Photoperiod and bromocriptine treatment effects on expression of prolactin receptor mRNA in bovine liver, mammary gland and peripheral blood lymphocytes

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Abstract

Recent evidence suggests that photoperiod influences immune function. Interestingly, photoperiod has profound effects on concentrations of prolactin (PRL), a hormone also known to be involved in fluctuations of the immune system. However, the impact of photoperiod on PRL receptor (PRL-R) expression is poorly understood, particularly in tissues of the immune system. Two experiments were performed to increase the general understanding of how photoperiod interacts with the immune system. Our first objective was to determine the effects of photoperiod on PRL-R mRNA expression and cellular immune function. Lymphocytes were isolated from blood collected from calves (n = 10) and PRL-R mRNA expression of both long and short forms was quantified using real-time PCR. Lymphocytes expressed PRL-R mRNA, suggesting that PRL could act directly on these cells. To determine the relationship between photoperiod and PRL-R mRNA expression in other tissues, hepatic and mammary biopsies were collected after calves were exposed to long days (LDPP; 16 h light:8 h darkness) or short days (SDPP; 8 h light:16 h darkness). Relative to LDPP, SDPP decreased circulating PRL, but increased expression of both forms of PRL-R mRNA in liver, mammary gland and lymphocytes. Short days also increased lymphocyte proliferation compared with long days. Reversal of photoperiodic treatments reversed the effects on circulating PRL, PRL-R mRNA expression and lymphocyte proliferation. Our second objective was to manipulate PRL concentration in photoperiod-treated animals, using bromocriptine. Concentrations of PRL in LDPP animals injected daily with bromocriptine for 1 week were decreased compared with LDPP controls, to a level similar to SDPP animals. Receptor expression was increased in LDPP+bromocriptine-treated animals relative to LDPP controls, as was lymphocyte proliferation. Overall, our results indicate that photoperiodic effects on PRL-R mRNA expression were inverse to those on circulating PRL, with short days stimulating expression of both forms of PRL-R mRNA. Expression of PRL-R mRNA changed in the same direction as lymphocyte proliferation with regard to photoperiod treatment, suggesting a link between photoperiodic effects on PRL sensitivity and immune function. Thus, PRL signaling may mediate photoperiodic effects on immune function.

Introduction

Many species use photoperiod as an environmental cue to time seasonal events associated with reproduction, growth and lactation (Nelson & Demas 1996, Dahl et al. 2000). Recent evidence suggests that shifts in immune competence may also be influenced by photoperiod (Nelson & Demas 1996, Dowell 2001, Bilbo et al. 2002a,b, Prendergast et al. 2002). For example, short-day photoperiod increases natural killer cell cytolytic capacity and spontaneous blastogenesis in lymphocytes of Siberian hamsters (Yellon et al. 1999). Of interest, prolactin (PRL) is considered a mediator of immune function (Reber 1993) as PRL is known to be secreted by peripheral blood mononuclear cells (PBMCs) (Sabharwal et al. 1992), lymphocytes express PRL receptor (PRL-R) in humans (Russell et al. 1984), rodents (Di Carlo et al. 1995), cattle (Schuler et al. 1997) and fish (Prunet et al. 2000) and PRL replacement in immunocompromised mice enhanced survival in response to Mycobacterium bovis challenge (Bernton et al. 1988). Because daylength has a marked effect on circulating PRL, it is possible that PRL is a mediator of photoperiodic effects on the immune system.

In addition to circulating PRL responses, the sensitivity of target cells to PRL may also be influenced by photoperiod, via changes in PRL-R expression in various
tissues. In that regard, the aforementioned effects of PRL manipulation on immune function must be considered in the context of recent studies in PRL-R knockout mice that fail to support a role for PRL in normal development and function of the immune system (Bouchard et al. 1999). But lack of essentiality should not be interpreted as no effect, as an immunomodulatory role for PRL may exist (Giffin et al. 2002) even in the PRL-R knockout mouse. Furthermore, species differences exist in PRL effects between cattle and mice with regard to mammary function, so consideration must be made with regard to immune system function as well.

To date, however, little is known about the response of PRL-R to photoperiodic treatment or physiological shifts in PRL secretion in general. In the first study in the paper, we determined the influence of photoperiod on PRL-R expression in three tissues: liver, mammary gland and peripheral blood lymphocytes. The objectives were to (i) establish the effects of photoperiod on PRL-R mRNA expression in liver, peripheral blood lymphocytes and mammary tissue, and (ii) evaluate the effects of photoperiod on lymphocyte proliferation in the bovine. To more specifically link shifts in PRL to the observed effects of photoperiod manipulations, a second study was completed in which serum PRL was manipulated by treatment with bromocriptine. Specifically the objective was to determine if abrogation of the long-day-induced increase in PRL secretion restored increases in PRL-R and immune function similar to observations under short-day photoperiod.

