FÁBIO LUIS VALÉRIO PINAFFI

Dinâmica hormonal durante o processo luteolítico nas espécies equina e bovina; com ênfase sobre o papel da prolactina

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VERSÃO CORRIGIDA

Dissertação apresentada ao Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Mestre em Ciências

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Orientador:

Prof. Dr. Luciano Andrade Silva

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Comitê de Ética em Pesquisa

COMITÊ DE ÉTICA EM PESQUISA – ÁREA: ANIMAL

Processo USP nº: 2012.1.1031.74.4

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Currículo(s) do(s) pesquisador(es): Os pesquisadores envolvidos neste projeto possuem currículo Lattes publicado e atualizado.

PARECER

Título do Projeto: Dinâmica hormonal durante o processo de luteólise nas espécies equina e bovina; com ênfase sobre o papel da prolactina.

Apreciação Geral: O Projeto tem importância dentro das áreas de Fisiologia da Reprodução e Reprodução Animal, baseia-se em bibliografia atualizada, a proposta é pertinente e a metodologia é factível.

Introdução: As bases teóricas da proposta estão fundamentadas em referências bibliográficas atualizadas.

Metodologia: Os pesquisadores avaliarão os efeitos da prolactina e da prostaglandina F2 alfa (níveis normais, com estimulação e supressão) sobre a luteólise, utilizando: éguas e vacas como modelos animais (mantidos em baias de 800 m^2, com alimentação e água à vontade), coletas seriadas de sangue (via inserção de cateter jugular, após sedação) e ultrassonografia transretal (com uso de gel lubrificante e contenção em tronco).

Aspectos Éticos: A proposta segue as recomendações da Resolução Nº 592 do CFMV e do COBEA, uma vez que contribuirá com a aquisição de conhecimento, os animais permanecerão em alojamentos adequados e será aplicado um sedativo para a realização dos procedimentos que envolverão dor (inserção do cateter).

Outros Comentários: Nada a declarar.

Esclarecimentos Necessários: Nada a declarar.

Pelo Comitê de Ética:

☒ **Referendado.**

☐ **Não referendado.**

☐ **Retirado de pauta.**

Pirassununga, 13 de setembro de 2012.

Notifique-se o interessado.

Prof. Dr. Marco Antonio Trindade - Presidente do CEP/FZEA

FOLHA DE AVALIAÇÃO

Nome: PINAFFI, Fábio Luis Valério.

Título: Dinâmica hormonal durante o processo luteolítico nas espécies equina e bovina; com ênfase sobre o papel da prolactina.

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Banca Examinadora

Prof. Dr. _____

Instituição: _____Julgamento_____

Prof. Dr. _____

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DEDICO

À minha família, em especial meus pais Wagner e Sônia

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Muito obrigado!!!

RESUMO

PINAFFI, F. L. V. **Dinâmica hormonal durante o processo luteolítico nas espécies equina e bovina; com ênfase sobre o papel da prolactina.** [Hormonal dynamics during the luteolytic period in equine and bovine species; with emphasis on the role of prolactin] 2012. 140 f. Dissertação (Mestrado em Ciência) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2012.

O presente estudo visou caracterizar a secreção de PRL e estudar suas interrelações com a PGFM durante a pré-luteólise, luteólise e pós-luteólise em éguas (Experimento 1); avaliar o efeito da inibição de PRL e PGF2α na luteólise e definir a sincronia entre PRL e PGFM em novilhas (Experimento 2); definir a sincronia entre PRL e PGFM em éguas (Experimento 3); e avaliar a constante estimulação da PRL durante o ciclo estral em éguas (Experimento 4). No **experimento 1** em éguas, amostras de sangue foram coletadas durante as 24 h da pré-luteólise, luteólise e pós-luteólise. As concentrações de PRL e PGFM foram rítmicas, sendo a duração dos pulsos de PRL de 5 h, com intervalos de 7,5 h entre pulsos e 12 h entre picos. Durante a luteólise e pós-luteólise, os pulsos de PRL foram mais proeminentes, as concentrações de PRL durante um pulso de PGFM foram maiores no pico de PGFM e notou-se uma maior sincronia entre picos de PRL e PGFM. No **experimento 2** em novilhas, as secreções de PRL e PGF2α foram inibidas durante a luteólise. A inibição da PRL associou-se a maiores concentrações de P4 e LH, sem efeito sobre a PGFM. Entretanto, a inibição da PGF2α associou-se a uma queda nas concentrações de PRL. A mensuração da área do CL mostrou-se eficiente em detectar a luteólise. No **experimento 3** em éguas, no verão e outono, inibiu-se a secreção de PGF2α e PRL no Dia 14. As concentrações de PGFM foram reduzidas com a inibição de PGF2α, mas não com a inibição da PRL. No verão, a inibição tanto de PRL quanto de PGF2α reduziu as concentrações de PRL. As concentrações de PGFM não diferiram entre o verão e o outono, enquanto que as concentrações de PRL foram menores no outono. No **experimento 4** em éguas, estimulou-se a secreção de PRL a cada 8 h. Amostras de sangue foram coletadas a cada 12 h do Dia 13 até a ovulação e a cada hora por 12 h no Dia 14. A estimulação repetida da PRL não aparentou manter as concentrações de PRL elevadas após o Dia 14. Nas amostras a cada hora, concentrações de PRL atingiram um valor máximo 4 horas após a estimulação e os pulsos de PRL foram aumentados. O aumento na PRL não afetou a PGFM, P4 e fluxo sanguíneo do CL. Entretanto, a estimulação da PRL quebrou a sincronia entre PGFM e PRL. Estão contidos nessa dissertação o primeiro relato em éguas sobre a caracterização e ritmicidade de pulsos de PRL, sincronia entre pulsos de PRL e PGFM

e maior atividade da PRL durante a luteólise e pós-luteólise. A inibição da PRL interferiu na secreção de P4 em novilhas, mas foi confundida pelo aumento de LH. A sincronia entre pulsos de PGFM e PRL representa um efeito positivo da PGF2α sobre a PRL, tanto em éguas quanto em novilhas.

Palavras-chave: Égua. Novilha. Luteólise. Prolactina. Pulsos.

ABSTRACT

PINAFFI, F. L. V. **Hormonal dynamics during the luteolytic period in equine and bovine species; with emphasis on the role of prolactin.** [Dinâmica hormonal durante o processo luteolítico nas espécies equina e bovina; com ênfase sobre o papel da prolactina]. 2012. 140 f. Dissertação (Mestrado em Ciência) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2012.

The aim of the present study was to characterize the PRL secretion and study the relationship between PRL and PGFM during preluteolysis, luteolysis and postluteolysis in mares (Experiment 1); evaluate the effect of PRL and PGF2α inhibition on luteolysis and define the synchrony between PRL and PGFM in heifers (Experiment 2); define the synchrony between PRL and PGFM in mares (Experiment 3); and evaluate the frequent stimulation of PRL during the estrous cycle in mares (Experiment 4). On **experiment 1** in mares, blood samples were collected during the 24 h of preluteolysis, luteolysis and postluteolysis. Concentrations of PRL and PGFM were rhythmic, prolactin pulses had 5h of duration, interval of 7,5 h between pulses, and 12 h between peaks. During luteolysis and postluteolysis, pulses of PRL were more prominent, concentrations of PRL during PGFM pulses were higher at the peak of PGFM, and were detected a greater synchrony between peaks of PRL and LH, and no effect was observed of PGFM. However, the inhibition of PGF2α was associated with a decrease on PRL concentrations. The CL area measurement was an efficient method to target luteolysis. On **experiment 3** in mares, in the summer and autumn, PGF2α and PRL secretion were inhibited on Day 14. Concentrations of PGFM were reduced after inhibition of PGF2α, but not after PRL inhibition. In the summer, the inhibition of both PRL and PGF2α reduced concentrations of PRL. Concentrations of PFGM were not different between summer and autumn, although concentrations of PRL were lower in the autumn. On **experiment 4** in mares, PRL secretion was stimulated every 8 h. Blood samples were collected every 12 h from Day 13 until ovulation, and hourly for 12 h on Day 14. The frequent stimulation of PRL did not appear to maintain higher concentrations of PRL after Day 14. On hourly samples, concentrations of PRL reached maximum value 4 h after stimulation and pulses of PRL were increased. The increase on PRL did not affect PGFM, P4, and blood flow of the CL. The synchrony between PGFM and PRL was partially disrupted by PRL stimulation. This was the first report on characterization and rhythm of PRL pulses, synchrony between PRL and PGFM pulses, and greater PRL activity during luteolysis and postluteolysis. The inhibition of PRL interfered with P4 secretion in heifers, but was confounded by the LH increase. In mares

and heifers, the synchrony between PGFM and PRL pulses represents a positive effect of PGF2α on PRL.

Key words: Mare. Heifer. Luteolysis. Prolactin. Pulses.

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1 INTRODUÇÃO

O ciclo estral é caracterizado pela ocorrência de ovulações sucessivas, sendo interrompido caso ocorra a fertilização do oócito seguida de gestação ou, no caso de espécies sazonais, quando se inicia a estação anovulatória. O corpo lúteo (CL) apresenta um papel central na regulação da duração do ciclo estral e na manutenção da gestação. Essas funções estão relacionadas à progesterona, o principal hormônio secretado pelo CL. A progesterona apresenta também funções importantes como favorecer um ambiente uterino propício para a chegada do embrião, assim como pela manutenção da gestação. Na ausência da fecundação, o CL regride, em um processo definido como luteólise, permitindo o início de um novo ciclo estral. A regressão do CL é resultado da ausência de sinalizações originárias do embrião para a manutenção da secreção de progesterona, ou seja, resultado da não ocorrência do reconhecimento materno-fetal da gestação.

Sendo constituída tanto de uma regressão funcional como estrutural do CL, a luteólise é um processo dinâmico envolvendo tanto mudanças hormonais sistêmicas quanto mudanças morfológicas. Para a avaliação dessas mudanças, pode ser utilizada a mensuração da concentração sanguínea de progesterona e escaneamento do CL por ultrassonografia transretal, respectivamente. Imagens ultrassonográficas possibilitam tanto uma análise visual como mensurações da área, volume e fluxo sanguíneo do tecido luteal.

O intervalo médio de uma ovulação a outra é semelhante entre equinos (22 dias) e bovinos (21 dias). Em contrapartida, o início da luteólise difere nas duas espécies, ocorrendo em éguas no dia 14 após a ovulação e em novilhas no Dia 17. O hormônio conhecidamente relacionado ao desencadeamento do processo de luteólise é a PGF2α secretada pelo endométrio, tanto em grandes animais quanto em outras espécies. Entretanto, outros hormônios também podem exercer papéis espécie-específicos nesse mecanismo.

A PRL é um hormônio protéico secretado predominantemente por lactotrófos da pituitária anterior. O controle da secreção de PRL se dá por meio da ligação da dopamina aos receptores de PRL nos lactotrófos, exercendo um bloqueio na secreção. Dependendo da espécie ou fase do ciclo, a PRL possui papel luteotrófico, luteolítico ou ambos. Em ratos, após o final da luteólise funcional, as células luteais são induzidas ao processo de apoptose pela PRL, a fim de se concluir a luteólise estrutural. Em carnívoros e roedores, a PRL faz parte do

complexo luteotrófico. Em suínos e bovinos, células luteais *in vitro* são estimuladas pela PRL a secretar P4, embora *in vivo* tal efeito não seja observado. Em equinos e bovinos foram encontrados receptores específicos para PRL no corpo lúteo, sugerindo o possível envolvimento da PRL na regulação do corpo lúteo. Em complementação a esses fatos, tem sido relatado em bovinos a possível ação vasoativa da PRL. Receptores de PRL foram encontrados na musculatura lisa de arteríolas intraluteais e células endoteliais, sendo notada *in vitro* a ação da PRL como mediador do desenvolvimento e regressão de vasos luteais.

O trabalho árduo e elevado custo para a realização de experimentos *in vivo* em equinos e bovinos dificultam a realização desse tipo de experimento. Desse modo, poucos pesquisadores se arriscaram a testar a hipótese de um papel da PRL sobre o processo luteolítico nessas espécies. Além disso, poucos destes trabalhos utilizaram-se de uma amostragem mais frequentes (ex: a cada hora), a qual proporciona uma interpretação mais detalhadas dos eventos estudados, sendo capaz de identificar interrelações mais sutis na ocorrência dos processos fisiológicos. Sem diminuir o mérito de experimentos *in vitro*, os quais são capazes de demonstrar possíveis papeis de certos hormônios em um processo fisiológico, somente experimentos *in vivo*, rodeados de inumeras interferências endógenas ao animal, possibilitam interpretar o que realmente ocorre no processo fisiológico.

No presente trabalho, o papel e mecanismos de ação da PRL durante a luteólise de éguas e novilhas foram estudados em quatro experimentos, sendo eles: (1) Caracterização da secreção de PRL e sua relação com o metabólito de PGF2α durante o processo de luteólise em éguas; (2-3) Análise do efeito da inibição de PRL e PGF2α durante a luteólise em éguas e em novilhas; (4) Análise da resposta ovariana e da PGF2α à estimulação de pulsos de PRL endógenos durante o ciclo estral em éguas. Como apêndice está um trabalho realizado fora dos experimentos da dissertação, no qual foi realizada a análise da relação intraovariana entre o futuro folículo ovulatório e o CL, assim como o efeito de um inibidor da PRL (Bromocriptina) na perfusão vascular e funcionalidade do CL em novilhas.

2 REVISÃO DE LITERATURA

2.1 Luteólise em bovinos e equinos

O início do processo luteolítico ocorre em média 17 dias após a ovulação em bovinos (GINTHER et al., 2010b) e 14 dias após a ovulação em éguas (GINTHER; BEG, 2011a). Embora se inicie em períodos diferentes, a duração da luteólise é similar entre as duas espécies, ocorrendo em um período médio de 24 h em bovinos (GINTHER et al., 2010b) e 23 h em éguas (GINTHER; BEG, 2011a). Ambos os parâmentros, início e duração, são baseados em avaliações diárias por ultrasonografia transretal e mensurações de progesterona (P4) em amostras de sangue coletadas a cada hora.

