



Rapid effects of estradiol on male aggression depend on photoperiod in reproductively non-responsive mice

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Abstract

In three genera and four species of rodents, housing in winter-like short days (8L:16D) increases male aggressive behavior. In all of these species, males undergo short-day induced regression of the reproductive system. Some studies, however, suggest that the effect of photoperiod on aggression may be independent of reproductive responses. We examined the effects of photoperiod on aggressive behavior in California mice (*Peromyscus californicus*), which do not display reproductive responsiveness to short days. As expected, short days had no effect on plasma testosterone. Estrogen receptor alpha and estrogen receptor beta immunostaining did not differ in the lateral septum, medial preoptic area, bed nucleus of the stria terminalis, or medial amygdala. However, males housed in short days were significantly more aggressive than males housed in long days. Similar to previous work in beach mice (*Peromyscus polionotus*), estradiol rapidly increased aggression when male California mice were housed in short days but not when housed in long days. These data suggest that the effects of photoperiod on aggression and estrogen signaling are independent of reproductive responses. The rapid action of estradiol on aggression in short-day mice also suggests that nongenomic mechanisms mediate the effects of estrogens in short days.

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Introduction

The physiological bases of aggressive behavior are typically examined under constant environmental conditions. However, the environment can have important effects on aggression and can even alter the effects of specific physiological systems. For example, adverse behavioral effects associated with the short form of the monoamine oxidase A (*MAOA*) gene are typically observed only in individuals who have been exposed to adverse environmental conditions (Caspi et al., 2003; Kim-Cohen et al., 2006; Frazzetto et al., 2007). In rodents, males are typically more aggressive when confronted in a familiar environment (e.g., a resident–intruder test) as compared to a neutral setting (Bester-Meredith et al., 1999), and males from some mouse strains are more aggressive when housed in environmentally enriched housing compared to the standard laboratory housing

conditions (Haemisch et al., 1994; Marashi et al., 2003). These studies document that the environmental context has important effects on the function of physiological pathways that regulate aggression.

Several laboratories have reported a consistent effect of photoperiod (day length) on male aggression in rodents. In Syrian hamsters (*Mesocricetus auratus*) (Garrett and Campbell, 1980; Jasnow et al., 2002; Caldwell and Albers, 2004), Siberian hamsters (*Phodopus sungorus*) (Jasnow et al., 2000; Demas et al., 2004; Wen et al., 2004), beach mice (*Peromyscus polionotus*) (Trainor et al., 2007a), and deer mice (*Peromyscus maniculatus*) (Trainor et al., 2007b), males are more aggressive in a resident–intruder test when tested in short days (8L:16D) as opposed to long days (16L:8D). This effect has been considered paradoxical, because in each of these species housing in short days causes regression of testes and a corresponding decrease in testosterone (Jasnow et al., 2000, 2002; Trainor et al., 2006c). In Siberian hamsters there is evidence that the effect of photoperiod on aggression is independent of gonadal responses (Demas et al., 2004). Photoperiod has no significant effect on reproductive

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physiology or aggression in CD-1 mice (Trainor et al., 2006a), but this stock of mice has been bred in captivity since at least the 1920s. Recent studies in *P. polionotus* suggest that changes in estrogen signaling are involved in mediating the effect of photoperiod on aggression in rodents.

Estrogens can affect aggression in male vertebrates because aromatase present in the brain can convert androgens to estrogens. The effects of estrogens on male aggression are variable, increasing aggression in some species and decreasing aggression in others (Trainor et al., 2006b). This could reflect differences in the expression of estrogen receptor (ER) subtypes. Selective deletion of ER α is associated with reduced male aggression in domestic mice (Ogawa et al., 1997; Scordalakes and Rissman, 2003). The deletion of ER β is generally associated with increased aggression (Ogawa et al., 1999; Nomura et al., 2006), although this effect appears to be context-dependent (Nomura et al., 2002). Deletion of both receptors is associated with increased male aggression (Ogawa et al., 2000). In male *P. polionotus*, and *P. maniculatus*, ER α expression in the lateral septum (LS) and bed nucleus of the stria terminalis (BNST) is increased in short days whereas ER β expression in the BNST and medial amygdala (MEA) is increased in long days (Trainor et al., 2007b). This led to the attractive hypothesis that changes in ER expression mediate the effect of photoperiod on aggression.

