RESEARCH ARTICLE

Short-Day Aggression is Independent of Changes in Cortisol or Glucocorticoid Receptors in Male Siberian Hamsters (*Phodopus sungorus*)

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ABSTRACT

Testosterone mediates aggression in many vertebrates. In some species, aggression remains high during the non-breeding season (e.g., winter), when testosterone levels are low. In Siberian hamsters (*Phodopus sungorus*), we have demonstrated photoperiodic changes in aggression with hamsters housed in short, "winter-like" days displaying significantly more territorial aggression than long-day animals, despite low levels of testosterone. The mechanisms by which photoperiod regulates aggression, however, remain largely unknown. Adrenocortical hormones (e.g., glucocorticoids) have been implicated in mediating seasonal aggression; circulating concentrations of these hormones have been correlated with aggression in some species. The goal of this study was to examine the role of cortisol and glucocorticoid receptors in mediating photoperiodic changes in aggression in male Siberian hamsters. Males were housed in long or short days and treated with either exogenous cortisol or vehicle. Circulating levels of cortisol, adrenal cortisol content, and aggression were quantified. Lastly, photoperiodic effects on glucocorticoid receptor (GR) protein levels were quantified in limbic brain regions associated with aggression, including medial prefrontal cortex, amygdala, and hippocampus. Short-day hamsters were more aggressive than long-day hamsters, however cortisol treatment did not affect aggression. Photoperiod had no effect on serum or adrenal cortisol or GR levels in the brain regions examined. Taken together, these data suggest that increases in cortisol levels do not cause increases associated with short-day aggression, and further that GR protein levels are not associated with photoperiodic changes in aggression. The results of this study contribute to our understanding of the role of adrenocortical steroids in mediating seasonal aggression. *J. Exp. Zool. 323A:331–342, 2015.* © 2015 Wiley Periodicals, Inc.

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Aggressive behaviors are known to promote the survival and fitness of individuals. As such, the suite of behaviors that have been categorized as aggressive have been relatively well investigated. Investigations aimed at elucidating the mechanisms that underlie aggression have focused primarily on the role of gonadal steroid hormones, specifically testosterone (T) in male animals. It is well known that castration reduces aggression and exogenous T increases aggression in some rodent species (Uhrich, '38; Beeman, '47; Leshner and Moyer, '75; Barkley and Goldman, '77; Wagner et al., '79; Haug et al., '86). Furthermore, aggression is significantly correlated with circulating T levels (Ehrenkranz et al., '74; Mazur and Booth, '98). The robust and reliable relationship between T and aggression has led to a predominant focus on the role of this hormone in the study of male aggression. More recent experimental evidence in a range of mammalian and avian species in both field and laboratory settings, however, suggests that the link between T and aggression may be more complex than originally assumed, and that many other hormones play an integral part in the expression of this social behavior (Simon, 2002; Demas et al., 2007; Soma et al., 2008, 2015).

Investigations of the hormonal regulation of aggression in seasonally breeding species that exhibit year-round aggression have found that non-breeding aggression is often independent of T (Canoine and Gwinner, 2002; Romeo et al., 2003; Soma, 2006). Males from several species of birds exhibit high levels of territorial aggression throughout the year (Canoine and Gwinner, 2002). Often the territoriality expressed during both the reproductive and non-reproductive seasons is quantitatively and qualitatively similar (Boonstra and McColl, 2000; Soma and Wingfield, 2001). During the breeding season aggression is regulated by T, whereas non-breeding aggression has been shown to be independent of T (Soma et al., 2000; Soma and Wingfield, 2001; Pinxten et al., 2003). Similar findings have been reported for rodents examined under controlled laboratory conditions (e.g., Demas et al., '99; Jasnow et al., 2002; Romeo et al., 2003; Trainor et al., 2007). For example, territorial aggression is uncoupled from T in hamsters. In Syrian (*Mesocrecitus auratus*) and Siberian (*Phodopus sungorus*) hamsters breeding occurs during long-day "summer-like" photoperiods, however, high levels of territorial aggression are expressed during short-day "winter-like" photoperiods, a time when gonads are regressed and circulating T levels are low (Garrett and Campbell, '80; Jasnow et al., 2000, 2002; Kramer et al., 2008). Thus, non-reproductive, gonadally regressed males with low levels of circulating T are more aggressive than males in reproductive condition. These results suggest that photoperiodic changes in aggression are independent or possibly inversely related to circulating concentrations of gonadal steroids (Flemming et al., '88; Jasnow et al., 2000; Scotti et al., 2007). These exceptions to the traditional link between gonadal steroids and aggression prompted the investigation of alternative hormonal mediators of aggressive behavior (Soma, 2006; Demas et al., 2007, Soma et al., 2015). Evidence for the role of adrenal hormones, specifically corticosteroids, as mediators of territorial aggression in rodents, has been widely reported (Haller and Kruk, 2003). For example, it has been demonstrated repeatedly that bilateral adrenalectomy reduces aggression (Brain et al., '71; Harding and Leshner, '72; Landau, '75; Paterson and Vickers, '81; Demas et al., 2004b). Especially relevant for species that maintain high levels of aggression year-round, or display non-breeding increases in aggressive behavior, are studies in which adrenalectomies have been performed on animals in which non-breeding condition has been induced. Bilateral removal of the adrenal glands reduced aggression in short-day like Siberian hamsters (Demas et al., 2004b). Demedullation alone, which disrupts the production of catecholamines but not steroids however, has no effect on short-day like aggression (Demas et al., 2004b). These results support the hypothesis that adrenocortical steroids play a key role in mediating territorial aggression; however, such studies have not tested which specific class of adrenocortical steroid is mediating the behavior (i.e., glucocorticoids or adrenal androgens). There are, several studies, across diverse taxa, that specifically implicate glucocorticoids and/or their cognate receptors as playing a substantial role in mediating aggression (Sigg et al., '66; Kostowski et al., '70; Banerjee, '71; Wingfield and Silverin, '86; Sapolsky, 2002; Wommack and Delville, 2003, 2007).

