

# Photoperiod affects estrogen receptor $\alpha$ , estrogen receptor $\beta$ and aggressive behavior

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**Keywords:** androgen, aromatase, hypothalamus, medial amygdala, *Peromyscus polionotus*, social behavior

## Abstract

Estrogens have important effects on male and female social behavior. Despite growing knowledge of the anatomy and behavioral effects of the two predominant estrogen receptor subtypes in mammals (ER $\alpha$  and ER $\beta$ ), relatively little is known about how these receptors respond to salient environmental stimuli. Many seasonally breeding species respond to changing photoperiods that predict seasonal changes in resource availability. We characterized the effects of photoperiod on aggressive behavior in two species of *Peromyscus* that exhibit gonadal regression in short days. *P. polionotus* (old field mice) were more aggressive than *P. maniculatus* (deer mice) and both species were more aggressive in short days. We used immunocytochemistry and real-time polymerase chain reaction to characterize the effects of photoperiod on ER $\alpha$  and ER $\beta$  expression. In both species ER $\alpha$ -immunoreactive staining in the posterior bed nucleus of the stria terminalis (BNST) was increased in short vs. long days. Both species had reduced ER $\beta$ -immunoreactive expression in the posterior BNST in short days. In the medial amygdala ER $\beta$  immunoreactivity was increased in long days for both species. Using real-time polymerase chain reaction on punch samples that included the BNST, we observed that ER $\alpha$  mRNA was increased and ER $\beta$  mRNA was decreased in short days. These data suggest that the effects of photoperiod on ER $\alpha$  and ER $\beta$  expression may thus have important behavioral consequences.

## Introduction

The effects of androgens (such as testosterone), on male behavior can occur via conversion to estrogens (such as estradiol), by aromatase within the brain. The discovery of multiple estrogen receptor (ER) subtypes resulted in many studies investigating the behavioral effects of ER $\alpha$  and ER $\beta$ . Generally, ER $\alpha$  is hypothesized to play a more important role than ER $\beta$  in regulating reproductive behaviors such as mating and parental behaviors (Ogawa *et al.*, 1997; Champagne *et al.*, 2006). Recent studies indicate that ER $\beta$  has a significant role in non-reproductive behaviors (Bodo & Rissman, 2006). In *Mus musculus*, selective deletion of ER $\alpha$  is associated with decreased male aggression (Ogawa *et al.*, 1997; Scordalakes & Rissman, 2003), whereas selective deletion of ER $\beta$  is associated with increased aggression (Ogawa *et al.*, 1999; Nomura *et al.*, 2002, 2006). Few data exist describing how ERs are regulated by salient environmental stimuli. In many species, seasonal fluctuations in estrogen-dependent behaviors are mediated by changes in photoperiod. Thus, understanding how ER subtypes are affected by photoperiod could provide insights into mechanisms of behavioral plasticity.

The effect of photoperiod on the reproductive system has received extensive attention. Many seasonally breeding rodents that mate in spring and summer respond to short photoperiods by reducing the size and function of the reproductive system (Prendergast *et al.*, 2001). In hamsters, short days increase male resident–intruder aggression

(*Phodopus sungorus*, Demas *et al.*, 2004; Wen *et al.*, 2004; *Mesocricetus auratus*, Garrett & Campbell, 1980; Jasnow *et al.*, 2000; Caldwell & Albers, 2004). This effect is paradoxical because increased aggression occurs when testosterone concentrations are at a nadir. Despite the lack of plasma androgens, estrogens may still be important. Adrenalectomy prevents increased aggression in short days in Siberian hamsters (Demas *et al.*, 2004). Studies of zebra finches (*Taeniopygia guttata*) show that the adrenal hormone dehydroepiandrosterone can be indirectly converted into estrogens within the brain (Soma *et al.*, 2004).

Immunocytochemistry studies show that ER $\alpha$  and ER $\beta$  have distinct but overlapping distributions in the brain (Shughrue & Merchenthaler, 2001; Greco *et al.*, 2003; Mitra *et al.*, 2003). Short-day housing decreases ER $\alpha$ -immunoreactive (ir) cell counts and mRNA in the medial pre-optic area (MPOA) and medial amygdala (MEA) of ovariectomized female hamsters housed in short days (Mangels *et al.*, 1998). To our knowledge, no previous study has observed the effect of photoperiod on ER expression in intact male rodents.

We examined the effect of photoperiod on ER expression in two closely related species of *Peromyscus* that inhabit different climates. Individuals of both species respond to winter-like short photoperiods by decreasing testes mass (Trainor *et al.*, 2006c). Using immunocytochemistry and real-time polymerase chain reaction (PCR) we comprehensively examined the effects of photoperiod on ER $\alpha$  and ER $\beta$  expression in *Peromyscus*. We focused our analyses on hypothalamic and limbic brain areas such as the lateral septum (LS) and bed nucleus of the stria terminalis (BNST) because these brain areas have been identified as important neural substrates for the control of social behaviors (Newman, 1999; Choi *et al.*, 2005; Goodson, 2005).

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Received 31 January 2007, revised 15 May 2007, accepted 23 May 2007

## Materials and methods

### Animals

We examined the effects of photoperiod on behavior and ER expression in two species of *Peromyscus* purchased from the *Peromyscus* Stock Center (Columbia, SC, USA). Old field mice, *Peromyscus polionotus*, are found primarily in the south-eastern United States. Field studies suggest that this rodent is monogamous (Foltz, 1981) and that breeding activity occurs throughout the year (Blair, 1951; Caldwell & Gentry, 1965). Deer mice, *Peromyscus maniculatus*, are distributed broadly and can be found as far north as the North-West Territories of Canada and as far south as northern Mexico. Populations of this species exhibit differential sensitivity of reproductive activity to photoperiod and the breeding season duration varies depending on the population (Bronson, 1985). Field studies indicate that *P. maniculatus* have a polygynous mating system (Ribble & Millar, 1996). Despite inhabiting a relatively tropical habitat, *P. polionotus* exhibit testicular regression when housed in short days (Trainor *et al.*, 2006c) as do *P. maniculatus* (Demas *et al.*, 1996). 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*.

### Experiments and behavioral testing

On arrival at our laboratory all males were individually housed and randomly assigned to be maintained in long (16 h light/8 h dark) or short (8 h light/16 h dark) days. In both long- and short-day treatments, lights were turned off at 14:00 h Eastern Standard Time. All males were between 4 and 8 months of age, sexually inexperienced and had not been tested in any behavioral tests. Animals were given access to food (Harlan Teklad 8640) and filtered tap water *ad libitum*. We used three different groups of animals to measure the effects of photoperiod on behavior, ER immunoreactivity and ER gene expression.

In Experiment I, males were tested in resident–intruder aggression tests after 8 weeks. For each test a group-housed sexually inexperienced male (conspecific) was introduced into each resident's home cage for 10 min under dim red light (between 15:00 and 18:00 h). Although it is possible that differences in phase angles between long- and short-day mice could contribute to differences in aggression, we took steps to minimize this possibility. Previously published studies on *Peromyscus* indicate that the effects of photoperiod on activity onset are typically less than 1 h (Johnston & Zucker, 1980; Majoy & Heideman, 2000) and we waited at least 1 h after lights out before testing mice to ensure that all mice had become active before testing. An individual who was unaware of treatment assignments scored videotapes and recorded the number of bites, bouts of boxing, bouts of allogrooming and attack latency. Boxing was defined as fighting with the forepaws. Allogrooming can be an antecedent to more intense aggression but can also function in a more pro-social context (Pellis & Pellis, 1997). The morning after behavioral tests (08:00–10:00 h), males were anesthetized with sodium pentobarbital (40 mg/kg; Nembutal, Sigma, St Louis, MO, USA) and both testes were removed with a sterile cautery for sperm measurements. Males were then immediately perfused through the heart with saline followed by 10% neutral buffered formalin. Brains were removed and post-fixed in formalin overnight at 4 °C. Each brain was then transferred to 30% sucrose in phosphate-buffered saline (PBS) for 24 h, frozen on dry ice

and stored at –80 °C. For *P. maniculatus*, 10 brains (long days,  $n = 5$ ; short days,  $n = 5$ ) were processed for ER $\alpha$  immunocytochemistry and 11 brains were processed for *P. polionotus* (long days,  $n = 6$ ; short days,  $n = 5$ ). Testes were removed from the tunica, minced with scissors, and ground in a saline solution containing 0.05% Triton-X and 0.025 mM thimerosal for 25 s. Spermatid nuclei in the resulting homogenate were then counted on a hemacytometer (Weil *et al.*, 2006).