Materials and Methods

General

The castrated male calves (steers) used in Experiment 1 were part of a larger study described by Kendall et al. (2003). Details on maintenance and performance of the steers can be found in Kendall et al. (2003). After 1 week of pretreatment acclimation (12 h light:12 h darkness), steers were randomly assigned to either long-day photoperiod (LDPP; 16 h light:8 h darkness) or short-day photoperiod (SDPP; 8 h light:16 h darkness). Lights came on at the same time for both treatment groups (0800 h) and went off at 1600 h for the SDPP steers and at 2400 h for the LDPP animals. Steers in Experiment 1 (n = 10) were maintained on their assigned lighting regime for 9 weeks and then switched to the opposite lighting regime (LDPP to SDPP and SDPP to LDPP) and maintained on those photoperiods until the end of the experiment (Fig. 1A). Average age (± s.d.) of the calves at the start of the experiment was 109 ± 8 ± 13·1 days. Steers used in Experiment 2 averaged 75·3 ± 8·5 days in age and 99·3 ± 6·2 kg in weight at the start of the experiment. Steers were individually fed a grain and alfalfa hay diet according to the guidelines of the National Research Council (1989). Every 2 weeks adjustments were made for body weight gain and intake was recorded daily. Water was accessible to the steers at all times. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

PRL assays

Blood (10 ml) was collected on a weekly basis for both experiments and also daily during bromocriptine treatment of Experiment 2. Blood was collected into sterile Vacutainer tubes containing sodium heparin (Becton Dickinson & Co., Franklin Lakes, NJ, USA) from the jugular vein of calves restrained individually in their pens. Collection occurred between 0830 and 1100 h on each day of sampling. Samples were placed on ice immediately after collection. Lymphocytes for subsequent RNA extraction were rapidly isolated to reduce RNA degradation. Within 2 h of collection, plasma for hormone determination was obtained from whole blood after centrifugation (1850 g, 20 min, 4 °C) and stored at −20 °C until assayed for PRL. Serum PRL concentrations were determined by RIA as described by Miller et al. (1999). Mean intraassay coefficients of variation (three assays) for medium (6·5 ng/ml) and high (11·8 ng/ml) control pools of sera were 3·3 and 9·6% respectively. Mean interassay coefficients of variation for medium and high control pools of sera were 5·6 and 7·9% respectively. Assay sensitivity averaged 0·94 ng/ml.

Lymphocyte isolation

Blood collected for lymphocyte RNA isolation and proliferation was taken at a consistent time before the biopsies of Experiment 1 so as not to confound the lymphocyte data with potential immunosuppression of the animals due to the biopsy procedure.

Bovine PBMCs were used as the source of lymphocyte mRNA for real-time PCR. Bovine PBMCs were isolated from blood samples collected on sodium heparin by density gradient centrifugation through Histopaque-1077 (density 1·077; Sigma, St Louis, MO, USA). The PBMCs were washed twice in RPMI-1640 (Sigma) and resuspended. For the lymphocyte proliferation assay (Morrow-Tesch et al. 1994), the cell concentration was adjusted to 5 × 10^6 cells/ml using RPMI supplemented with 10% fetal bovine serum (FBS) (Sigma) and 50 μg/ml gentamycin (Sigma). Lymphocytes were washed only once in RPMI before RNA isolation to minimize the degradation of RNA.

Lymphocyte proliferation assay

Diluted bovine lymphocytes (100 μl) were added in triplicate to 96-well flat-bottom sterile plates. The mitogens concanavalin A (ConA), phytohemagglutinin (PHA) and
pokeweed mitogen (PWM) (all from Sigma) were added (100 µl) in triplicate at various doses (PHA, PWM: 0, 0.1, 1, 10 µg/ml; ConA: 0, 0.2, 2, 20 µg/ml). Only one mitogen was used per plate. Cells (total volume 200 µl) were then incubated for 48 h at 37°C. At that point, 100 µl supernatant were removed, after centrifugation of the plates, and replaced with 100 µl fresh RPMI supplemented with 10% FBS, containing the appropriate mitogen. Approximately 24 h later, 20 µl MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; optimum concentration of 1 mg/ml MTT in RPMI) were added to each well and the cells were incubated for a further 4 h. After incubation, 100 µl acidic isopropanol were added to each well and the wells were mixed thoroughly by repeated pipetting. The optical density (OD) of each well was measured at 600 nm within 1 h. Proliferation is expressed as the stimulation index (percentage of the unstimulated control), which is the OD of the treated wells/OD of the unstimulated control wells (cells receiving no mitogen).