Elevated cortisol (CORT) levels (Bilbo and Nelson, 2003), as well as an increase in adrenal mass (Scotti et al., 2008), have been observed in male Siberian hamsters housed in short- compared with long-days. Additionally, photoperiodic changes in glucocorticoid receptors (GRs) have been shown in Syrian hamsters (Ronchi et al., '98). Based on these data, as well as the published studies that report a role for glucocorticoids in aggression, we hypothesized that photoperiodic changes in CORT, and likely GRs, contribute to seasonal changes in aggressive behavior. To test this hypothesis, serum CORT levels of long- and short-day housed male Siberian hamsters were experimentally elevated via exogenous hormone treatment. Aggressive behavior was then assessed using a resident-intruder model of aggression (Jasnow et al., 2000; Scotti et al., 2007). Additionally, differences in GR protein levels in limbic brain regions associated with aggression, including medial prefrontal cortex, amygdala, and hippocampus, in long- and short-day housed hamsters were quantified via both western blot and immunohistochemistry to determine if seasonal changes in receptor levels are associated with

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seasonal changes in aggression, a previously untested possibility. We predicted that short-day animals would be more aggressive than long-day animals and that CORT treatment would increase aggression in both photoperiods. We also predicted increased GR levels in short-day animals exhibiting increased aggression.

MATERIALS AND METHODS

Animals and Housing Conditions

Adult (>60 days of age) male Siberian hamsters (*P. sungorus*) were obtained from a breeding colony at Indiana University. One week before the start of the experiments, hamsters were housed individually in polypropylene cages (40 cm × 20 cm × 20 cm) in rooms with a 24 h light: dark 16:8 cycle (lights off 1800 h EST). Temperature was kept constant at 20 ± 2°C and relative humidity was maintained at 50 ± 5%. Food (Purina Rat Chow) and tap water were available ad libitum throughout the experiment. Additional animals were used as non-aggressive intruders during aggression testing and were group-housed (3–4 animals per cage) in long days (light: dark 16:8) to keep aggression to a minimum (Brain, '72; Svare and Leshner, '73). These animals were approximately 2 months younger than experimental animals and thus, weighed less. Siberian hamsters lose approximately 10% of their body weight when moved into a short-day photoperiod (Fine and Bartness, '96), therefore, in the case of such interactions the intruder is always younger than the resident, but may in fact be larger. These non-aggressive intruders were chosen to prompt aggression from the resident (Jasnow et al., 2000), and were used no more than twice per testing day. All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and a protocol approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC).

Experimental Design

Hamsters ($n = 117$) were weighed and individually housed under either a light: dark 16:8 h ($n = 40$) or a light: dark 8:16 h cycle ($n = 77$) at the beginning of the experiment. At the end of 7 weeks, the animals were re-weighed. In many seasonally breeding rodents, including Siberian hamsters, there is a small subset of individuals that are non-responsive to short-day photoperiods. These photoperiodic "non-responders" do not undergo gonadal regression and generally respond physiologically and behaviorally like long-day animals (Goldman, 2001). A Siberian hamster that is responsive to short-day photoperiod loses at least ten percent of its body weight (Fine and Bartness, '96). Any animals that did not lose weight or gained weight while housed in a short-day photoperiod during that 7-week period were designated as non-responders and removed from the study ($n = 35$). Individuals that were long-day housed ($n = 40$) and short-day responders ($n = 42$) were then randomly assigned to receive either a control treatment or CORT treatment. At the end of the experiment, necropsies were performed on all animals to ensure that all non-responders were correctly identified. Short-day males whose paired testes were less than 0.25 g were classified as a responder (Greives et al., 2007).

All animals were tested using a resident-intruder model of aggression by introducing a non-aggressive intruder into the home cage of an experimental animal for 5 min to assess territorial aggression (Jasnow et al., 2000). To increase territorial aggression, the bedding material of experimental animals remained unchanged for at least 1 week prior to the behavioral encounter (Jasnow et al., 2000). Behavioral testing began at lights-off (i.e., 1,400 for short-days and 1,800 for long days) and the testing did not exceed 2 hr in order to control for circadian rhythmicity of both behavior and hormone levels. All trials were performed under low illumination/red light conditions, which allowed sufficient light for video recording and observation without disturbing the natural behavior of the hamsters. Behavioral interactions were videotaped and scored using ODlog™ (Macropod Software, Eden Prairie, MN) by an observer naïve to experimental conditions. Intruders were identified by small patches of shaved fur on their dorsal surfaces. Twenty-four hours prior to behavioral testing, and 2 hr following daily CORT injection (see below), resident hamsters were lightly anesthetized with diethyl ether (VWR, Indianapolis, IN) and blood samples were drawn from the retro-orbital sinus. Serum was extracted following centrifugation.