In Experiment II we collected brains from *P. polionotus* and *P. maniculatus* housed in either long or short days that had not been tested in behavioral tests. Between 13:00 and 15:00 h males were anesthetized with isoflurane and decapitated. Brains were quickly removed and transferred to 5% acrolein in PBS overnight at 4 °C. We used acrolein fixation for these animals because we determined in pilot studies that acrolein fixation resulted in improved ER staining compared with formalin. Each brain was then transferred to 30% sucrose in PBS for 24 h, frozen on dry ice and stored at –80 °C for ER $\alpha$  and ER $\beta$  immunocytochemistry. For *P. maniculatus*, eight brains (long days,  $n = 4$ ; short days,  $n = 4$ ) were processed for ER $\alpha$  and ER $\beta$  immunocytochemistry and 10 brains were processed for *P. polionotus* (long days,  $n = 5$ ; short days,  $n = 5$ ).

In Experiment III, we collected micropunch samples from *P. polionotus* and *P. maniculatus* males that had been housed in long or short days. Males were anesthetized with isoflurane and decapitated between 08:00 and 10:00 h. Brains were quickly dissected with the use of a brain matrix to generate coronal slices. A slice starting at the optic chiasm and ending 2 mm anterior was collected, immediately transferred to RNAlater (Ambion, Austin, TX, USA) and kept at 4 °C overnight. Bilateral samples containing the LS/BNST (these brain areas are contained in the same punch sample), MPOA and ventromedial hypothalamus (VMH) were collected the next day with 1000- $\mu$ m punches. Punch samples were kept in RNAlater at –20 °C for RNA extraction. For *P. maniculatus*, punch samples from eight brains (long days,  $n = 4$ ; short days,  $n = 4$ ) were processed for ER $\alpha$  and ER $\beta$  gene expression and 12 brains were processed for *P. polionotus* (long days,  $n = 6$ ; short days,  $n = 6$ ).

### Immunocytochemistry

In Experiment I, formalin-fixed brains were sectioned at 40  $\mu$ m on a cryostat and free-floating sections were processed for ER $\alpha$  immunocytochemistry. Sections were washed three times in PBS and then incubated in 1% sodium borohydride in PBS for 10 min. Sections were then rinsed in 20% normal goat serum and 0.3% hydrogen peroxide in PBS for 20 min. Sections were incubated in primary ER $\alpha$  antibody (1 : 50 000, C1355, Upstate Biotechnology, Chicago, IL, USA) in 1% normal goat serum at 4 °C for 48 h. The ER $\alpha$  antibody is well characterized (Friend *et al.*, 1997; Greco *et al.*, 2001) and has been previously used in *Peromyscus* (Kramer *et al.*, 2005). Titration experiments indicated that the 1 : 50 000 dilution was optimal for the lot of primary antibody used in this study. Sections were rinsed in PBS and incubated for 2 h with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) in PBS + Triton X (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 with nickel for 2 min. Sections were mounted on gel-coated slides, dehydrated and coverslipped.

In Experiment II, sections of acrolein-fixed brains were processed as described above except that alternate sections were incubated in

either primary ER $\alpha$  (1 : 20 000, C1355, Upstate Biotechnology) or primary ER $\beta$  (1 : 400, D7N, Invitrogen, Carlsbad, CA, USA) antibody in 1% normal goat serum in 0.5% Triton-X PBS (PBS + TX) for 48 h at 4 °C. Although previously used in studies of human breast tissue (Skliris *et al.*, 2001), to our knowledge the D7N antibody has not been previously used in brain tissue. In control experiments on *Peromyscus* brain tissue, the omission of primary antibody resulted in no positive staining and pre-incubation with ER $\beta$  peptide (1 : 500) completely abolished positive staining (Fig. 1). The dilutions used in Experiment II were chosen based on titration experiments using the lots of ER $\alpha$  and ER $\beta$  primary antibody available for this experiment.

### Image analysis

In Experiment I, we used a Nikon E800 microscope to capture representative photomicrographs of each of the following brain areas using a mouse brain atlas (Paxinos & Franklin, 2002): ventral LS (bregma 0.26 mm), MPOA (bregma 0.02 mm) and VMH (bregma -1.70 mm). In these areas the number of ER $\alpha$ -ir cells within a 305  $\times$  365  $\mu$ m box was counted with the aid of NEUROLUCIDA software (Microbrightfield, Williston, VT, USA) by an observer unaware of treatment assignments. We used a less conservative strategy to ensure that all cells in a given nucleus were counted, although this may have resulted in the inclusion of cells outside the regions of interest. When using brains fixed with acrolein, we detected ER $\alpha$  immunoreactivity in some areas that could not be observed in formalin fixed brains. In Experiment II we sampled the number of ER $\alpha$ -ir and ER $\beta$ -ir cells in the ventral LS, MPOA and VMH, and also in the posterior BNST (bregma 0.02 mm), paraventricular nucleus (PVN) (bregma -1.22 mm) and MEA (bregma -1.82 mm). For Experiment II we used a more conservative strategy for quantification. We used a 140  $\times$  170  $\mu$ m box, which ensured that our quantification was strictly limited to the regions of interest (Fig. 2). This approach has been used numerous times to quantify the expression of steroid receptors in hypothalamic and limbic brain areas (Lonstein *et al.*, 2000; Scordalakes *et al.*, 2002; Chung *et al.*, 2006) and has also been used extensively to quantify immediate early gene expression in the brain (Gammie & Nelson, 2001; Kollack-Walker & Newman, 1995). We also quantified these same regions using a larger box (305  $\times$  365  $\mu$ m) and the results were essentially identical to the results presented below (data not shown).

### Quantitative real-time polymerase chain reaction

RNA was extracted from punch samples using RNeasy (Ambion) kits. RNA samples were precipitated with lithium chloride and reconstituted in 20  $\mu$ L of elution solution before spectrographic analysis. For each sample, 1  $\mu$ g of RNA was reverse transcribed with Superscript (Invitrogen). Using cDNA pools of ovary tissue, we obtained partial sequences of the *Peromyscus* ER $\alpha$  and ER $\beta$  cDNAs via PCRs with degenerate primers based on sequences from mouse, rat and human. We visualized bands of approximately 400 bp for ER $\alpha$  and 450 bp for ER $\beta$  on 2% TAE-agarose gels containing ethidium bromide. The PCR products were purified and directly sequenced, revealing partial cDNA sequences for ER $\alpha$  (GenBank accession no. DQ357060) and ER $\beta$  (GenBank accession no. DQ357061), respectively.

