restrict reproduction to a discrete period and are reproductively quiescent the rest of

the year (Bronson 1985; Bronson & Heideman 1994). Fluctuations in reproductive phenotype are likely due to natural selection favouring the birth of young to coincide with the time of year when the maximum amount of food is available (Bronson 1985; Bronson & Heideman 1994;

*Correspondence author. E-mail: nrendon@indiana.edu

Prendergast, Kriegsfeld & Nelson 2001). Further, selection is stronger on females than males due to the asymmetry in the cost of reproduction – females bear the cost of a developing foetus during gestation and high energetic demands of lactation postnatally (Bronson 1985; Kiltie 1988; Bronson & Heideman 1994; Prendergast, Kriegsfeld & Nelson 2001). Energetic bottlenecks make it critical for individuals to predict seasonal changes in optimal breeding conditions in advance of their occurrence. To accomplish this, seasonal breeders rely on fluctuations in environmental variables such as day length, temperature and food availability to appropriately time alterations in seasonal phenotypes (Bronson & Heideman 1994; Goldman 2001; Walton, Weil & Nelson 2011).

Many seasonally breeding mammals use ambient day length (i.e. photoperiod) as the primary cue to coordinate reproduction with the appropriate breeding season (Bronson & Heideman 1994; Goldman 2001; Walton, Weil & Nelson 2011). Within controlled photoperiodic conditions, transitions from long ‘summer’ days to short ‘winter’ days results in decreased secretion of gonadal steroids, regression of the gonads and loss of sexual behaviour. In addition to these changes, females transition from an ovulatory cycle to anovulation, becoming acyclic in short days (Bronson & Heideman 1994; Goldman 2001; Walton, Weil & Nelson 2011). Following prolonged exposure to short days, however, seasonal breeders become refractory to inhibitory short photoperiods (i.e. lose sensitivity to the signal) and undergo spontaneous gonadal recrudescence by reverting to the reproductively competent long-day phenotype (likely in anticipation of the upcoming spring breeding season). Recrudescence involves gonadal regrowth, the restoration of gametogenesis and resumption of ovulation.

In addition to marked physiological changes occurring on a yearly basis, there are profound fluctuations in social behaviour observed across a number of species. For example, song sparrows (*Melospiza melodia*) and spotted antbirds (*Hylophylax naevoides*) display high aggression during the breeding season when gonads are functional, but also maintain aggression when gonads are regressed and circulating levels of gonadal hormones are low (Soma & Wingfield 1999; Hau, Stoddard & Soma 2004). Gonadal hormones do not appear to underlie non-breeding aggression. Siberian hamsters (*Phodopus sungorus*) (Fig. 1) also display marked increases in aggression when reproductively inactive (i.e. short days) compared with reproductively active morphs (i.e. long days) (Jasnow *et al.* 2000; Scotti, Place & Demas 2007; Bedrosian *et al.* 2012; Rendon *et al.* 2015b). From a functional perspective, short-day aggression might confer an evolutionary advantage for this solitary animal when food availability is relatively low and competition for limited resources is high – food restriction increases aggression in a photoperiod regime that mimics the transition between breeding and non-breeding seasons (Bailey *et al.* 2016). Aggression during the breeding season co-occurs with elevated testosterone



Fig 1. Siberian hamsters (*Phodopus sungorus*) housed in long ‘summer-like’ days exhibit thin, brown/grey coats (left) and hamsters housed in short ‘winter-like’ days exhibit thick, white coats (right).

concentrations in males and 17β -oestradiol (oestradiol) in females (Jasnow *et al.* 2000; Scotti, Place & Demas 2007). In contrast, aggression that persists during the non-breeding season (when hormone levels are low) suggests that the same behaviour may be regulated independent of gonadal steroids during the non-breeding season (Jasnow *et al.* 2000; Scotti, Place & Demas 2007), as is the case for the antbird and sparrow examples. Experimental elevation of gonadal steroids that mimic long-day levels in males (i.e. testosterone) and females (i.e. oestradiol) does not reduce aggression, showing that aggression is not suppressed by high hormone levels (Jasnow *et al.* 2000; Scotti, Place & Demas 2007). Further, aggression is not reduced when gonadal steroids are virtually depleted via ovariectomies, strongly suggesting that the regulation of female aggression may be independent of gonadal steroids (Scotti, Place & Demas 2007). The lack of a relationship in a season-dependent manner provides support for the hypothesis that alternate neuroendocrine mechanisms underlie aggression across seasons.

Two alternative, but not mutually exclusive, mechanisms have been proposed across a variety of species to describe how high levels of aggression despite relatively low levels of a specific hormone could persist. First, low hormone levels could be accommodated by extra-gonadal hormonal precursors. For example, the adrenal androgen dehydroepiandrosterone (DHEA) has been implicated as an important hormonal precursor in the regulation of winter-like aggression, but not summer-like aggression, via metabolic conversion to testosterone and oestradiol (e.g. birds: Soma & Wingfield 1999; Pradhan, Yu & Soma 2008; mammals: Gutzler *et al.* 2009; Rendon *et al.* 2015b; Rendon & Demas 2016; reviewed in: Soma *et al.* 2015). Second, high levels of aggression could be accommodated by shifts in target tissue sensitivity (e.g. upregulation of hormone receptors in the brain) across the seasons. This has been reported as a compensatory mechanism in a number of species (birds: Canoine *et al.* 2007; mammals: Kramer, Simmons & Freeman 2008), including species that display

a sex role reversal in aggression (Voigt & Goymann 2007; Goymann *et al.* 2008). Females of sex role reversed species typically display low levels of gonadal hormones, but are predicted to have heightened sensitivity to hormones in areas of the brain associated with aggression (reviewed in: Eens & Pinxten 2000; Rosvall 2013b). These examples are consistent with the overarching hypothesis that seasonal changes in mechanisms of aggression map onto shifts between sources of hormones and their location of action to facilitate the acquisition of limited resources (e.g. food), while also avoiding the cost of maintaining reproductive tissues (i.e. costs of high levels of sex steroids) and not siring young out of season (Prendergast, Kriegsfeld & Nelson 2001; Wingfield, Lynn & Soma 2001).