**Neutrophil chemotaxis (Experiment 2)**

Blood (7 ml) for neutrophil isolation was collected using Vacutainer tubes containing EDTA. After centrifugation, the plasma layer, buffy coat and approximately one quarter of the red cell layer were removed. Ten milliliters of cold water were added to the remaining cell layer and mixed thoroughly. Isotonicity was restored after 1 minute by adding 5 ml of 3/2 PBS. The cells were centrifuged for 10 min at 475 g. The supernatant was poured off and the red blood cell lysis was repeated as needed. Cell pellets were resuspended in 2 ml PBS and neutrophil numbers determined using a Coulter counter (Coulter Electronics, Miami, FL, USA). The chemoattractants used in these experiments were human interleukin-8 (100 ng/ml) and...
human complement 5a (10^{-8} M). Approximately 30 µl chemoattractant or control media were placed in each well of the bottom chamber of the chemotaxis chamber. Prior to loading the top chamber with neutrophils, the chamber was equilibrated in an incubator for 10−15 min at 5% CO₂ and 37 °C. During the equilibration, the cell concentrations were adjusted using RPMI to 3 × 10⁶ cells/ml. The adjusted cell solution was added to the top chamber at 50 µl per well and the chemotaxis chamber was incubated for 1 h. Following incubation, the non-cell side of the filter was wiped with 3 × PBS and then dried. The cell side was then dipped in methanol eight times and dried. Finally, the filter was stained with Diff-Quick (Fisher Scientific, Pittsburgh, PA, USA), placed on a microscope slide and allowed to dry. Five fields per well were counted to determine the number of neutrophils that migrated in response to the chemoattractants.

Bromocriptine treatment (Experiment 2)
This experiment consisted of three treatments, LDPP plus a vehicle (ethanol:methanol in saline) injection (LDPP+v), LDPP plus a bromocriptine injection (LDPP+b) and SDPP plus a vehicle injection (SDPP+v). Each animal was given each treatment in a randomized manner and thus each animal was used as its own control. Animals were allowed 10 days to acclimate to new photoperiod treatments before the bromocriptine treatment period began. Treatments (bromocriptine or vehicle) were administered via s.c. injections daily for 1 week. Injections occurred after daily blood samples were collected, between 1000 and 1130 h (see Fig. 3A). Bromocriptine was solubilized in 1:1 ethanol:methanol at an initial concentration of 40 mg/ml. The final dose of bromocriptine (in saline) was 0.05 mg/kg. This dose was chosen based on previous observations of Barrington et al. (1999), in which their dose of 0.11 mg/kg essentially suppressed PRL concentrations. Our dose was designed to suppress PRL concentrations of LDPP animals to the level of SDPP animals.

Liver and mammary biopsies (Experiment 1)
Liver biopsies were performed as described by Swanson et al. (2000) and Kendall et al. (2003). Briefly, animals were sedated with Xylazine (0.2 mg/kg; Vedco, Inc., St Joseph, MO, USA) and placed on a surgical table. An incision was made between the ninth and tenth ribs after s.c. application of local anesthetic (Lidocaine; Abbott Laboratories, North Chicago, IL, USA). A biopsy trocar was inserted into the incision, through the body wall and peritoneum and finally into the liver. Liver tissue samples (approximately 0.6 g) were placed in 1.5 ml Eppendorf tubes, immediately frozen in liquid nitrogen and stored at −80 °C until isolation of RNA.

On the final day of treatment in Experiment 1, mammary tissue samples were obtained while calves were sedated for liver biopsy as described above. Local anesthetic (Lidocaine; 2 ml) was administered in a line block, superior to the left mammary gland and teat. Palpable mammary epithelial tissue was present as the lining of the teat canal and a pea-size nodule (~0.2–0.5 cm³) at the base of the teat. An incision was made at the base of the teat and the teat and underlying parenchymal tissue were dissected and removed. Subsequently, epithelial tissue was dissected and was frozen in liquid nitrogen and stored at −80 °C until isolation of RNA.