The estrogen receptor hypothesis was tested with hormone manipulation experiments (Trainor et al., 2007a). In male *P. polionotus*, treatment with the aromatase inhibitor fadrozole increased aggression in long days but decreased aggression in short days. The estrogen receptor hypothesis was not supported because the effects of fadrozole were reversed by co-treatment with either ER α or ER β selective agonists, regardless of photoperiod. These data suggested that differential expression of estrogen receptors could not explain the effect of photoperiod on aggression. Instead, it appears that photoperiod changes the molecular activity of estrogen receptors. Results from a microarray experiment showed that estrogen-dependent gene expression in the BNST was decreased in short days compared to long days (Trainor et al., 2007a). Furthermore, estradiol injections increased aggression within 15 min in male *P. polionotus* housed in short days, but had no effect on males housed in long days. These data suggested that estrogens increase aggression in short-day mice by activating nongenomic mechanisms, as it is generally thought that 15 min is insufficient time for changes in estrogen-dependent changes in gene expression to occur (Vasudevan and Pfaff, 2006). Together, these results suggest that the effect of photoperiod on aggression is independent of changes in estrogen receptor expression and is mediated instead by changes in receptor activity (genomic or nongenomic).

In the present study we examined the effect of photoperiod on aggression in California mice (*P. californicus*), a species in which males do not regress testes when housed in short days (Nelson et al., 1995). We hypothesized that if the effect of photoperiod is indeed independent of gonadal responses to short days, then male California mice would increase aggressive behavior in short days. We also examined whether photoperiod influences the effects of estrogen receptor expression and how

estrogens regulate aggressive behavior. Estrogen receptor alpha and beta are expressed in the LS, medial preoptic area (MPOA), BNST, ventromedial hypothalamus (VMH), and MEA. These brain areas are part of a circuit that regulates social behavior (Newman, 1999; Goodson, 2005), including aggression (Nelson and Trainor, 2007). It was previously reported that, as in *P. polionotus*, fadrozole increased aggression in *P. californicus* housed in long days (Trainor et al., 2004). In the current study, we tested whether estradiol acts rapidly to increase aggression in short-day *P. californicus*.

Methods

Animals

P. californicus were obtained from Dr. Catherine Marler (University of Wisconsin, Madison, WI, USA). California mice form monogamous mating pairs in the field (Ribble and Salvioni, 1990) and males do not respond to short days by reducing testes mass (Nelson et al., 1995). Males were individually housed and provided with filtered tap water and Teklad 8640 food (Harlan, Madison, WI) ad libitum. Field studies show that male *P. californicus* defend exclusive territories (Ribble and Salvioni, 1990), which indicates that to a certain extent, single housing approximates the social organization of young unpaired males in this species. All experimental procedures were approved by the Ohio State University Institutional Animal Care and Use Committee and animals were maintained in accordance with the recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Effect of photoperiod on behavior and physiology

Males (residents) were randomly assigned to be housed in long ($n=9$, LD 16:8) or short ($n=7$, LD 8:16) days for 8 weeks. One week before resident–intruder aggression tests, retroorbital blood samples were collected under light isoflurane anesthesia. Plasma samples were frozen at -80°C . Resident–intruder tests were conducted 1 h after lights out (1500 EDT) under dim red light. A group-housed, sexually inexperienced male intruder (weight matched to within 4 g) was introduced into each resident's cage for 7 min. Observations were videotaped and scored offline by an observer blind to treatment assignments. One hour after aggression tests residents were anesthetized with isoflurane and rapidly decapitated. This time course was chosen because previous studies have reported that maximal c-fos expression occurs between 1 and 2 h after stimulation (Kovacs and Sawchenko, 1996; Hoffman and Lyo, 2002). Brains were quickly removed and fixed in 5% acrolein overnight at 4°C . Brains were then transferred to 30% sucrose for 24 h and frozen for immunocytochemistry. Plasma was collected from trunk blood samples to measure post-encounter testosterone and testes were dissected and weighed. Total testosterone was measured with a ^{125}I testosterone RIA kit (DSL-4100; Diagnostic Systems Laboratories, Webster, TX). The intra-assay coefficient of variation was 2.8%. Hormone concentrations were not normally distributed and were log transformed for statistical analyses.