Based on these sequences we designed the following primers and probes for ER $\alpha$  and ER $\beta$ :

ER $\alpha$  forward, 5'-GAACAGCCCCGCTTGT-3';

ER $\alpha$  reverse, 5'-GCATCCAGCAAGGCACTGA-3';

ER $\alpha$  probe, 5'-TGACAGCTGACCAGATG-3';

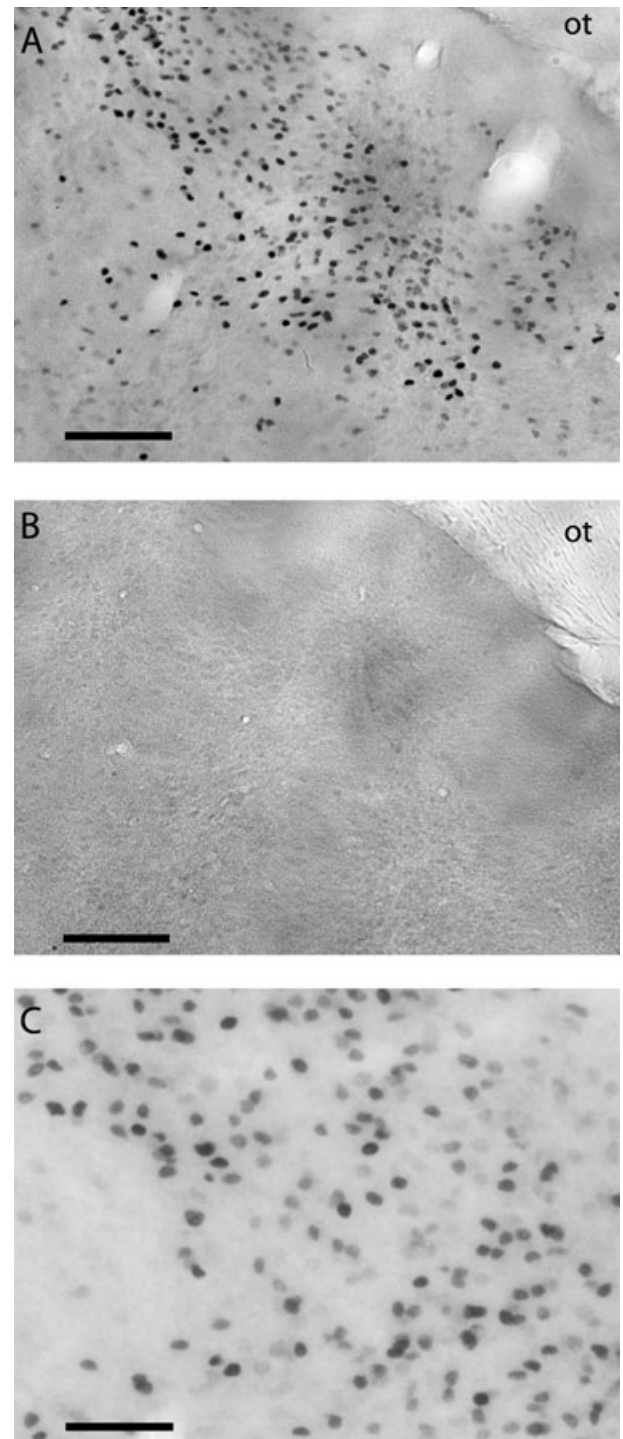


FIG. 1. Estrogen receptor  $\beta$ -immunoreactive staining in the medial amygdala without (A) and with (B) pre-incubation with immunizing peptide. The optic tract (ot) is visible in the top right corner of each panel. The high-power photomicrograph in C shows the nuclear localization of the immunoreactivity. Scale bars: 100  $\mu$ m, A and B; 50  $\mu$ m, C.

ER $\beta$  forward, 5'-GCTGATGTGGCGCTCGAT-3';

ER $\beta$  reverse, 5'-CCCTCATCCCTGTCCAGAAC-3' and

ER $\beta$  probe, 5'-ACCACCCTGGCAAGCTCATCTTT-3'.

The ER $\beta$  gene has multiple splice variants (Price *et al.*, 2000) and we designed our ER $\beta$  primers to exclude the ER $\beta$ 2 isoform that contains a 117 bp insertion between exons 5 and 6. Probes were

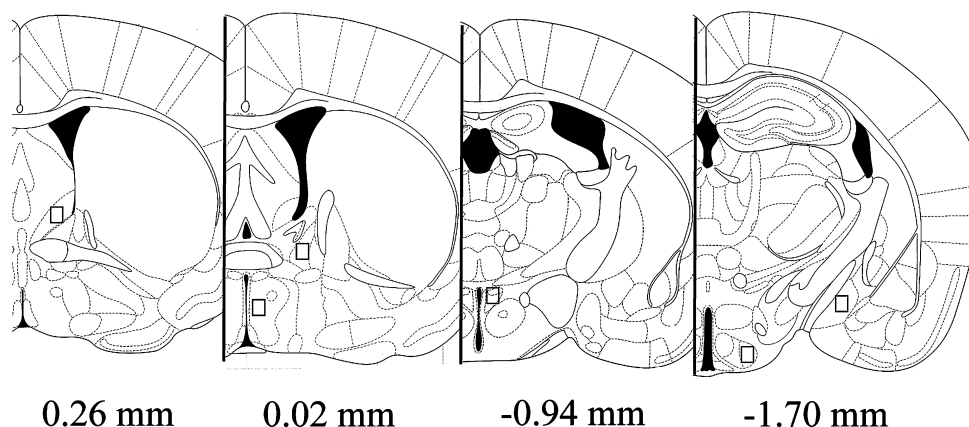


FIG. 2. Representation of the quantification areas used for microscopic analyses in Experiment II. Reproduced from Paxinos & Franklin (2002), with permission from Academic Press. Figures 29 (ventral lateral septum), 31 (bed nucleus of stria terminalis and medial pre-optic area), 39 (paraventricular nucleus) and 45 (ventromedial hypothalamus and medial amygdala).

labeled with the 6-FAM dye and MGB (non-fluorescent quencher) at the 5' and 3' ends, respectively. A TaqMan 18S ribosomal RNA primer and probe set (labeled with VIC dye; Applied Biosystems, Foster City, CA, USA) was used as a control gene for relative quantification. Amplification was performed on an ABI 7000 Sequencing Detection System with the Taqman® System. The universal two-step PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression of duplicate individual samples was calculated by comparison to standard curves consisting of serial dilutions of pooled *P. polionotus* ovary cDNA ( $1 : 10^2$ ,  $1 : 10^3$ ,  $1 : 10^4$  and  $1 : 10^5$ ) followed by normalization to 18S rRNA gene expression.

### Statistical analyses

Aggressive and mating behaviors were square root transformed for parametric statistical analyses to minimize any effects of outliers and to achieve homogeneity of variances between treatment groups (Zar, 1996). We used two-way ANOVA to examine species differences and test for effects of photoperiod on aggression, ER-ir cell number and ER gene expression. We used planned comparisons to test for effects of photoperiod within each species. In Experiment I, for each species we used a principle component analysis on aggressive behavior to facilitate correlations with ER expression (see Results).

## Results

### Species differences and effects of photoperiod on behavior

In male–male resident–intruder aggression tests, *P. polionotus* were more aggressive than *P. maniculatus* (Table 1). Male *P. polionotus* exhibited higher levels of biting ( $F_{1,26} = 7.9$ ,  $P < 0.01$ ) and boxing ( $F_{1,26} = 43.1$ ,  $P < 0.001$ ), and had shorter attack latencies ( $F_{1,26} = 22.3$ ,  $P < 0.001$ ) than *P. maniculatus*. In general, aggression was increased in short compared with long days. Male *P. polionotus* showed increased biting and boxing behavior in short compared with long days (Table 1). Male *P. polionotus* housed in long days engaged intruders primarily via increased allogrooming (Table 1). We often observed that bouts of boxing could be initiated by the intruder following extended periods of the resident grooming the intruder, which occurred primarily in long days. In male *P. maniculatus*, boxing was increased in short-day males and there was a non-significant increase in biting in short-day males (Table 1). In *P. polionotus*, a principle component analysis identified one component that explained 67% of the variance in behavior. This component consisted of biting (component score = 0.68), boxing (0.79) and attack latency (−0.89). A similar component explaining 73% of the variance in behavior was identified in *P. maniculatus*. This component consisted of biting (component score = 0.82), boxing (0.77) and attack latency (−0.96). We refer to these variables below as the aggression composite score.

