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# Photoperiod-dependent effects of neuronal nitric oxide synthase inhibition on aggression in Siberian hamsters

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ABSTRACT

Many nontropical species undergo physiological and behavioral adaptations in response to seasonal changes in photoperiod, or day length. In most rodent species, short winter photoperiods reduce testosterone concentrations, which provoke gonadal regression and reduce testosterone-dependent behaviors such as mating and aggression. Seasonally-breeding Siberian hamsters, however, are paradoxically more aggressive in short-days, despite much reduced reproductive activity and testosterone concentrations. Nitric oxide (NO) signaling has been proposed as part of an alternate mechanism underlying this phenomenon. A reduction in neuronal nitric oxide synthase (nNOS), the enzyme responsible for synthesizing NO in the brain, is associated with increased aggression in male short-day hamsters. In the present study, we hypothesized that pharmacological inhibition of nNOS would increase aggressive behavior in long days, but not in short days because nNOS is already reduced. Adult male Siberian hamsters were housed in either long (LD 16:8 h) or short (LD 8:16 h) photoperiods for 8 weeks, then treated with either the selective nNOS inhibitor, 3-bromo-7-nitroindazole (3BrN) or oil vehicle, and subsequently tested for aggression in a resident-intruder test. Treatment with 3BrN increased attack frequency and duration in long days, but had no effect in short days. Short days also reduced testosterone concentrations, without any effect of treatment. These data provide further evidence linking reduced nNOS to elevated short-day aggression and support a role for NO signaling in this phenomenon.



## Introduction

Many nontropical species undergo physiological and behavioral adaptations in response to seasonal changes in the environment; the most salient of these cues is photoperiod, or day length (Goldman, 2001; Prendergast et al., 2002). In the laboratory, photoperiodic rodents exposed to changes in day length can be induced to undergo seasonal changes in physiology and behavior. Siberian hamsters (*Phodopus sungorus*) housed in short-days (<13 h light/day), for example, decrease body mass, reduce gonadal size and gonadal steroid hormone concentrations, cease mating behavior, and develop white pelage color (Hoffmann, 1982). This species offers a unique naturalistic model for studying seasonal adaptations in physiology and behavior.

In most rodent species, short winter photoperiods reduce gonadal steroid hormone concentrations which coincide with the wintertime reduction in mating and aggressive behaviors (Knol and Egberink-Alink, 1989; Rubinow and Schmidt, 1996). However, some photoperiodic species, including Siberian and Syrian (*Mesocricetus auratus*) hamsters, undergo seasonal changes in aggressive behavior, in contrast with the direction that would be expected. Hamsters housed in winter-like short days are highly aggressive, despite gonadal regression and virtually undetectable testosterone concentrations (Garrett and Campbell, 1980; Badura and Nunez, 1989; Jasnow et al., 2000). Testosterone, or its estrogenic metabolites, can facilitate aggression in a variety of species and was one of the first mechanisms of aggression delineated (Wingfield et al., 1990; Simon and Lu, 2005). Interestingly, however, exogenous administration of testosterone to short-day male Siberian hamsters does not increase aggression as it does in males of some species (Jasnow et al., 2000). In contrast, Siberian hamsters housed in summer-like long day lengths are less aggressive, despite having high testosterone concentrations and being reproductively active. Discrete environmental signals can contribute to the effects of hormones in California mice as well. Activation of estrogen receptors increases aggression in short days but decreases aggression in long days (Trainor et al., 2008; Trainor et al., 2007a, 2007b). These observations led to the hypothesis that aggression is regulated independently of gonadal steroids in this species.

Nitric oxide (NO) signaling has been proposed as part of an alternate mechanism underlying the paradoxical winter aggression in Siberian hamsters (Wen et al., 2004). Mice with targeted deletions of the gene encoding neuronal nitric oxide synthase (nNOS), the synthetic enzyme responsible for converting L-arginine into NO and its byproduct, citrulline, in the brain, are highly aggressive (Nelson

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et al., 1995). Pharmacological inhibition of nNOS produces heightened aggression in wild type mice (Demas et al., 1997), in addition to decreased social investigation (Trainor et al., 2007a, 2007b). Furthermore, in Siberian hamsters, short-day elevation in aggression is negatively correlated with nNOS expression in the anterior amygdala and paraventricular nucleus of the hypothalamus, suggesting that reduced NO in short days contributes to the aggressive phenotype among these hamsters (Wen et al., 2004).