Tanto em bovinos como em equinos, a luteólise é constituída por uma regressão funcional e estrutural do CL (MCCRACKEN; CUSTER; LAMSA, 1999; STOCCO; TELLERIA; GIBORI, 2007). A secreção pulsátil de PGF2α pelo endométrio apresenta um papel fundamental na luteólise funcional tanto em bovinos (GINTHER et al., 2007) quanto em outros mamíferos (MCCRACKEN; CUSTER; LAMSA, 1999). Na ausência do reconhecimento materno da gestação, o processo de luteólise é necessário para dar início ao ciclo estral subsequente. O termo "reconhecimento materno da gestação" (SHORT et al., 1969) é comumente usado para descrever os eventos que resultam na gestação, referindo-se ao processo fisiológico que remete ao prolongamento da funcionalidade do CL.

O crescimento, manutenção e regressão (luteólise) do corpo lúteo são avaliados pela dosagem da concentração sanguínea de P4. Tanto na espécie equina quanto bovina, a luteólise pode ser dividida em trê períodos: *pré-luteólise, luteólise, e pós-luteólise* (Revisado em GINTHER; BEG, 2012a), baseando-se em determinações da concentração de P4 a cada hora. *Pré-luteólise* é o período anterior ao início da queda progressiva de P4, *luteólise* é o período de declínio de P4 até atingir concentrações inferiores a 1ng/mL, enquanto que a *pós-luteólise* é o período em que a P4 está em concentrações abaixo de 1 ng/mL. Entre o fim da *pré-luteólise* e início da *luteólise* há o momento chamado de *transição*, de grande relevância para o estudo do início do processo luteolítico.

Em bovinos há uma alta correlação entre a concentração plasmática de P4 e a área (cm^2) de tecido luteal (KARG; SCHAMS; REINHARDT, 1972; SIQUEIRA et al., 2009). Em

estudo recente em novilhas, a área do CL foi mensurada por ultrasonografia a cada 8 h a partir do Dia 14, sendo que no momento em que a área do CL reduziu em 25% da área do Dia 14, 52 % das novilhas estavam em luteólise funcional, 43% em pós-luteólise, e 5 % em pré-luteólise (PUGLIESI et al., 2012b). Essa técnica mostrou-se eficiente para a detecção imediata de animais em luteólise, quando comparada com a mensuração de P4 plasmática. A baixa eficiência e inviabilidade da mensuração de P4 plasmática se devem ao longo período para a análise hormonal e a alta flutuação da concentração de P4 durante a luteólise em bovinos (GINTHER et al., 2011d). Outro ponto positivo do uso de mensurações frequentes da área do CL seria a detecção de estágios específicos da luteólise, possibilitando um estudo mais aprofundado de cada parte desse processo.

Em bovinos, o padrão da secreção de P4 é dinâmico, refletindo períodos de atuação de pulsos de LH secretados pela pituitária e pulsos de PGF2α secretados pelo endométrio (GINTHER; BEG, 2011). Em éguas, o perfil da P4 é menos dinâmico, pois o LH não atua nas flutuações de P4. O efeito positivo de pulsos de LH sobre a secreção de P4 em bovinos foi demonstrado em diversos estudos. Pulsos de LH apresentam-se em sincronia com flutuações de P4 (GINTHER et al., 2011c), sendo notada uma redução no número e proeminência de flutuações de P4 em novilhas tratadas com um inibidor de LH (GINTHER et al., 2011b). Além disso, pulsos de LH aparentam estimular a funcionalidade e ter um papel no desenvolvimento do CL (QUINTAL-FRANCO et al., 1999), notando-se um prolongamento da vida luteal após a administração exógena de LH (DONALDSON;HANSEL, 1965).

A secreção episódica de PGF2α é necessária para completar a luteólise em várias espécies (GINTHER et al., 2009; GINTHER; SIDIQUI; BEG, 2009; SCHRAMM et al., 1983). Essa secreção episódica tem sido demonstrada em bovinos pela maior proeminência de pulsos de PGFM durante a luteólise, se comparados com as fases pré e pós-luteólise (GINTHER et al., 2010c). Além disso, o tempo para a finalização da luteólise é, pelo menos em parte, dependente da proeminência da secreção de PGF2α (PUGLIESI et al, 2012a; SCHRAMM et al., 1983), representado pelas concentrações do metabólito de prostaglandina F2α (PGFM). Um pulso sequencial de PGFM menor ou ausente, em período apropriado durante o período luteolítico, pode resultar em um aumento da secreção de P4 (ressurgência) por células luteais (SCHRAMM et al., 1983).

Na espécie bovina em particular, durante cada pulso de PGFM a progesterona sofre uma redução da concentração seguida de um restabelecimento à concentração inicial, sendo esse fenômeno denominado de rebote (MANN et al., 1975). Foi relatada a ação de pulsos LH

sobre o fenômeno de rebote, sendo que tais picos ocorrem 1,5 h após o pico de PGFM, em média. O intervalo entre os picos de PGFM e LH possibilitou comprovar que o pico de LH age positivamente na secreção de P4, sendo o responsável pelo fenômeno de rebote, sem interferência da queda de PGFM (GINTHER et al., 2012). Diferentemente do que ocorre em bovinos, em éguas o fenômeno de rebote não é detectado.

Agindo como uma citoquina potente, a PGF2α endógena está presente em todos os tecidos dos animais (GINTHER, 1992). A fim de evitar os possíveis efeitos colaterais da PGF2α na circulação sistêmica, a natureza manteve mecanismos estratégicos semelhantes ou particulares a cada espécie. Uma das estratégias universais é a curta meia vida da PGF2α na circulação sistêmica, sendo 64% da PGF2α metabolizada na primeira passagem nos pulmões em vacas (DAVIS et al., 1985). Uma segunda estratégia protetora seria a eficiente captura de moléculas de PGF2α pelo CL, sendo 10 vezes maior em equinos do que em bovinos (KIMBALL; WYNGARDEN, 1977). Uma terceira estratégia, universal, é a secreção de PGF2α em pulsos, sendo que a indução e finalização da luteólise é feita por dois a quatro pulsos sequenciais de PGF2α tanto em vacas (GINTHER et al., 2009) quanto em éguas (GINTHER et al., 2008). Uma ultima estratégia é a forma de transporte da PGF2α do endométrio aos ovários, sendo em bovinos por via unilateral ou local, através do contato da veia uterina com a artéria ovariana (GINTHER, 1981), enquanto que em éguas este transporte é sistêmico, pois não há contato entre vasos uterinos e ovarianos (GINTHER, 1992). A via unilateral em éguas é aparentemente desnecessária devido à extrema sensibilidade do CL à PGF2α. Devido à curta meia vida em ambas as espécies equina e bovina, as concentrações de PGF2α são usualmente acessadas por mensuração do metabólito de PGF2α (KINDAHL et al., 1976).

Estudos descreveram a hemodinâmica do CL tanto em éguas como em vacas, utilizando-se da técnica não invasiva da ultrassonografia transrretal no modo Doppler colorido (GINTHER et al., 2007). A porcentagem de área de tecido luteal com sinais de fluxo sanguíneo aumenta em paralelo com o aumento das dimensões do CL e concentração sistêmica de progesterona (ACOSTA et al., 2003; GINTHER et al., 2007). Desse modo, a consideração da hemodinâmica e dimensões luteais são mensurações importantes em estudos envolvendo manutenção e regressão do CL. Durante o desenvolvimento do CL, um gradual aumento na vascularização foi concominate com o aumento de volume e concentração plasmática de P4 (ACOSTA et al., 2003), estando, portanto, associados à produção e liberação de P4. Durante a luteólise espontânea há um aumento seguido de diminuição da

vascularização do CL, temporalmente associados a pulsos individuais de um metabólito da prostaglandina F2α (PGFM) (GINTHER et al., 2007). Tal aumento do fluxo sanguíneo durante a porção ascendente do pulso de PGFM mantém o fluxo sanguíneo elevado por 2 h após o pico de PGFM, ocorrendo um posterior decréscimo.

2.2 Frequência de amostragens de sangue para estudos hormonais durante a luteólise

A análise mais frequente (e.g., horária) das interrelações entre mudanças hormonais e eventos reprodutivos propiciou um avanço no conhecimento de eventos relacionados à reprodução (GINTHER; SHRESTHA; BEG, 2010). A maioria dos estudos em animais de produção baseou-se em coletas de sangue pouco frequentes para a detectação e caracterização tanto de pulsos hormonais como da duração de processos reprodutivos específicos, os quais podem ocorrer em poucas horas. Como exemplo, amostras a cada hora são necessárias para identificar tanto pulsos de PGFM em equinos (GINTHER et al., 2008) quanto pulsos de PGFM e LH em bovinos (GINTHER et al., 2011a). Amostras diárias não detectaram flutuações de PRL em éguas cíclicas (JOHNSON, 1986). Foram detectados entre um a três aumentos da concentração de PRL próximos ao fim da luteólise em amostras diárias (WORTHY et al., 1987), a cada 6 h (KING et al., 2010a) e a cada 4h (SHAND et al., 2000), sendo caracterizados como flutuações (KING et al., 2010a). Embora seja um trabalho mais árduo, amostragens mais frequentes propiciam uma descrição mais acurada da dinâmica hormonal, possibilitando um novo entendimento dos processos fisiológicos reprodutivos.

Outro ponto positivo de uma amostragem mais frequente está na detecção e descrição de fases específicas do ciclo estral. Como exemplo, a duração média do período luteolítico em bovinos foi de 24 h quando coletas de sangue foram feitas a cada hora (GINTHER; BEG, 2011b). Em contrapartida, considerando as amostras a cada 6 h, a aparente duração média da luteólise seria de 54 h, devido à ocorrência da luteólise em diferentes horas entre indivíduos. Em estudo prévio sobre a interrelação entre a dinâmica hormonal e foliculogênese, utilizou-se a amostragem de sangue a cada hora e avaliações ultrasonográficas cada 6 h para a detecção do momento da ovulação (HAUGHIAN et al., 2004). Neste trabalho, embora as mudanças hormonais tenham sido bem detalhadas, a interpretação da relação temporal entre as mudanças hormonais e o momento da ovulação foi enfraquecida, já que a ovulação é um

evento imediato. Desse modo, o uso de amostragens mais frequentes se mostra crucial para se detectar e descrever eventos com curto intervalo de tempo.

2.3 Envolvimento da prolactina na reprodução

A PRL é um hormônio protéico secretado por lactotrófos na pituitária anterior e possui papéis especializados na reprodução de mamíferos (GREGERSON, 2006). Entretanto, outros locais extrapituitários também são capazes de secretar PRL, como as glândulas mamárias (MALVEN; KEENAN, 1983) e útero (GOLANDER et al., 1978). O controle da secreção de PRL se dá principalmente pela ligação da dopamina (DA) proveniente do hipotálamo, através um sistema chamado de feed-back loop curto (GRATTAN, 2002). O feed-back loop curto é caracterizado por uma auto regulação da secreção de PRL intermediada pela DA, representado por uma relação negativa entre concentrações de PRL e DA. Em um breve exemplo, concentrações elevadas de PRL estimulam a secreção de DA, a qual inibe a secreção de PRL. A atuação da DA como inibidor da PRL é notada tanto *in vitro* (KOCH; LU; MEITES, 1970) quanto *in vivo* (MACLEOD; FONTHAM; LEHMEYER, 1970). Em alguns mamíferos, a PRL apresenta um papeis importantes na funcionalidade do CL. Em roedores e carnívoros, a PRL é um constituinte do complexo luteotrófico (MURPHY; RAJKUMAR, 1985). Em ratos, após o fim da luteólise funcional, a PRL induz apoptose de células luteais, sendo necessária para completar a regressão estrutural do CL (BERNICHTEIN;TOURAINE;GOFFIN, 2010; KIYA et al., 1998). Em suínos, a PRL estimula a secreção de P4 em cultura de células luteais *in vitro* (CIERESZKO et al., 2002). Em ovinos, em consonância com o LH, a PRL contribui para o complexo luteotrófico (DENAMUR;MARTINET;SHORT, 1973).

Em bovinos, não foram detectados papeis da PRL sobre a função luteal *in vivo*, sendo o LH o fator luteotrófico dominante (SMITH; MCSHAN; CASIDA, 1957; HOFFMAN et al., 1974; BEVERS; DIELEMAN, 1987). Entretanto, estudos *in vitro* identificaram um aumento da secreção de P4 após perfusão de PRL em ovários bovinos com CL (BARTOSIK et al., 1967) e um papel vasoativo da PRL no tecido luteal (ERDMAN et al., 2007). No tecido luteal *in* vitro, a PRL é clivada por catepsina D gerando fragmentos antiangiogênicos, os quais podem mediar a regressão luteal. Os receptores para PRL estão presentes tanto na

musculatura lisa de artérias luteais quanto em células endoteliais (SHIBAYA et al., 2006). Em éguas, não há indicação de um envolvimento da PRL sobre a função luteal (BECKER; JOHNSON,1990). Tanto em bovinos quanto em equinos, receptores para PRL estão presentes no CL (POINDEXTER et al., 1979; SAITO; SAXENA, 1975; KING et al., 2010a), embora não tenha sido identificado nenhum papel direto da PRL na funcionalidade do CL *in vivo*. Além disso, em bovinos foi identificada a expressão de PRL e mRNA de receptores de PRL (SHIBAYA et al., 2006), sugerindo uma possível regulação tanto local quanto sistêmica do CL pela PRL (THOMPSON et al., 2011).