TABLE 1. Effects of photoperiod on male–male resident–intruder aggression

	<i>Peromyscus maniculatus</i>		<i>Peromyscus polionotus</i>	
	Long day	Short day	Long day	Short day
Bites (per 10 min)	0.1 ± 0.1	2.0 ± 1.0	4.7 ± 2.4 <sup>†</sup>	13.5 ± 7.0*
Boxing (bouts per 10 min)	0.3 ± 0.3	5.7 ± 2.4*	8.33 ± 2.38 <sup>†</sup>	27.12 ± 5.0*
Allogrooming (bouts per 10 min)	0 ± 0	1.28 ± 1.0	26.2 ± 5.0 <sup>†</sup>	11.4 ± 3.5*
Attack latency (s)	544.3 ± 55.5	423.1 ± 75.6	191 ± 73.3 <sup>†</sup>	112.2 ± 61
Sperm count (sperm/mg testes)	1.52 ± 0.10 × 10 <sup>5</sup>	0.7 ± 0.08 × 10 <sup>5</sup> *	1.12 ± 0.13 × 10 <sup>5</sup>	0.88 ± 0.12 × 10 <sup>5</sup>
(n)	(7)	(7)	(9)	(8)

\*Effect of photoperiod,  $P < 0.05$ ; <sup>†</sup>overall species difference,  $P < 0.05$ .

### Effects of photoperiod and species differences on estrogen receptor $\alpha$ immunoreactivity

In Experiment I, observations were conducted on formalin-fixed brains from animals that had been tested in aggression tests. In the ventral LS *P. polionotus* had significantly more ER $\alpha$ -ir cells than *P. maniculatus* ( $F_{1,17} = 27.3$ ,  $P < 0.001$ ) and both species had significantly more ER $\alpha$ -ir cells in short compared with long days (Fig. 3A). There was no significant interaction between photoperiod and species. In the MPOA, the effect of photoperiod on ER $\alpha$  differed between the two species (interaction,  $F_{1,17} = 4.48$ ,  $P < 0.05$ ). In *P. polionotus*, ER $\alpha$  immunoreactivity was increased in short compared with long days, whereas in *P. maniculatus*, ER $\alpha$  immunoreactivity was increased in long compared with short days (Fig. 3B). In the VMH there were no apparent species differences, effect of photoperiod or interaction on ER $\alpha$  immunoreactivity (Fig. 3C). In *P. polionotus*, ER $\alpha$  immunoreactivity in the ventral LS was positively correlated with the aggression composite score (Spearman  $\rho = 0.60$ ,  $P < 0.05$ , Fig. 3D). In neither the MPOA nor VMH was ER $\alpha$  immunoreactivity correlated with the aggression composite score in *P. polionotus*. In *P. maniculatus*, there were no significant correlations between ER $\alpha$  immunoreactivity and the aggression composite score.

In Experiment II, observations were conducted on acrolein-fixed brains from animals that had not been tested in behavioral tests. Acrolein fixation allowed the detection of ER $\alpha$  immunoreactivity in several regions that were undetectable in formalin-fixed brains (Figs 4 and 6, Table 2). In the ventral LS, *P. polionotus* had significantly more ER $\alpha$ -ir cells than *P. maniculatus* (Table 2,  $F_{1,14} = 7.42$ ,  $P < 0.05$ ) and both species had significantly more ER $\alpha$ -ir cells in short days (Fig. 4). In the posterior BNST, *P. polionotus* had significantly more ER $\alpha$ -ir cells than *P. maniculatus* (Table 2,  $F_{1,14} = 5.25$ ,  $P < 0.05$ ). Additionally, in *P. polionotus* there were more ER $\alpha$ -ir cells in short-day mice compared with long-day mice, whereas this difference was not significant in *P. maniculatus* (Fig. 4, Table 2). In the MPOA, *P. polionotus* had more ER $\alpha$ -ir cells than *P. maniculatus* (Fig. 5,  $F_{1,14} = 11.3$ ,  $P < 0.01$ ) but there was no effect of photoperiod (Table 2). In the PVN, there was no effect of photoperiod on ER $\alpha$  immunoreactivity but *P. polionotus* had more ER $\alpha$ -ir cells than *P. maniculatus* (Fig. 6, Table 2,  $F_{1,14} = 13.0$ ,  $P < 0.01$ ). In the VMH there was no species difference in ER $\alpha$  immunoreactivity (Table 2) and both species had reduced ER $\alpha$  immunoreactivity in short days (Table 2,  $F_{1,14} = 5.30$ ,  $P < 0.05$ ). In the MEA there was no species difference or effect of photoperiod on ER $\alpha$  immunoreactivity (Table 2). There were no significant species by photoperiod interactions in any of the brain areas examined.

The physiological and endocrine data for the animals used in Experiment II have been published elsewhere (Trainor *et al.*, 2006c). In both species, testes mass and testosterone were reduced in short days and there were no significant species differences in testosterone or testes mass ( $P > 0.05$  in each case).

### Effects of photoperiod and species differences on estrogen receptor $\beta$ immunoreactivity

Few ER $\beta$ -ir cells were detected in the ventral LS and *P. polionotus* had more ER $\beta$ -ir cells when housed in long vs. short days (Table 2). In contrast, large numbers of ER $\beta$ -ir cells were observed in the posterior BNST of both species. Both species had increased ER $\beta$  immunoreactivity in long compared with short days (Fig. 4,  $F_{1,14} = 13.0$ ,  $P < 0.01$ ). There was no effect of photoperiod or any species differences in ER $\beta$  immunoreactivity in the MPOA (Fig. 5, Table 2). There was no species difference or effect of photoperiod on ER $\beta$

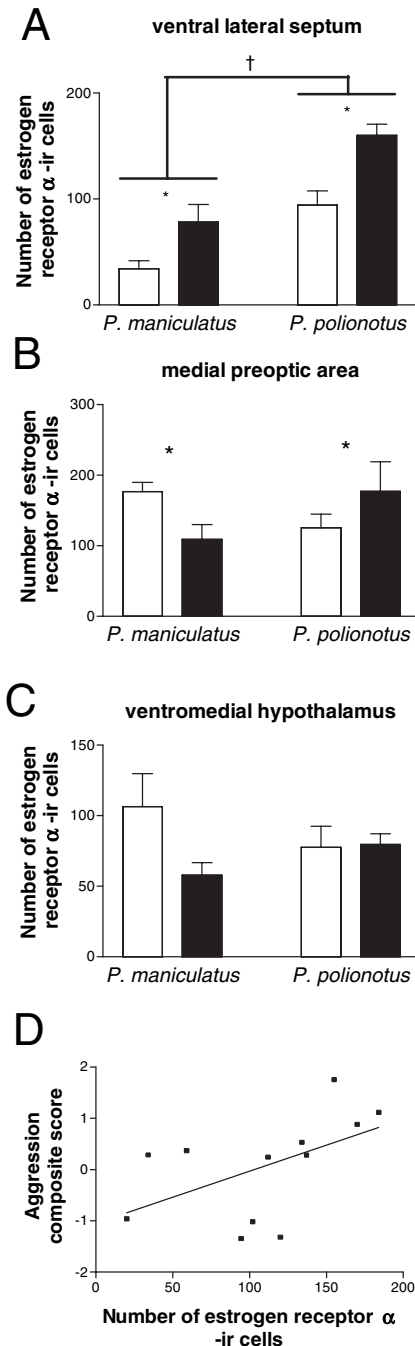


FIG. 3. Estrogen receptor (ER) $\alpha$ -immunoreactive (ir) cell counts in ventral lateral septum (A), medial pre-optic area (B) and ventromedial hypothalamus (C) from formalin-fixed brains. *Peromyscus maniculatus*: long days ( $n = 5$ ) and short days ( $n = 5$ ). *P. polionotus*: long days ( $n = 6$ ) and short days ( $n = 5$ ). Open bars, long days; filled bars, short days. \*Photoperiod effect,  $P < 0.05$ ; †species difference,  $P < 0.05$ . In *P. polionotus* ER $\alpha$ -ir in the ventral lateral septum was positively correlated with the aggression composite score (D) (Spearman  $\rho = 0.60$ ,  $P < 0.05$ ).

immunoreactivity in the PVN (Fig. 6, Table 2). In the VMH, ER $\beta$  immunoreactivity was increased in *P. polionotus* vs. *P. maniculatus* (Table 2,  $F_{1,14} = 21.7$ ,  $P < 0.001$ ). Also in the VMH, both species had significantly more in ER $\beta$ -ir cells in long days (Table 2,  $F_{1,14} = 15.0$ ,  $P < 0.01$ ). In the MEA, both species had increased ER $\beta$  immunoreactivity in long compared with short days (Fig. 4,