Hormonal Manipulations

Hamsters in the CORT treatment groups received a 100 µl subcutaneous injection of 10mg/kg of cortisol (Sigma Chemical #H4001, St. Louis, MO, USA), the primary glucocorticoid in Siberian hamsters (Reburn and Wynne-Edwards, '99), dissolved in peanut oil. Control animals received 100 µl of vehicle only (i.e., peanut oil). Each animal received a single injection for seven consecutive days beginning on their 7th week of photoperiodic treatment. Each injection was given at the same time each day (8 hr prior to lights-off). The final injection was given on the day of the aggression trials, which coincided with the animals' 8th week of photoperiodic treatment.

Behavioral Testing and Scoring

The following behaviors were scored over the 5 min testing period: aggressive behaviors (i.e., attacking, latency to attack), investigative behavior (i.e., anogenital investigation) and maintenance behavior (i.e., grooming). We defined an attack as physical contact between the resident and intruder that was initiated by the resident and resulted in biting and/or pinning. Anogenital investigation (AGI) was scored as the resident sniffing the anogenital region of the intruder.

Perfusions and Necropsies

Upon completion of behavioral trials animals were euthanized via 0.3 cc of a 1:10 ketamine cocktail comprised of ketamine (100 mg/ml; Henry Schein, Melville, NY, USA), xylazine (20 mg/ml; Henry

Schein), and 0.9% saline. Following euthanasia, brains to be processed for western blots were removed and immediately frozen on dry ice. Once frozen, each brain was maintained at −80°C until processed. Brains to be processed for immunohistochemistry were perfused. For perfusions, animals were given a 0.3 cc of a 1:10 ketamine cocktail and perfused transcardially with 100 ml of 0.9% saline, followed by 150 ml of 4% paraformaldehyde in 0.1 M PBS, pH = 7.3. Brains were post-fixed for 2 h at room temperature in 4% paraformaldehyde, and cryoprotected in 20% sucrose in 0.1 M PB for at least 48 hr. The brains were then stored in 0.1 M PB at 4°C until processed. Necropsies were performed on all animals and paired testes were collected, cleaned of fat and connective tissue and weighed. Animals that, after 8 weeks in short days, had paired testes weighing >0.25 g were classified as short-day non-responders ($n = 35$); animals with paired-testes weighing <0.25 were classified as short-day responders ($n = 42$).

Tissue Punches

Brains were sectioned on a cryostat (Leica Jung Frigocut 2600E), and tissue punches were collected using a 0.5 mm diameter sample corer tool (Fine Science Tools; Foster City, CA). Tissue punches from coronal sections (400 μm thick) were taken through the medial prefrontal cortex (mPFC) and medial amygdala (mAMY). Punches were immediately snap frozen on powdered dry ice and stored at −70°C until protein assays and western blots were conducted.

Protein Assays, Western Blotting and Densitometry

To prepare lysates for protein assays and western blots, tissues were homogenized with a sonifier pestle in lysis buffer (1% SDS in dH_2O with Roche complete, Mini, EDTA-free protease inhibitor cocktail; Roche, Diagnostics, Basal, Switzerland) and then incubated in a boiling water bath for 10 min. Lysates were then spun in a microfuge (13,000 rpm for 30 sec) to remove insoluble material. The protein concentration of the cleared supernatant was determined by the BCA method (Pierce, Rockford, IL). Western blots were used to measure GR protein levels in mPFC and mAMY. Lysates were subjected to SDS gel electrophoresis, blotted to a nitrocellulose membrane (Invitrogen, Carlsbad, CA), and probed with anti-GR (1:5,000; M-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins were visualized by chemiluminescence (Pierce) according to the manufacturer's instructions on Kodak XAR film. After membranes were probed for glucocorticoid receptors, the membranes were stripped using SDS/2-mercapto-ethanol for 20 min at 50°C and re-probed with actin (1:50,000, monoclonal; Sigma-Aldrich, Saint Louis, MO, Chemical) as a loading control. After stripping, membranes were washed and incubated with chemiluminescence to make sure the original signal was removed. The relative optical densities (RODs) of the immunoreactive bands were measured using computerized image analysis software (MCID-M4). The experimenter making the density measures was blind to the experimental conditions of the samples.

Glucocorticoid Receptor Immunohistochemistry

Free-floating sections (40 μm) were washed three times for 10 min in 0.1M PB and incubated for 10 min in 0.05% H_2O_2 in 0.1M phosphate buffered saline (PBS). Sections were then washed three times for 10 min in 0.1M PB with 0.1% Triton X-100 (PBT), blocked in 2% normal goat serum (NGS) in PBT for 1 h, and incubated in rabbit anti-GR raised against the N-terminus (1:10,000, GR (M-20), Santa Cruz Biotechnology, Santa Cruz, CA) in 2% NGS in PBT for 48 h at 4°C. Sections were then washed three times for 10 min in PBT and incubated in biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA) in PBT for 1 h at room temperature (RT). Sections were then washed 3 times for 10 min in PBT and incubated in avidin–biotin horseradish peroxidase complex (1:200; Vectastain ABC Kit, Vector Labs, Burlingame, CA, USA) in PBT for 1 h at RT. After three 10 min washes in PBS, horseradish peroxidase was visualized with 0.02% 3,3′diaminobenzidine (DAB) in a 3 M sodium acetate buffer containing 0.05% H_2O_2. Sections were then washed 5 times for 10 min in PBS, mounted onto Fisher Brand Plus slides (Fisher Scientific, Pittsburgh, PA, USA), dried, dehydrated in increasing concentrations of ethanol (70%, 95%, and 100%), cleared in xylenes, and coverslipped with DPX Mountant (Sigma Aldrich Chemical, St. Louis, MO, USA). Processing tissue in the presence of both the GR (M-20) antibody and the blocking peptide (sc-1004P, 10 μg/ml; Santa Cruz Biotechnology, Dallas, TX) resulted in no detectable immunostaining by the secondary antibody (Fig. 3A and B).