Real-time PCR
Total RNA was extracted from liver samples and mammary tissue (Experiment 1), as well as lymphocytes (Experiments 1 and 2) using Trizol reagent (Gibco BRL, Grand Island, NY, USA) and stored at −80 °C until further processing. Total RNA (10 µg) was reverse transcribed to cDNA using the ProSTAR First-Strand RT-PCR kit (Stratagene, La Jolla, CA, USA). Real-time PCR was performed on the cDNA using a probe and primers designed for PRL-R using the Primer Express software (Applied Biosystems, Foster City, CA, USA) and 18S rRNA was amplified as the endogenous reference. Sequences of the long form PRL-R probe, forward primer and reverse primer were 5’-AAGGCCCAGTCGACCTGGAGGCT-3’, 5’-ACACACGGAGGACAGGCGT-3’ and 5’-TGAAGCCCCCGCCGCTG-5’ respectively. Sequences of the short form PRL-R probe, forward primer and reverse primer were 5’-CAAGTCGTTCCATCTCCAGGGCAGCAGC-3’, 5’-GAACCTCTAGCCCATCCTCCT-3’ and 5’-TCCCTGCAGGATCGCTT-5’ respectively. Sequences of the 18S probe, forward primer and reverse primer were 5’-TGGGAGCAGGCGGTATACTC-3’, 5’-GATCCATTGAGGGCCAAGTCT-3’ and 5’-TGAGACCGGCCCCGT-5’ respectively. Detection was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems). The PRL-R TaqMan probe was labeled at the 5’-end with the reporter dye, FAM and at the 3’-end with the quencher dye, TAMRA. The 18S TaqMan probe was labeled with the reporter dye, VIC and the quencher dye, TAMRA. Amplification mixes (25 µl) contained 2.5 µl cDNA, 300 (18S) or 900 (PRL-R) nM of each primer, 0.05 pmol probe and 2 × TaqMan Universal PCR Master mix. Reactions were run in triplicate, with PRL-R and 18S run in separate wells and included five dilutions of cDNA pooled from 12 animals to obtain the relative standard curve for calculations.

The formula used to calculate the input amounts of both PRL-R and 18S was \((C_T - b)/m = \log\) input amount, where \(C_T\) is the threshold cycle; \(b\) is the \(y\)-intercept of the standard curve line; and \(m\) is the slope of the standard curve line. The log input amount was then converted to input amount by the formula \(10^\log\) (log input amount) and the input amounts were normalized to the 18S
(endogenous control) values. The final values are reported as expression values relative to a calibrator cDNA within animal and as percentage of baseline within treatment.

Statistical analyses
Statistical analyses were performed using the SAS System v. 8.2 (SAS Inst. Inc., Cary, NC, USA). In Experiment 1, a mixed model was used to analyze repeated-measures data, specifically comparing PRL concentrations, long and short form PRL-R mRNA expression and lymphocyte proliferation between LDPP- and SDPP-treated animals and across time. In Experiment 2, repeated-measures evaluation used a mixed model comparing PRL concentrations, long and short form PRL-R mRNA expression, neutrophil chemotaxis and lymphocyte proliferation between LDPP+v, LDPP+b and SDPP+v treatments and across time. Evaluation used a mixed model comparing PRL concentrations, long and short form PRL-R mRNA expression, neutrophil chemotaxis and lymphocyte proliferation between LDPP- and SDPP-treated animals and across time. Differences were considered significant at \( P < 0.05 \).

Results

Experiment 1
There was no difference in average daily gain or dry matter intake of the calves on the different photoperiod treatments in this experiment, as reported by Kendall et al. (2003). Concentrations of PRL did not differ on day 0 of the experiment (\( P = 0.33 \)). Within 1 week, concentrations of PRL in plasma were higher (\( P = 0.02 \)) in animals on LDPP as compared with animals kept on SDPP (Fig. 1B) for the first 8 weeks of the experiment, after which the photoperiod treatments were reversed (at week 9). Within 1 week of acclimation to the opposite photoperiod treatments, the PRL concentration of the calves once again showed a difference between treatments with the calves now on LDPP (previously on SDPP, represented by LDPP:SDPP) having higher (\( P = 0.05 \)) PRL concentrations than the calves now on SDPP (previously on LDPP, represented by LDPP:SDPP; Fig. 1B). The difference in PRL concentrations persisted through the end of the experiment.

Due to the lack of sensitivity of Northern blots to measure low expression of lymphocyte PRL-R mRNA, we used real-time quantitative RT-PCR to test the hypothesis that the expression of PRL-R mRNA is influenced by photoperiod treatment. Agarose gel electrophoresis demonstrated PCR products of the size expected for PRL-R. Expression of the long form of PRL-R mRNA in the liver of the steers did not differ (\( P = 0.45 \)) prior to the start of the experiment, when the animals were on the same photoperiod.