Em equinos, a maioria dos estudos envolvendo a PRL está relacionada à prenhez (AURICH et al., 1995; IRELAND et al., 1991), parto (WORTHY et al., 1986) e sazonalidade reprodutiva (KING et al., 2010b; GREGORY et al., 2000; THOMPSON; HOFFMAN Jr; DEPEW, 1997). Os níveis da concentração de PRL são elevados no verão e baixos no inverno (THOMPSON et al., 1986). Durante o período transicional entre a estação anovulatória para a ovulatória, a estimulação da secreção de PRL acelera o crescimento folicular e antecipa a primeira ovulação da estação ovulatória (BESOGNET;HANSEN;DAELS, 1995; NEQUIN et al., 1993; THOMPSON et al., 1986), agindo como um potencial regulador da secreção de gonadotrofinas (GREGORY et al., 2000). Na estação ovulatória, os níveis de PRL são mais elevados no estro do que no diestro (WORTHY et al., 1987; SHAND et al., 2000), sendo observado um efeito positivo da concentração de estradiol sobre a PRL no início da fase luteal (ROSER et al., 1987). O aumento da PRL plasmática está também associado a um aumento da PRL intrafolicular (KING; ROSER; JONES, 2008).

Próximos ao fim da luteólise, foram detectados de um a três aumentos na concentração de PRL (WORTHY et al., 1987; KING et al., 2010a; SHAND et al., 2000), caracterizados como flutuações (KING et al., 2010a). Tais flutuações estavam relacionadas a aumentos na concentração de PGFM (WORTHY et al., 1987; SHAND et al., 2000). Um aumento na concentração de PRL também foi observado após tratamento de éguas em meio de diestro com PGF2α (SHAND et al., 2000). Além disso, em bovinos, pulsos espontâneos de PGFM associam-se temporalmente à PRL (GINTHER; BEG, 2009), sendo que a concentração de PRL é mantida por 1 a 2 h após o pico de PGFM até iniciar uma queda. No Dia 15 pós-ovulação, foi feita a descrição da secreção de PRL em pulsos, obtendo-se um pulso a cada 4/8h, baseando-se em amostragens de sangue a cada 3 a 6 min por 6 h (ROSER et al., 1987). Desse modo, coletas de sangue de hora em hora mostram-se eficientes em detectar pulsos de PRL em um período mínimo de 8 h em bovinos.

A concentração de PRL e proeminência de pulsos de PRL em bovinos variam consideravelmente durante o ciclo estral (GINTHER; BEG, 2011b; SWANSON; HAFS, 971; SINHA; TUCKER, 1968). Durante o estro, as concentrações de PRL apresentam-se elevadas tanto em bovinos (SWANSON; HAFS; MORROW, 1972; RZEPKOWSKI et al., 1982) quanto em ovinos (MCNEILLY, 1987; KANN; DENAMUR, 1974). A frequência e características da PRL circulatória foi descrita recentemente no fim do diestro (GINTHER; BEG, 2011b), sendo que pulsos de PRL apresentam uma duração média de 4 h, são sequenciais, mais proeminentes e rítmicos nas 12 h antes e 12 h após o fim da luteólise e associam-se à pulsos de PGFM após o início da luteólise. A associação entre pulsos de PRL e PGFM é sugestiva de um efeito estimulador de um hormônio sobre o outro, ou algum fator externo está estimulando ambos, necessitando de novos estudos. Em equinos a descrição da secreção de pulsos de PRL durante a luteólise não foi realizada.

Em várias espécies, a PRL foi demonstrada como inibidora da secreção de LH (MILENKOVIĆ et al., 1994). Em ratos e humanos, altas concentrações de PRL resultam em infertilidade, sendo essa relacionada à inibição de LH (SARTÓRIO et al., 2000; COHEN-BECKER; SELMANOFF; WISE; 1986), sendo que o restabelecimento das concentrações de PRL a níveis fisiológicos capaz de restaurar a ciclicidade menstrual e pulsatilidade de LH em mulheres (SARTÓRIO et al., 2000). Em vacas pós-parto, é mais evidente um efeito negativo de peptídeos opióides endógenos nas concentrações de GnRH e LH do que um efeito direto da PRL (CHANG;GIMENEZ;HENRICKS, 1981; GREGG et al., 1986; MONTIEL; AHUJA, 2005). Em galinhas, foi reportado um aumento da produção de ovos após à supressão da PRL, resultante da estimulação da secreção de LH (REDDY; DAVID; RAJU, 2007). O efeito negativo da PRL sobre o LH nas referidas espécies é, pelo menos em parte, mediado pela ação direta da PRL em neurônios de GnRH (GRATTAN et al., 2007). Estudos com fragmentos de pituitária (SMITH, 1978; CHEUNG, 1983) e secção do pedículo da pituitária (ANDERSON et al., 1999) demonstraram que gonadotrófos e lactotrófos estão funcionalmente conectados por um mecanismo parácrino, comunicando-se por meio da PRL secretada por lactotrófos no interior da glândula pituitária (HOVATH; KOVACS; EZRIN, 1977; JIN et al., 1997; TORTONESE et al., 1998). Entretanto, em bovinos não foi realizado nenhum estudo foi feito sobre a relação entre a secreção de gonadotrofinas e concentrações séricas de PRL.

2.4 Fármacos que atuam na secreção de prolactina e prostaglandina

A secreção de PRL é inibida pela ligação da DA secretada no hipotálamo, ligando-se a receptores dopaminérgicos tipo D_2 presentes na pituitária. A ligação de DA à tais tipos de receptores resulta na inibição da acumulação intracelular de AMPc, resultando no bloqueio da secreção de PRL. Fármacos angonistas de DA são, portanto, inibidores da secreção de PRL, enquanto que antagonistas de DA são estimuladores. Alguns exemplos de agonistas de DA são: bromocriptina, apomorfina, lisuride, pergolide, cabergolide, pepinirole e prampexole.

A bromocriptina (Bc) é um agonista de receptor de DA e atua como um potente inibidor da secreção de PRL em bovinos (BEVERS; DIELEMAN, 1987; WILLIAMS; RAY, 1980; MILLS; LEMENAGER; HORSTMAN, 1989) e na mulher (THORNER et al., 1981). As doses de Bc utilizadas em experimentos prévios variaram de 15 a 80 mg/animal e a frequência de tratamentos variou entre duas vezes ao dia a uma vez a cada dois dias (BEVERS; DIELEMAN,, 1987; WILLIAMS; RAY, 1980; KARG; SCHAMS; REINHARDT, 1972; SCHAMS; REINHARDT; KARG, 1972). Entretanto, a dose mínima de Bc e a meia vida ainda não foram estabelecidas para bovinos.

Em éguas, foram estudados os efeitos de vários fármacos na secreção de PRL (BECKER;JOHNSON,1990). O tratamento de éguas durante a estação anovulatória reduz as concentrações de PRL e durante a transição da estação anovulatória para a ovulatória, Bc aparenta atrasar o crescimento folicular do folículo pré-ovulatório, mas não atrasa o momento da ovulação (BENNET-WIMBUSH et al., 1998). Uma baixa dose de Bc (10 mg) reduz a concentração de PRL na estação ovulatória, mas não na anovulatória, quando as concentrações estão naturalmente baixas (BECKER;JOHNSON,1990). Em éguas em final de gestação, no momento em que a placenta é a fonte principal de P4, a concentração de P4 é reduzida pela Bc (IRELAND et al., 1991). Entretanto, aparentemente não foram realizados estudos sobre os efeitos da redução de concentração de PRL por Bc durante o ciclo estral em éguas.

A PGF2α, assim como a PGE_2, são sintetizadas a partir do ácido araquidônico proveniente de fosfolipídeos de membrana, sendo a reação catalizada pela enzima citosólica fosfolipase A_2 responsiva a hormônio (CLARK et al., 1991). A síntese de PGF2α pode ser inibida pela utilização de antiinflamatórios não esteroidais, como a flunixina meglumina, a qual atua como competidor do ácido araquidônico por sítios de ligação na enzima

ciclooxigenase (ODENSVIK; GUSTAFSSON; KINDAHL, 1998; SIMMONS; BOTTING; HLA, 2004). A enzima cicloxigenase converte ácido araquidônico em PGH_2, a qual é convertida em prostaglandinas, como PGF2α e PGE_2 (SIMMONS; BOTTING; HLA, 2004).

O tratamento de novilhas com 2,5 mg/kg de peso vivo no Dia 16 é capaz de reduzir a proeminência de pulsos e concentração de PGF2α por até 9 h, baseado em coletas de sangue de hora em hora (PUGLIESI et al., 2011b). Em éguas, FM tem sido utilizado para a indução da formação de folículos anovulatórios (CUERVO-ARANGO; BEG; GINTHER, 2011; GINTHER; CUERVO-ARANGO; BEG, 2011), resultante da queda da concentração de PGFM no momento da ovulação, de mecanismo ainda desconhecido. Aparentemente, não foram realizados estudos sobre o tratamento com FM antes e durante a luteólise, a fim de analizar os efeitos da redução de PGF2α nesse período do ciclo estral em éguas.

3 TEMPORAL RELATIONSHIPS OF A PULSE OF PROLACTIN (PRL) TO A PULSE OF A METABOLITE OF PGF2α IN MARES

3.1 Synopsis

Hourly blood samples were collected from 10 mares during 24 h of each of the preluteolytic, luteolytic, and postluteolytic periods. The autocorrelation function R was used to detect pulse rhythmicity, and the intra-assay CV was used to locate and characterize individual PRL and PGFM pulses. Rhythmicity of PRL and PGFM concentrations was detected in 67% and 89% of mares, respectively. Combined for the three periods (no difference among periods), the intervals for PRL pulses were 5.2 ± 0.4 h at the base, 7.5 ± 1.5 h between nadirs of adjacent pulses, and 12.3 ± 1.5 h from peak to peak. The peaks of PRL pulses were greater ($P < 0.05$) during the luteolytic period (46 ± 14 ng/mL) and postluteolytic period (52 ± 15 ng/mL) than during the preluteolytic period (17 ± 3 ng/mL). Concentration of PRL during the hours of a PGFM pulse were different ($P < 0.003$) within the luteolytic period and postluteolytic period and was greatest at the PGFM peak; PRL concentrations during a PGFM pulse were not different during the preluteolytic period. The frequency of the peak of PRL and PGFM pulses occurring at the same hour (synchrony) was greater for the luteolytic period (65%, $P < 0.01$) and postluteolytic period (50%, $P < 0.001$) than for the preluteolytic period (17%). This is the first report in mares on characterization and rhythmicity of PRL pulses, synchrony between PRL and PGFM pulses, and greater PRL activity during the luteolytic and postluteolytic periods than during the preluteolytic period.

3.2 Introduction

Prolactin (PRL) is a proteinaceous hormone secreted by the anterior pituitary by lactotrophic cells and has specialized roles in mammalian female reproduction (GREGERSON, 2006). Although PRL is primarily produced by the anterior pituitary, it has been detected in several extrapituitary sites, including the mammary gland (MALVEN; KEENAN, 1983) and the uterus (GOLANDER et al., 1978). The secretion of PRL is

controlled by the binding of dopamine to receptors on the lactotrophs, resulting in inhibition of secretory activity (BEN-JONATHAN; HNASKO, 2001).

Prolactin is believed to be the most important constituent of the luteotropic complex in rodents and carnivores (MURPHY; RAJKUMAR, 1985). In sows, PRL stimulates progesterone production of luteal cells and may be involved in the beginning of the luteal phase (CIERESZKO et al., 2002). In ewes, PRL does not appear to be necessary for luteal function (LOUWN et al., 1974) but acts in consonance with LH for maintenance of the CL and thereby contributes to the luteotrophic complex (DENAMUR; MARTINET; SHORT, 1973). In cows, LH is the dominant luteotrophic factor, whereas PRL has little or no luteal activity (HOFFMAN et al., 1974). However, binding sites for PRL are present in CL of cows (POINDEXTER et al., 1979; SAITO; SAXENA, 1975). In mares, there has been no direct indication that the PRL is involved in luteal function (BECKER; JOHNSON,1990), but specific receptors in the CL for equine PRL have been reported (KING et al., 2010a). The PRL receptors were uniformly distributed in the mature luteal tissue, suggesting a capacity for *de novo* PRL synthesis by luteal cells and a direct influence of PRL on CL function in mares.

In horses, the majority of the published studies on PRL are related to pregnancy (AURICH et al., 1995; IRELAND et al., 1991) parturition (WORTHY et al., 1986), and seasonality of reproduction (KING et al., 2010b; JOHNSON, 1986; GREGORY et al., 2000; BESOGNET;HANSEN;DAELS, 1995; EVANS et al., 1991; THOMPSON et al., 1986; THOMPSON; HOFFMAN Jr; DEPEW, 1997). There is a rise in serum and pituitary PRL concentration in the summer, whereas concentrations are low in winter (THOMPSON et al., 1986). During the transitional period between the anovulatory and ovulatory seasons, increasing concentration of PRL accelerates follicular growth (BESOGNET; HANSEN; DAELS, 1995; NEQUIN et al., 1993), hastens the ovulatory season (THOMPSON et al., 1986), and plays a potential role in regulation of gonadotropin concentrations (GREGORY et al., 2000). During the early luteal phase, estradiol has a positive effect in regulating PRL release, based on a correlation between estradiol and PRL concentrations in mares (ROSER et al., 1987) and greater PRL concentration during estrus than during diestrus in several species as cattle (SWANSON; HAFS; MORROW, 1972; RZEPKOWSKI et al., 1982), sheep (MCNEILLY , 1987; KANN; DENAMUR, 1974), and horses (WORTHY et al., 1987; SHAND et al., 2000).