In addition to metabolic conversion of hormonal precursors to active androgens and oestrogens (reviewed in: Soma *et al.* 2015), the variation in aggression seen across seasons may depend on changes in abundance and distribution of oestrogen receptors in the brain. A variety of vertebrate species have a dense distribution of oestrogen receptors that are localised to many of the same brain regions in the 'social behaviour network', consisting of six neuroanatomical regions involved in social behaviours, including reproduction and aggression (Newman 1999; Goodson 2005; O'Connell & Hofmann 2012). Generally speaking for mammals, the anterior hypothalamus (AH; referred to as 'attack area'), periaqueductal grey (PAG) and ventromedial hypothalamus (VMH) are thought to mostly be involved in the regulation of aggression (Lin *et al.* 2011; Hashikawa *et al.* 2016) and the arcuate (ARC) and anteroventral periventricular (AVPV) regions of the hypothalamus regulate reproduction (Micevych, May Wong & Mittelman-Smith 2015). The bed nucleus of the stria terminalis (BnST), lateral septum (LS), medial amygdala (MeAD) and preoptic area (POA) have been shown to be associated with sexual behaviours and aggression (Micevych, May Wong & Mittelman-Smith 2015; Hashikawa *et al.* 2016; see also: Trainor, Greiwe & Nelson 2006; Trainor *et al.* 2007). Mosaic changes in brain receptor abundance throughout this network changes seasonally and likely predict temporal fluctuations in physiology and behaviour.

For many females, including hamsters, oestradiol is the predominant gonadal steroid that shows dynamic seasonal variation; therefore, site-specific changes in oestrogen receptors within behaviourally relevant regions could serve as a compensatory mechanism by which significant increases in aggression are maintained during periods of gonadal regression when levels of oestradiol are relatively low. Oestradiol can act on oestrogen receptor-alpha (ER α) and oestrogen receptor-beta (ER β), however, knock out studies that functionally disrupt these receptor subtypes reveal causal support for ER α regulating aggression. Specifically, ER α KOs demonstrated reduced aggression (Ogawa *et al.* 1997, 1998), whereas ER β KOs show no change or a slight elevation in aggression (Ogawa *et al.* 1997, 1998; see also: Trainor *et al.* 2007). Because of the

strong association between ER α and aggression and our aim of examining neuroendocrine mechanisms that change seasonally, we have chosen to focus on the α -subtype. Comparative analyses of hormones, receptors and aggression in both sexes are rare, therefore such a comprehensive approach is a necessary step to understanding mechanisms regulating female seasonal aggression.

The goal here was to test the hypothesis that increased sensitivity to oestradiol in brain regions associated with aggression, but not reproduction, regulates high aggression in seasonal phenotypes where levels of oestradiol are low. Specifically, we manipulated photoperiodic regimes; female hamsters were housed in long or short days in order to induce variation in seasonal phenotype following gonadal regression (mimicking winter), a transition back to a reproductively functional state (late winter/early spring) and full gonadal recrudescence (spring). Long-day animals maintained functional reproductive physiology (summer) across all time points. Across seasonal phenotypes, we examined changes in circulating oestradiol and abundance of ER α in the brain across varying degrees of aggression.

Materials and methods

ANIMALS AND HOUSING

Adult female hamsters (>60 days of age) were reared and maintained in a breeding colony at Indiana University under long days (LD, light : dark, 16 : 8 h) and group-housed at weaning (postnatal day 18) in polypropylene cages (40 × 20 × 20 cm). Sani-chip bedding (Laboratory Grade, Harlan Teklad) was used (see: Landeros *et al.* 2011) and hamsters were given *ad libitum* access to standard laboratory rodent chow (Lab Diet 5001, PMI Nutrition) and water. Ambient temperature was maintained at 20 ± 2 °C and relative humidity was maintained at 55 ± 5%. All procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) at Indiana University.

PHOTOPERIODIC MANIPULATIONS

Prior to start of the photoperiodic manipulation, experimental (resident) female hamsters were individually housed ($n = 102$) for a 1-week acclimation period on a long-day light cycle (LD). Intruder hamsters, used as stimulus animals during behavioural testing, were pair-housed with a sibling ($n = 40$) and remained in long days for the duration of the experiment. Following acclimation, resident hamsters were transferred to a room on a short-day light cycle (SD, light : dark, 8 : 16 h) or were relocated to a new room on a long-day light cycle. Hamsters remained in photoperiodic regimens for 10 (LD: $n = 10$; SD: $n = 19$), 24 (LD: $n = 14$; SD: $n = 34$) or 30 (LD: $n = 10$; SD: $n = 15$) weeks in order to capture variation in female seasonal phenotype following (1) gonadal regression, (2) transition to a reproductively functional state and (3) full gonadal recrudescence, respectively.

SEASONAL PHENOTYPES

Metrics to determine suites of changes in seasonal phenotype (i.e. long-day like vs. short-day like) and reproductive functionality (i.e. intact reproductive tissues and oestrous cyclicity vs. regressed