## Long day

## Short day

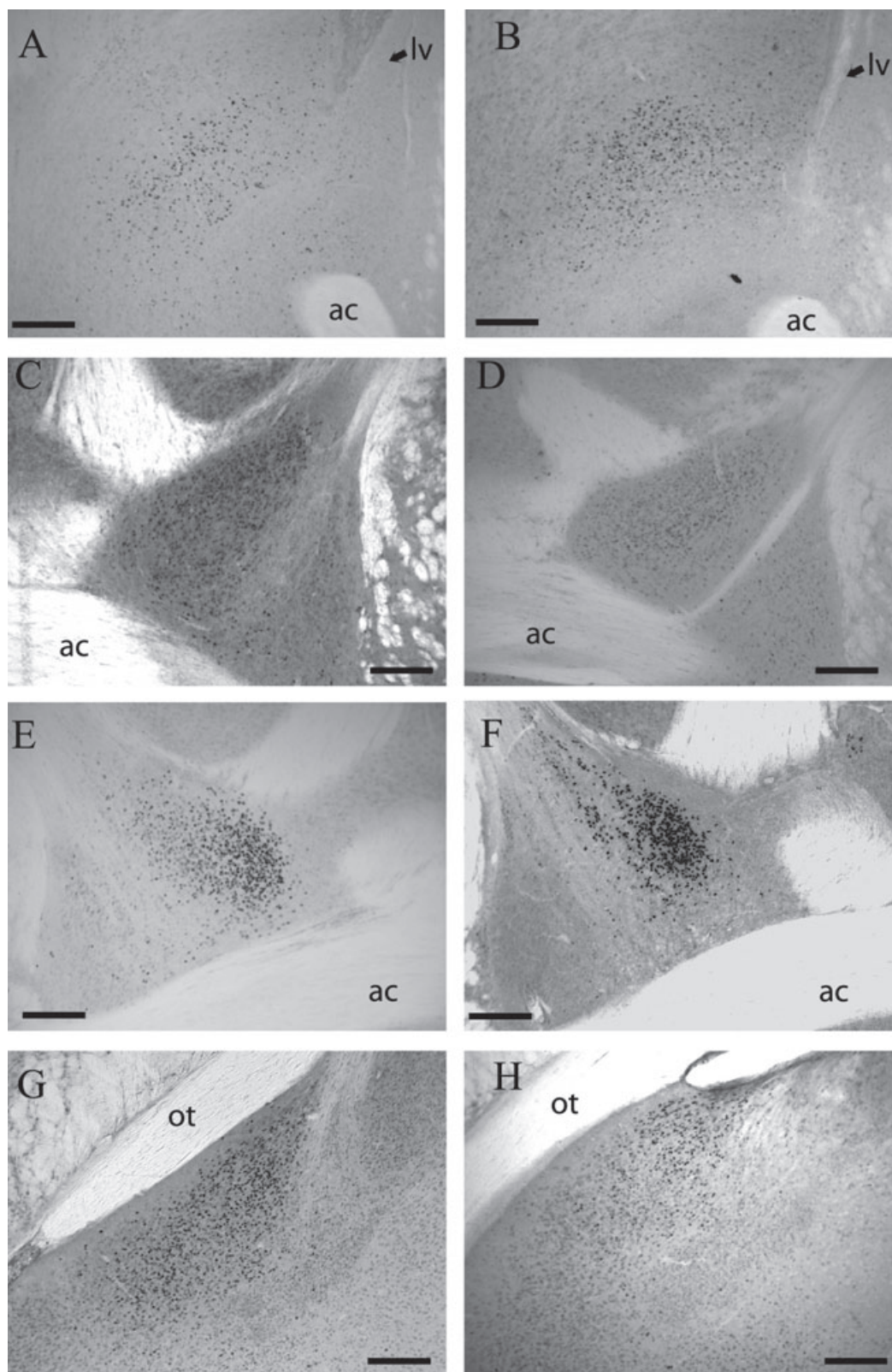


FIG. 4. Photomicrographs of estrogen receptor (ER) $\alpha$ - and ER $\beta$ -immunoreactive staining in *Peromyscus polionotus* in long (left column) and short (right column) days. There were more ER $\alpha$ -immunoreactive cells in the ventral lateral septum (A and B) and bed nucleus of the stria terminalis (C and D) in short compared with long days. There were more ER $\beta$ -immunoreactive cells in the bed nucleus of the stria terminalis (E and F) and medial amygdala (G and H) in long compared with short days. ac, anterior commissure; lv, lateral ventricle; ot, optic tract. Scale bars, 170  $\mu$ m.

TABLE 2. Effects of photoperiod on estrogen receptor (ER) $\alpha$ - and ER $\beta$ -immunoreactive cells/mm<sup>2</sup> ( $n = 4-5$ /group)

	ER $\alpha$				ER $\beta$			
	<i>Peromyscus maniculatus</i>		<i>Peromyscus polionotus</i>		<i>Peromyscus maniculatus</i>		<i>Peromyscus polionotus</i>	
	Long day	Short day	Long day	Short day	Long day	Short day	Long day	Short day
vLS	2668 $\pm$ 87	3015 $\pm$ 213	2454 $\pm$ 250	3375 $\pm$ 323*	832 $\pm$ 301	210 $\pm$ 210	1143 $\pm$ 187	269 $\pm$ 212*
MPOA	2910 $\pm$ 259	3256 $\pm$ 479	3897 $\pm$ 229 <sup>†</sup>	4521 $\pm$ 309	2889 $\pm$ 255	3036 $\pm$ 443	3504 $\pm$ 209	3550 $\pm$ 220
BNST	2437 $\pm$ 45	2878 $\pm$ 175*	3521 $\pm$ 256 <sup>†</sup>	3924 $\pm$ 142*	4034 $\pm$ 349	2941 $\pm$ 375*	4218 $\pm$ 95 <sup>†</sup>	3429 $\pm$ 161*
PVN	1681 $\pm$ 212	1345 $\pm$ 357	2387 $\pm$ 224 <sup>†</sup>	2361 $\pm$ 153	1387 $\pm$ 250	1345 $\pm$ 280	1992 $\pm$ 191	1555 $\pm$ 351
VMH	2952 $\pm$ 150	2300 $\pm$ 203*	2697 $\pm$ 244	2420 $\pm$ 169	1922 $\pm$ 405	742 $\pm$ 137*	3235 $\pm$ 210 <sup>†</sup>	2153 $\pm$ 267*
MEA	2342 $\pm$ 206	2423 $\pm$ 303	2218 $\pm$ 344	2294 $\pm$ 206	3831 $\pm$ 241	2489 $\pm$ 638*	4579 $\pm$ 774	3134 $\pm$ 151*

\*Effect of photoperiod within species,  $P < 0.05$ ; <sup>†</sup>overall species difference,  $P < 0.05$ . BNST, bed nucleus of the stria terminalis; MEA, medial amygdala; MPOA, medial pre-optic area; PVN, paraventricular nucleus; LS, ventral lateral septum; VMH, ventromedial hypothalamus.