Microscopic Analysis of Glucocorticoid Receptor Immunohistochemistry

The areal density (cells per unit area) of GR-immunoreactive (ir) cell profiles was quantified in the prelimbic (PL) and anterior cingulate cortex (ACC) of the medial prefrontal cortex (mPFC), hippocampal formation (CA1, CA3, and dentate gyrus), and medial amygdala (mAMY). These areas were chosen for examination because they are highly sensitive to CORT and are involved in 3A aggressive behavior (i.e., mPFC, AMY) or have been reported to show differences in GR levels across photoperiod (HIPP). Brain sections were inspected under light microscopy using a Zeiss Axiovert 200 microscope. Two sections through each brain region, separated by 120 μm and anatomically matched across animals, were used for analysis. Each brain region was centered in the field of view at 10×, and the magnification was increased to 20×. Bilateral counts were made for each brain area. A cell was considered immunopositive when reaction product was observed in the nucleus. All data are expressed as mean number of GR-ir cells/62,500 μm^2 (mPFC PL and ACC), /25,000 μm^2 (HIPP CA1, CA3, and DG), and hippcampus) or /40,000 μm^2 (AMY). One experimenter, blind to the experimental conditions of the animals, quantified all GR-ir areal density measures.

Cortisol Enzyme Immunoassays

We measured total serum CORT to determine if there was an effect of photoperiod and if exogenous hormone treatment elevated circulating levels of CORT. We also measured total paired adrenal CORT (see below for details) content using this assay. Serum CORT concentrations were measured in duplicate using a commercially available kit produced by Assay Designs (Ann Arbor, MI). This assay was previously validated for use in Siberian hamsters (Demas et al., 2004a), and is highly specific for CORT; sensitivity for this assay is 56.72 pg/ml. Cross-reactivity with corticosterone is 27.7% and <4.0% for other steroid hormones. Samples were diluted 1:40 with assay buffer. Intra-assay variation was 1.21% and inter-assay variation was 2.85%.

Adrenal Cortisol Content

To determine potential photoperiodic changes in total CORT content of the adrenal glands, we housed an additional group of male hamsters in long ($n = 6$) or short ($n = 6$) days to determine total paired adrenal content of CORT via a modification of previously described methods (Maayan et al., 2005). After 8 weeks, animals were euthanized, and paired adrenals were removed and weighed. Paired adrenals were minced then sonicated for 5 min on ice in 2 ml of 95% ethanol and stored at −20°C for 48 hr to allow for protein precipitation. Ethanol was then separated via centrifugation (3,500 rpm for 30 min at 4°C), the supernatant was drawn off and ethanol was allowed to evaporate. The residue was eluted in 120 μl of standard 0 buffer from CORT EIA kit, and content was determined via EIA as described above.

Statistical Analyses

Analyses were performed using the SPSS v.14 (SPSS Inc., Chicago, IL) or JMP 11 (SAS Institute Inc., Cary, NC) statistical packages, and a value of $P < 0.05$ was considered to be statistically significant. Data were transformed when they violated the assumptions of being normally distributed and/or having homogeneous variances. If data transformation did not remedy such violations, Wilcoxen signed-rank tests were used. We used a two-way analysis of variance (ANOVA) to examine the effects of photoperiod, CORT treatment and the interaction of the two on number of attacks, and latency to first attack. A two-tailed *t*-test was used to compare LD and SD levels of total paired adrenal CORT content. Pearson's product correlations were used to examine relationships between behavior and CORT.

RESULTS

Short-day males had significantly smaller testes masses than long-day males ($Z = -7.793$, $P < 0.001$; Fig. 1A) regardless of whether or not they received CORT ($Z = -0.107$, $P > 0.05$; Fig. 1A). CORT injections significantly increased serum CORT levels ($Z = -6.233$, $P < 0.001$; Fig. 1B), but there was no photoperiodic effect on serum CORT levels ($Z = -0.843$, $P > 0.05$; Fig. 1B). Photoperiod did not significantly alter absolute ($F_{1,10} = 0.86$;