reproductive tissues and anoestrous/oestrous acyclicity) were based on *a priori* criteria previously established for Siberian hamsters (Jasnow *et al.* 2000; Scotti, Place & Demas 2007; Rendon *et al.* 2015a, b). Throughout the duration of the experiment, body mass (weekly), coat colour (weekly) and oestrous cyclicity via vaginal cytology (weekly and 5 days prior to behavioural trials, Supporting Information) were assessed and reproductive tissues (ovaries and uterine horns) were collected at 10, 24 or 30 weeks. Reproductive tissues and oestrous cycles were the primary means of assessing group designations (criteria matched in all instances); body mass and coat colour were used to confirm categorisation. Hamsters were characterised as exhibiting a long-day phenotype if they had functional reproductive tissues, displayed no significant change in body mass (<10%) and maintained a brown/grey coat colour (long days, LD; week 10: $n = 10$, week 24: $n = 14$, week 30: $n = 10$; short-day non-responder, SD-NR; week 10: $n = 10$, week 24: $n = 8$, week 30: $n = 0$). Females exhibiting a short-day like phenotype were characterised by regressed reproductive tissues, a significant decrease in body mass (>10%) and a white coat colour (short-day responder, SD-R; week 10: $n = 9$, week 24: $n = 26$, week 30: $n = 15$). Short-day non-responders (SD-NR) did not respond reproductively to short days for the duration of the experiment (Fig. 3), a natural phenotype affecting ~30% of the population of this and many rodent species (Lynch, Lynch & Kliman 1989; Gorman & Zucker 1995; Goldman 2001). Although in inhibitory short days, *non-responsive* SD-NR hamsters display patterns of melatonin similar to LD animals. Melatonin, the hormonal correlate of photoperiod, is secreted during darkness and inhibited by light (Puchalski & Lynch 1986; Prendergast, Kriegsfeld & Nelson 2001). SD-NRs were included in statistical analyses for weeks 10 and 24, but not for week 30 due to lack of sufficient animals. In addition to classifying females at static time points, we examined temporal patterns of seasonal transitions using body mass and oestrous cycles as a proxy of seasonal phenotype (see Fig. 2). Females were considered long-day like if they were cycling and did not show a change in body mass (<10%), whereas females were considered short-day like if they were acyclic/anoestrous and showed a decrease in body mass (>10%) (see Fig. 2). If both criteria were not met (e.g. cycling, but showed a decrease in body mass), that female was excluded from proxy data.

QUANTIFICATION OF AGGRESSIVE BEHAVIOUR

Same-sex aggressive encounters were staged, recorded and analysed for all animals after 10, 24 or 30 weeks in their respective photoperiodic treatments, using a 5-min female–female resident–intruder paradigm outlined previously (Rendon *et al.* 2015b, 2016a, b; Supporting Information). Latency to first attack (seconds) as well as number and duration (seconds) of attacks and chases were quantified for our suite of aggressive behaviours. Aggression data were reduced to a composite ‘aggression score’ using principal component analyses (PCA), with the extracted component explaining 73.54% of the total variance (Table S1, Supporting Information).

BLOOD SAMPLING, OESTRADIOL QUANTIFICATION AND TISSUE COLLECTION

After 10 and 30 weeks of photoperiodic manipulation, blood samples were drawn 24 h prior to behavioural trials. Animals were slightly anaesthetised, using isoflurane (Isothesia; 50562; Butler Schein, Dublin, OH) and blood samples from the retro-orbital sinus were drawn within 1 min (3 min total). Blood samples were processed and serum oestradiol concentrations were quantified using a commercially available enzyme immunoassay (Supporting

Information). Tissues were collected at necropsy immediately following aggression trials.

IMMUNOLABELLING AND QUANTIFICATION OF OESTROGEN RECEPTOR-ALPHA (ER α)

Brain oestrogen receptor-alpha immunoreactive cells (ER α) were fluorescently labelled via a standard protocol on free-floating coronal sections (40 μ m) of paraformaldehyde fixed tissue (Bharati & Goodson 2006; Goodson *et al.* 2009) using a rabbit polyclonal antibody (anti-ER α C1355; 06-935; EMD Millipore, Billerica, MA) diluted at 1 : 400 and a secondary antibody conjugated to Alexa Fluor[®] 488 (S11223; Life Technologies, Carlsbad, CA). Samples were counterstained with a nuclear stain, DAPI (P36935; Life Technologies, Carlsbad, CA), in order to localize brain regions of interest and to confirm if receptors were localised within the nucleus (Fig. S1). Regions, including sub-regions (i.e. medial portion, m; ventral portion, v) analysed for ER α include the anterior hypothalamus (AH; Bregma –0.94), arcuate nucleus of the hypothalamus (ARC; Bregma –1.46), anteroventral periventricular nucleus of the hypothalamus (AVPV; Bregma 0.02), bed nucleus of the stria terminalis (BnST; Bregma 0.14), lateral septum (LS; Bregma 0.14), medial nucleus of the amygdala (MeAD; Bregma –1.70), periaqueductal grey of the midbrain (PAG; Bregma –2.70), preoptic area of the hypothalamus (POA; Bregma 0.02), paraventricular nucleus of the hypothalamus (PVN; Bregma –0.94) and the ventromedial nucleus of the hypothalamus (VMH; Bregma –1.46); each were identified using a brain atlas (Paxinos & Franklin 2001). Nuclear-bound labelled ER α cells were counted bilaterally for each region of interest, corrected for size, Abercrombie corrected and expressed as cells per unit area (Abercrombie 1946).

STATISTICAL ANALYSES

All statistical analyses were run in JMP v. 11.0.0 (SAS Institute, Inc., Cary, NC) and statistical significance was attributed if $P < 0.05$ after adjusted to control for false discovery rate when making multiple comparisons (Verhoeven, Simonsen & McIntyre 2005). Data were transformed to attain normality and equal variances. Two-way analyses of variances (ANOVAs) were used to examine the effects of photoperiod (i.e. LD and SD) and week (i.e. 10, 24 and 30) on aggression variables, reproductive physiology, circulating oestradiol and ER α counts. ER α counts were analysed in separate models for each brain area. Tukey's HSD post hoc analyses were conducted if there was a statistically significant interaction of photoperiod and week. Spearman's rank correlations were used to quantitatively assess relationships between aggression and ER α counts.