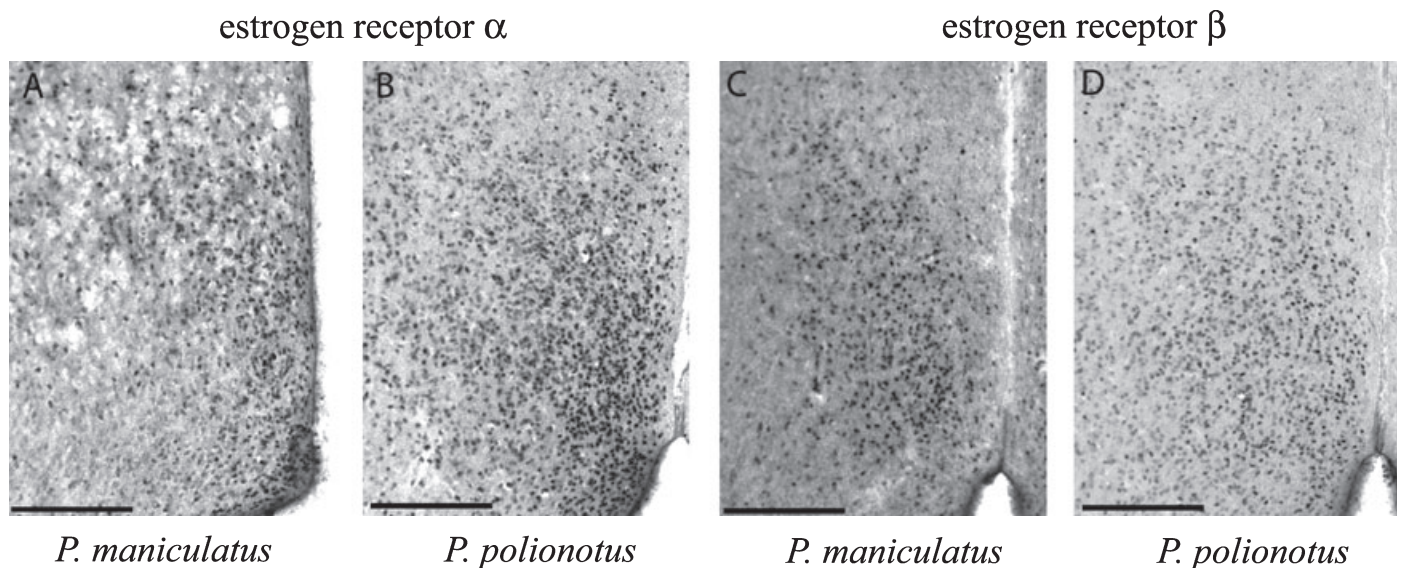


FIG. 5. Estrogen receptor (ER) immunoreactivity in medial pre-optic area. ER $\alpha$  immunoreactivity in *Peromyscus maniculatus* (A) and *P. polionotus* (B). ER $\beta$  immunoreactivity in *P. maniculatus* (C) and *P. polionotus* (D). The third ventricle is visible in the lower right corner of each panel. Scale bars, 200  $\mu$ m.

Table 2,  $F_{1,14} = 8.8$ ,  $P < 0.01$ ). There were no significant species by photoperiod interactions in any of the brain areas examined.

#### Species differences and effects of photoperiod on estrogen receptor mRNA

In Experiment III, ER mRNA was measured with quantitative real-time PCR from punch samples that included both the ventral LS and posterior BNST. In these punch samples, both species had increased ER $\alpha$  mRNA in short compared with long days (Fig. 7A,  $F_{1,16} = 14.62$ ,  $P < 0.01$ ). No significant species differences in ER $\alpha$  mRNA were detected. In short days, both species had significantly lower ER $\beta$  gene expression (Fig. 7B). Again, no significant species differences were observed. In the MPOA, there was a marginal effect of photoperiod ( $F_{1,16} = 4.44$ ,  $P = 0.05$ ) and non-significant interaction on ER $\alpha$  mRNA ( $F_{1,16} = 3.65$ ,  $P = 0.07$ ). In *P. polionotus*, ER $\alpha$  mRNA was significantly increased in short as compared with long days (Fig. 7C) but this photoperiod effect was not significant in *P. maniculatus*. Also in the MPOA, there was a marginal species by photoperiod interaction ( $F_{1,16} = 4.5$ ,  $P = 0.05$ ) for ER $\beta$  expression. This reflected a significant up-regulation of ER $\beta$  gene expression in short days of *P. maniculatus* and no significant effect of photoperiod

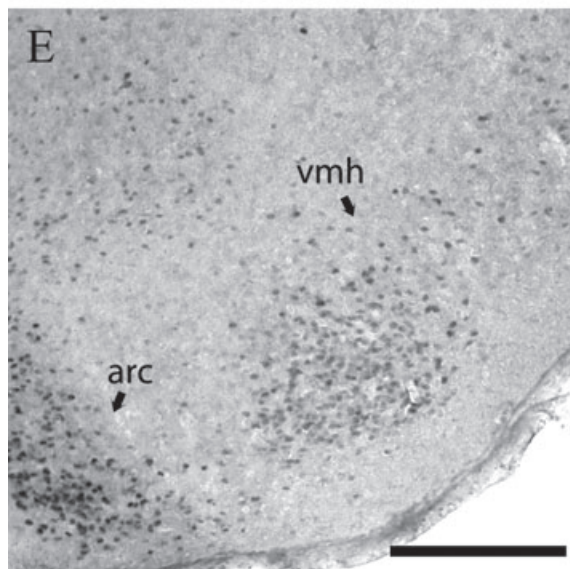
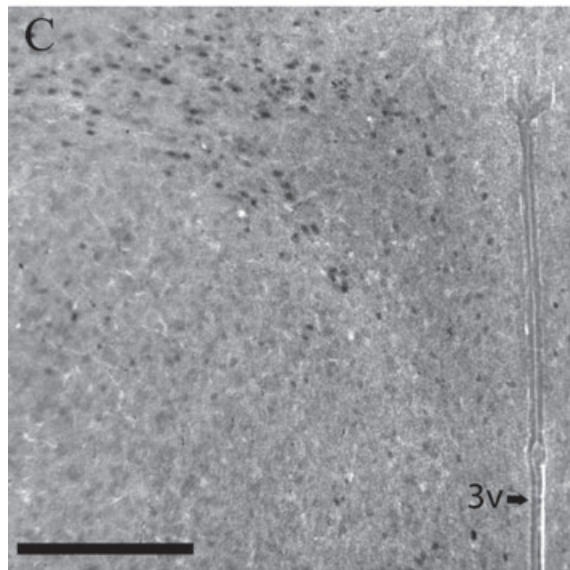
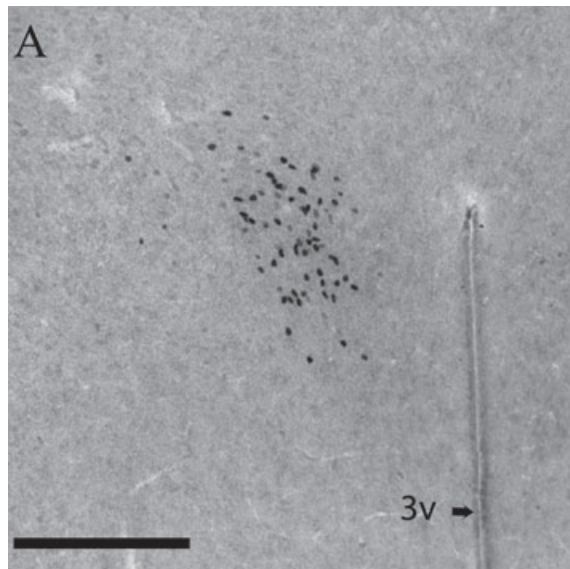
in *P. polionotus*. In the VMH, no species differences or effects of photoperiod on ER $\alpha$  or ER $\beta$  mRNA expression were observed. No significant species differences, effects of photoperiod or interactions on the cycle thresholds of 18s RNA samples were detected, suggesting that this gene was not differentially regulated across species or photoperiods.