In almost all studies in farm species, blood was collected too infrequently to detect and characterize pulses of PRL, assuming that PRL pulses are only a few hours in duration, similar to the duration of PGFM and LH pulses. Sampling at least every hour is necessary for

detecting PGFM pulses in horses (GINTHER at al., 2008) and PGFM and LH pulses in cattle (GINTHER et al., 2011a). In gilts during sexual development, blood was sampled every 20 min for 6 h and an episodic pattern was detected for LH but not for PRL. Daily sampling did not detect fluctuations in PRL during the estrous cycle in mares (JOHNSON, 1986). Other studies have described one to three transient increases in PRL toward the end of luteolysis in blood samples collected daily (WORTHY et al., 1987), every 6 h (KING et al., 2010a), or every 4 h (SHAND et al., 2000). These transient increases have been described as surges (KING et al., 2010a) and seemed related to increases in PGFM (WORTHY et al., 1987; SHAND et al., 2000). An increase in plasma PRL also is related to an increase PRL in follicular fluid in mares (KING; ROSER; JONES, 2008). In an abbreviated report with blood collection every 3 to 6 min for 6 h, the frequency of PRL peaks was about 4/8 h on day 15 postovulation; in one mare, an episode was described for day 3 postovulation that apparently involved several hours (ROSER et al., 1987).

The current study was done to determine PRL plasma concentration in mares before, during, and after luteolysis. Emphasis was on detecting PRL pulses in hourly samples and characterizing the pulses. Data were examined for synchrony between PRL concentrations and pulses with PGFM pulses. The goal was to provide rationale that could be used for development of hypotheses for future experiments, especially in relation to a role for PRL in luteal function.

3.3 Materials and methods

3.3.1Animals

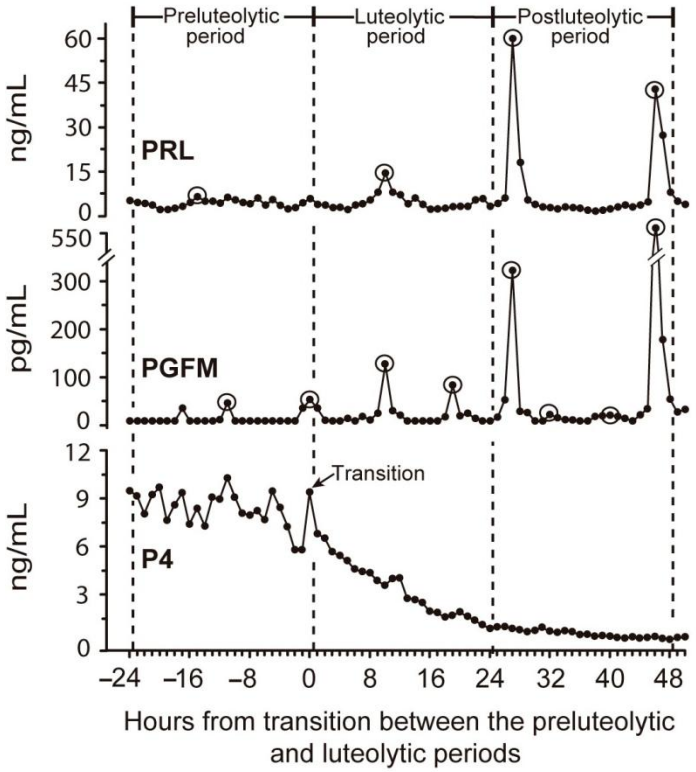
Mixed breeds of pony mares and apparent pony-horse crosses weighing 300 to 490 kg and aged 2 to 12 were used during June in the northern temperate zone. Mares with no apparent abnormality of the reproductive tract were used, as determined by ultrasound examinations (GINTHER, 1995). Mares were kept under natural light in an open shelter and outdoor paddock and were maintained by free access to water, trace-mineralized salt, and a mixture of alfalfa and grass hay. All mares (n = 10) remained healthy and in good body condition throughout the study. Ultrasonic monitoring for ovulation detection was done daily,

and the day of disappearance of the preovulatory follicle was designated the day of ovulation (GINTHER, 1995). Animals with two CL or an apparent undersized CL (< 2.0 cm^2) were not used. Handling of the mares was in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research.

3.3.2 Source of plasma samples and partitioning of data

Hourly blood samples were collected through an indwelling catheter in a jugular vein. Collection of blood samples began at 7 AM on day 12 postovulation and continued to 6 PM on day 16 or until the CL area was < 2.0 cm^2. The mares had free access to hay and water between sample collections. The hourly samples were partitioned on the basis of P4 concentrations into the preluteolytic, luteolytic, and postluteolytic periods. The common hour at the end of the preluteolytic period and beginning the luteolytic period was defined as the transitional hour (Hour 0). Transition was based on the beginning of a progressive decrease in the peaks of P4 fluctuations in the hourly blood samples as described for heifers (Fig. 3.1; GINTHER et al., 2011d). The end of the luteolytic period in individuals was defined as the last hour that P4 concentration was 1.0 ng/mL, and the postluteolytic period was defined as the hours when P4 was < 1.0 ng/mL. This definition of the end of luteolysis has been used in cattle (GINTHER et al., 2007). An interval of 24 h was chosen for the three periods and was based on the mean length of luteolysis. The preluteolytic period was 24 h beginning retroactively at the transitional hour (Hours –23 to 0). The luteolytic period was 24 h after the transitional hour, and the postluteolytic period was a mean of 24 h after the end of luteolysis.

Figure 3.1 – Concentrations of PRL, PGFM, and Progesterone (P4) in an individual mare, demonstrating the hour of P4 transition and the partitioning into periods.



The ring at a mean indicates a CV-identified PRL and PGFM pulse. In this mare the PRL pulses are synchronized with a PGFM pulses during luteolytic and postluteolytic periods but not during the preluteolytic period. However, there are PGFM pulses without temporally associated PRL pulses

3.3.3 Definition of pulses

To distinguish between fluctuations and pulses of PRL and PGFM, the intra-assay coefficient of variation (CV) was used as described (GINTHER et al., 2011d; GINTHER et al., 2007). Briefly, a PRL or PGFM fluctuation was an increase and decrease encompassing at least four hourly values, including nadirs. A fluctuation with a CV at least three times greater than the mean intra-assay CV was defined as a pulse. The peak of an identified PRL or PGFM pulse was designated as 0 h, and the pulse was assigned to the 24-h period that included the peak.

3.3.4 Comparisons of hormones and pulses

In mares, a sampling interval of 1 h has been used to detect PGFM pulses and is supported by a mean interval of 5 h between the beginning and ending nadirs during both luteolysis and postluteolysis (GINTHER et al., 2008). The adequacy of the 1-h interval for detection of PRL pulses seemed justified retrospectively by the results (about 5 h from nadir to nadir). Plasma concentrations of PRL, PGFM, and P4 were centralized to the transitional hour (Hour 0) and partitioned into the 24-h preluteolytic, luteolytic, and postluteolytic periods. The mean PRL hourly concentrations were compared among periods initially without regard to the location of fluctuations or pulses. A pulsatility program (see Section 3.3.6) was used separately in each mare to evaluate the rhythmicity of PRL and PGFM pulses (GINTHER et al., 2011a). The pulsatility test was done for all periods combined, owing to an inadequate number of pulses for considering each period separately. For comparison of PRL pulses among periods, each pulse was used in the analyses to a maximum of three/period. Only one mare had > 3 pulses/period and the three pulses for the analyses were chosen at random. For comparison of PGFM pulses among periods, the pulse with the greatest concentration at the peak/period was used.

Discrete end points for both PRL and PGFM pulses were number of pulses/24-h period; mean concentration/period; concentration at the beginning nadir (Nadir 1), peak, ending nadir (Nadir 2), and amplitude (concentrations at peak minus Nadir 2); area under the curve; and interval (elapsed number of hours) from Nadir 1 to Nadir 2 and from peak to peak and Nadir 2 to Nadir 1 of adjacent pulses.

Temporal relationships were studied between PRL and PGFM by centralizing PRL concentrations to the peak of a PGFM pulse and comparing the PRL profile during the hours of the PGFM pulse among the three 24-h periods. The peak of PGFM was designated as 0 h and the PRL concentration was evaluated between –3 h and 3 h. Differences were compared among periods in PRL concentrations and area under curve, and the hour of the PRL maximum value during a PGFM pulse was assessed. The extent of synchrony between a PRL peak and a PGFM peak was also evaluated.

3.3.5 Hormone assays

Blood samples were collected into heparinized tubes and immediately placed in ice water for 10 min before centrifuging (2000 X *g* for 10 min). The plasma was decanted and immediately stored (–20 ^{o}C) until assay. Plasma concentrations of PRL were determined by RIA as validated and described for equine plasma in our laboratory (GINTHER; BEG, 2009). The intra- and interassay CV and sensitivity were 13.7%, 8.7% and 0.1 ng/mL, respectively. Concentrations of PGFM were determined by an enzyme-linked immunosorbent assay that was developed in our laboratory for bovine plasma and has been described in detail (GINTHER; SHRESTHA; BEG, 2010). The assay was adapted and validated for use in equine plasma, using plasma from banamine-treated mares for preparing the standards. Serial volumes of a pool of equine plasma (7.5 to 100 µL) from mares treated with PGF2α were processed as for the experimental samples and resulted in a displacement curve that was similar to the standard curve. The intra- and interassay CV and sensitivity were 8.2%, 18.2%, and 8.0 pg/mL, respectively. Progesterone concentrations were measured by a validated radioimmunoassay, as described for mare plasma in our laboratory (GINTHER at al., 2005). The intra- and interassay CV and sensitivity were 5.4%, 4.8% and 0.03 ng/mL, respectively.

3.3.6 Statistics

Data that were not normally distributed were transformed to natural logarithms or ranks. Differences among pulses were analyzed for main effects of period (preluteolytic, luteolytic, postluteolytic) and hour and the interaction of period by hour. The SAS MIXED procedure (Version 9.2) was used with a REPEATED statement to account for autocorrelation between sequential measurements. When an hour effect was significant or approached significance, difference among hours were further analyzed by a Least Significant Difference (LSD) test. Student's unpaired *t*-tests were used to locate differences among periods within an hour when an effect of hour or an interaction was obtained. Selected comparisons were also examined by Student's paired *t*-tests. Differences among or within periods for the discrete PGFM characteristics were analyzed by ANOVA. The pulsatility (rhythmicity) in

concentrations of PRL and PGFM was determined for each mare by estimating the autocorrelation function in the statistical program R (R Development Core Team, 2010). If the value of the autocorrelation function was different ($P < 0.05$) from zero for any interval, the presence of pulsatility (peak-to-peak) equal to that interval was inferred. The pulsatility test was used only for mares with identified pulses during each period. Chi-square tests of independence were used for frequency data. A probability of $P \leq 0.05$ indicated that a difference was significant. When a probability was $P > 0.05$ to $P \leq 0.1$, approaching significance was inferred. Data are presented as the mean ± SEM, unless otherwise indicated.

3.4 Results

Complete data (24 h) were available for six, nine, and four mares in the preluteolytic, luteolytic, and postluteolytic periods, respectively. In addition, partial data (10 to 20 h) were available for four mares in each of the preluteolytic and postluteolytic periods. The prominence of PRL pulses varied widely, as indicated by concentration at the peak (7.7 to 149.7 ng/mL). Concentration differences that approached significance were found for the peak ($P < 0.08$), Nadir 2 ($P < 0.06$), and area under the curve ($P < 0.07$). Despite the wide variation, the concentration at the peak and the area under the curve were greater ($P < 0.05$) during the luteolytic and postluteolytic periods than during the preluteolytic period (Table 3.1).

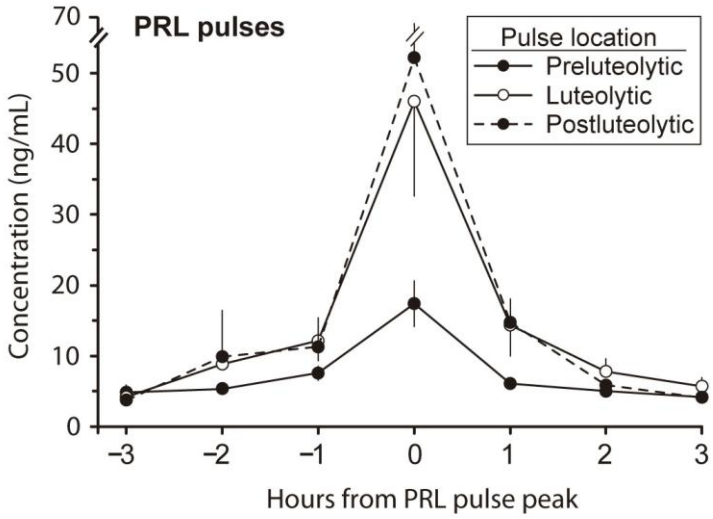
Table 3.1 – Mean ± SEM characteristics of PRL pulses during the preluteolytic, luteolytic, and postluteolytic periods in mares

Periods	Preluteolytic	Luteolytic	Postluteolytic
PRL characteristics	(n = 12)	(n = 16)	(n = 10)
Number of pulses/24 h	1.6 ± 0.4	2.0 ± 0.5	1.5 ± 0.3
Concentrations (ng/mL)			
Pulse mean concentration[w]	7.9 ± 1.1	14.3 ± 3.3	14.1 ± 3.6
Nadir 1[x]	4.1 ± 0.4	6.7 ± 2.2	3.6 ± 0.3
Peak	17.4 ± 3.2[a]	46.0 ± 14.1[b]	52.2 ± 15.0[b]
Nadir 2[x]	4.6 ± 0.4[a]	4.6 ± 1.0[a]	2.9 ± 0.4[b]
Amplitude[y]	13.3 ± 3.1	39.3 ± 14.1	37.9 ± 11.6
Area under curve (ng/mL/h)	20.0 ± 3.6[a]	63.0 ± 20.3[b]	74.4 ± 21.4[b]
Intervals (h)			
Nadir 1 to Nadir 2	4.6 ± 0.6	5.4 ± 0.7	5.6 ± 0.7
Peak to Peak[z]	13.1 ± 3.1	10.1 ± 1.6	15.8 ± 3.8
Nadir 2 to Nadir 1[z]	8.8 ± 2.8	5.0 ± 1.9	10.7 ± 3.7

[ab] Means in each row or end point with no common superscript are different ($P < 0.05$).
[w] Mean for all PRL concentration values in a pulse. Number of pulses/period was not different among period (1.3 ± 0.3; n = 38).
[x] Nadir 1 is at the beginning and Nadir 2 is at the end of a PRL pulse.
[y] Concentration at peak minus Nadir 1 (beginning nadir).
[z] Nadir 2 to Nadir 1 and peak to peak refers to adjacent pulses
n = Number of pulses during the period, using only the most prominent pulse/period in each mare.