Results

SEASONAL PHENOTYPES AND AGGRESSIVE BEHAVIOUR DIFFERED ACROSS PHOTOPERIODS

Using the seasonal phenotype proxy, temporal patterns of photoperiodic responsiveness among groups was tracked. In response to short days, SD-Rs exhibited a short-day like phenotype by undergoing gonadal regression (weeks 5–14), spontaneously gonadally recrudescing (weeks 24–30) and achieving gonadal recrudescence (Week 30). In contrast, LDs and SD-NRs maintained a long-day phenotype (weeks 0–30) (Fig. 2). SD-Rs displayed decreased

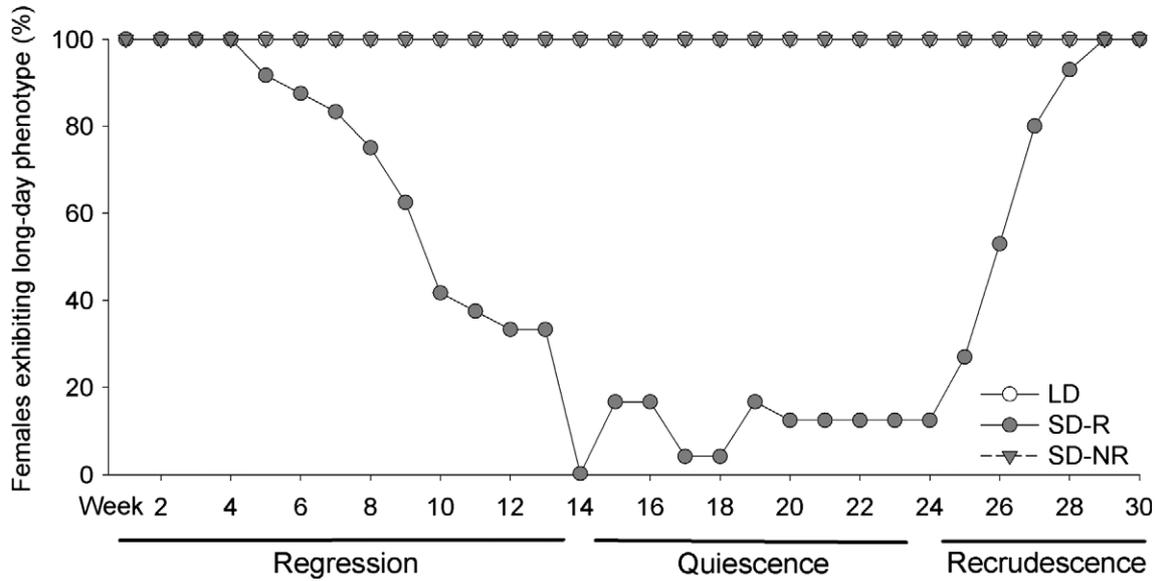


Fig 2. Fluctuations in seasonal phenotype differ across photoperiods. Females were placed in either long days (LD) or short days (SD; Week 0; displayed estrous cyclicity and high body mass). In response to short-day exposure (SD-R), females underwent gonadal regression (weeks 5–14; displayed estrous acyclicity and low body mass) and transitioned from a long-day-like to a short-day-like phenotype. Following prolonged exposure, females became insensitive to the short-day signal and underwent spontaneous gonadal recrudescence (weeks 24–30) and transitioned back to a long-day-like phenotype (week 30). Long-day females and short-day non-responders (SD-NR) maintained their seasonal phenotype regardless of photoperiod (weeks 0–30). White symbols depict long days; grey symbols depict short days.

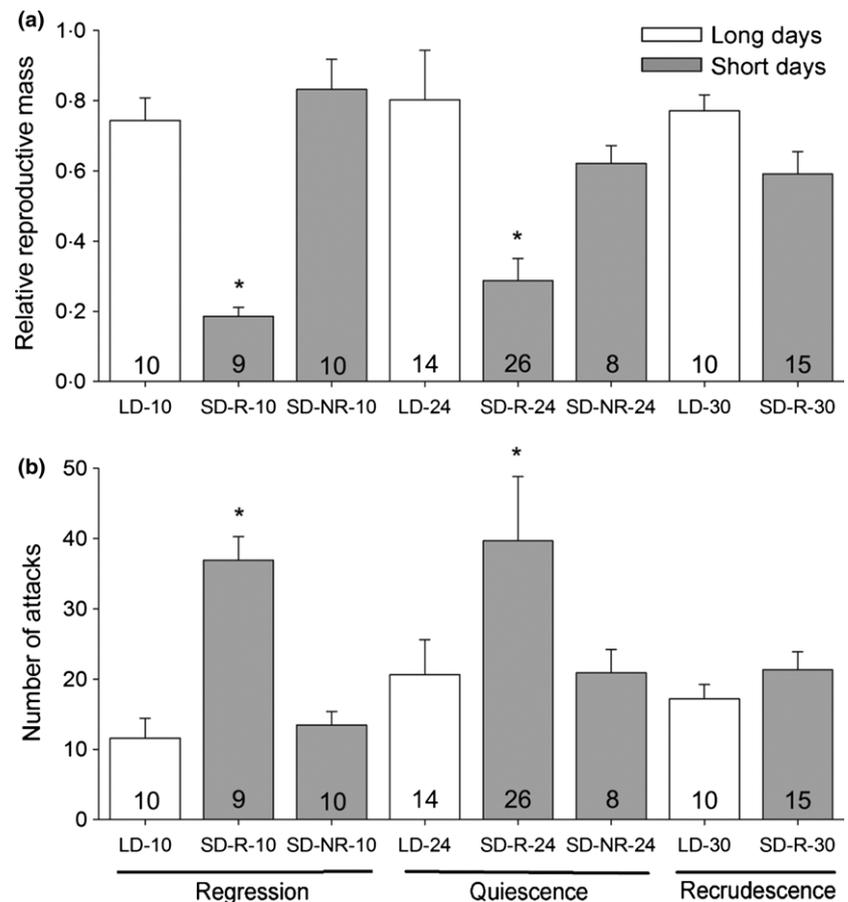


Fig 3. Reproductive physiology and aggressive behaviour changed across groups. (a) Relative reproductive mass and (b) Number of attacks. Bar heights represent means \pm SEM and sample sizes are indicated by numbers at base of each bar. Gonadal regression (week 10); reproductive quiescence (week 24); gonadal recrudescence (week 30). *Indicates statistically significant differences between group means (two-way ANOVAs with Tukey's HSD post hoc analyses, $P < 0.05$).