Immunocytochemistry for ER α and ER β was conducted using the methods of Trainor et al. (2007b). Briefly, six brains from each group were sectioned at $40\ \mu\text{m}$ on a cryostat and free-floating sections were then incubated in 1% sodium borohydride in 0.1 M phosphate-buffered saline (PBS) for 10 min. Sections were then rinsed in 20% normal goat serum and 0.3% hydrogen peroxide in PBS for 20 min. Alternate sections were incubated in either primary ER α antibody (C1355, Upstate Biotechnology, concentration 1:20 K), primary ER β (D7N, Invitrogen, Carlsbad, CA, concentration 1:400), or primary c-fos (rabbit anti c-fos, Chemicon 1:10 K) in 1% normal goat serum in 0.5% PBS+triton X (PBS TX) for 40 h at 4°C . Sections were rinsed in PBS and incubated for 2 h with biotinylated goat–anti-rabbit antibody (1:500, Vector Laboratories, Burlingame, CA) in PBS+TX. The sections were rinsed in PBS and then incubated for 30 min in avidin–biotin complex (ABC Elite kit, Vector Laboratories). After rinses in PBS, the sections were developed in hydrogen peroxide and diaminobenzidine for 2 min. Sections were mounted on gel-coated slides, dehydrated and

coverslipped. A Nikon E800 microscope was used to capture representative photomicrographs of each of the following brain areas using a mouse brain atlas (Paxinos and Franklin, 2002): ventral lateral septum (bregma 0.26), BNST (bregma 0.02), MPOA (bregma 0.02), VMH (bregma -1.70), paraventricular nucleus (PVN, bregma -1.22), and posterodorsal MEA (bregma -1.82). In these areas the number of ER and c-fos immunoreactive (-ir) cells was counted using Image J (NIMH, Bethesda, MD) by an observer unaware of treatment assignments. We counted the number of cells in a box in the MPOA ($1 \times w$, $400 \times 250 \mu\text{m}$), LS ($330 \times 480 \mu\text{m}$), BNST ($500 \times 350 \mu\text{m}$), PVN ($330 \times 480 \mu\text{m}$), and MEA ($450 \times 450 \mu\text{m}$) similar to our previous study quantifying estrogen receptor expression in *P. polionotus* and *P. maniculatus* (Trainor et al., 2007b). In the VMH, the number of ER α and c-fos positive cells in a circle with a radius of $180 \mu\text{m}$ was counted. In the anterior hypothalamus (bregma -1.22) we counted c-fos positive cells (box size, $520 \times 520 \mu\text{m}$) but not ER positive cells because there are no ER α or ER β cells in this region of the brain in this species. The aggressive behavior data and cell counts from the males assigned to the long-day group in this study were also analyzed (as virgin males) in the accompanying paper examining the effect of parental experience on aggression and estrogen receptor expression (Trainor et al., 2008-this issue).

Effects of acute estradiol injections in long days and short days

Male California mice were randomly assigned to be housed in long or short days for 8 weeks. All males were then bilaterally castrated under isoflurane anesthesia. Each male was implanted with a Silastic implant (1.47 mm i.d.; 1.96 mm o.d.) packed with 1 mm of testosterone. All males were also implanted with an osmotic minipump (model 2002, Alzet, Palo Alto, CA) containing fadrozole (0.25 mg/kg/day), a potent aromatase inhibitor. After surgery all animals were treated with an s.c. injection of buprenorphine (0.38 mg/kg). After 10 days, all males were tested in a resident-intruder aggression test. Fifteen minutes before testing, each male was injected with either saline or 100 $\mu\text{g}/\text{kg}$ cyclodextrin conjugated estradiol (cE₂). The genomic effects of estrogens

generally occur on a time scale of hours or days, whereas nongenomic effects of estrogens can occur within minutes or seconds (Nabekura et al., 1986). Previous studies have used the delivery of water soluble cE₂ to study the nongenomic effects of estrogens on reproductive behavior (Cross and Roselli, 1999; Cornil et al., 2006). Fifteen minutes after injections males were tested in resident-intruder aggression tests as described above.