The concentration at Nadir 2 was greater ($P < 0.05$) for the preluteolytic and luteolytic periods than for the postluteolytic period. Concentration of PRL at Nadir 1 and the intervals between events during a PRL pulse did not differ among periods (Table 3.1). Combined for all periods, the interval at the pulse base (Nadir 1 to Nadir 2) was 5.2 ± 0.4 h. The combined interpulse intervals were 7.5 ± 1.5 h from Nadir 2 to Nadir 1 of the next pulse and 12.3 ± 1.5 h from peak to peak. For nine mares with adequate data, the R program detected ($P < 0.05$) rhythmicity of PRL pulses in three mares and approaching rhythmicity ($P < 0.1$) in three mares. The mean profiles of PRL pulses during each of the three periods are shown (Figure 3.2).

Figure 3.2 – Mean ± SEM concentrations of PRL in pulses detected during the preluteolytic, luteolytic, and postluteolytic periods in mares (n = 8 to 10).



Concentration at the peak and area under the curve were greater ($P < 0.05$) for the luteolytic and postluteolytic periods than for the preluteolytic period.

The characteristics of the most prominent PGFM pulse/period are shown (Table 3.2). The number of pulses/24-h period was greater ($P< 0.05$) for the luteolytic and postluteolytic periods than for the preluteolytic period. Concentration differences ($P < 0.0001$) among periods were found for the mean/pulse, peak, and amplitude. For each of these three end points, concentration was greater ($P < 0.05$) during the luteolytic and postluteolytic periods than during the preluteolytic period. Concentration at the peak approached being greater ($P < 0.06$) for the postluteolytic period than for the luteolytic period. The peak and amplitude of PGFM pulses were greater in four of eight mares in the postluteolytic period than for each mare in the luteolytic period (4/8 vs 0/9, $P < 0.03$). Area under the curve was different among periods ($P < 0.0001$). The area was greater ($P < 0.05$) in the postluteolytic period than in each of the other two periods.

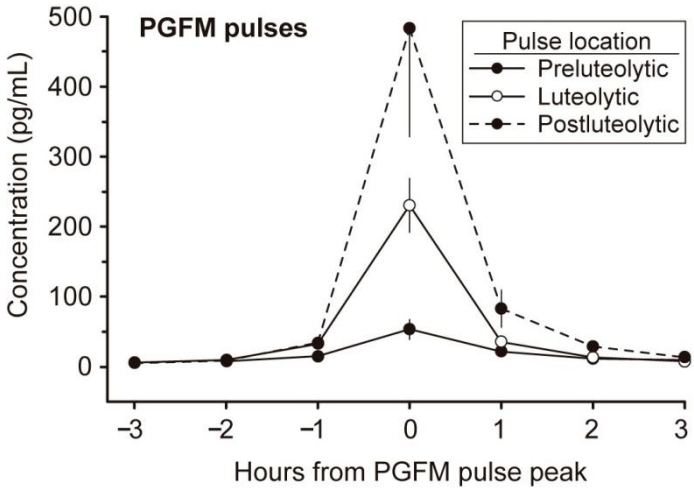
Table 3.2 – Mean ± SEM characteristics of the most prominent PGFM pulse during the preluteolytic, luteolytic, and postluteolytic periods in mares

Periods	Preluteolytic	Luteolytic	Postluteolytic
PGFM characteristics	(n = 8)	(n = 9)	(n = 8)
Number of pulses/24 h	1.4 ± 0.4[b]	2.3 ± 0.2[a]	2.1 ± 0.2[a]
Concentrations (ng/mL)			
Pulse mean concentration[w]	19.7 ± 3.4[b]	61.3 ± 9.3[a]	183.6 ± 54.5[a]
Nadir 1[x]	7.8 ± 0.9	9.2 ± 2.8	19.4 ± 7.3
Peak	53.5 ± 14.1[b]	231.0 ± 38.3[a]	483.5 ± 155.0[a]
Nadir 2[x]	6.7 ± 1.2	17.3 ± 5.8	16.6 ± 5.3
Amplitude[y]	45.6 ± 14.4[b]	221.9 ± 38.4[a]	464.1 ± 156.5[a]
Area under curve (ng/mL/h)	74.9 ± 15.5[b]	284.8 ± 40.3[b]	585.4 ± 165.9[a]
Intervals (h)			
Nadir 1 to Nadir 2	5.1 ± 0.6	4.8 ± 0.5	4.6 ± 0.5
Peak to Peak[z]	10.5 ± 1.3	9.1 ± 0.9	10.5 ± 2.2
Nadir 2 to Nadir 1[z]	3.8 ± 1.6	1.9 ± 0.6	2.6 ± 1.6

[ab] Means in each row or end point with no common superscript are different ($P < 0.05$).
[w] Mean for all PRL concentration values in a pulse.
[x] Nadir 1 is at the beginning and Nadir 2 is at the end of a PRL pulse.
[y] Concentration at peak minus Nadir 1 (beginning nadir).
[z] Nadir 2 to Nadir 1 and peak to peak refers to the prominent pulse and the next pulse.
n = Number of pulses during the period, using only the most prominent pulse/period in each mare.

In the factorial analysis of PGFM pulse concentrations, the main effects of period ($P < 0.006$) and hour ($P < 0.0001$) and the interaction of period by hour ($P < 0.0009$) were significant (Figure. 3.3). The interaction reflected differences in peak concentration and hours in the luteolytic period ($P < 0.003$) and postluteolytic period ($P < 0.001$) but not in the preluteolytic period. The intervals between events within the most prominent pulse/period and between the prominent pulse and the next pulse were not different among periods. The R program detected ($P < 0.05$) PGFM rhythmicity in eight of the nine mares with adequate data.

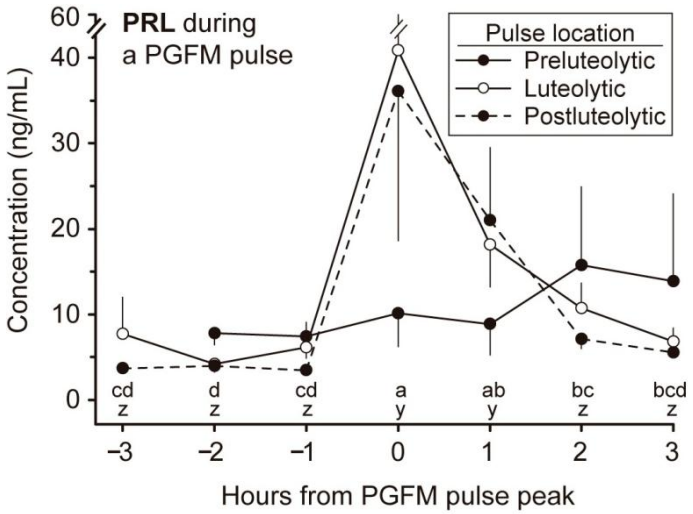
Figure 3.3 – Mean ± SEM concentrations of the most prominent PGFM pulses during the preluteolytic, luteolytic, and postluteolytic periods in mares (n = 8 to 10).



The interaction of period and hour was significant ($P < 0.0009$) and the area under the curve was different ($P < 0.0001$) among periods.

Concentrations of PRL at each hour of a PGFM pulse showed a main effect of hour ($P < 0.0001$) and an interaction of period by hour ($P < 0.05$; Figure.3.4). The interaction represented a difference in PRL among hours of the PGFM pulse for the luteolytic period ($P < 0.003$) and for the postluteolytic period ($P < 0.001$) but not for the preluteolytic period. For the luteolytic period, the concentration of PRL was greater ($P < 0.05$) at the PGFM peak (0 h) than at –3 to –1 h and 2 and 3 h. For the postluteolytic period, the concentration of PRL at 0 h of the PGFM pulse and at 1 h was greater ($P < 0.05$) than for all other hours.

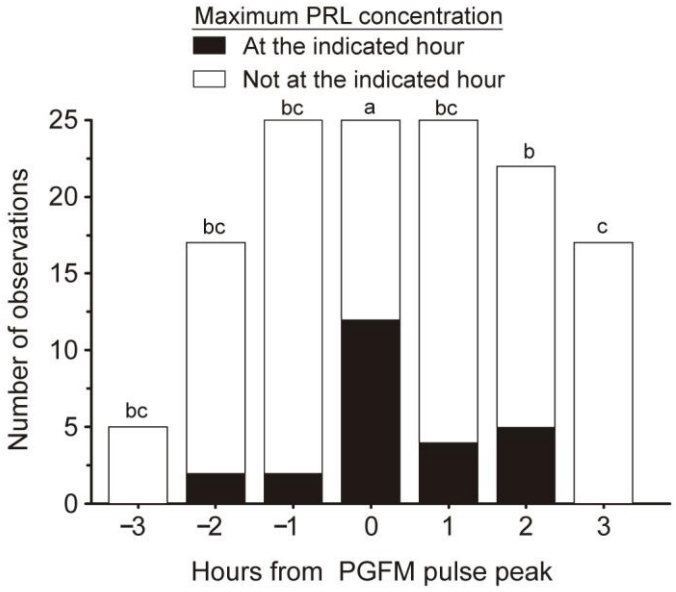
Figure 3.4 – Mean ± SEM concentrations of PRL within the hours of a PGFM pulse during the preluteolytic, luteolytic, and postluteolytic periods (n = 8 to 10).



An interaction ($P < 0.05$) represented differences in PRL among the hours of a PGFM pulse for the luteolytic ($P < 0.003$) and postluteolytic ($P < 0.003$) periods but not for the preluteolytic period.

The frequency of a peak of a PRL pulse occurring at the same hour as the peak of a PGFM pulse (synchrony) was different ($P < 0.0009$) among the preluteolytic, luteolytic, and postluteolytic periods. The frequency of synchrony between pulse peaks was greater for the luteolytic period (11/17, 65%; $P < 0.01$) and for the postluteolytic period (4/8, 50%; $P < 0.001$) than for the preluteolytic period (2/12, 17%). The luteolytic and postluteolytic periods did not differ. In each of the four remaining mares in the postluteolytic period, the PRL peak occurred 1 h after the PGFM peak. When the interval from PRL peak to PGFM peak was 1 h (n = 6), the PRL peak occurred after the PGFM peak in each interval. The number of hours between PRL and PGFM peaks, averaged for each mare and period, was different among periods ($P < 0.02$). The interval from the PRL peak to PGFM peak was shorter ($P < 0.05$) in the postluteolytic period (0.5 ± 0.2 h) than in the preluteolytic period (3.0 ± 0.5 h) and the luteolytic period (2.1 ± 1.1 h). The frequency of the maximum concentration of PRL during a PGFM pulse occurring at various hours of the PGFM pulse combined for the three periods differed ($P < 0.001$) among hours (Figure. 3.5).

Figure 3.5 – Frequency of the maximum concentration of PRL occurring during various hours of a PGFM pulse.



abc = any two hours with no common letter are different ($P < 0.05$) in frequency.

Maximum concentration of PRL occurred more frequently ($P < 0.05$) at the PGFM pulse peak than at any other hour of the PGFM pulse. Combined for the luteolytic and postluteolytic periods, the number of PGFM pulses/24 h (2.2 ± 0.2) was greater ($P < 0.05$) than the number of PRL pulses/24 h (1.8 ± 0.3).

3.5 Discussion

Pulses of PRL were detected for the first time in mares throughout 24 h of the preluteolytic period, during the mean 24 h of the luteolytic period, and 24 h of the postluteolytic period. Rhythmicity of PRL pulses throughout the three periods was indicated in 67% of the mares. In previous studies, transient increases in PRL concentrations were detected toward the end of luteolysis, but the increases cannot be characterized as pulses, owing to the long intervals between blood samples as 1 d (WORTHY et al., 1987), 6 h (KING et al., 2010a), and 4 h (SHAND et al., 2000). According to the current finding of a mean 5-h nadir-to-nadir interval for PRL pulses and the 12-h interval between pulse peaks, detection of a PRL increase with infrequent sampling (e.g., every 4 or 6 h) would depend on chance

sampling during a major portion of a pulse. In the current study, it is likely that most pulses were detected by the 1-h sampling interval, considering the 5-h PRL-pulse base (beginning nadir to ending nadir) for each of the preluteolytic, luteolytic, and postluteolytic periods. In this regard, a 1-h sampling interval detects PGFM pulses (GINTHER et al., 2008), and the PGFM pulses in the current study also had a base of about 5 h. In addition, comparisons between 15-min and 1-h sampling for LH pulses with a 4-h pulse base during preluteolysis in heifers indicated that 1-h sampling was adequate for pulse detection (GINTHER et al., 2011a). However, for PRL in the current study, the interval from the end of a pulse and beginning of the next pulse was 7 h, and other pulses of short duration and nondetectable by the 1-h interval could have occurred during the interim between the detected pulses. In addition, small pulses could have been superimposed on the detected pulses or between pulses similar to what occurs during PGFM concentrations in heifers (GINTHER et al., 2011a). A future study with more frequent sampling (10 or 15 min) will be needed for further characterizing the PRL pulsatility in the systemic circulation in mares.

The PRL pulses were more prominent during the luteolytic and postluteolytic periods than during the preluteolytic period as shown by the greater peak concentration and area under the curve. The luteolytic and postluteolytic periods did not differ. Greater PGF2α activity also occurred during the luteolytic and postluteolytic periods than during the preluteolytic period as indicated by the mean, peak, and amplitude in the concentrations of PGFM during the detected PGFM pulses. In addition, greater PGF2α activity during the postluteolytic period than during the previous two periods was indicated by the greater area under the curve of PGFM pulses. The rhythmicity of PGFM pulses was indicated by the R program in 89% of mares. Thus, the periods of greater activity were similar for PRL and PGF2α.