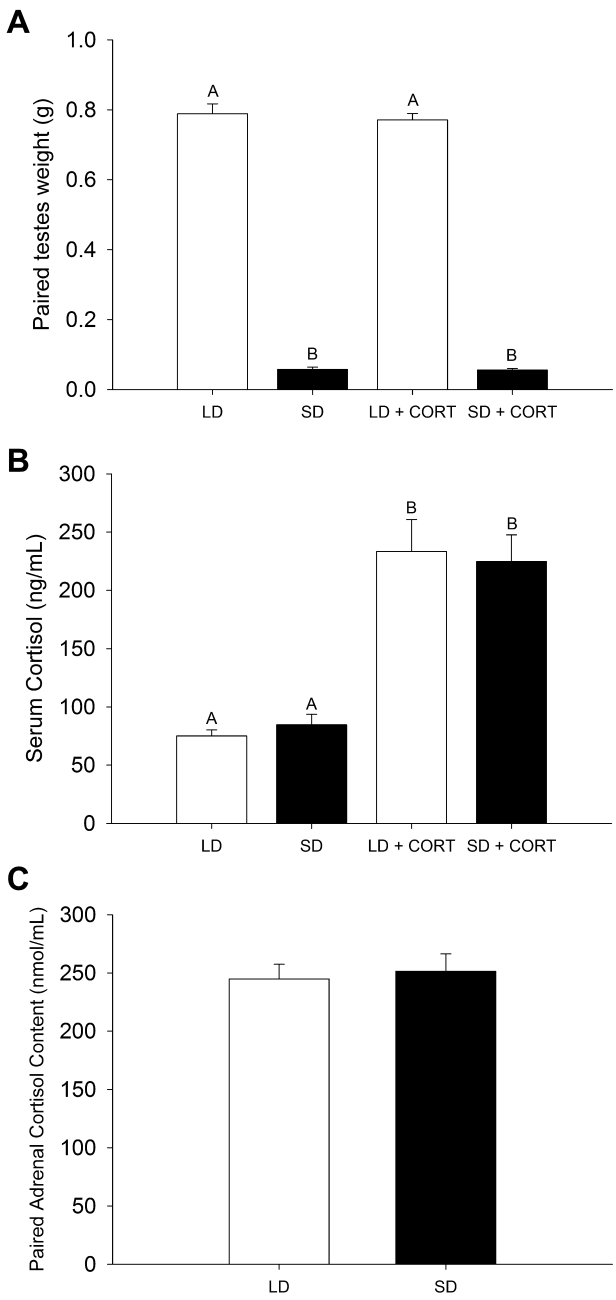

Figure 1. (A) Mean (±SEM) paired testes weights for long-day (LD), short-day (SD), long-day animals that received cortisol (LD + CORT), and short-day animals that received cortisol (SD + CORT). (B) Mean (±SEM) serum cortisol levels. (C) Mean (±SEM) paired adrenal CORT content. Groups with different letters indicate statistically significant differences between group means ($P < 0.05$); groups sharing the same letters are statistically equivalent ($P > 0.05$).

P = 0.38), but did alter relative ($F_{1,10} = 2.13$; P = 0.18) adrenal weights of adrenals that we used to quantify adrenal CORT content. There was no difference in paired adrenal CORT content between males in either photoperiod ($F_{1,10} = 0.11$; P = 0.74; Fig. 1C).

There was an overall effect of photoperiod on number of attacks ($F_{1,42} = 6.67$; P = 0.004; Fig. 2A), however, CORT treatment, did not affect number of attacks ($F_{1,42} = 14.76$; P = 0.11; Fig. 2A). Because there was no effect of treatment, we compared number of attacks across photoperiods in control animals only; short-day males attacked significantly more than long-day males ($t_{17} = 3.85$; P = 0.001). Long-day ($t_{17} = 0.38$; P = 0.71; Fig. 2A) and short-day ($t_{17} = 1.83$; P = 0.08; Fig. 2A) males did not differ in number of attacks across CORT treatment. There was no effect of either photoperiod ($F_{1,42} = 0.44$; P = 0.51; Table 1) or CORT treatment ($F_{1,42} = 0.50$; P = 0.49; Table 1) on latency to first attack, however, latency to first attack was negatively correlated with number of attacks ($R^2 = -0.557$, $P < 0.05$). The number of AGIs performed by the resident was also not affected by photoperiod ($Z = -0.638$, $P > 0.05$; Fig. 2B) or CORT treatment ($Z = -0.592$, $P > 0.05$; Fig. 2B). The number of AGIs, however, are negatively correlated with number of attacks ($R^2 = -0.223$, P = 0.044). There was no effect of photoperiod ($Z = -1.074$, $P > 0.05$; Table 1) or CORT treatment ($Z = -0.047$, $P > 0.05$; Table 1) on the number of self-grooming bouts performed by residents. The number of attacks and serum levels of CORT are related ($R^2 = 0.13$; P = 0.02). This relationship is driven by LD + CORT males ($R^2 = 0.49$; P = 0.04; Fig. 2C), as there is no relationship between number of attacks and CORT in any of the other groups (LD: $R^2 = 0.005$; P = 0.86, SD: $R^2 = 0.005$; P = 0.81, SD + CORT: $R^2 = 0.12$; P = 0.29; Fig. 2C).

There was no effect of photoperiod on GR protein levels in any of the brain regions measured via immunohistochemistry [mPFC PL ($F_{1,10} = 1.333$, $P > 0.05$; Table 1), mPFC ACC ($F_{1,10} = 0.892$, $P > 0.05$; Table 1), HIPP CA1 ($Z = -1.278$, $P > 0.05$; Table 1), HIPP CA3 ($F_{1,10} = 0.053$, $P > 0.05$; Table 1), HIPP DG ($F_{1,10} = 0.115$, $P > 0.05$; Table 1), mAMY $F_{1,9} = 2.664$, $P > 0.05$; Table 1; Fig. 3C and D)] and western blots [Amygdala ($Z = -0.577$, $P > 0.05$; Table 1), Prefrontal cortex ($Z = -0.289$, $P > 0.05$; Table 1)]. Representative photomicrographs of glucocorticoid receptor immunoreactive (GR-ir) cells in the CA1 pyramidal cell layer of the hippocampal formation are shown in Figure 3.