In the present study, we directly manipulated NO levels using the selective nNOS inhibitor, 3-bromo-7-nitroindazole (3BrN), in both long and short day lengths in order to determine a direct role for nitric oxide signaling in photoperiodic aggression. We also assessed photoperiod response by measuring body and testis mass, as well as serum testosterone, dehydroepiandrosterone (DHEA), and 17β-estradiol concentrations. We hypothesized that pharmacological inhibition of nNOS would increase aggressive behavior in long-days, but not in short-day responders or non-responders because nNOS is already reduced in the brains of these individuals.

## Materials and methods

### *Ethics statement*

All animals were maintained and tested in accordance with the Institutional Animal Care and Use Committee of The Ohio State University and NIH guidelines. The OSU IACUC approved all procedures.

### *Animals*

28 adult (>8 weeks) male Siberian hamsters (*Phodopus sungorus*) were obtained from our in-house breeding colony at the Ohio State University. The colony was originally started with 30 breeding pairs obtained from Dr. Katherine Wynne-Edwards in 2000; the ancestors of this colony were descendants of a colony established by Dr. Wynne-Edwards and Dr. Alexei B. Surov from 30 wild-caught individuals repeatedly out-bred with wild animals trapped near Karasuk and Beya, Siberia. Animals were singly housed in polypropylene cages (28×17×12 cm) with food and water available *ad libitum*, and cotton nesting material available in the cage. Ambient room temperature was 22±2 °C and relative humidity was maintained at 50±5%. All animals were born and maintained in long day lengths (L:D 16:8 h, lights on at 22:00 h EST) until 8 weeks of age, at which time they were moved to experimental lighting conditions (LD, N=10; SD, N=18). Short-day hamsters were further subdivided into reponders and non-responders. Animals that, after 8 wk in short days, had paired testes weighing more than 0.15 g were classified as short-day nonresponders (N=6); animals with paired testes weighing 0.15 g or less were classified as short-day responders (N=12) for purposes of statistical analyses. Additional hamsters used as non-aggressive intruders during behavioral testing were size- and age-matched group housed siblings maintained in long-day conditions.

### *Photoperiod manipulation*

At 8 weeks of age, hamsters were randomly assigned to either a short-day group (SD; L:D 8:16 h, lights on at 06:00 h EST) or a long-day group (LD; L:D 16:8 h, lights on at 22:00 h EST). Hamsters were maintained in experimental light schedules for 8 weeks, which is sufficient to provoke seasonal adaptations in this species (Prendergast et al., 2000). Photoperiodic responsiveness was determined by changes in body mass and postmortem paired testes mass. Short-day hamsters that did not show reduction of body mass and testicular regression were categorized as non-responders and were separated in subsequent statistical analysis.

### *Effect of 3BrN on aggression*

Prior to behavioral testing, hamsters were randomly assigned to receive subcutaneous injections of either corn oil vehicle (0.1 mL) or the selective nNOS inhibitor, 3-bromo-7-nitroindazole (3BrN; Cayman Chemicals, Ann Arbor, MI; 20 mg/kg) dissolved in 0.1 mL corn oil. Injections were administered twice a day (between 0800–0900 h and 1500–1700 h) for four consecutive days. On day 4, aggressive behavior was assessed in the resident-intruder test exactly 30 min following the afternoon injection. All testing was performed within the first 3 h after lights off to control for circadian phase and all cages were left unchanged 7 days prior to testing to provoke territoriality in the experimental animal. Briefly, a group-housed non-aggressive intruder was introduced into the home cage of the experimental hamster and behavior was videotaped for 10 min. Intruder animals were identified by a small patch of shaved skin on the back. Aggressive behaviors (duration of attacks and total number of attacks) were later scored by an observer uninformed of the treatment groups.