relative reproductive mass (ovaries + uterine horns adjusted by body mass) at weeks 10 and 24 when compared with LDs and SD-NRs indicative of gonadal regression and statistically indistinguishable relative reproductive mass with LDs and SD-NRs at week 30 indicative of gonadal recrudescence (photo*week: $F_{2,96} = 12.16$; $P = 0.02$; Fig. 3a). Further, SD-Rs displayed increased aggression during gonadal regression and reproductive quiescence (weeks 10 and 24) when compared with LD and SD-NRs and statistically indistinguishable aggression with LD and SD-NRs during gonadal recrudescence (week 30) (number of attacks: photo*week: $F_{2,96} = 14.44$; $P = 0.04$; Fig. 3b; additional behaviours in Supporting Information).

DIFFERENTIAL TIMING IN FLUCTUATIONS OF REPRODUCTIVE COMPETENCE AFFECTED AGGRESSIVE BEHAVIOUR

Female SD-Rs displayed significant variation in time to reproductively respond to inhibitory short days via gonadal regression (weeks 5–14; Fig. 2) and inception of refractoriness via gonadal recrudescence (weeks 24–30; Fig. 2). Week 24 was a transitional period for SD-Rs and captured differential timing of reproductive functionality; some females were gonadally regressed during this period (short-day delayed responders, SD-DR-24), whereas others were gonadally recrudescing (short-day recrudescing, SD-REC-24; $t_{24} = 6.48$, $P = 0.007$; Fig. 4a). During week 24, gonadally regressed females (SD-DR-24) displayed increased levels of aggression when compared with gonadally recrudescing females (SD-REC-24; $t_{24} = -3.95$, $P = 0.004$; Fig. 4b).

Changes in aggression paralleled seasonal phenotype such that aggression increased in hamsters transferred

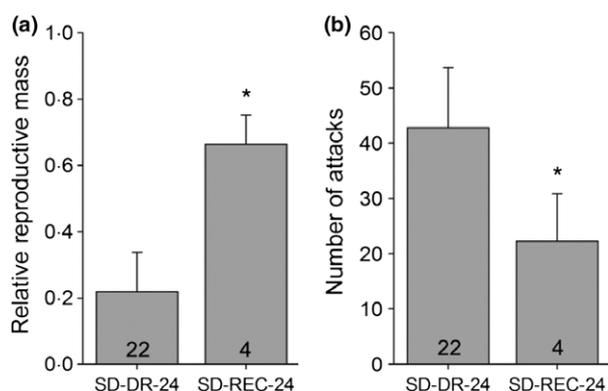


Fig 4. Differential timing of gonadal regression and gonadal recrudescence among short-day responders affects variation in aggression. During a transitional period (week 24) there is variation in (a) Relative reproductive mass and (b) Number of attacks among short-day delayed responders (SD-DR-24) and short-day recrudescing (SD-REC-24) females. Bar heights represent means \pm SEM and sample sizes are indicated by numbers at base of each bar. *Indicates statistically significant differences between group means (two-tailed t -test, $P < 0.05$).

from long to short days until the point that refractoriness to short days was observed. During week 10, gonadally functional females (LD and SD-NR) grouped together with high relative reproductive mass and a low number of attacks when compared with gonadally regressed females

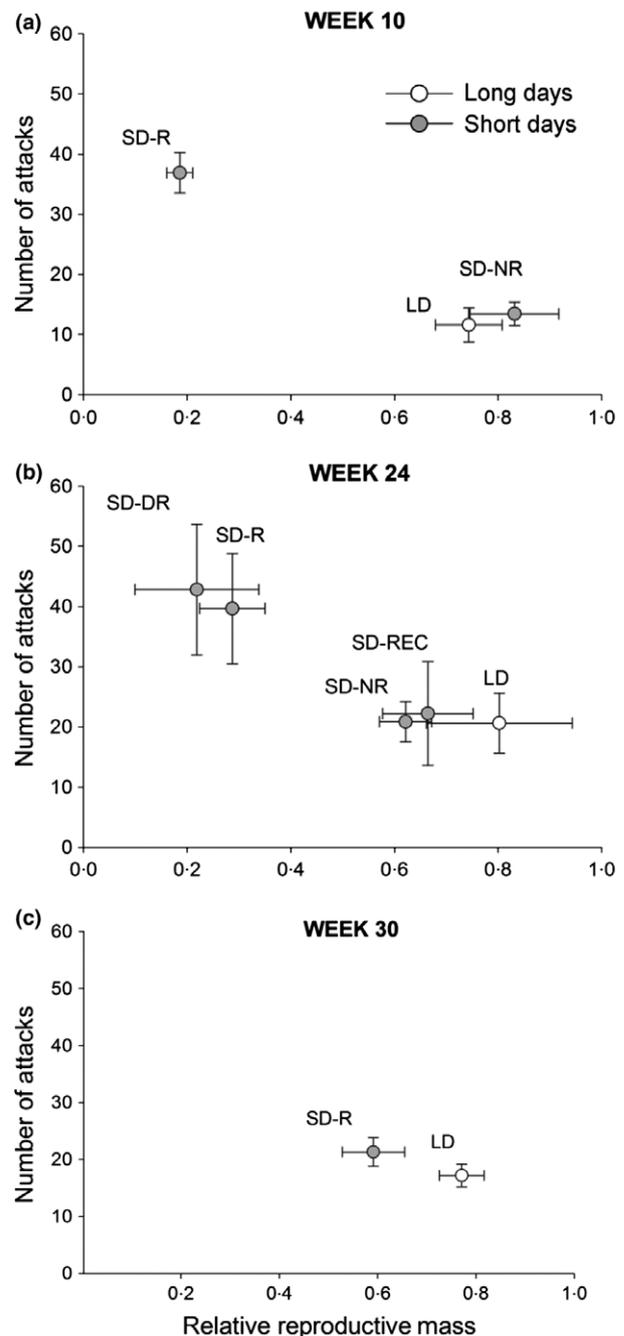


Fig 5. Relationships between aggressive behaviour and reproductive physiology differ across seasonal phenotypes. Number of attacks (y -axis) and relative reproductive mass (x -axis) are plotted across (a) Gonadal regression, (b) Transitional period and (c) Gonadal recrudescence. Circles represent means \pm SEM. LD, long day; SD-R, short-day responder; SD-NR, short-day non-responder; SD-DR, short-day delayed responder; SD-REC, short-day recrudescing.