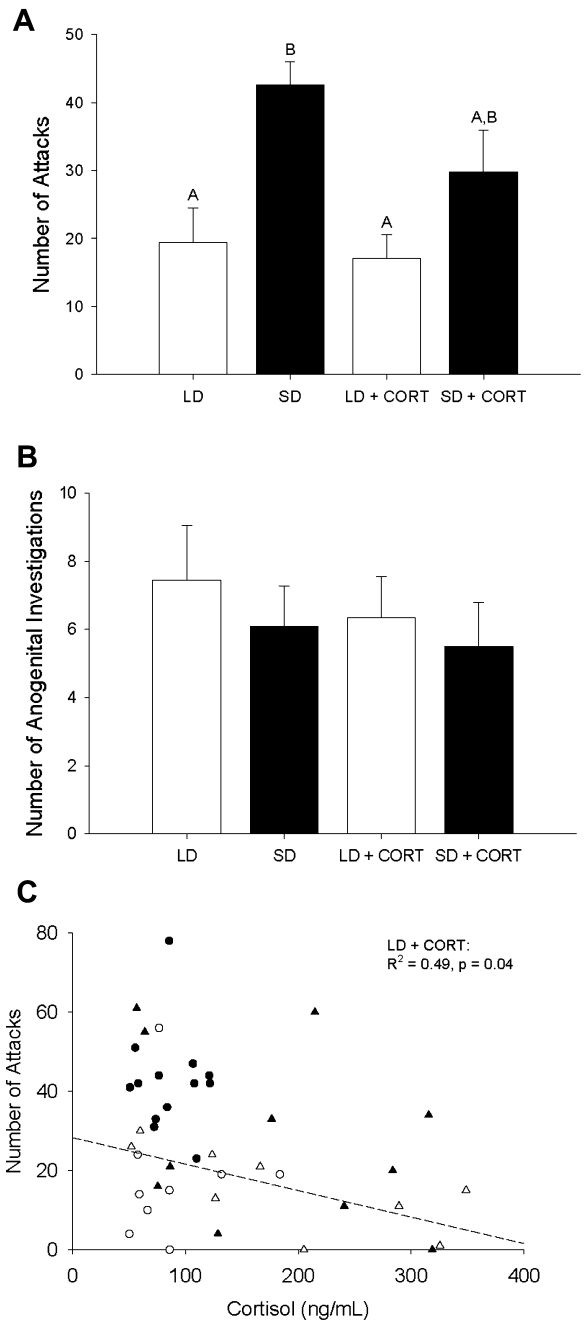

Figure 2. (A) Mean (±SEM) number of attacks performed by the resident. (B) Mean (±SEM) number of anogenital investigations performed by resident. Groups with different letters indicate statistically significant difference between group means ($P < 0.05$); groups sharing the same letters are statistically equivalent ($P > 0.05$). (C) Relationships between number of attacks and cortisol for LD (◯), SD (●), LD + CORT (△), SD + CORT (▲) males. Dotted line denotes statistically significant correlation ($P < 0.05$) in LD + CORT hamsters only.

DISCUSSION

Consistent with previous findings, short-day hamsters displayed significantly increased aggression compared with long-day hamsters (Jasnow et al., 2000; Demas et al., 2004b; Scotti et al., 2007). In contrast to our hypothesis, experimental elevation of CORT attenuated aggression, but only in short-day animals; CORT had no effect on long-day aggression. Lastly, photoperiod did not affect GR protein levels in any of the brain regions examined. Collectively, these results do not support our hypothesis that

Table 1. Mean (±SEM) number (#) of behaviors performed by long-day (LD), short-day (SD), long-day + cortisol (LD + CORT) and short-day + cortisol (SD + CORT) animals, relative optical densities (ROD) for the amygdala (AMY) and prefrontal cortex (PFC), and GR positive cell numbers in the prelimbic (PL) and anterior cingulated cortex (ACC) of the medial prefrontal cortex (mPFC), Hippocampal formation HIPP (CA1), (CA3) and dentate gyrus HIPP (DG), and medial amygdala (mAMY).

	LD	SD	LD + CORT	SD + CORT
Behaviors (#)				
Latency to first attack (s)	70.90 ± 29.24	39.43 ± 11.62	71.21 ± 27.86	71.92 ± 25.46
AGI	7.45 ± 1.59	6.10 ± 1.18	6.35 ± 1.20	5.50 ± 1.28
Grooming	3.65 ± 0.83	5.35 ± 1.01	4.05 ± 0.64	4.23 ± 0.74
GR ROD				
AMY	0.43 ± 0.05	0.34 ± 0.07		
PFC	0.26 ± 0.02	0.24 ± 0.02		
GR positive cells				
mPFC (PL)	42.27 ± 1.07	48.13 ± 1.30		
mPFC (ACC)	35.00 ± 0.68	36.15 ± 0.67		
HIPP (CA1)	45.35 ± 1.15	43.12 ± 0.77		
HIPP (CA3)	24.27 ± 0.51	24.05 ± 0.63		
HIPP (DG)	57.37 ± 1.05	57.40 ± 1.52		
mAMY	132.06 ± 1.61	141.22 ± 2.19		

short-day increases in aggression in Siberian hamsters are mediated by increases in the glucocorticoid CORT or levels of GR in behaviorally relevant brain regions. The results of the present experiment, however, suggest that short-day Siberian hamsters may display increased behavioral sensitivity to glucocorticoids.