#### Discussion

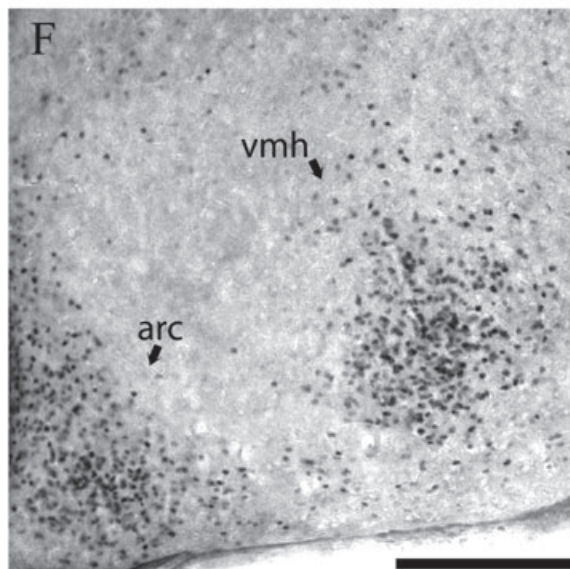
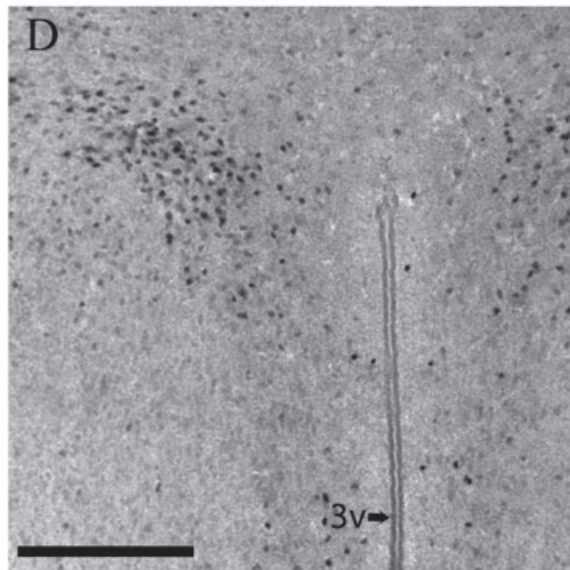
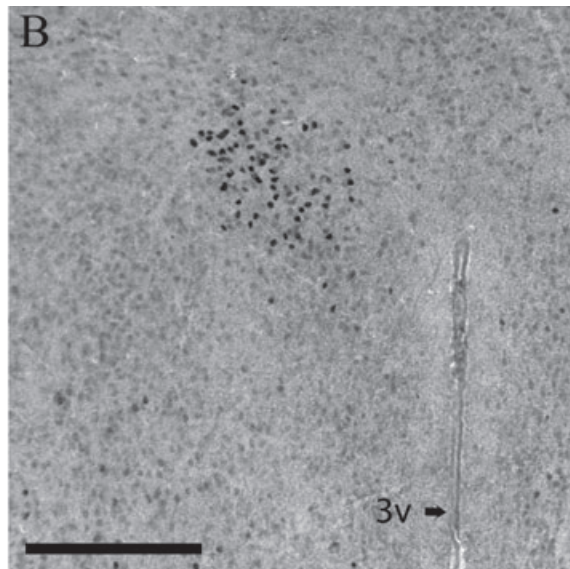
We demonstrated by using immunocytochemistry and real-time PCR that photoperiod has differential effects on ER expression and that these effects are anatomically specific. The ability to measure both ER subtypes separately proved to be important because ER $\alpha$  and ER $\beta$  were often (but not always) inversely expressed. In short-day-housed animals we observed a consistent increase in ER $\alpha$  expression in the ventral LS and a corresponding decrease in ER $\beta$  expression in the posterior BNST. Increased ER $\alpha$  immunoreactivity in short days was observed in both naive animals and animals that were tested in aggression tests, indicating that the effects of photoperiod on ER $\alpha$  and ER $\beta$  was not mediated by experience in aggression tests. Additionally, these observations are supported by a real-time PCR experiment that demonstrated that short-day mice had increased ER $\alpha$  mRNA and



*P. maniculatus*



*P. polionotus*





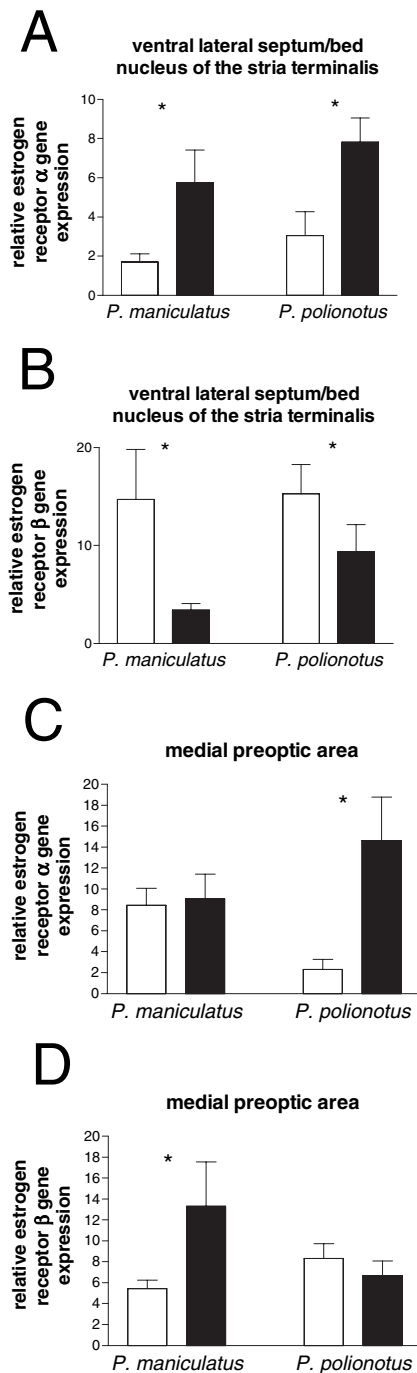


FIG. 7. Estrogen receptor (ER) $\alpha$  and ER $\beta$  mRNA as measured by quantitative real-time polymerase chain reaction (PCR) in mice housed in long (open bars) and short (filled bars) days. *Peromyscus maniculatus*: long days ( $n = 4$ ) and short days ( $n = 4$ ). *P. polionotus*: long days ( $n = 6$ ) and short days ( $n = 6$ ). ER $\alpha$  (A) and ER $\beta$  (B) gene expression was measured in punch samples that included the ventral lateral septum and posterior bed nucleus of the stria terminalis (BNST). ER $\alpha$  (C) and ER $\beta$  (D) were measured in punch samples that included the medial pre-optic area (MPOA). Gene expression is normalized relative to 18s mRNA expression. \*Effect of photoperiod,  $P < 0.05$ .

decreased ER $\beta$  mRNA in LS/BNST punch samples. The effects of photoperiod in the MPOA were less consistent. In general, ER $\alpha$  expression in the MPOA of *P. polionotus* was up-regulated in short days. In contrast, both species had fewer ER $\beta$ -ir cells in the posterior BNST, MEA and VMH when housed in short days. These data suggest that photoperiod regulation of receptor expression in the brain could have important consequences for estrogen-sensitive behaviors.

#### Effects of photoperiod on estrogen receptor $\alpha$ and social behavior

We observed that, in the ventral LS, short days increased both ER $\alpha$  mRNA and the number of ER $\alpha$ -ir cells. In both *P. maniculatus* and *P. polionotus* we observed increased aggressive behavior in short days, although this effect was stronger in *P. polionotus*. In addition, the number of ER $\alpha$ -ir cells in the ventral LS (but not MPOA or VMH) was positively correlated with aggression in *P. polionotus*. In CD-1 mice (*M. musculus*), the number of ER $\alpha$ -ir cells in the LS is positively correlated with male aggression in resident-intruder tests (Trainor *et al.*, 2006b) and numerous studies have demonstrated increased c-fos in the LS following male-male aggression tests (Kollack-Walker & Newman, 1995; Delville *et al.*, 2000). Most studies in rodents have observed that estrogens increase aggression (Hilakivi-Clarke, 1999; Simon, 2002; Trainor *et al.*, 2006b), presumably through activation of ER $\alpha$ .

There was some suggestion that male *P. polionotus* had increased ER $\alpha$  in MPOA when housed in short days, although this difference was not consistently observed in all experiments. There were no consistent effects of photoperiod on ER $\alpha$  immunoreactivity or mRNA in *P. maniculatus*. The MPOA is a critical brain area regulating male reproductive behavior (Hull *et al.*, 2002). In particular, estrogens appear to promote mating behavior by binding to ER $\alpha$  (Ogawa *et al.*, 1997; Wersinger *et al.*, 1997). Thus, it seems counterintuitive that ER $\alpha$  should be increased in short days when testes are regressed. Although these changes may simply reflect effects of negative feedback on receptor expression, field observations on *P. polionotus* indicate that this species breeds throughout the year (Blair, 1951). Consistent with these observations, the relative decrease in testicular sperm production of short-day *P. polionotus* was much smaller than the observed decrease in sperm of short-day *P. maniculatus*.