### *Hormone assays*

Immediately following the aggressive encounter, hamsters were anesthetized with isoflurane vapors and blood samples were drawn from the retro-orbital sinus. The blood samples were centrifuged for 30 min at 4 °C and 6000 r.p.m. and then frozen at −80 °C. Testosterone (T), dehydroepiandrosterone (DHEA), and 17β-estradiol (E2) were measured via commercial EIA kits using unextracted serum (Correlate-EIA Kit; Assay Designs, Ann Arbor, MI). The antibodies used in this kit are highly specific; cross-reactivity of T was 7.20% for androstenedione, 0.72% for DHEA, 0.4% for 17β-estradiol and <0.01% for other steroids. Cross-reactivity of E2 was <0.02% for other steroids. These assays have been previously validated for use in Siberian hamsters (Schum and Wynne-Edwards, 2005; Scotti et al., 2008; Scotti et al., 2009). Samples were diluted (1:20 for T and 1:2 for DHEA and E2) with assay buffer and run in duplicate for each sample. The sensitivity of these assays was as follows: 5.67 pg/ml for T, 2.90 pg/ml for DHEA and 28.5 pg/ml for E2. All samples were run on a single plate per hormone; intra-assay variation was <10% for all assays. All procedures were followed as per the guidelines provided by the manufacturer.

### *Statistical analysis*

Body mass, testis mass, hormone, and behavioral data were analyzed using two-way ANOVA with photoperiod and drug treatment as the independent variables. Main effects were followed up with Fisher's PLSD for post-hoc comparisons. In the case of behavior data, planned comparisons were used to assess the effect of drug treatment within each lighting condition using Student's *t*-test. Correlations between testosterone and aggressive behavior were also calculated. All statistics were performed using Statview 5.0.1 for Windows. Data are represented as mean±SEM. Mean differences were considered statistically significant when p was <0.05.

## Results

### *Body and paired testes masses*

Eight weeks of housing in short day lengths reduced body mass ($F_{1,25}=49.294$, $p<0.0001$; Fig. 1A), regardless of drug treatment condition ($p>0.05$). Short day lengths also reduced testes mass ($F_{1,25}=16.195$, $p<0.001$; Fig. 1B), without any effect of drug treatment ($p>0.05$). Among the hamsters housed in short-days, several non-responders failed to reduce testes mass compared to responders ($t_{17}=-8.742$, $p<0.0001$; Fig. 1C).

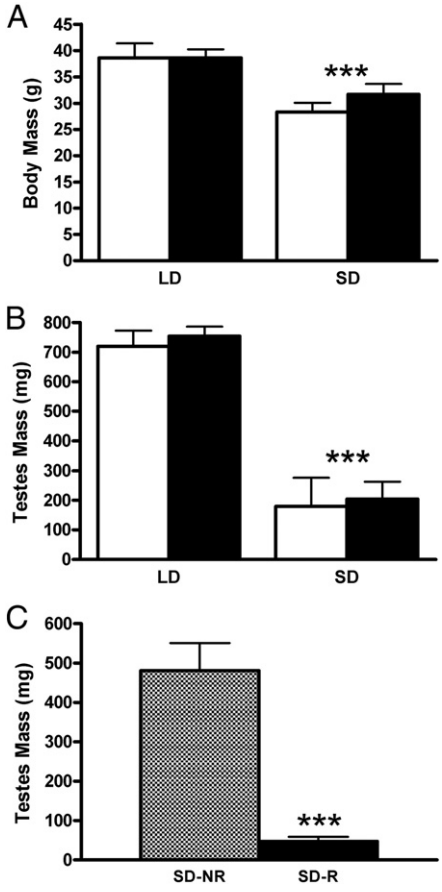


**Fig. 1.** Body and testes mass in long-day versus short-day hamsters. Open bars represent vehicle treatment and closed bars represent 3BrN treatment (A–B). Short-days reduced body mass (A), and testis mass (B), independent of treatment group. Some short-day hamsters failed to reduce testes mass and were classified as non-responders (C). *** — main effect of photoperiod $p<0.0001$.