(SD-R) that displayed high attack rates (Fig. 5a). In week 24, the same pattern was seen, with SD-RECs grouping with gonadally functional females and SD-DRs grouping with gonadally regressed females (Fig. 5b). By week 30, all short-day responsive females had gonadally recrudescenced, grouping with LDs (Fig. 5c). Overall, a negative relationship between reproductive physiology and aggression persisted into week 24 (transitional period) but was abolished by week 30 (recrudescence). Week 24 was a transitional period, comprised of two seasonal phenotypes, therefore, we focused on weeks 10 and 30 that captured gonadal regression and gonadal recrudescence in order to probe the underlying neuroendocrine mechanisms regulating this behaviour.

CIRCULATING OESTRADIOL AND NEURAL ER α DIFFERED ACROSS SEASONAL PHENOTYPES

Circulating concentrations of oestradiol paralleled changes in reproductive mass; SD-Rs displayed decreased oestradiol at weeks 10 when compared with LD and SD-NRs and statistically indistinguishable oestradiol levels with LDs and SD-NRs at week 30 (photo \times week: $F_{2,96} = 13.78$; $P = 0.02$; Fig. 6). ER α abundance paralleled number of attacks in some regions associated with aggression, PAG (photo \times week: $F_{2,40} = 4.69$, $P = 0.03$; Fig. 7a), BSTm (m, medial portion of BnST; photo \times week: $F_{2,40} = 1.28$, $P = 0.03$; Fig. 7b), LSv (v, ventral portion of LS; photo \times week: $F_{2,40} = 3.93$, $P = 0.04$; Fig. 7c), but not all, MeAD (photo \times week: $F_{2,40} = 3.68$, $P = 0.20$; Fig. 7d). In contrast, ER α abundance did not parallel number of attacks in brain regions associated with reproduction, POA (photo \times week: $F_{2,40} = 3.50$, $P = 0.12$; Fig. 7e) and ARC (photo \times week: $F_{2,40} = 1.47$, $P = 0.49$; Fig. 7f). In addition, ER α abundance either did not differ among groups or did not parallel number of attacks in

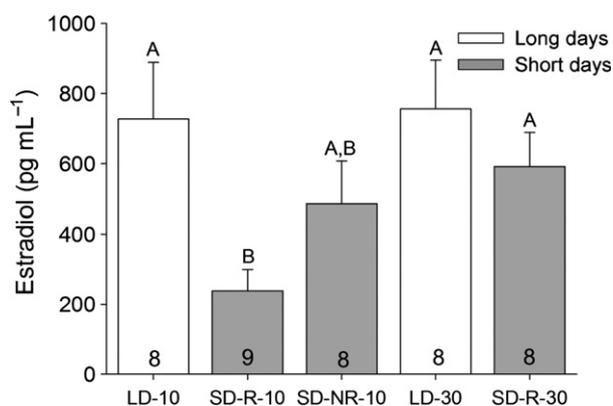


Fig 6. Circulating levels of oestradiol change across gonadal regression (week 10) and gonadal recrudescence (week 30). Bar heights represent means \pm SEM and sample sizes are indicated by numbers at base of each bar. Groups with different letters indicate statistically significant differences between group means, whereas groups sharing the same letter are statistically equivalent (two-way ANOVA with Tukey's HSD post hoc analysis, $P < 0.05$).

other brain regions examined (Fig. S3). Cross-sectional area did not differ across groups for any regions where ER α was quantified ($P > 0.05$ in all cases).

INDIVIDUAL LEVELS OF AGGRESSION AND ER α WERE ASSOCIATED IN DISCRETE BRAIN REGIONS

Reproductively functional females displayed positive associations between number of attacks and ER α in the AH (LDs and SD-NRs), BnST (SD-RECs) and PAG (SD-NRs). In contrast, reproductively quiescent females displayed negative associations between aggression and ER α in the AH (SD-Rs) and MeAD (SD-Rs) (Table 1). There were no significant relationships between aggression and ER α in the ARC, POA and LS.

Discussion

This is the first study to our knowledge to examine changes in aggression, hormone levels and hormone receptors across seasonal (i.e. photoperiodic) transitions in a mammal. We show that aggression tracks phenotype over seasonal reproductive development. Further, we show site-specific upregulation of ER α in brain areas associated with aggression, consistent with the hypothesis for a season-dependent compensatory mechanism underlying robust displays of aggression during periods when oestradiol is relatively low. These results broaden our understanding of how breeding phenology is an important driver of associated changes in mechanisms of social behaviour and such insights may apply to other seasonal breeders.

AGGRESSIVE BEHAVIOURS TRACK SEASONAL PHENOTYPE ACROSS TRANSITIONS

By comparing seasonal phenotypes we found that reproductively quiescent females displayed increased aggression during gonadal regression (i.e. 'winter') and the transition back to reproductive competence (i.e. 'late winter/early spring'). In contrast, following gonadal recrudescence ('spring'), reproductively functional short-day females displayed decreased aggression, similar to long-day hamsters. Further, in response to inhibitory photoperiods, females displayed considerable variation in the onset of reproductive incompetence (between 5 and 14 weeks) and subsequent refractoriness, observed by the onset of reproduction (as late as week 28). Such findings greatly expand our knowledge of female seasonal aggression (see Badura & Nunez 1989; Scotti, Place & Demas 2007; Rendon *et al.* 2015a). It is predicted that anoestrous females would not mate during periods of gonadal regression and the transitional period (i.e. periods of infertility), but would mate during gonadal recrudescence (i.e. when fertility resumes) (Beery *et al.* 2007), suggesting that the absence of copulation precedes increases in winter aggression and that sexual behaviour subsequently follows decreases in aggression during gonadal regrowth.