#### Possible mechanisms of estrogen receptor $\alpha$ regulation

An obvious possible factor influencing ER $\alpha$  expression in short-day mice is reduced testosterone. Both *P. maniculatus* (Demas *et al.*, 1996) and *P. polionotus* (Trainor *et al.*, 2006b) have reduced testosterone concentrations in short compared with long days. Reduced testosterone almost certainly reduces estrogens in the brain by decreasing available substrate and also aromatase activity in areas of the brain such as the MPOA (Roselli *et al.*, 1996). Previous studies suggest that the effect of castration on receptor immunoreactivity may depend on the antibody used. For example, castration in male rats increased the number of observed ER $\alpha$ -ir cells when antibodies raised to the ligand-binding domain of ER $\alpha$  were used but not when antibodies raised outside the ligand-binding domain

FIG. 6. Photomicrographs of estrogen receptor (ER) $\alpha$ -immunoreactive staining in *Peromyscus maniculatus* (left column) and *P. polionotus* (right column) in the paraventricular nucleus (A and B) and ventromedial hypothalamus (E and F). Photomicrographs of ER $\beta$ -immunoreactive staining in *P. maniculatus* and *P. polionotus* in the paraventricular nucleus (C and D), arc, arcuate nucleus; 3v, third ventricle; vmh, ventromedial hypothalamus. Scale bars, 200  $\mu$ m.

were used (Clancy & Michael, 1994). Similar results have been reported in female rats (Weiland *et al.*, 1997). The C1355 ER $\alpha$  antibody used in this study binds outside the ligand-binding domain (Friend *et al.*, 1997), which suggests that the increased number of ER $\alpha$ -ir cells observed in the ventral LS of short-day mice is not due to competitive binding with endogenous estrogen. Thus, any possible effects of testosterone on ER $\alpha$  immunoreactivity in this study should have occurred at the transcriptional or translational levels. A role for testosterone is supported by observations in male *P. californicus*, in which photoperiod does not affect ER $\alpha$  or ER $\beta$  immunoreactivity in hypothalamic and limbic brain areas (B. C. Trainor, M. S. Finy & R. J. Nelson, unpublished). Males of this species do not decrease testes mass (Nelson *et al.*, 1995) or testosterone concentrations (B. C. Trainor, M. S. Finy & R. J. Nelson, unpublished) when housed in short days.

Recent research suggests anatomical specificity in the regulation of ER $\alpha$ . When researchers created transgenic rats that expressed green fluorescent protein under the control of the ER $\alpha$  O/B promoter, they observed green fluorescent protein in forebrain regions (including BNST and MPOA) but not the VMH (Hamada *et al.*, 2005). In *P. polionotus* we observed that ER $\alpha$  immunoreactivity and mRNA were increased in short days in ventral LS, posterior BNST and MPOA but not in VMH, PVN or MEA. These data suggest that the O/B promoter may mediate the effects of testosterone in the ventral forebrain but not other hypothalamic and limbic areas such as the VMH and MEA. Tissue-specific regulation of aromatase activity has also been observed in *P. californicus*. Reproductive experience altered aromatase activity in MPOA and ventral LS/BNST punch samples but not in VMH or MEA punch samples (Trainor *et al.*, 2003). These findings suggest that similar mechanisms may regulate ER $\alpha$  expression and aromatase activity in an anatomically specific manner.

#### Photoperiod regulation of estrogen receptor $\beta$

This study is the first to report the effects of photoperiod on ER $\beta$  immunoreactivity and mRNA in the brain. In both *P. polionotus* and *P. maniculatus* ER $\beta$  immunoreactivity in the posterior BNST, MEA and VMH ER $\beta$  was decreased in short days. In *M. musculus*, castration increases ER $\beta$  immunoreactivity in the MPOA, BNST, VMH and PVN (Nomura *et al.*, 2003) and in male *Rattus norvegicus* castration increases ER $\beta$  immunoreactivity in the VMH but not the MEA (Orikasa & Sakuma, 2004). In contrast to these previous observations, *Peromyscus* mice exhibited up-regulated ER $\beta$  in the posterior BNST in long days when testosterone is elevated. These data suggest that, if testosterone does have an effect on ER $\beta$  expression, it is positive as in prostate tissue (Asano *et al.*, 2003). However, ER $\beta$  was also increased in the MEA in long-day mice, a tissue in which ERs typically do not respond to castration. This suggests that there may be some non-androgen-based mechanisms that may mediate the effect of photoperiod on ER $\beta$  in the MEA.

Several recent studies have demonstrated that ER $\beta$  activation can reduce anxiety-like behavior in female rats and mice (Imwalle *et al.*, 2005; Lund *et al.*, 2005; Walf & Frye, 2005). Recent studies on male Siberian hamsters (*Ph. sungorus*) have demonstrated that anxiety-like and depressive-like behaviors are increased in short days (Prendergast & Nelson, 2005; Pyter & Nelson, 2006). The amygdala and its projections to the BNST are thought to play an important role in modulating affective states (Phelps & LeDoux, 2005), so a decrease in ER $\beta$  activity in these regions during short days could contribute to increased anxiety-like behavior.

#### Species differences in estrogen receptor expression

In immunocytochemistry experiments, *P. polionotus* had significantly more ER $\alpha$ -ir and ER $\beta$ -ir cells than *P. maniculatus* in several brain areas. A previous study also reported increased ER $\alpha$  immunoreactivity in the PVN of *P. polionotus* compared with other species of *Peromyscus* (Kramer *et al.*, 2005). At present the mechanistic bases and functional consequences of these differences are unclear. Our real-time PCR measurements did not detect any species differences in either ER $\alpha$  or ER $\beta$  mRNA. This could be due to species differences in post-translational processes or species differences in antibody-receptor binding. It is tempting to speculate that the increased ER $\alpha$  immunoreactivity expression in numerous brain regions in *P. polionotus* may contribute to the increased aggressive behavior relative to *P. maniculatus* or may be related to species differences in mating systems. It is unlikely that species differences in body size could account for increased ER immunoreactivity because the smaller species (*P. polionotus*) consistently exhibited more ER $\alpha$ -ir and ER $\beta$ -ir cells than *P. maniculatus*. Further study of ER function and regulation in *Peromyscus* is needed.

#### Conclusions

We have demonstrated that photoperiod has differential effects on ER $\alpha$  and ER $\beta$  expression, and that these effects are anatomically specific. For *P. polionotus*, animals that were tested in aggression tests and naive animals had increased ER $\alpha$  immunoreactivity in the ventral LS when housed in short days. Additionally, ER $\alpha$  mRNA in ventral LS/BNST punch samples was increased in short days, whereas ER $\beta$  mRNA was increased in long days. Photoperiod regulation of ERs in the ventral LS and BNST reflected a general pattern of increased ER $\alpha$  in short days and increased ER $\beta$  in long days, although not every brain area responded to photoperiod in this way. These changes in receptor expression may have important consequences for the control of estrogen-dependent processes including aggressive, mating and affective behaviors. Hormone manipulation experiments are needed to examine the behavioral consequences of these differences in ER expression.

#### Acknowledgements

We thank G. A. Bishop, J. D. Blaustein, L.B. Martin II, N. S. Hasen, L.M. Pyter and Z.M. Weil for helpful discussions, K.M. Greiwe, K. M. Kassouf, S.L. Kidder, J. R. Kuhlman, A.G. Trainor and J. E. West for technical assistance, and Invitrogen for generously donating ER $\beta$  blocking peptide. This work was supported by NIH MH076313 (B.C.T.) and NIH MH57535 (R.J.N.).

#### Abbreviations

BNST, bed nucleus of the stria terminalis; ER, estrogen receptor; ir, immunoreactive; LS, lateral septum; MEA, medial amygdala; MPOA, medial pre-optic area; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVN, paraventricular nucleus; TX, Triton X; VMH, ventromedial hypothalamus.

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