There was no effect of CORT on aggression, however, there was a trend towards a decreased incidence of attacks by short-day males. This trend suggests a potential for differential sensitivity to CORT, perhaps dependent on reproductive condition, as in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Breuner and Wingfield, 2000). Furthermore, Siberian hamsters that are photoperiodic non-responders (i.e., individuals that maintain reproductive status while housed in short days) respond similarly, with respect to aggressive behavior, to long-day individuals when treated with CORT (M.L. Scotti, and G.E. Demas, unpublished results). Short-day non-responders, like long-day animals, are also less aggressive than short-day responsive animals (Bedrosian et al., 2012). This trend should be followed up by exploring changes in reproductive state to determine the mechanism of action of potential photoperiodic changes in behavioral sensitivity. The effects of photoperiod appear to be specific to aggression, and there was no effect of exogenous CORT on any other behavior measured. There was no effect of either photoperiod or hormone treatment on any AGIs and self-grooming bouts; long-day animals did not perform AGIs or bouts of self grooming any more or less frequently than short-day animals, nor did manipulation of CORT levels seem to affect these behaviors. Importantly, the lack of a significant effect of our experimental manipulations on non-aggressive behaviors support the idea that the effects of increased CORT levels on aggression is not a result of a general change in activity; CORT did not make the animals more or less active. Evidence from this study suggests that this behavioral sensitivity is not mediated by changes in brain GR, at least in the regions examined in the present study.

Previous studies in another hamster species, Syrian hamsters (*M. auratus*) reported photoperiodic changes in mineralcorticoid receptors (MR) (i.e., Type I corticoid receptors) (Ronchi et al., '98). However, GRS (i.e., Type II corticoid receptors) are more widely distributed throughout the hamster brain, including regions that are associated with aggressive behavior, than MRs which are localized to the hippocampus (Sutanto and De Kloet, '87), and GRs have also been implicated in the development of agonistic behavior (Wommack and Delville, 2007). These receptors, therefore, seemed to be a likely candidate for the regulation of short-day increases in aggression. The results of both western blot and immunohistochemical analysis of brain regions thought to play a role in aggressive behavior, however, did not suggest a photoperiodic change in receptor level. The lack of a difference in receptor level, coupled with the absence of an increase in serum CORT levels in short-day animals and the differential behavioral response to CORT treatment between photoperiods suggests several possibilities. First, negative feedback on GRs is not likely the reason that exogenous CORT caused a decrease in aggression in short-day animals, because no effect was seen in long-day males and serum levels of CORT did not differ between photoperiod. Second, the actions of glucocorticoids are mediated by both MRs and GRs, and it has been previously shown that CORT has a higher affinity for MRs than GRs (Haller and Kruk, 2003). Further, previously reported data in Syrian hamsters show that MR

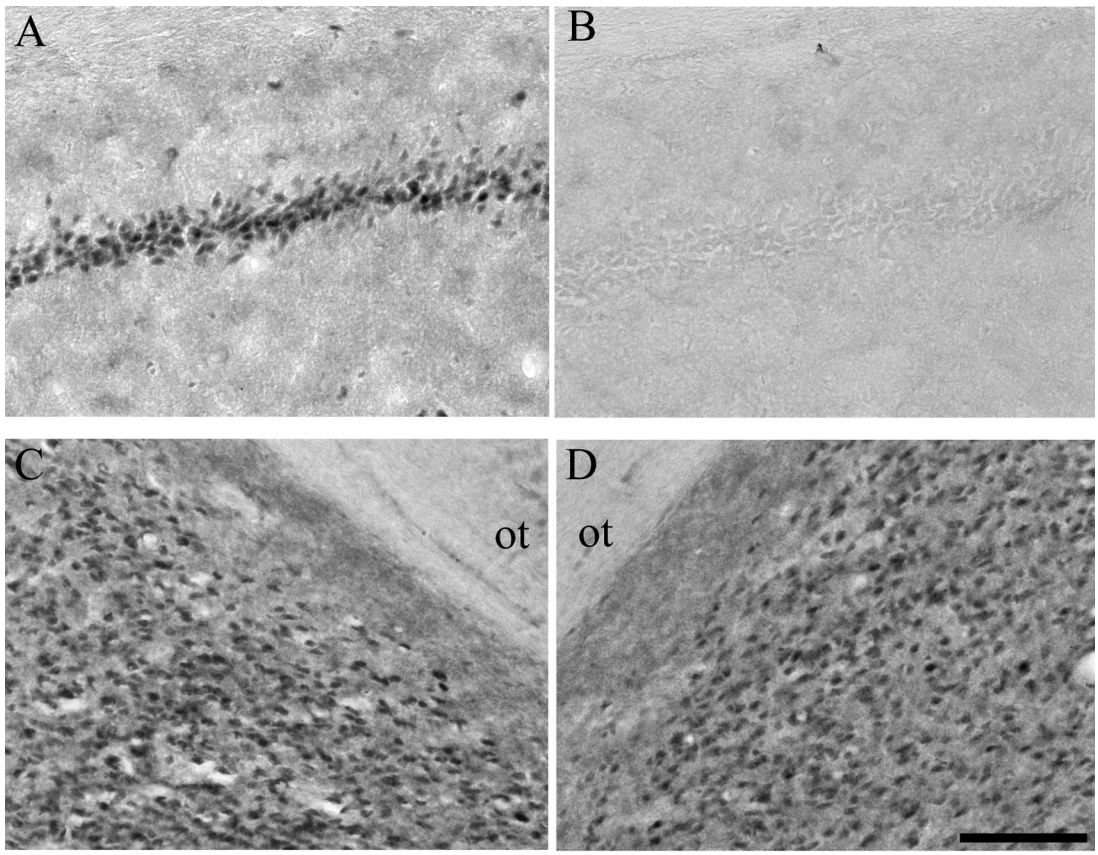

Figure 3. Photomicrographs of glucocorticoid receptor immunoreactive (GR-ir) cells in the CA1 pyramidal cell layer of the hippocampal formation without (A) or with (B) glucocorticoid receptor blocking peptide. Representative photomicrograph of medial amygdala of a long-day (C) and short day (D) Siberian hamster. Bar = 100 μm. ot = optic tract.