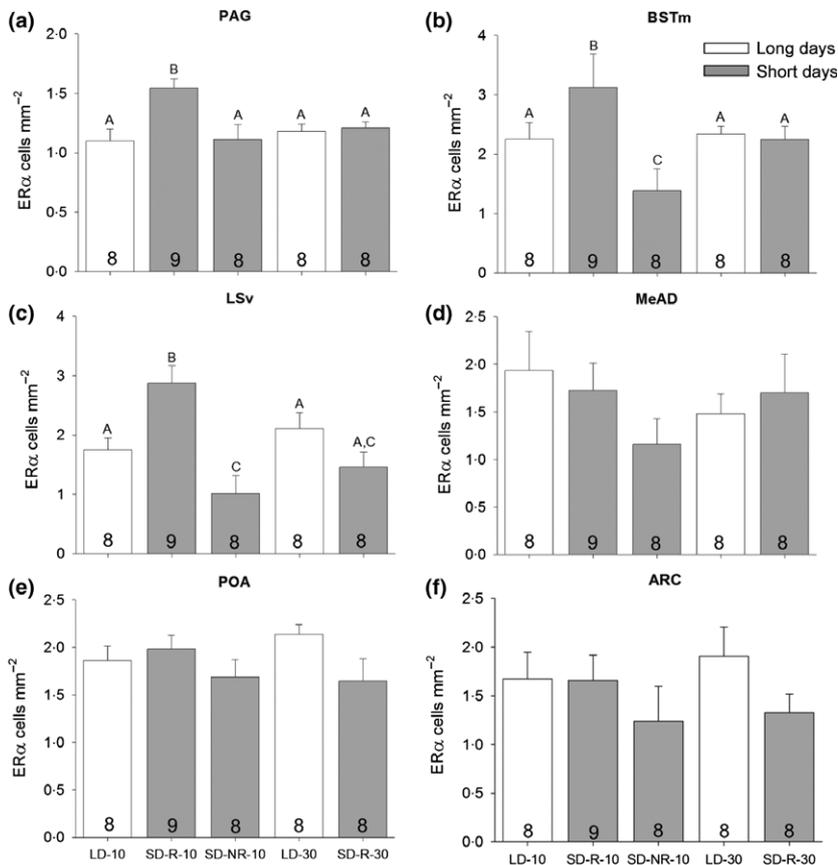


Fig 7. Neural ER α abundance changed across gonadal regression (week 10) and gonadal recrudescence (week 30). ER α changed in some regions associated with aggression, but not all: (a) PAG, (b) BnST (medial portion), (c) LS (ventral portion) and (d) MeAD. In contrast, ER did not change in regions associated with reproduction: (e) POA and (f) ARC. Bar heights represent means \pm SEM and sample sizes are indicated by numbers at base of each bar. Groups with different letters indicate statistically significant differences between group means, whereas groups sharing the same letter are statistically equivalent (two-way ANOVAs with Tukey's HSD post hoc analyses, $P < 0.05$).

Table 1. Relationships between aggression (number of attacks) and neural ER α (cells per mm 2) in brain regions associated with aggressive behaviour across groups

Group	<i>n</i>	Attacks and AH ER α		Attacks and BnST ER α		Attacks and LS ER α		Attacks and MeAD ER α		Attacks and PAG ER α	
		ρ	<i>P</i>	ρ	<i>P</i>	ρ	<i>P</i>	ρ	<i>P</i>	ρ	<i>P</i>
LD	16	0.27	0.03	-0.18	0.49	0.25	0.35	-0.06	0.84	0.31	0.25
SD-R	9	-0.87	0.002	-0.03	0.93	-0.23	0.55	-0.62	0.008	0.13	0.73
SD-NR	8	0.54	0.03	0.18	0.67	0.31	0.45	-0.19	0.65	0.66	0.03
SD-REC	8	-0.47	0.24	0.55	0.004	-0.12	0.78	0.29	0.49	-0.08	0.84

Bold values indicate statistical significance using Spearman's rank correlations within groups, $P < 0.05$.

ALTERNATIVE SOURCES OF OESTRADIOL DURING GONADAL REGRESSION

Marked increases in aggression occur during gonadal regression when oestradiol secretion is basal, suggesting that short-day aggression is independent of oestradiol or that these animals rely on another hormonal precursor to regulate aggression. In support of the latter hypothesis, we have recently shown that adrenal androgen dehydroepiandrosterone (DHEA) concentrations mirror seasonal aggression (Rendon *et al.* 2015b) and that the adrenal gland is a key hormonal source during periods of reproductive quiescence, indicative of a 'seasonal switch' from gonadal to adrenal regulation of aggression (Rendon *et al.* 2015b, see also: Soma & Wingfield 1999; Demas,

Soma & Albers 2011; Soma *et al.* 2015; Prough, Clark & Klinge 2016; Rendon & Demas 2016). DHEA can serve as a precursor to testosterone and oestradiol, therefore metabolic conversion makes it possible for relatively low levels of circulating hormones to have dynamic behavioural effects across seasons, as observed across numerous mammals and birds (Hau, Stoddard & Soma 2004; Soma *et al.* 2005, 2015; Labrie 2010, 2015; Pradhan *et al.* 2010; Demas, Soma & Albers 2011). In further support of this hypothesis, we have shown rapid metabolic conversion of DHEA and a subsequent surge in oestradiol induced by aggression in short-day but not long-day females (Soma *et al.* 2015). Natural selection, therefore, has likely favoured a 'seasonal switch' in endocrine source as well as the metabolic conversion of precursor hormones to

bioactive forms during the non-breeding season to avoid the cost of maintaining reproductive tissues and siring young outside of the appropriate time of year (Prendergast, Kriegsfeld & Nelson 2001; Wingfield, Lynn & Soma 2001). Moreover, costs of elevated levels of circulating T have been shown to be detrimental to females [e.g. infertility, delaying breeding, reducing maternal care, interfering with mate choice, altering immune function (Barracough & Gorski 1962; Clotfelter *et al.* 2004; McGlothlin *et al.* 2004; Rutkowska *et al.* 2005; Gerlach & Ketterson 2013; Rosvall 2013a)].