Results

Effects of photoperiod on physiology, receptor expression, and behavior

Photoperiod did not affect testes mass (Fig. 1A; $t_{14}=1.15$, $p=0.27$), baseline testosterone concentrations (Fig. 1B, $t_{14}=0.83$, $p=0.42$), or the number of ER α -ir cells in the LS (Figs. 2A and B, $t_{10}=0.28$, $p=0.80$), BNST ($t_{10}=1.26$, $p=0.26$), MPOA (Figs. 2C and D, $t_{10}=0.17$, $p=0.90$), MEA ($t_{10}=1.1$, $p=0.28$), PVN ($t_{10}=0.28$, $p=0.80$), or VMH ($t_{10}=1.40$, $p=0.2$). There was also no effect of photoperiod on the number of ER β immunostained cells in the BNST (Figs. 2E and F, $t_{10}=0.96$, $p=0.36$), MPOA ($t_{10}=0.74$, $p=0.48$), PVN ($t_{10}=1.11$, $p=0.26$), or MEA (Figs. 2G and H, $t_{10}=0.54$, $p=0.60$).

Despite the absence of differences in estrogen receptor expression or testosterone concentrations, males housed in short days were significantly more aggressive than males housed in long days. Males housed in short days showed shorter attack latencies (Fig. 1C, $t_{14}=2.90$, $p=0.01$) and more offensive attacks (Fig. 1D, $t_{14}=2.23$, $p=0.04$) in aggression

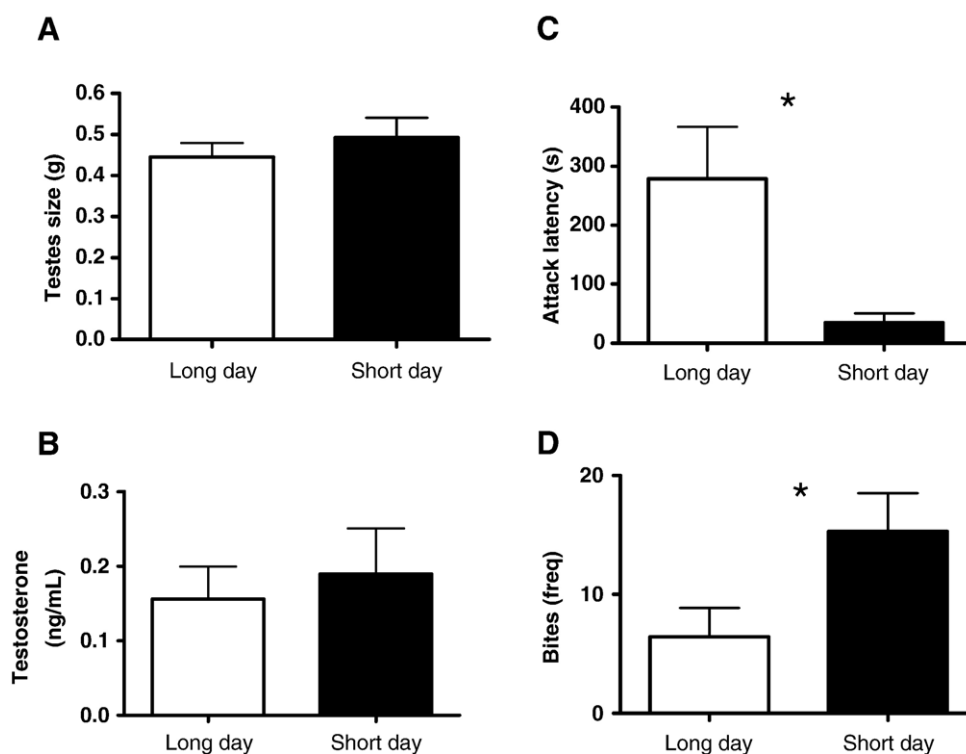


Fig. 1. Effect of photoperiod on physiology and behavior. There was no effect of photoperiod on testes mass (A) or baseline plasma testosterone (B). In resident-intruder aggression tests, males housed in short days showed significantly shorter mean attack latency (C) and increased bites (D) compared to males housed in long days. * $p < 0.05$. For all panels; long days $n=9$, short days $n=7$.