numbers and mRNA expression increase in specific brain regions during short-days (Ronchi et al., '98; Lance et al., '98). Due to methodological constraints, we were unable to investigate the role of MRs and the possibility that they may play a role in mediating changes in aggression. Future research will investigate this possibility in Siberian hamsters. Finally, it is possible that although GR levels did not increase in short-day animals, changes in receptor affinity occurred in response to photoperiod. This possibility was not investigated here, but may be an important area of research to pursue. It is important to note that although our data do not support the hypothesis that GRs mediate seasonal changes in aggression, this is the first study to examine GRs in this species in any context.

Although our results do not support the hypothesis that CORT and GRs mediate seasonal increases in aggression in Siberian hamsters, glucocorticoids and their receptors cannot be fully dismissed as playing a role in these behaviors. Studies investigating the effects of glucocorticoids on aggression have been difficult to interpret seemingly because the results of such work depend on the species, length of exposure, and the experimental model used. For example, acute reduction of corticosterone (via a single injection of metyrapone) decreases aggression, and chronic reduction of corticosterone (via adrenalectomy) causes abnormally intense aggression in rats, unlike hamsters and mice (Paterson and Vickers, '81; Haller et al., 2004; Demas et al., 2004b). Conversely, elevating CORT via exogenous injection has been shown to increase aggressiveness in mice (Kostowski et al., '70; Banerjee, '71). The current experiment utilized only a single dose of CORT (10 mg/kg) which elevated serum CORT levels to supra-physiological levels, which might account for the drop in aggression observed in short-day males that were treated with CORT. It is difficult to reconcile this, however, with the lack of effect seen in long-day animals treated with the same dose. This is especially true considering the lack of a photoperiodic effect on serum CORT levels in control animals and the lack of a photoperiod effect on GR protein levels.

Many studies have also investigated the potential role of adrenocorticotropic hormone (ACTH) in aggressive behavior.

ACTH, the anterior pituitary tropic hormone that regulates glucocorticoid release from the adrenal glands, is elevated in adrenalectomized animals, due to a lack of negative feedback, and levels of the hormone decrease when animals are treated with exogenous glucocorticoids. It has been suggested that these changes in ACTH and not downstream changes in cortisol or corticosterone are responsible for changes in aggression (Leshner et al., '73; Moyer and Leshner, '76; Brain and Evans, '77). For example, exogenous ACTH administration decreased the aggressiveness of mice in which glucocorticoid levels had been experimentally controlled, suggesting that ACTH, rather than glucocorticoid levels are critical in the control of aggressiveness (Leshner et al., '73). Although we cannot completely eliminate this as a possibility in our study because we did not measure levels of ACTH, it is not likely. It is probable that ACTH concentrations in the present study were reduced in CORT-treated hamsters due to steroid negative feedback; however, there was a trend towards *reduced* aggression in CORT-treated animals, at least in short days, findings that are inconsistent with elevated ACTH inhibiting aggression.

We tested the hypothesis that the adrenocortical hormone CORT plays a role in the mediation of seasonal aggression in the Siberian hamster. The adrenal cortex, however, also produces the androgen dehydroepiandrosterone (DHEA). We did not investigate if DHEA affects aggression in this study, however, DHEA is a likely candidate as it has been implicated in the mediation of non-breeding aggression in both mammalian and avian systems (Demas et al., 2011; Soma et al., 2015). DHEA has been implicated in both males (Scotti et al., 2009) and females (N.M. Rendon and G. E. Demas, unpublished results) of this species, however, additional experiments should be conducted to determine the exact neuroendocrine mechanisms by which DHEA regulates aggression in this species. Blocking the conversion of DHEA from its sulfated precursor (DHEA-S) increases aggression (Nicolas et al., 2001) as does the injection of DHEA-S in mice (Nicolas et al., 2001). Other experiments have shown that administration of DHEA decreases aggression in male and female mice (Haug et al., '83, '89; Perche et al., 2001). DHEA also mediates non-breeding aggression in several bird species. For example, song sparrows (*Melospiza melodia morphna*) that are treated with physiologically relevant doses of DHEA in the non-breeding season increase the number of songs and significantly reduced song latency in response to a simulated territorial intrusion (Soma and Wingfield, 2001; Soma et al., 2002). These data suggest that the role of adrenal androgens, DHEA and DHEA-S in the regulation of seasonal aggression in Siberian hamsters should be further investigated.

CORT does not mediate seasonal increases in aggression, although short-day animals do appear more sensitive to increases in CORT levels, as exogenous CORT abolishes short-day increases in aggression. Lastly, theses effects do not appear to be mediated by changes in the GR protein levels, at least in the brain regions examined. Thus, the neuroendocrine mechanisms underlying the differences in the response to elevated CORT across photoperiod remains unknown, but may be due, at least in part, to changes in receptor affinity or other actions "downstream" of receptor binding (e.g., intracellular transcription). Alternatively, ongoing investigations will explore the possible role of DHEA, in the control of seasonal changes in aggressive behavior, as in a range of other vertebrate species (Demas et al., 2011; Soma et al., 2015). Collectively, the findings of the present study provide greater insight regarding the neuroendocrine regulation of seasonal aggression in mammals.

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