Expression of hepatic long form PRL-R mRNA, as determined by real-time PCR, was higher (\( P = 0.03 \)) in the calves on SDPP at weeks 5 and 9 of the experiment as compared with the calves on LDPP (Fig. 1C). Four weeks after the calves were switched to the opposite photoperiods, the SDPP calves (reversed from their original LDPP treatment) had greater expression of hepatic long form PRL-R mRNA than the LDPP calves (reversed from their original SDPP treatment; Fig. 1C). Calves on SDPP had increased (\( P = 0.05 \)) long form PRL-R mRNA expression, relative to baseline values, at week 5, with a similar trend for an increase over baseline at week 9. Conversely, LDPP calves had a tendency for decreased expression of long form PRL-R mRNA compared with baseline values. After the switch in photoperiod treatment, the LDPP:SDPP calves had a tendency (\( P = 0.11 \)) for higher expression of long form PRL-R mRNA relative to baseline values, with the SDPP:LDPP calves tending toward a decrease from baseline (Fig. 1C). Expression of short form PRL-R mRNA followed similar trends to the long form of the receptor. At weeks 5 and 9 of the experiment, SDPP steers had higher short form PRL-R mRNA expression (196·5 and 191·2% respectively; \( P = 0.05 \)) compared with LDPP steers (152·6 and 142·3% respectively). After photoperiod treatments were reversed, the short form of PRL-R showed higher expression in LDPP:SDPP calves (175·3%; \( P < 0.05 \)) than SDPP:LDPP calves (126·4%).

Mammary and lymphocyte PRL-R mRNA expression followed a similar pattern to that of the liver (Table 1). Calves on SDPP had greater expression of PRL-R mRNA in lymphocytes than calves on LDPP both before and after (\( P < 0.05 \)) the switch of treatments, regardless of the form of the receptor. Expression of both forms of PRL-R mRNA in mammary tissue was also greater (\( P < 0.001 \)) in SDPP-treated animals relative to those on long days.

Table 1 Long and short form PRL-R mRNA expression in bovine lymphocytes and mammary tissue is altered by photoperiod treatment (Experiment 1)

<table>
<thead>
<tr>
<th>Week</th>
<th>Form</th>
<th>LDPP:SDPP</th>
<th>SDPP:LDPP</th>
<th>S.E.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>Long</td>
<td>152.2d</td>
<td>224.6</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>129.3c</td>
<td>160.5</td>
<td>10.1</td>
</tr>
<tr>
<td>9</td>
<td>Long</td>
<td>145.2d</td>
<td>284.0</td>
<td>89.1</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>170.0e</td>
<td>32.2</td>
<td>5.7</td>
</tr>
<tr>
<td>13</td>
<td>Long</td>
<td>265.3c</td>
<td>125.2</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>39.4d</td>
<td>10.5</td>
<td>16.3</td>
</tr>
<tr>
<td>Mammary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Long</td>
<td>261.1c</td>
<td>157.3</td>
<td>56.5</td>
</tr>
<tr>
<td></td>
<td>Short</td>
<td>103.8d</td>
<td>45.3</td>
<td>27.0</td>
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\( n = 10. \)

\( \text{s.E.D.}, \) standard error of the difference.

\( a \)Relative abundance of mRNA (PRL-R normalized to 18S; percent of baseline), expressed as mean values, as detected by real-time PCR; SDPP:LDPP=long days before switch, short days after; SDPP:LDPP=short days before switch, long days after.

\( b \)Weeks 2 and 9 are prior to switching the photoperiod treatments (first treatment) and week 13 is after the switch of photoperiod treatments (second treatment).

\( \text{P} < 0.001, \) compared with SDPP:LDPP animals.

\( \text{P} < 0.01, \) compared with SDPP:LDPP animals.

\( \text{P} < 0.05, \) compared with SDPP:LDPP animals.
Various concentrations of the mitogens ConA, PHA and PWM, were used to establish a dose–response in the lymphocyte proliferation assay. A dose–response was observed (data not shown; \( P < 0.05 \)) and the optimal doses of the mitogens were determined to be 20 \( \mu \)g/ml for ConA and 10 \( \mu \)g/ml for PHA and PWM. Proliferation in response to these optimal doses was used in the statistical analyses. Baseline values of proliferation did not differ \( (P=0.43) \) between treatments. Compared with LDPP-treated animals, lymphocytes of SDPP calves had higher proliferation, reflecting greater metabolic activity, when lymphocytes were stimulated with each of the three mitogens (Fig. 2). This was consistent at week 3 and also at the end of week 12, a time point after the animals were switched to opposite photoperiod treatments.

**Experiment 2**

Average daily gain and dry matter intake did not differ between photoperiod or bromocriptine treatment in the steers in this experiment. Concentrations of PRL did not differ on day 0 of the experiment \( (P=0.62) \); however, within 1 week, LDPP animals had greater concentrations of PRL \( (P<0.05) \) relative to SDPP animals. At the start of the bromocriptine treatment, there was no difference in PRL concentration \( (P=0.23) \) between LDPP+b and LDPP+v animals, but by the completion of the week of daily bromocriptine injections, PRL concentrations of LDPP+b animals had decreased to concentrations similar \( (P=0.04) \) to SDPP+v animals, significantly decreased \( (P=0.05) \) from LDPP+v animals (Fig. 3B).