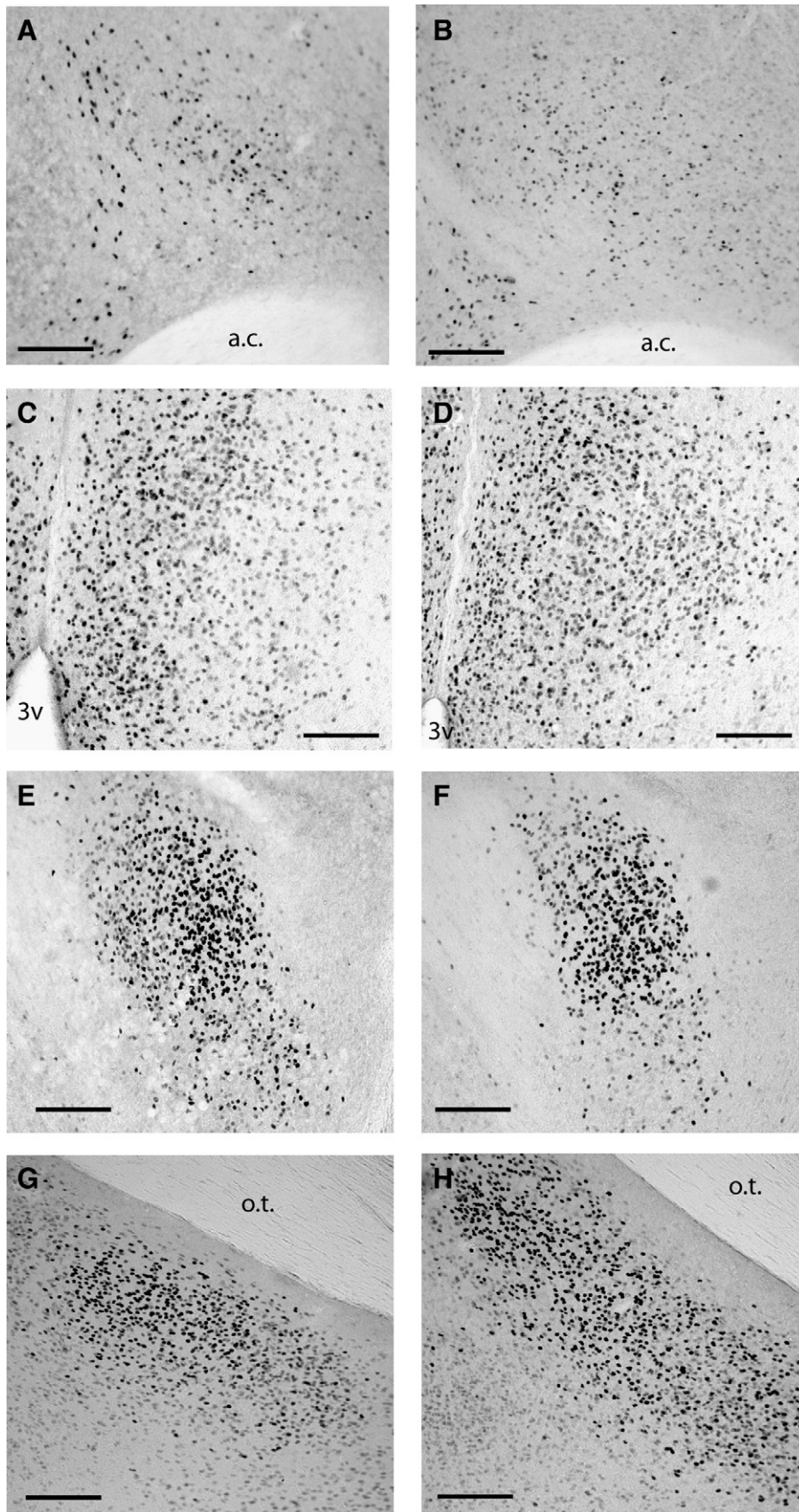


Fig. 2. Representative photomicrographs of estrogen receptor alpha immunoreactivity in long days (A, C) and short days (B, D) in the lateral septum (A, B) and medial preoptic area (C, D). Representative photomicrographs of estrogen receptor beta immunoreactivity in long days (E, G) and short days (F, H) in the bed nucleus of the stria terminalis (E, F) and medial amygdala (G, H). Abbreviations: anterior commissure (ac), third ventricle (3v). Scale bars = 100 μm.

tests than males housed in long days. In general the frequency of boxing in long-day (mean±SE, 6.8 ± 2.4) and short-day (5.4 ± 2.2) mice was low, and unaffected by photoperiod ($t_{14}=0.69$, $p=0.41$).

One hour after aggression tests, males housed in short days (0.38 ± 0.1 ng/ml) had significantly higher testosterone concentrations compared to long-day males (0.12 ± 0.2 ng/ml, $t_{14}=2.26$, $p=0.04$). In the MPOA there were more c-fos positive cells in short-day males compared to long-day males (Supplementary Table 1, $t_{10}=2.80$, $p=0.02$). There were no significant differences in c-fos positive cells in any other brain region.

Rapid effects of estradiol on aggression