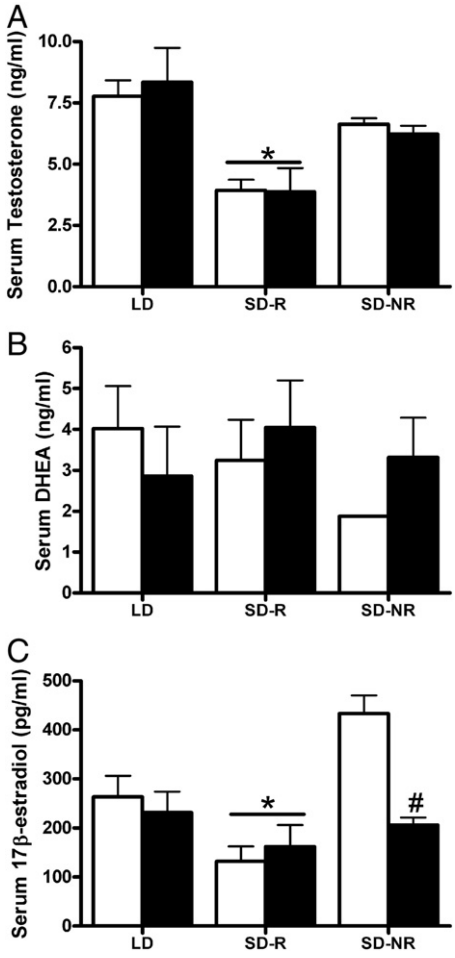


**Fig. 2.** Serum testosterone, DHEA, and estradiol concentrations in long-day versus short-day hamsters. Open bars represent vehicle treatment and closed bars represent 3BrN treatment (A–C). Short-day responders had reduced testosterone concentrations relative to other groups (A). Photoperiod did not affect serum DHEA concentrations (B). Short-day responders had reduced estradiol concentrations compared to the other two groups, and 3BrN treatment reduced estradiol relative to vehicle treatment in the non-responder group only. (C). * — main effect of photoperiod $p<0.05$; # — post-hoc relative to untreated SD-NR $p<0.01$.

*Hormone concentrations*

Serum testosterone, DHEA, and 17β-estradiol concentrations were measured after 8 weeks in photoperiod. Short-days reduced testosterone concentrations ($F_{2,22}=13.726$, $p<0.0001$; Fig. 2A), with no effect of drug ($p>0.05$) and no drug x photoperiod interaction ($p>0.05$). Short-days reduced testosterone relative to hamsters housed in long-days (post-hoc, $p<0.0001$), as well as short-day non-responders (post-hoc, $p<0.02$). Long-day hamsters had equivalent testosterone concentrations to short-day non-responders (post-hoc, $p>0.05$). Serum DHEA concentrations were equivalent across all groups ($p>0.05$ for all comparisons; Fig. 2B). Estradiol concentrations were affected by photoperiod ($F_{2,22}=8.835$, $p<0.01$; Fig. 2C) and treatment ($F_{2,22}=4.841$, $p<0.05$), with a significant interaction of the two variables ($F_{2,22}=4.389$, $p<0.05$). Short-days reduced estradiol relative to hamsters housed in long-days (post-hoc, $p=0.01$) and 3BrN reduced estradiol concentrations within the SD-NR group (post-hoc, $p<0.01$). Estradiol concentrations were elevated in long-day hamsters vs. short-day non-responders (post-hoc, $p<0.05$).

*Aggressive behavior*

There was an effect of photoperiod on attack duration in the resident-intruder test ($F_{2,23}=12.018$, $p<0.001$; Fig. 3A), as short-day hamsters spent more time attacking a novel intruder relative to long-day hamsters (post-hoc, $p<0.0001$) and short-day non-responders (post-hoc, $p<0.01$). There was no significant effect of treatment and no photoperiod by treatment interaction. Long-day hamsters had an equivalent attack duration to short-day non-responders (post-hoc, $p>0.05$). Treatment with 3BrN increased attack duration in long-days ($t_6=3.173$, $p<0.0001$), but had no effect in short-days ($p>0.05$ for both responders and non-responders). Photoperiod also affected the number of attacks ($F_{2,23}=18.137$, $p<0.0001$; Fig. 3B), as short-day hamsters attacked more frequently than long-day hamsters (post-hoc, $p<0.0001$) and short-day non-responders (post-hoc, $p<0.01$). Treatment with 3BrN increased attack frequency in long-days ($t_6=3.284$, $p<0.05$).

*Hormone–behavior relationships*

Serum testosterone concentrations were significantly negatively correlated with attack duration ($R^2=0.39$, $p<0.001$; Fig. 4A) and number of attacks ($R^2=0.52$, $p<0.0001$; Fig. 4B) in the resident–intruder test. When treated subjects were pulled out from that analysis and examined separately, there was no correlation with testosterone in either duration ($R^2=0.05$, $p>0.05$) or number of attacks ($R^2=0.06$, $p>0.05$), suggesting 3BrN treatment changes aggression behavior without changes in testosterone concentrations. Measures of aggression, however, were not significantly correlated with either DHEA or 17β-estradiol concentrations in either long- nor shor-day animals ($p>0.05$ in all cases).