There was no difference \( (P=0.32) \) in PRL-R mRNA expression (either form) between SDPP and LDPP animals prior to the start of photoperiod treatment (data not shown). However, within 10 days, SDPP+v animals had significantly greater PRL-R mRNA expression of both long and short forms of the receptor than either LDPP+b or LDPP+v animals (Fig. 3C; Table 2). After 1 week of daily bromocriptine injections, LDPP+b animals had increased receptor expression relative to LDPP+v animals, similar to SDPP+v animals. This was observed for both long and short forms of the receptor (Table 2).

Lymphocyte proliferation did not differ among animals before the start of the experiment \( (P=0.76) \); however, at the beginning of the first injection period, SDPP+v animals had greater proliferation in response to all three mitogens compared with both LDPP+v and LDPP+b animals (Fig. 4). The LDPP-treated animals did not differ from each other. After the week of bromocriptine/vehicle injections, LDPP+b animals had an increase in proliferation to all three mitogens compared with LDPP+v animals, up to a similar level of proliferation to that of the SDPP+v animals (Fig. 4).

This experiment had an additional measure of cellular immune function in the form of neutrophil chemotaxis. Represented in Table 3, SDPP+v animals had greater migration of neutrophils towards both chemotactants compared with the LDPP+v and LDPP+b animals prior to the injection period. After 1 week of daily bromocriptine injections, LDPP+b animals had increased neutrophil chemotaxis compared with LDPP+v animals.

**Discussion**

In the first study, we demonstrated expression of the long and short forms of PRL-R mRNA in bovine lymphocytes using real-time PCR. These results are consistent with those of Schuler et al. (1997) in cattle as well as those of other species, including rat and human (Russell et al. 1984, Di Carlo et al. 1995). We confirm the influence of photoperiod on circulating PRL concentrations and extend previous studies by showing that PRL-R mRNA expression in multiple tissues is influenced by photoperiod manipulation, potentially through the effect of photoperiod on circulating PRL. Further, we provide initial evidence that lymphocyte PRL-R mRNA expression has potential for use as a proxy for PRL-R expression in liver and mammary gland, particularly as it offers a less-invasive approach to monitor PRL-R expression in those tissues.

Results of the second study provide support for the concept that photoperiod-induced shifts in circulating PRL are causally related to changes in PRL-R expression and cellular immune function. Indeed, elimination of
long-day-induced increases in PRL by concurrent bromocriptine treatment restored elevated expression of PRL-R mRNA. Following bromocriptine treatment, multiple indices of immune function, including lymphocyte proliferation and neutrophil chemotaxis, were restored to levels consistent with a short-day photoperiod.

Generally speaking, PRL causes a down-regulation of its receptor (Di Carlo et al. 1995, Gratton et al. 2001, Goffin et al. 2002) although there is some evidence that acute increases in PRL up-regulate PRL-R (Telleria et al. 1997, Ling et al. 2000). Therefore, it is not surprising that the increase in PRL concentration in LDPP animals is associated with a decrease in PRL-R mRNA expression, relative to SDPP animals, in each of the tissues studied. Bromocriptine treatment of calves on LDPP prevented the increase of PRL and likewise eliminated the depression of PRL-R mRNA expression. This is consistent with the results of Di Carlo et al. (1995), in which expression of both the long and short form of the PRL-R were increased after bromocriptine injections in rat peripheral lymphocytes. Although we did not directly measure PRL-R protein, many studies indicate that changes in PRL-R mRNA parallel shifts in PRL-R protein expression (Royster et al. 1995, Gunes & Mastro 1997, Schuler et al. 1997, Telleria et al. 1997, Guillaumot & Benahmed 1999). Thus, the observed inverse relationship of PRL-R and circulating PRL in response to differing photophase duration or bromocriptine suggests that photoperiod evokes a classic negative feedback loop to regulate PRL sensitivity in multiple tissues.