We detected significant main effects of estradiol injections on biting ($F_{1,32}=4.94$, $p<0.05$) and attack latency ($F_{1,32}=4.37$, $p<0.05$), but not boxing ($F_{1,32}=0.1$, $p>0.05$). Although there

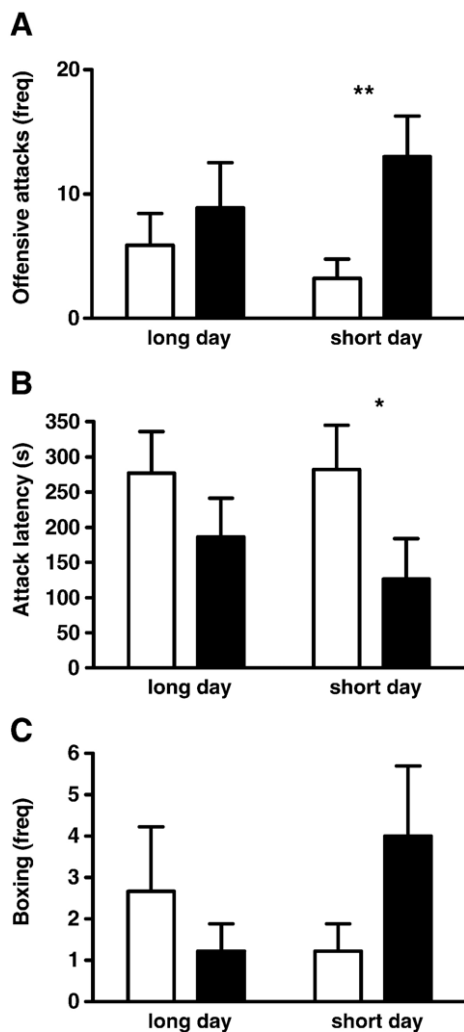


Fig. 3. Rapid effects of estradiol on aggression in a resident–intruder test. Estradiol injections (100 µg/kg) administered 15 min before aggression tests caused a significant increase in bites (A) and a decrease in attack latency (B) in short-day males but not long-day males. There was no effect of photoperiod or estradiol injections on boxing behavior (C). ** $p<0.01$, * $p<0.05$.

were no significant photoperiod by injection interactions (all p 's >0.16), planned comparisons showed that the effects of estradiol injections were stronger when mice were housed in short days. Estradiol injections caused a significant increase in bites in short (Fig. 3A, $p<0.01$), but not long days ($p>0.05$). Similarly, estradiol injections caused a significant decrease in attack latency in short days (Fig. 3B, $p<0.05$) but not long days ($p>0.05$). There were no significant differences for boxing behavior (Fig. 3C).