A COMPENSATORY MECHANISM REGULATING AGGRESSION DURING REPRODUCTIVE QUIESCENCE

While the seasonal switch hypothesis above can explain how short-day females utilize alternative endocrine mechanisms to regulate non-breeding aggression, it cannot account for how reproduction and aggression become dissociated in short days. Siberian hamsters are simultaneously reproductively active and aggressive during the breeding season and both behaviours are regulated by oestradiol (Prendergast, Nelson & Zucker 2002; Walton, Weil & Nelson 2011). In short days, however, there is a marked dissociation between reproduction and aggression at a time when oestradiol is at a seasonal nadir. One hypothesis is that there are concomitant seasonal changes in hormone receptors in brain areas associated with aggression, but not in regions associated with reproduction. If true, this would allow for dissociation of the effects of oestradiol on aggression versus reproduction; relatively low levels of oestradiol could enhance aggression due to increased target tissue sensitivity (see Voigt & Goymann 2007). Consistent with this hypothesis, our data demonstrate that gonadally regressed females had low levels of oestradiol that co-occurred with site-specific elevations in abundance of ER α in the PAG, BSTm and LSv, areas associated with aggression (Newman 1999; Goodson 2005; Nelson 2006; O'Connell & Hofmann 2011; Hashikawa *et al.* 2016; see also Trainor *et al.* 2007; Kramer, Simmons & Freeman 2008). In contrast, we did observe increases in abundance of ER α in the POA, ARC and AVPV, areas associated with reproduction (Prendergast, Nelson & Zucker 2002; Walton, Weil & Nelson 2011; Hashikawa *et al.* 2016; see also Kramer, Simmons & Freeman 2008). It should be noted that we report negative correlations between aggression and ER α abundance within the AH for SD-Rs, but positive correlations within the AH for LDs (see also Trainor, Greiwe & Nelson 2006). Therefore, it seems that ER α is downregulated within the AH during the transition from summer to winter, but is upregulated within the PAG, BSTm and LSv. These findings support the idea that non-breeding animals increase oestradiol sensitivity through upregulation of ER α , thereby maintaining an aggressive phenotype, consistent with previous studies in a number of mammalian and avian species (Trainor, Greiwe & Nelson 2006; Canoine *et al.* 2007; Trainor *et al.*

2007; Voigt & Goymann 2007; Kramer, Simmons & Freeman 2008; Rosvall *et al.* 2012; Burns, Rosvall & Ketterson 2013). We also report positive correlations between aggression and ER α abundance within the BSTm and PAG for SD-REC and SD-NR respectively. These seasonal morphs experience short days but the cue is not transduced biochemically to target tissues, therefore, they display melatonin profiles that are indistinguishable from long-day animals. This suggests that the dynamic increases in aggression and increased abundance of ER α seen in SD-Rs is largely driven by long-duration melatonin exposure (Jasnow *et al.* 2002; Rendon *et al.* 2015b; N.M. Rendon, C.L. Petersen, A.C. Amez, D.L. Boyes, M.A. Kingsbury & G.E. Demas, unpubl. data). In addition, we report photoperiodic differences in oestradiol concentrations, consistent with Bedrosian *et al.* (2012), however, no photoperiodic changes have likewise been observed (Scotti, Place & Demas 2007). This inconsistency in oestradiol warrants further examination (see Phalen *et al.* 2010), however, the hypothesis that aggression during short days is independent of circulating hormones is supported. Although the precise mechanisms underlying seasonal aggression are not fully known, future studies should address the potential interplay between melatonin and hormonal profiles, the role of brain-derived steroids, non-genomic responses to hormones and seasonal shifts in receptor affinity (do Rego & Vaudry 2015; Soma *et al.* 2015; Prough, Clark & Klinge 2016). Additional neuroendocrine mechanisms may be involved in the regulation of aggression across seasonal transitions [e.g. progesterone (Rubenstein & Wikelski 2005; Goymann *et al.* 2008), prolactin (Eens & Pinxten 2000), cortisol (Newman & Soma 2011); reviewed in Rosvall 2013b). Nonetheless, a compensatory upregulation of ER α likely plays an important role in aggression during periods of reproductive quiescence for hamsters, similar to what has been reported in other vertebrate species (Trainor, Greiwe & Nelson 2006; Canoine *et al.* 2007; Trainor *et al.* 2007; Voigt & Goymann 2007; Kramer, Simmons & Freeman 2008; Rosvall *et al.* 2012; Burns, Rosvall & Ketterson 2013).

Conclusions

Taken together, the findings from this study suggest that animals that express aggression outside of breeding conditions facilitate this behaviour through a compensatory receptor mechanism. We provide support for the hypothesis that increased brain sensitivity in a non-reproductive brain can fuel aggression despite decreased circulating oestradiol, via site-specific changes in abundance of ER α . This work complement previous work detailing a shift to an extra-gonadal hormone source to regulate non-breeding aggression across mammal and bird species. More broadly, this work contributes meaningful insights to the evolution of hormone-mediated phenotypes and to a more comprehensive understanding of neuroendocrine mechanisms regulating seasonal behaviours.

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Data accessibility

Data deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.m466p> (Rendon et al. 2016a).

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Supporting Information

Details of electronic Supporting Information are provided below.

Table S1. Loading and eigenvalues of principal components analysis for aggression data.

Fig. S1. Representative photomicrograph of ER α labelling.

Fig. S2. Aggression score across groups.

Fig. S3. ER α across groups.