Figure 3 Effect of photoperiod and bromocriptine treatment on PRL concentration and long form PRL-R mRNA expression in lymphocytes (Experiment 2). (A) Photoperiod design of the experiment; black arrows indicate start and stop of daily bromocriptine/vehicle injections. Note: each of the animals (n=6) received each of the three treatments. (B) Concentrations of PRL from daily samples. (C) Representative values of relative abundance of PRL-R mRNA expression (long form), as percent of baseline values (before photoperiod treatment started), taken prior to bromocriptine treatment and after bromocriptine treatment period. Black bars or squares, SDPP+v; white bars or squares, LDPP+v; hatched bars or gray circles, LDPP+b. S.E. of the difference for the PRL concentrations and PRL-R expression are 5.4 and 38.7 respectively; *P<0.05 compared with SDPP+v values.
Table 2 Expression of long and short form PRL-R mRNA before and after s.c. injection of bromocriptine or vehicle in steers on long or short day photoperiod treatment (Experiment 2)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Before injection</th>
<th>After injection</th>
<th>S.E.D.</th>
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<td>Long form</td>
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<tr>
<td>LDPP +v</td>
<td>119.6 ± 6</td>
<td>126.8 ± 6</td>
<td>12.4</td>
</tr>
<tr>
<td>LDPP +b</td>
<td>117.3 ± 5</td>
<td>194.6 ± 6</td>
<td>32.5</td>
</tr>
<tr>
<td>SDPP +v</td>
<td>228.3 ± 4</td>
<td>214.9 ± 4</td>
<td>9.6</td>
</tr>
<tr>
<td>Short form</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LDPP +v</td>
<td>112.8 ± 8</td>
<td>111.1 ± 7</td>
<td>3.2</td>
</tr>
<tr>
<td>LDPP +b</td>
<td>113.5 ± 5</td>
<td>168.4 ± 1</td>
<td>45.6</td>
</tr>
<tr>
<td>SDPP +v</td>
<td>190.6 ± 6</td>
<td>206.8 ± 6</td>
<td>11.7</td>
</tr>
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</table>

n=6; standard error of the difference.

aRelative abundance of mRNA (PRL-R normalized to 18S; percent of baseline), expressed as mean values, as detected by real-time PCR prior to start of bromocriptine treatment.

bRelative abundance of mRNA (PRL-R normalized to 18S; percent of baseline) as detected by real-time PCR after one week of daily subcutaneous bromocriptine injections.

cValues with different superscripts are different at P<0.05.

d,e,f,gValues with different superscripts are different at P<0.05.

Exogenous PRL is considered to be immunostimulatory because of its similarity to interleukins in being co-mitogenic (Clevenger et al. 1998, Goffin et al. 2002). In this study, we observed that animals with relatively lower PRL concentrations (i.e. those on SDPP and on LDPP plus bromocriptine) had enhanced lymphocyte proliferation and neutrophil chemotaxis, as compared with animals on LDPP only. It is possible that a shift in PRL sensitivity, as evidenced by increased PRL-R mRNA expression in SDPP and LDPP+b animals, may facilitate this enhancement. Indeed, the observation that reduction of circulating PRL, regardless of photoperiod enhanced PRL-R mRNA expression, lymphocyte proliferation and neutrophil chemotaxis, suggests that photoperiodic shifts in PRL sensitivity are directly linked to immune function responses. But a direct action of bromocriptine on immune function cannot be fully excluded. A mode of bromocriptine action via PRL secretion would be consistent with our observations with regard to photoperiodic effects, and with earlier studies in cattle wherein PRL replacement to bromocriptine-treated cows during the periparturient period was sufficient to reverse profound reductions in mammary epithelial cell differentiation (Knight 2001). Additional studies involving lymphocyte and neutrophil responses to PRL and bromocriptine in vitro are underway to clarify these speculations regarding independent effects of bromocriptine on immune function.

Immune function of many species appears to be influenced by changes in the seasons (Nelson et al. 1995, Nelson & Demas 1996, Dowell 2001). Epidemiological studies in humans show seasonal cycles of infectious diseases such as rubella and influenza in the last four decades (Dowell 2001). A variety of species of rodents show seasonal fluctuations in lymphoid tissue mass as well as immune cell counts (Nelson et al. 1995). Photoperiod is one of the most invariant of the environmental cues that are associated with the progression of the seasons. In the present study, we manipulated the photoperiod of steers and observed that lymphocyte proliferation and neutrophil chemotaxis were enhanced in SDPP-treated animals as compared with LDPP animals. Further, bromocriptine treatment that blocked long-day-induced increases in PRL also blocked the suppression of cellular immune function. It is possible that the PRL/PRL-R system mediates the effect of photoperiod on lymphocyte proliferation and neutrophil chemotaxis. Yet research in

Figure 4 Effect of photoperiod and bromocriptine treatment on lymphocyte proliferation (Experiment 2). Bromocriptine treatment of long-day-treated animals enhanced proliferation to a level similar to that of short-day-treated animals. Lymphocytes from each group of animals were stimulated with each of three mitogens (ConA, 20 μg/ml; PHA, 10 μg/ml; PWM, 10 μg/ml). Average ± of the difference=29.3; *P<0.05.