Discussion

The biology of *P. californicus* allowed us to test hypotheses on the relationship between photoperiod and aggression from a unique perspective. The effects of photoperiod on aggression in *P. californicus* occur in the absence of gonadal regression, a result that supports the hypothesis that the effect of photoperiod on aggression is independent of changes in gonadal hormones (Demas et al., 2004; Wen et al., 2004). We also observed that photoperiod affected aggression in the absence of changes in ER α and ER β expression. This result supports the hypothesis developed in *P. polionotus* that the effects of photoperiod on aggression are independent of changes in ER expression (Trainor et al., 2007a). Finally, we observed that in *P. californicus* estradiol injections act rapidly to increase aggression in mice housed in short days but not long days. These data suggest that estrogens increase aggression by activating nongenomic pathways in short, but not long days. These data indicate that the environment has important effects on how estrogens regulate aggression.

Previously, the effect of photoperiod on aggression had only been observed in species that undergo gonadal regression and reduced testosterone when housed in short days (Matochik et al., 1986; Jasnow et al., 2000, 2002; Caldwell and Albers, 2004; Trainor et al., 2007b). Our results demonstrate that the effect of photoperiod on aggression can be dissociated from reproductive responses, because *P. californicus* were more aggressive in short days in the absence of reproductive changes. A previous study reported that exogenous testosterone administered to short-day Siberian hamsters decreased aggressive behavior in a resident–intruder test (Jasnow et al., 2000), but a subsequent report showed that short-day non-responders (which do not decrease testes size or testosterone production in short days) are just as aggressive as short-day males with regressed testes (Wen et al., 2004). Our results suggest that the effect of photoperiod on aggression occurs independently of changes in gonadal testosterone. Studies in hamsters suggest that the primary source of aromatizable androgen in *Peromyscus* may be produced in the brain, most likely from adrenal steroids such as dehydroepiandrosterone (DHEA) (Demas et al., 2004). DHEA can be converted to the aromatizable androgen androsteindione by 3 β -hydroxysteroid dehydrogenase.

In closely related *P. polionotus* and *P. maniculatus*, increased aggression in short days is associated with increased expression of ER α and decreased expression of ER β (Trainor et al., 2007b). In contrast, we observed in the present study that increased aggression in short days occurs in the absence of differences in ER α and ER β expression in the hypothalamus

and limbic system. It has been hypothesized that the effect of photoperiod on aggression is independent of changes in ER α or ER β in the brain (Trainor et al., 2007a), and the current experimental results are consistent with this hypothesis. Although we have not tested this directly, we suspect that the effect of photoperiod on ER α and ER β expression in *P. polionotus* is related to changes in testosterone and resulting negative feedback (Clancy and Michael, 1994). We observed no effect of estradiol injections on aggression in *P. californicus* housed in long days whereas studies of wild-type *Mus musculus* (housed in a 12-h light cycle) report that a similar dose of estradiol increases aggression in males (Nomura et al., 2006). These contrasting results could be attributed to several factors. First, in the Nomura study estradiol was administered over a 3-week period via implants whereas we conducted our tests 15 min after a subcutaneous injection. A 3-week time period is sufficient to induce genomic changes mediated by estrogen receptors, and most researchers agree that 15 min is not enough time for genomic effects to occur. In *P. californicus*, treatment with fadrozole for 10 days is associated with increased aggression. This suggests that estrogens may indeed affect aggression in *P. californicus* by affecting gene expression, but in the opposite direction observed in *M. musculus*. This raises a second possibility. Species differences in estrogen receptor expression may contribute to differences in how estrogens affect aggression. For example, ER α positive cells are present in the PVN of *P. californicus* but not *M. musculus*, whereas ER α positive cells are present in the AHA of *M. musculus* but not *P. californicus* (Merchenthaler et al., 2004). The AHA is known to have important effects on aggressive behavior in rodents (Ferris et al., 1997), so the absence of ER α in this area may influence how estrogens affect aggression in *Peromyscus*. The availability of selective ER agonists should facilitate examination of the effects of the two ER subtypes on behavior in different species that exhibit different distributions of estrogen receptor expression.