A temporal association between PRL concentrations and pulses with PGFM pulses was well documented in the current study. The temporality was indicated by three closely related end points: (1) maximum concentration of PRL during PGFM pulses, (2) synchrony between the peaks of PRL and PGFM pulses, and (3) length of the interval between the peak of a PRL pulse and the peak of a PGFM pulse. Concentration of PRL increased during the hours of a PGFM pulse with the maximum PRL concentration occurring at the hour of the PGFM peak. Concentrations of PRL also were greater the hour after the PGFM peak but not during the hours before the peak. Similarly, the peaks of PRL pulses and PGFM pulses occurred at the same hour in 50 or 65% of the temporal associations between pulses. These indicators of a temporal association between PRL concentrations and pulses and PGFM

pulses were greater during the luteolytic period and postluteolytic period than during the preluteolytic period. Only two of 12 PRL pulses peaks during the preluteolytic period occurred at the same hour as a peak of a PGFM pulse. For each of the eight PRL/PGFM temporal relationships during the postluteolytic period, the PRL pulse peak occurred at either the same hour as the PGF peak or 1 h after the PGFM peak. The temporal association between PRL and PGFM pulses is consistent with the finding in mares that a spontaneous PGFM pulse during luteolysis was associated with an increase in PRL (GINTHER; BEG, 2009). The reported increase in PRL occurred during the ascending portion of a PGFM pulse, but PRL did not decrease until 1 or 2 h after the PGFM peak.

The synchrony between PRL and PGFM pulses did not demonstrate whether PGF2α stimulates PRL or whether PRL stimulates PGF2α. In addition, other factors may be involved that stimulates both pulses. Although not conclusive, the following indicate that PGF2α stimulates PRL: (1) the temporal relationships in the current study indicated that the PRL peak sometimes occurred after the PGFM peak, (2) the decline in PRL after the peak of a spontaneous PGFM pulse in mares is delayed for 1 or 2 h after the PGFM peak (GINTHER; BEG, 2009), treatment of mid-diestrus mares with PGF2α induces an increase in PRL concentrations (SHAND et al., 2000).

The increased prominence of the PRL pulses and the increased synchrony between PRL and PGFM pulses during the luteolytic and postluteolytic periods are compatible with an influence of decreasing or low concentrations of progesterone and increasing or high concentration of estradiol on the temporality of the PRL and PGFM. In this regard, estradiol has a positive effect on PRL or a temporal association with PRL in mares as indicated by the following: (1) a positive correlation between estradiol and PRL concentrations (ROSER et al., 1987), (2) greater PRL concentrations during estrus than during diestrus (WORTHY et al., 1987), (3) interference with opioid inhibition of PRL release by estrogen treatment in ovariectomized mares (AURICH et al., 1995), and (4) increases in both estradiol and PRL concentrations during the ascending portion of a spontaneous PGFM pulse (GINTHER; BEG, 2009). Further study will be needed to clarify the functional interrelationship among PRL and PGF2α and the role of estradiol in the PRL/PGF2α relationship.

In conclusion, hourly blood sampling detected and characterized for the first time pulses of PRL throughout 24 h of the preluteolytic period, the mean 24 h of the luteolytic period, and 24 h of the postluteolytic period. For the three periods, the PRL pulse base was an interval of about 5 h, the interval between nadirs of adjacent pulses was 7 h, and the peak-to-peak interval was 12 h. The pulses were more prominent during the luteolytic and

postluteolytic periods than during the preluteolytic period. An increase in PRL concentration during the hours of a PGFM pulse demonstrated a temporal association between the PRL and PGF2α secretion, especially after the preluteolytic period.

4 DIRECT EFFECT OF PGF2A PULSES ON PRL PULSES, BASED ON INHIBITION OF PRL OR PGF2A SECRETION IN HEIFERS

4.1 Synopsis

Heifers were treated with a dopamine-receptor antagonist (Bromocriptine; BC) and a non-steroidal anti-inflammatory drug (Flunixin meglumine; FM) to inhibit PRL and PGF2α, respectively. The BC was given (Hour 0) when ongoing luteolysis was indicated by a 12.5% reduction in CL area (cm^2) from the area on Day 14 postovulation, and FM was given at Hours 0, 4, and 8. Blood samples were collected every 8-h beginning on Day 14 until Hour 48 and hourly for Hours 0 to 12. Three groups of heifers in ongoing luteolysis were used: control (n=7), BC (n=7), and FM (n=4). Treatment with BC decreased ($P< 0.003$) the PRL concentrations averaged over Hours 1 to 12. During the greatest decrease in PRL (Hours 2 to 6), LH concentrations were increased. Progesterone concentrations averaged over hours were greater ($P< 0.05$) in the BC group than in the controls. In the FM group, no PGFM pulses were detected, and PRL concentrations were reduced. Concentrations of PGFM were not reduced in the BC group, despite the reduction in PRL. Results indicated that measurement of CL area (cm^2) was more efficient method in detecting ongoing luteolysis (63%) than select any day from Days 14 to ≥19 (efficiency/day, 10 to 24%). The hypothesis that PRL has a role in luteolysis was supported but was confounded by the known positive effect of LH on progesterone. The hypothesis that the synchrony of PGFM and PRL pulses represents a positive effect of PGF2α on PRL rather than an effect of PRL on PGF2α was supported.

4.2 Introduction

The beginning of luteolysis in cattle ranges from Days 16 to 19 (Day 0 = ovulation; GINTHER; CUERVO-ARANGO; BEG, 2011). The wide range precludes targeting an individual for a specific stage of luteolysis for initiating an experiment. The long turn-around time for progesterone (P4) assay and the great fluctuations in P4 concentration from hour to hour (GINTHER et al., 2011d) hampers the use of P4 concentration for this purpose. A decrease in CL area (cm^2) of 25% from the area on Day 14, based on transrectal

ultrasonography, has been used to indicate that luteolysis is underway (PUGLIESI et al., 2012b). However, based on retrospective P4 concentrations, 52% of the heifers were in late luteolysis and 43% were after luteolysis (P4, ≤ 0.9 ng/mL). A better indicator is needed for detecting ongoing luteolysis in individual heifers.

Luteolysis consists of both functional (decrease in progesterone) and morphological regression of the CL (MCCRACKEN; CUSTER; LAMSA, 1999; STOCCO; TELLERIA; GIBORI, 2007). Pulsatile secretion of PGF2α by the endometrium has a fundamental role in functional luteolysis in cattle (GINTHER et al., 2007) and other mammals (MCCRACKEN; CUSTER; LAMSA, 1999). However, other hormones may have specific roles in the luteolytic mechanism. In rats, after the end of functional luteolysis, PRL induces apoptosis of luteal cells to complete the morphological regression (BERNICHTEIN; TOURAINE; GOFFIN, 2010; KIYA et al., 1998). In rodents and carnivores, PRL is a constituent of the luteotropic complex (MURPHY; RAJKUMAR, 1985). In pigs, PRL stimulates P4 secretion by luteal cells *in vitro* (CIERESZKO et al., 2002). Prolactin perfusion of bovine ovaries increases the secretion of P4 *in vitro* (BARTOSIK et al., 1967), but no effect on P4 was observed *in vivo* (SMITH; MCSHAN; CASIDA, 1957; HOFFMAN et al., 1974; BEVERS; DIELEMAN, 1987). The bovine CL has binding sites for PRL (POINDEXTER et al., 1979) and expression of PRL and PRL-receptor mRNA (SHIBAYA et al., 2006), suggesting that PRL may be involved in systemic and local regulation of the CL (THOMPSON et al., 2011). Moreover, PRL protein acts as a vasoactive factor (ERDMAN et al., 2007), and PRL proteins are present in the smooth muscle of intraluteal arterioles and endothelial cells (SHIBAYA et al., 2006), suggesting that PRL may mediate development and regression of luteal vessels.

The frequency and characteristics of circulatory PRL pulses have been reported for heifers (GINTHER; BEG, 2011b) and mares (GINTHER et al., 2012b). In heifers, pulses of PRL are most prominent and rhythmic during the 12 h before the end of luteolysis and the first 12 h of postluteolysis. The length of a PRL pulse from nadir to nadir is 4 h, and the ending nadir of one pulse usually occurs at the same hour as the beginning nadir of the next pulse. The PRL pulses after the beginning of luteolysis are temporally associated with pulses of a PGF2α metabolite (PGFM) in heifers and mares. However, whether there is a direct positive effect of one hormone on the other or other factors are involved in the temporal relationship is unknown.

The regulation of PRL secretion involves a short-loop feedback system in the hypothalamus (GRATTAN, 2002). Dopamine is secreted by the hypothalamus and is

considered to be the major prolactin-inhibiting factor (FREEMAN et al., 2000). Dopamine decreases PRL secretion *in vitro* (KOCH; LU; MEITES, 1970) and *in vivo* (MACLEOD; FONTHAM; LEHMEYER, 1970). Bromocriptine (BC), a dopamine receptor agonist, is a potent PRL inhibitor used to treat hyperprolactinaemia in women (THORNER et al., 1981). In cattle, BC is an efficient PRL inhibitor (BEVERS; DIELEMAN, 1987; WILLIAMS; RAY, 1980), but the lowest effective dose and half-life have not been established. Doses of BC have ranged from 15 to 80 mg/animal, and treatment frequencies have ranged from twice a day to every second day (BEVERS; DIELEMAN, 1987; WILLIAMS; RAY, 1980; KARG; SCHAMS; REINHARDT, 1972; SCHAMS; REINHARDT; KARG, 1972). Flunixin meglumine (FM), a nonsteroidal anti-inflammatory drug, blocks PGF2α synthesis by acting on cyclooxygenase isoforms-1 and -2 (ODENSVIK; GUSTAFSSON; KINDAHL, 1998). A single FM treatment (2.5 mg/kg body weight) at 16 d after ovulation inhibits transient PGFM increase for at least 9 h in heifers (PUGLIESI et al., 2011b).

The aim of the present study in heifers was to evaluate the effect of circulatory PRL and PGF2α suppression on functional and morphological regression of the CL and to define the temporal relationships between PRL and PGFM. The following hypotheses were tested: (1) ongoing CL regression is detectable at 8-h intervals by a 12.5% reduction in CL area (cm^2) from the area on Day 14, (2) circulatory PRL has a role in luteolysis, and (3) the temporal relationship between PGFM and PRL pulses during luteolysis represents a positive effect of PGF2α on PRL secretion rather than an effect of PRL on PGF2α.

4.3 Material and methods

4.3.1 Animals and ultrasound scanning

Holstein heifers (n = 46) aged 23 to 28 mo and weighing 500 to 600 kg were used during May and June in the northern temperate zone. Animals were selected with no abnormality of the reproductive tract and without double or undersized CL (< 3 cm^2), as determined by ultrasound examinations (GINTHER, 1998). The heifers were kept under natural light and maintained by *ad libitum* access to a mixture of alfalfa and grass hay, water, and trace-mineralized salt. Ovulation (Day 0) was detected by daily transrectal ultrasound

examinations (GINTHER, 1998). Handling of animals was in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

A duplex ultrasound scanner (Aloka SSD-3500; Aloka America, Wallingford, CT, USA) equipped with a 7.5 MHz transrectal linear probe was used. For the gray-scale mode, the brightness and contrast controls of the monitor and the gain controls of the scanner were standardized to constant settings (GASTAL; GASTAL; GINTHER, 2006). The largest follicles were monitored daily to determine the day of ovulation.

4.3.2 Experiment 1: Dose titration study for bromocriptine (Bc)

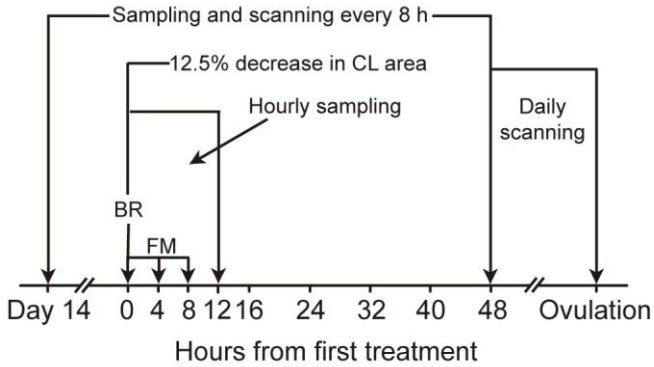
Heifers in late-luteal and periovulatory stages of the cycle (6 d before to 2 d after ovulation) were assigned randomly by replication into four groups (n=4 heifers/group): vehicle, BC-8 (8 mg of Bc), BC-16 (16 mg of BC) and BC-32 (32 mg of BC). Bromocriptine (2-bromo-α-ergocryptine methanesulfonate salt, catalog # B2134; Sigma Aldrich Co, St. Louis MO 63103 USA) was dissolved in absolute ethyl alcohol and an equal volume of saline was added to make a solution of 3.2 mg/mL. A single intramuscular administration of vehicle (ethyl alcohol:saline solution, 1:1) or Bc was given at 7:00 AM immediately after collection of a blood sample. The hour of the treatment was designated as Hour 0. Blood samples were collected hourly from the coccygeal vessels during Hours 0 to 8 and at Hours 12 and 24. Plasma samples were assayed for PRL concentration to determine the minimal effective dose of Bc. The mean overall PRL concentration and transient increases in PRL after Hour 0 were used as indicators of the effectiveness of the Bc doses.