Table 3 Neutrophil chemotaxis in response to interleukin-8 (IL-8) and complement 5a (C5a) in animals treated with photoperiod and bromocriptine (Experiment 2)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Before injection</th>
<th>After injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDPP +v</td>
<td>Media 8.9 ± 8.4</td>
<td>22.5 ± 2.3</td>
</tr>
<tr>
<td>IL-8</td>
<td>125.0 ± 15.4</td>
<td>119.7 ± 21.6</td>
</tr>
<tr>
<td>C5a</td>
<td>7.5 ± 2.5</td>
<td>89.3 ± 10.1</td>
</tr>
<tr>
<td>LDPP +b</td>
<td>Media 12.9 ± 2.4</td>
<td>7.3 ± 2.2</td>
</tr>
<tr>
<td>IL-8</td>
<td>115.8 ± 21.5</td>
<td>217.6 ± 20.7</td>
</tr>
<tr>
<td>C5a</td>
<td>6.2 ± 2.5</td>
<td>191.2 ± 17.0</td>
</tr>
<tr>
<td>SDPP +v</td>
<td>Media 8.8 ± 6.8</td>
<td>3.7 ± 3.4</td>
</tr>
<tr>
<td>IL-8</td>
<td>24.4 ± 15.3</td>
<td>220.3 ± 23.1</td>
</tr>
<tr>
<td>C5a</td>
<td>187.9 ± 20.6</td>
<td>182.4 ± 9.5</td>
</tr>
</tbody>
</table>

aValues are the average number of neutrophils (± s.d.) that migrated in response to the media or chemoattractant; five fields per well were counted; n=6.

bCounts taken prior to start of bromocriptine treatment.

cCounts taken after one week of daily s.c. bromocriptine or vehicle injections.

d,e,f,gValues with different superscripts are different at P<0.05.
other species, particularly knockout models in mice, does not support the concept that PRL is critical to immune responses. Whereas PRL-R knockout mice have clear deficiencies in mammary gland development (Brisken et al. 1999) and reproductive competence (Binart et al. 2000), PRL-R knockout mice did not differ from controls in their ability to display lymphocyte proliferation or natural killer cell cytotoxicity, or to generate normal immunoglobulin levels (Bouchard et al. 1999). In addition, Kelly et al. (2001) note that PRL-R knockout mice have an apparently normal immune system. Although our data initially appear inconsistent with studies in PRL-R knockout mice, there are potential explanations to reconcile the observations made in the murine and bovine models. For example, our results clearly show that PRL manipulation in cattle (Tucker 1994). Complete resolution of these differences may not occur until suitable PRL-R knockout models are available for cattle.

The influence of photoperiod, and therefore PRL, on immune function may be more closely linked in the longer-lived cow relative to the short-lived mouse. It is also important to compare more general aspects of PRL physiology between rodent and ruminant models. PRL, for example, is clearly galactopoietic in the mouse whereas growth hormone functions as the galactopoietic hormone in cattle (Tucker 1994). Complete resolution of these apparent differences may not occur until suitable PRL-R knockout models are available for cattle.

Both the long and short form of the PRL-R have been characterized in the bovine (Schuler et al. 1997). Although it is speculated that the short form of the PRL-R plays a role in such functions as reproduction and lactation in the ewe (Cassy et al. 1998, Symonds et al. 1998) and the rat (Telleria et al. 1997, Feng et al. 1998), no studies done to date have found a specific role for the short form of the receptor in the bovine. However, the present study identifies shifts in short form that occur in the same trend as the long form of the receptor, potentially indicating a role for the short form PRL-R in the bovine. In addition, it appears that the ratio of long to short form may be critical to processes such as pregnancy and lactation in the ruminant (Cassy et al. 1998). Comparison of PRL-R mRNA expression in liver, lymphocytes and mammary parenchyma on the final day of photoperiod treatment gives initial support to the speculation that PRL-R expression shifts in parallel in these tissues. There are similar results in multiple tissue studies in other species (Jahn et al. 1991, Feng et al. 1998). Therefore, lymphocyte PRL-R may be a suitable index of PRL-R expression in other tissues. This is of considerable interest as the procedure for obtaining lymphocytes is considerably less invasive than those required to obtain liver and mammary tissue. Such an approach would also allow for greater temporal resolution because sampling frequency could be increased while reducing animal discomfort.

In conclusion, bovine lymphocytes express PRL-R mRNA and the expression is influenced by photoperiod and circulating PRL. The change in expression of PRL-R mRNA in animals receiving short-day photoperiod treatment relative to those on long-day photoperiod is inversely related to the PRL concentration in these animals. In addition, in vitro lymphocyte proliferation and neutrophil chemotaxis are increased in short-day-treated animals, an indication of photoperiodic modulation of immune function in the bovine. This effect may be mediated through PRL and its receptor. Indeed, reversal of the long-day-induced increase in PRL by concurrent bromocriptine treatment eliminates the long-day effect on PRL-R mRNA expression and cellular immune function.

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