Estradiol acts within 15 min to increase aggression in *P. californicus* housed in short days, suggesting that estrogens act nongenomically to increase aggression. This result is consistent with a previous study in *P. polionotus* which reported that estradiol injections increased bites in short- but not long-day mice (Trainor et al., 2007a). It is thought that such rapid behavioral effects of estradiol must be mediated by nongenomic processes (Nilsson et al., 2001; Vasudevan and Pfaff, 2006). Previous studies showed that estradiol acts rapidly to increase male mating behavior in rats (Cross and Roselli, 1999) and quail (Cornil et al., 2006), but these effects did not differ from those observed in response to systemic estradiol treatment. Nongenomic effects of glucocorticoids on behavior have been reported in several species. In rough skinned newts (*Taricha granulosa*), corticosterone rapidly inhibits male mating behavior (Moore and Miller, 1984), presumably by binding to glucocorticoid receptors positioned at the membrane (Orchinik et al., 1991). Corticosterone also acts rapidly to increase aggression in rats (Mikics et al., 2004) and mice (Poole and Brain, 1974). In contrast, over longer time frames corticosterone appears to inhibit rat aggression (Haller et al., 2001, 2004).

These findings suggest that corticosterone might inhibit aggression in rats via genomic processes and increase aggression via nongenomic processes, similar to how estrogens appear to regulate aggression in *Peromyscus*. An intriguing possibility is that nongenomic estrogen receptor and glucocorticoid receptor activity may tap into similar second messenger systems to facilitate aggressive behavior. Presently, it is unclear which pathways mediate the rapid actions of estrogens or glucocorticoids on aggressive behavior.

We have demonstrated in two species of *Peromyscus* that estrogens act rapidly to increase aggression in short days and that this effect is weaker or absent in long days. A previous study reported that estrogens inhibited aggression in *P. californicus* housed in long days (14 L) (Trainor et al., 2004), indicating that a photoperiod-mediated reversal of the effects of estrogens on aggression in *Peromyscus* is not limited to a single species. It remains unspecified how differences in day length can exert such a profound effect on hormone action. One intriguing possibility is suggested by *in vitro* studies. In cell culture, melatonin interacts with the DNA binding domain of ER α and inhibits its transcriptional activity (Rato et al., 1999; Kiefer et al., 2002, 2005). Mice housed in short days have increased melatonin concentrations in the brain for prolonged periods of time compared to mice housed in long days, and recent data suggest that classical estrogen receptors can have nongenomic effects (Abraham et al., 2004). One possible explanation for our results is that melatonin may inhibit the transcriptional activity of classical estrogen receptors without interfering with nongenomic activity. This would explain why nongenomic action of estradiol is more prevalent in short days. Another possibility is that melatonin could affect the secretion of adrenal hormones that could provide the substrate for aromatizable androgen in the brain. Studies in *M. musculus* (Paterson and Vickers, 1981) and *P. sungorus* (Demas et al., 2004) have demonstrated that the facilitating effect of melatonin on male aggression can be blocked via adrenalectomy. It is also possible (and perhaps likely) that melatonin affects aggressive behavior by working at multiple levels of hormone function simultaneously.

The effects of photoperiod on aggressive behavior were first described in species that exhibit reproductive suppression in short days (Garrett and Campbell, 1980; Jasnow et al., 2000). Our observations in *P. californicus*, a species that does not exhibit reproductive inhibition in short days, suggest that these findings may be applicable to a wider array of species, including humans. There is some evidence that components of aggression in humans exhibit seasonal rhythms. Patients diagnosed with seasonal affective disorder tend to be more likely to exhibit anger attacks compared to patients diagnosed with non-seasonal depression (Winkler et al., 2006). Anger and hostility scores, as measured using Emotion Rating scales (Beck et al., 1961), have also reported seasonal variation, with higher scores being recorded in winter (Harmatz et al., 2000). Accumulating evidence indicates that the effect of photoperiod on aggression is decoupled from reproductive responses, suggesting that hamsters and *Peromyscus* may be effective models for studying seasonal changes in aspects of human behavior.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yhbeh.2007.09.016.

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