4.3.3 Experiment 2: Inhibition of PRL and PGF2α during luteolysis

Heifers were randomized into three groups (n = 10/group): control, bromocriptine-treated (Bc group) and flunixin-meglumine-treated (FM group). The maximum CL area was measured using a B-mode still image and the tracing function. If an anechoic fluid-filled cavity was present in the CL, the area of the cavity was subtracted from the total

area (PUGLIESI et al., 2012a). The first detection at 8-h intervals of ongoing morphological CL regression was based on the hour when maximum CL area (cm^2) decreased 12.5 % from the area on Day 14 and was designated Hour 0 (Figure. 4.1). The Bc treatment was administered only at Hour 0 and FM treatments at Hours 0, 4, and 8. The Bc treatment dose was based on the findings in Experiment 1 that the optimum single dose for decreasing the PRL concentration for at least 12 h was 16 mg. For the FM treatment, the dose of FM (FluMeglumine®, 50 mg/mL, Phoenix Pharmaceutical, Inc., St. Joseph, MO, USA) was 2.5 mg/kg body weight, based on a previous titration study (PUGLIESI et al., 2011b). Ultrasound scanning to determine CL area (cm^2) and blood sampling were done every 8 h from Day 14 to Hour 48. Hourly blood samples were taken for 12 h from Hours 0 to 12. Heifers had access to water and hay between collections of blood samples.

Figure 4.1 – Diagram of experimental design for Experiment 2



Heifers were scanned every 8 h by transrectal ultrasound to determine when CL area (cm^2) decreased 12.5 % (Hour 0) from the area at Day 14. Heifers served as controls or were treated with bromocriptine (Bc; a prolactin antagonist) or with flunixin meglumine (FM; a prostaglandin antagonist) at the indicated hours (n = 10/group).

The beginning of luteolysis was defined as occurring at the 8-h sample before the P4 concentration decreased ≥ 1 ng/mL between 8-h intervals, followed by a progressive decrease in concentrations until the end of luteolysis (PUGLIESI et al, 2011b). The end of luteolysis was defined by the 8-h sample before P4 decreased to < 1 ng/mL (GINTHER et al., 2010c). For heifers in ongoing luteolysis at the hour of the first treatment (Hour 0) based on CL area (cm^2), the hourly P4 concentrations were also used to confirm a progressive P4 decrease. Heifers with complete luteolysis (P4 < 1 ng/mL) at Hour 0 or did not begin luteolysis until after Hour 12 on the basis of the 8-h or 1-h samples, were excluded from the analyses. One heifer in the FM group showed high values of PGFM concentration

periodically during the hourly samples, indicating ineffectiveness of the FM treatment, and was also excluded. After exclusion of heifers, the analyses during ongoing luteolysis were done with seven heifers in the control group, seven in the Bc group, and four in the FM group. Data that showed significant or apparent differences among groups at Hour 0 were transformed to percentage change from Hour 0.

4.3.4 Hormone assay

Blood samples were collected into heparinized tubes and immediately placed in ice water for 10 min before centrifuging (2000 x *g* for 10 min). The plasma was decanted and stored (−20 ºC) until assay. Plasma progesterone was measured by a solid-phase RIA kit containing antibody-coated tubes and I^{125}-labeled progesterone (Coat-A-Count Progesterone; Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure has been validated and described for bovine plasma in our laboratory (GINTHER et al., 2007). The intra-assay CV and sensitivity were 6.2% and 0.02 ng/mL, respectively. Plasma concentrations of PRL were determined by a RIA that was validated and described for use in bovine plasma in our laboratory (SHRESTHA et al., 2010). The intra- and interassay CV and sensitivity were 8.9%, 8.3%, and 0.28 ng/mL, respectively. Concentrations of PGFM were assayed by ELISA that was developed and validated in our laboratory for use in bovine plasma (GINTHER et al., 2010). The intra- and interassay CV and sensitivity were 11.5%, 13.2%, and 5.2 pg/mL, respectively. Concentrations of LH were determined by RIA as described (PALHAO et al., 2009) and modified (HANNAN et al., 2010) for bovine plasma in our laboratory. The intra- and interassay CV and sensitivity were 6.1%, 15.8%, and 0.06 ng/mL, respectively.

Fluctuations of PRL, PGFM and LH concentrations were evaluated by the CV method as described (GINTHER et al., 2007). When CV of the values comprising a fluctuation was at least three times greater than the mean intra-assay CV, the fluctuation was considered to be a pulse. The adequacy of detecting PRL (GINTHER; BEG, 2011b), PGFM and LH (GINTHER; BEG, 2011b; GINTHER et al 2011c) pulses by hourly blood sampling in heifers for this approximate stage of the estrous cycle has been reported.

4.3.5 Statistical analyses

Data that were not normally distributed were transformed to natural logarithms or ranks, and data that showed differences at Hour 0 were transformed into percentage change from Hour 0. The statistical analyses were done by using SAS PROC MIXED (Version 9.2; SAS Institute Inc., Cary, NC, USA) with a REPEATED statement to minimize autocorrelation between sequential measurements. Spatial power was used to account for uneven intervals between samples. When the interaction between group and hour was significant or approached significance, differences between groups at each hour were examined by Student's unpaired *t*-tests. The LSD test was used to locate differences among hours within groups or hours. Student's paired *t*-tests were used for selected comparisons of means within a group. Differences between groups for discrete characteristics were analyzed by ANOVA. Chi square or Fisher's exact test was used for comparison of the frequency of pulses among groups. A probability of $P \leq 0.05$ indicated a difference was significant, and a probability of $P > 0.05$ to ≤ 0.1 indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

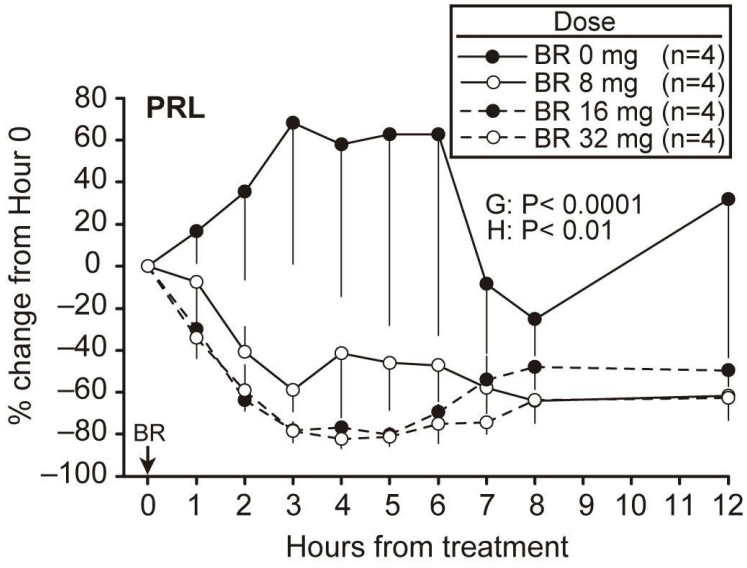
4.4 Results

4.4.1 Experiment 1

Concentrations of PRL decreased ($P < 0.05$) between Hour 0 (Bc treatment) and Hours 1 or 2 and remained lower ($P < 0.05$) until Hour 12 in Bc-8, Bc-16, and Bc-32 groups. The PRL concentrations were restored by Hour 24 (no significant difference between Hours 0 and 24). Owing to a difference ($P < 0.05$) in PRL concentrations among groups at Hour 0, data were transformed into percentage change from Hour 0. The percentage change was determined at each of Hours 1 to 8 and at Hour 12 for each heifer and was used in the analyses of doses (0, 8, 16, and 32 mg). The percentage change in PRL concentrations showed main effects of dose and hour (Figure. 4.2). The dose effect reflected the following percentage change in PRL concentration averaged over Hours 1 to 12: 0 mg (33.6 ± 19.3%a),

8 mg (–43.3 ± 6.0%a,b), 16 mg (–61.1 ± 3.5%b), and 32 mg (–68.0 ± 3.5%b); means with a different superscript are different ($P < 0.05$). The corresponding PRL concentrations averaged over Hours 1 to 12 were 17.3 ± 1.9, 9.5 ± 1.1, 6.1 ± 1.0, and 8.0 ± 1.1 ng/mL for the four doses, respectively. An increase in PRL concentration occurred between Hours 5 and 8 for the 16-mg dose ($P < 0.05$) and 32-mg dose (approached significance, $P < 0.06$).

Figure 4.2 – Mean ± SEM percentage change in PRL concentration from the hour of treatment (Hour 0) for four indicated doses of bromocriptine (Bc; a PRL antagonist).



Probabilities for main effects of group (G) and hour (H) are shown. Experiment 1.

4.4.2 Experiment 2

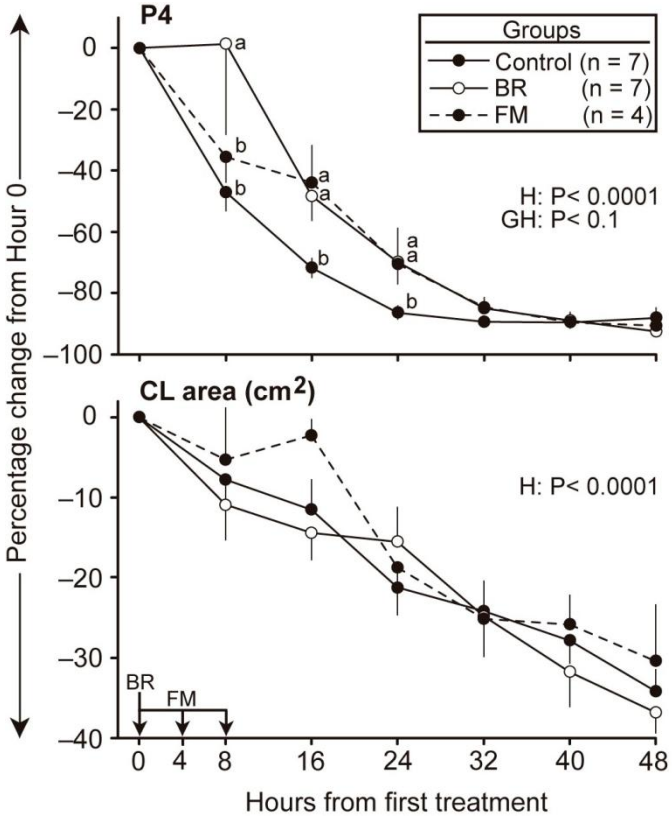
The 12.5% CL area (cm^2) decrease from Day 14 occurred on Day 17.4 ± 0.3 in the 29 heifers. Based on P4 concentrations in blood samples taken at 8-h intervals, the percentage of heifers in preluteolysis, luteolysis, and postluteolysis at Hour 0 (hour of treatment) was 10%, 63%, and 27% respectively. Blood samples for eight heifers in postluteolysis at Hour 0 were not assayed for PRL, PGFM, and LH, and the heifers were not considered further. Three heifers, one in each group remained in preluteolysis during the hourly sampling as indicated by apparent maintenance of P4 concentrations. The remaining heifers (seven, seven, and four in the control, Bc, and FM groups, respectively) were in

ongoing luteolysis beginning before or during the hourly sampling and were used for testing the hypotheses. The mean P4 in the 18 heifers in the luteolytic stage was 3.6 ± 0.6 ng/mL at Hour 0. Completion of luteolysis was reached in 7 of 18 (39%) heifers by Hour 12. For the 18 heifers, the interval as determined every 8-h from Hour 0 (first treatment) to the end of luteolysis was affected by treatment. The interval was shorter ($P < 0.04$) in the controls (16.0 ± 2.1 h) than in the Bc group (26.3 ± 5.4 h) and approached being shorter ($P < 0.08$) in controls than in the FM group (24.0 ± 6.5 h). The length of the interovulatory interval was not different among groups (combined length, 21.2 ± 0.5 d).

The probabilities for a group effect, hour effect, and a group-by-hour interaction for the factorial analyses are given in the figures and the probabilities for differences in discrete end points are given in the table or text. Owing to apparent differences among groups at Hour 0, the percentage change in P4 concentration and in CL area (cm^2) from Hour 0 was used to analyze differences at 8-h intervals for Hours 0 to 48. Percentage change in concentrations of P4 showed a significant effect of hour and an interaction that approached significance (Figure. 4.3). The group effect was not significant in the factorial analysis, but difference among groups was indicated by one-way ANOVA in the average percentage decrease during 8-h intervals. There was a greater ($P < 0.05$) average decrease in the controls (–59.4 ± 4.7%) than in the Bc group (–23.4 ± 16.3%); the FM group was intermediate (–39.8 ± 7.5%) and was not different from either of the other groups. The interaction represented an approaching greater ($P < 0.06$) percentage decrease in the control and FM groups than in the Bc group at Hour 8 and a greater ($P < 0.05$) percentage decrease in the controls than in the Bc and FM groups at Hours 16 and 24 (Figure. 4.3). The P4 concentration (ng/mL) between Hours 0 and 8 decreased in the controls (3.1 ± 0.6 vs 1.7 ± 0.4; $P < 0.001$) and in the FM group (3.3 ± 0.8 vs 2.2 ± 0;8; $P< 0.01$), but did not decrease in the Bc group (4.2 ± 0.9 vs 4.2 ± 1.0; $P > 0.1$). The P4 concentrations (ng/mL) between Hours 8 and 16 decreased in the controls (1.7 ± 0.9 vs 0.9 ± 0.2; $P< 0.008$) and Bc group (4.2 ± 1.0 vs 2.2 ± 0.15; $P< 0.04$), but did not decrease in the FM group (2.2 ± 0.8 vs 2.1 ± 0.9; $P > 0.1$).

The percentage change from Hour 0 for CL area (cm^2) at 8-h intervals over Hours 0 to 48 showed only a significant hour effect (Figure. 4.3). The interaction ($P < 0.13$) did not approach significance, although the percentage decrease in the CL area at Hour 16 was less ($P < 0.05$) in the FM group than in the other two groups.