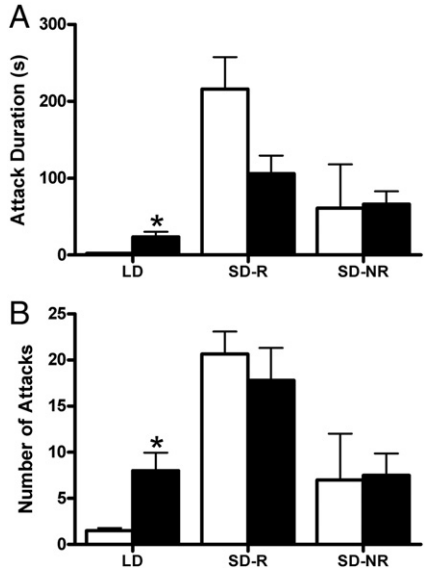


**Fig. 3.** Aggressive behavior in the resident–intruder test. Open bars represent vehicle treatment and closed bars represent 3BrN treatment (A–B).Short-day responders spent more time attacking than long-day hamsters; treatment with 3BrN increased attack duration in long-days, but not short-days (A). Short-day responders also attacked more frequently than long-day hamsters; treatment with 3BrN increase attack frequency in long-days, but not short-days (B). * — relative to untreated LD $p<0.05$.

## Discussion

In the present study, we report evidence for a photoperiod-dependent effect of nNOS inhibition on aggression in Siberian hamsters. Treatment with the selective nNOS inhibitor, 3BrN, increased aggression in long, but not short days nor in SD-nonresponders, and did not alter serum testosterone concentrations. A previous study presented correlational evidence to suggest a role for short-day reductions in nNOS in photoperiod-dependent aggression (Wen et al., 2004); however, no study has since directly linked the two factors. This is the first study to directly manipulate nNOS and aggressive behavior in the context of photoperiod.

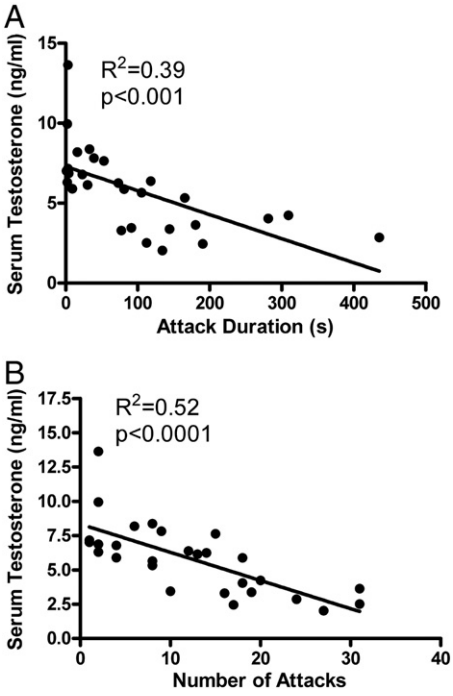


**Fig. 4.** Serum testosterone correlates with aggression. Serum testosterone concentration is negatively correlated with attack duration (A) and attack number (B).

In many seasonally-breeding rodents, aggression peaks during the spring and summer months when competition for mates is high, thus offering an adaptive advantage to the most dominant individuals. For Siberian hamsters, however, aggression peaks after exposure to short days. Similarly, this phenomenon has recently been reported in both male and female California mice as well (Silva et al., 2010; Trainor et al., 2010). It is possible that the environmental selective pressures of winter, such as competition for food or shelter, drive short-day aggression in this species. Competition for scarce resources and food during the winter months likely affords an adaptive advantage to aggressive individuals. Other examples of species exhibiting high aggression during the non-breeding season include red squirrels and arctic ground squirrels; in these animals territorial aggression is exhibited in the field during both the breeding and non-breeding seasons, independent of seasonal variation in androgenic steroids (Buck and Barnes, 2003; Boonstra et al., 2008).

In the majority of species studied, testosterone regulates aggression, making short-day aggression in Siberian hamsters somewhat paradoxical. It is possible that photoperiod directly regulates aggression in this species to some extent. It is well-established that the pattern of pineal melatonin secretion serves as the biochemical signal of photoperiodic information in rodents. Consistent with this idea, melatonin administration that mimics the pattern of melatonin secretion in short-days increases aggression in both Siberian and Syrian hamsters independent of changes in gonadal steroids Jasnow et al., 200 ref (Demas et al., 2004). The results of the present study additionally support a direct role for nNOS in regulating short-day aggression. nNOS is generally thought to be regulated by gonadal steroids (Scordalakes et al., 2002). Testosterone down-regulates nNOS through androgen receptors (Hull et al., 1997), whereas estradiol upregulates nNOS via estrogen receptors (Warembourg et al., 1999). Photoperiod, however, may either directly regulate nNOS or regulate it via the hypothalamo-pituitary-adrenal (HPA) axis in Siberian hamsters. Adrenalectomy blocks reduced nNOS activity (Jahng et al., 2004). and also blocks short-day increases in aggression (Demas et al., 2004), suggesting a role for adrenal hormones in nNOS regulation DHEA, however, was not altered by photoperiod or 3BrN in our experiment. nNOS positive cells are localized in the limbic system in high density, particularly the lateral septum, posterior hypothalamus, entorhinal cortex, and amygdala (Albert and Walsh, 1984; Vincent and Kimura, 1992). Reduced NO activity in these regions may drive exaggerated aggression (Wen et al., 2004). Interestingly, short-day aggression was limited to reproductively responsive animals (i.e., responders); short-day non-responders did not display enhanced aggression in the present study. Although one previous study demonstrated that short-day aggression was independent of photoperiodic responsiveness (Wen et al., 2004), the present results are consistent with other studies (e.g., Demas et al., 2004). Given the evidence that short-day aggression is mediated by increased duration of melatonin secretion, the lack of increased aggression in nonresponders is consistent with a direct action of melatonin on behavior. Unlike other rodents, short-day nonresponsiveness is mediated by pre-pineal mechanisms in which nonresponders fail to undergo an expansion in the duration of melatonin secretion and maintain long-day like patterns of melatonin secretion. If prolonged exposure to melatonin is critical to increased aggression, then it is expected that both LD and SD nonresponders would display decreased aggression, a prediction consistent with the present findings.

As noted, treatment with the nNOS inhibitor, 3BrN, increased aggression in long days, but not short days. This result may be attributed to the observation that nNOS is already reduced in short-day hamsters, possibly below a threshold where any further reductions would have a behavioral effect. Alternatively, levels of aggression are already so high in short-day hamsters; it is possible that our behavioral assay is not sufficiently sensitive to detect any further increases in aggressive behavior. Although treatment with 3BrN increased aggression in long-

days, it is important to note that it did not increase it to levels equivalent to untreated short-day hamsters. This finding suggests that nNOS reduction on its own does not fully drive the short-day increase in aggression. Long-day and short-day aggression may work by different additional physiological mechanisms, despite similar behavioral phenotypes.

By the same token, the hypothesis that additional mechanisms converge to determine photoperiod aggression levels may help to explain our somewhat unexpected finding that 3BrN treatment did not affect short-day non-responders in the same way as long-day hamsters. Short-day non-responders maintain gonadal size and testosterone concentrations, making them physiologically similar to long-day hamsters. Nevertheless, short-day non-responders did not exhibit the same heightened aggression with 3BrN treatment as long-day hamsters. There may be other physiological changes occurring with photoperiod that are separate from the gonadal axis, yet put short-day non-responders at a different physiological baseline than long-day hamsters, thus leading to different behavioral responses. Interestingly, estradiol concentrations were elevated in short-day non-responsive hamsters relative to the other groups. While the precise reason for this increase is unknown, it is possible that short-day nonresponders display elevated aromatase activity while also maintaining their gonadal function (and thus high levels of gonadal steroids compared with short-day nonresonders). If true, then this might explain the elevated levels of estradiol in these animals. Previous findings are consistent with the notion that short days increase steroid hormone conversion (Scotti et al., 2009); however this idea will require further testing.

## Conclusions

Taken together, our data demonstrate a direct role for nNOS in photoperiodic aggression. Pharmacological inhibition of nNOS enhances aggression in long-days, but not short-days, and does so without changes in testosterone concentrations. This evidence supports a role for NO signaling in the non-steroid-dependent context of photoperiodic aggression.

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