Figure 4.3 – Mean ± SEM percentage change from Hour 0 in concentrations of P4 and CL area (cm^2) in controls and in heifers treated with a PRL antagonist (bromocriptine; Bc) or a prostaglandin antagonist (flunixin meglumine; FM) at the indicated hours during luteolysis (Hour 0 = 12.5% CL-area decrease from Day 14).



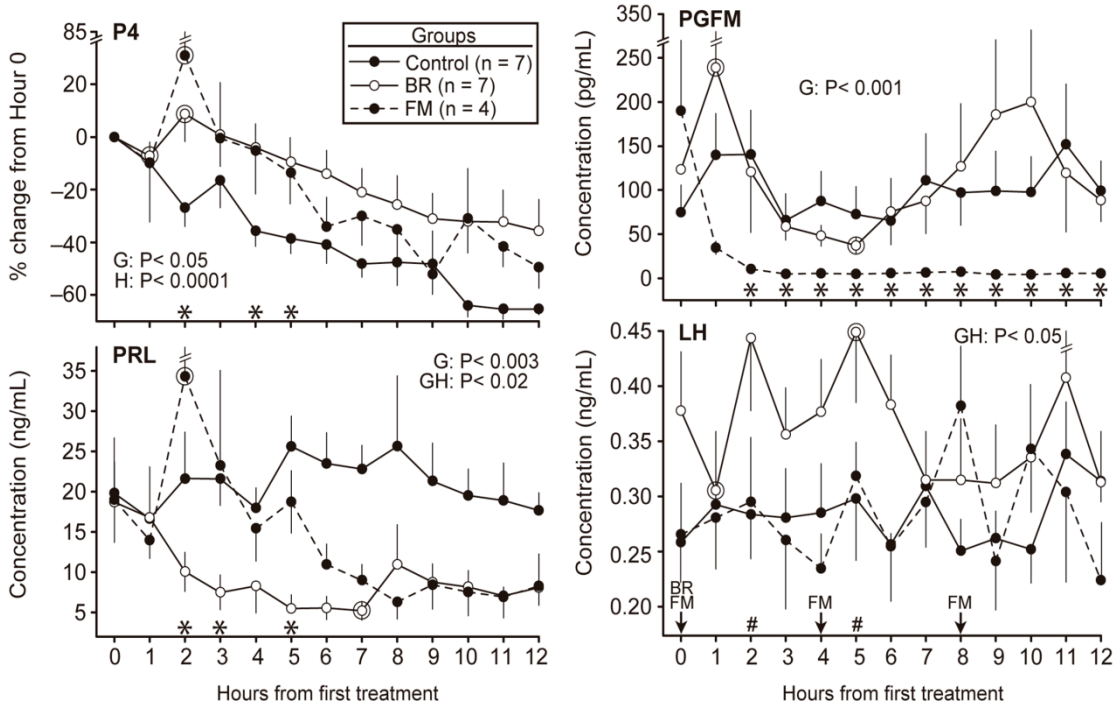
Probabilities that were significant or approaching significance are indicated for main effect of hour (H) and the interaction (GH). ab = means within an hour with different letters are different ($P < 0.05$), except at Hour 8 when the differences approached significance ($P < 0.06$). Concentration (ng/mL) of P4 decreased between Hour 0 and Hour 8 in the controls ($P < 0.001$) and FM group ($P < 0.05$) but not in the Bc group. Experiment 2.

The percentage change in P4 concentrations from Hour 0 for the hourly samples (Hours 0 to 12) had a group effect and an hour effect (Figure. 4.4). The group effect was from differences in percentage change averaged over all hours as follows: controls (–42.3 ± 3.0%a), Bc group (–16.9 ± 3.2%b), and FM group (–22.6 ± 6.2%c); means with a different superscript letter are different ($P < 0.05$). The corresponding overall mean P4 concentrations were: controls (1.9 ± 0.1 ng/mL), Bc group (3.6 ± 0.2 ng/mL), and FM group (2.7 ± 0.3 ng/mL). Although the interaction was not significant, differences ($P < 0.05$) among groups were detected at Hours 2, 4, and 5 from less percentage change in P4 concentrations in the Bc and FM groups than in the controls (Figure. 4.4). Concentrations of P4 increased ($P < 0.03$) between Hour 1 (3.9 ± 0.7 ng/mL) and Hour 2 (4.7 ± 1.0 ng/mL) in the Bc group.

Hourly concentrations of PRL from Hours 0 to 12 showed a group effect and an interaction of group by hour (Figure. 4.4). The group effect reflected the following PRL concentration averaged over Hours 1 to 12: controls (21.0 ± 1.2a ng/mL), Bc group (9.3 ± 0.9b ng/mL), FM group (14.0 ± 2.1c ng/mL); means with a different superscript letter are different ($P < 0.05$). The interaction primarily represented lower ($P < 0.05$) concentrations in the Bc group than in the control and FM groups at Hours 2, 3, and 5. Concentration of PRL was lower ($P < 0.05$) in the Bc group than in the controls for each of Hours 2 to 12. Concentrations were lower ($P < 0.05$) in the FM group than in the controls at each of Hours 7 to 12. In the FM group, the concentrations decreased ($P < 0.03$) after Hour 2 and reached the lowest concentration at Hour 8. Number of PRL pulses/12 h was not different among the three groups (Table 4.1), but there was an approaching lower frequency ($P < 0.1$) of prominent pulses (peak > 10 ng/mL) in the Bc group (3 of 5 pulses) than in the control group (9 of 9). The overall mean PRL concentration/pulse was lowest in the Bc group, intermediate in the FM group, and greatest in the controls. Concentrations at Nadirs 1 and 2 were greater ($P < 0.05$) in the controls than in the Bc and FM groups. Concentration at the pulse peak was greater ($P < 0.05$) in the controls than in the Bc group.

Hourly PGFM concentrations showed only a group effect from lower overall concentration in the FM group (23.0 ± 9.1 pg/mL) than in the control (100.2 ± 16.3 pg/mL) and Bc (116.2 ± 17.4 pg/mL) groups. Although the interaction was not significant, the lower concentration in the FM group occurred throughout Hours 2 to12. In the Bc group, the decrease ($P < 0.05$) between Hours 1 and 4 was followed by an increase ($P < 0.05$) and was similar to the pretreatment concentration by Hour 8. Number of PGFM pulses detected in a 12-h hourly session was similar between the controls (2.1 ± 0.3) and Bc group (1.9 ± 0.3), but no PGFM pulses were detected in the FM group. The concentration for the characteristics of PGFM pulses were not different between the controls and Bc group (not shown).

Figure 4.4 – Mean ± SEM percentage change from Hour 0 in concentrations of P4 and CL area (cm2) in controls and in heifers treated with a PRL antagonist (bromocriptine; Bc) or a prostaglandin antagonist (flunixin meglumine; FM) at the indicated hours during luteolysis (Hour 0 = 12.5% CL-area decrease from Day 14).



Probabilities that were significant or approaching significance are indicated for main effect of hour (H) and the interaction (GH). ab = means within an hour with different letters are different ($P < 0.05$), except at Hour 8 when the differences approached significance ($P < 0.06$). Concentration (ng/mL) of P4 decreased between Hour 0 and Hour 8 in the controls ($P < 0.001$) and FM group ($P < 0.05$) but not in the Bc group. Experiment 2.

Hourly LH concentrations showed an interaction of group and hour (Figure. 4.4). The interaction appeared to represent greater concentrations in the Bc group than in the other two groups over Hours 2 to 6; concentrations were significantly greater at Hours 2 and 5. In addition, concentrations were greater ($P < 0.05$) in FM group than in the other two groups at Hour 8. In the Bc group, LH concentrations increased ($P < 0.05$) between Hours 1 and 2 and decreased ($P< 0.05$) between Hours 5 and 7. The number of LH pulses/12 h was greater ($P < 0.05$) in the FM group than in the other two groups (Table 4.1). Concentrations of LH for the overall mean/pulse, at the peak, and for the amplitude were greater ($P < 0.05$) in the Bc group than in the other two groups. Concentration at each nadir was greater ($P < 0.05$) in the Bc group than in the FM group, but the controls did not differ from either group.

Table 4.1 – Mean ± SEM characteristics of PRL and LH pulses during luteolysis in control heifers (n = 7) and heifers treated with bromocriptine (BR; n = 7), or flunixin meglumine (FM; n = 4)

	Prolactin			Luteinizing hormone		
Pulse characteristics	Control	Bc	FM	Control	Bc	FM
Total number of pulses	9	5	4	12	16	14
Number of pulses/12h	1.2 ± 0.4	0.7 ± 0.29	1.0 ± 0.0	1.71 ± 0.42^{a}	2.29 ± 0.42^{a}	3.50 ± 0.50^{b}
Concentrations (ng/mL)						
Pulse mean^{x}	25.1 ± 3.6^{a}	9.4 ± 4.8^{b}	19.4 ± 5.3^{c}	0.30 ± 0.03^{a}	0.43 ± 0.22^{b}	0.29 ± 0.23^{a}
At Nadir 1^{y}	17.2 ± 5.0^{a}	5.7 ± 1.4^{b}	8.7 ± 2.5^{b}	0.23 ± 0.03^{ab}	0.28 ± 0.03^{a}	0.22 ± 0.02^{b}
At peak	35.7 ± 7.4^{a}	15.4 ± 6.1^{b}	38.1 ± 19.6^{ab}	0.38 ± 0.03^{a}	0.54 ± 0.03^{b}	0.38 ± 0.02^{a}
At Nadir 2^{y}	15.6 ± 2.5^{a}	5.0 ± 1.0^{b}	5.8 ± 1.9^{b}	0.24 ± 0.03^{ab}	0.29 ± 0.03^{a}	0.22 ± 0.02^{b}
Amplitude^{z}	8.3 ± 4.9	9.6 ± 5.5	29.4 ± 20.2	0.15 ± 0.02^{a}	0.26 ± 0.02^{b}	0.17 ± 0.02^{a}

^{ab} Means within each hormone and each characteristic with no common superscript are different ($P < 0.05$)

^{x} Mean for all concentration values in a pulse.

^{y} Nadir 1 is at the beginning and Nadir 2 is at the end of a pulse.

^{z} Concentration at peak minus Nadir 1.

4.5 Discussion

The titration study (Experiment 1) indicated that the 16 mg of Bc was the lowest effective dose for inhibition of PRL secretion in heifers during the late luteal and periovulatory phases. A dose of 32 mg was similarly effective and 8 mg was partially effective. For the 16-mg dose, PRL concentrations were suppressed by Hour 2 (Hour 0 = hour of treatment), and PRL inhibition continued thereafter until the end of the experiment at Hour 12. There was an increase in PRL concentration between Hours 5 and 8, but the concentrations remained lower than at Hour 0. These results confirmed the reported potent PRL-inhibiting activity of Bc in cattle (BEVERS; DIELEMAN, 1987; WILLIAMS; RAY, 1980; MILLS; LEMENAGER; HORSTMAN, 1989) and also determined an optimal dose and length of effectiveness for at least 12 h. In this regard, PRL concentration (GINTHER; BEG, 2011b; SWANSON; HAFS, 1971; SINHA; TUCKER, 1968) and prominence of PRL pulses (GINTHER; BEG, 2011b) vary considerably during the estrous cycle in heifers.

Hypothesis 1— that ongoing CL regression is detectable at 8-h intervals by a 12.5% reduction in CL area (cm^2) from the area on Day 14— was supported. This criterion

was successful in that it detected ongoing luteolysis in 63% of heifers for use in testing the hypotheses. The CL-reduction approach was a considerable improvement over using a day postovulation for obtaining heifers in the luteolytic phase. In this regard, 10%, 21%, 24%, 17%, 17%, and 11% of the heifers were in ongoing luteolysis on Days 14, 15, 16, 17, 18, or ≥19, respectively. Previous experiments have shown high correlation between plasma P4 concentration and area (cm^2) of luteal tissue (KARG; SCHAMS; REINHARDT, 1972; SIQUEIRA et al., 2009), but criteria for detecting the beginning of morphological CL regression were not reported. In a recent study (PUGLIESI et al., 2012b), 8 h after a reduction of 25% of CL area from the area at Day 14, 20% of the heifers were in functional luteolysis, 75% in postluteolysis and 5% in preluteolysis.

An inhibitory effect of Bc on PRL was indicated by the lower PRL concentration averaged over Hours 1 to 12, a group-by-hour interaction that reflected lower concentrations at Hours 2, 3, and 5, and reduced prominence of PRL pulses in the Bc group compared to the controls. The reduced PRL prominence of pulses was indicated by lower concentration at the pulse peak and nadirs. The treatment with FM inhibited PGF2α secretion, as indicated by no detection of CV-identified PGFM pulses and lower PGFM concentrations during hourly samples in the FM group than in the other groups. Reports on the use of Bc and FM for altering the concentrations of PRL and PGF2α, respectively, are noted in the Introduction.

Hypothesis 2— that circulatory PRL has a role in luteolysis— was supported for functional luteolysis but not for morphological luteolysis. Furthermore, the support for the hypothesis by the P4 increase when PRL decreased was confounded by an increase in LH. The effect of reduced PRL on functional luteolysis was indicated in the 8-h samples by the lower P4 concentration decrease between Hours 0 and 8 and the greater concentration at Hours 8, 16, and 24 in the Bc group than in the controls. In contrast, there were no morphological differences between the two groups as indicated by CL area during the luteolytic period. The hypothesis was also supported for functional luteolysis by the P4 concentration in hourly samples for Hours 0 to 12, as indicated by a lower overall average percentage P4 decrease in the Bc group (PRL inhibited) than in the controls. A role of PRL in functional regression of the CL has not been reported for cattle. The present study found a relationship between an experimental decrease in PRL and an increase in P4, which would be compatible with a luteolytic effect of PRL. In this regard, PRL has luteotropic effects in some species and also a luteolytic effect in rats; in addition, the presence of PRL and PRL binding sites in bovine luteal cells and PRL proteins in the wall of luteal vessels are compatible with a role for PRL in control of the CL in cattle (see Introduction).

The apparent effect of reduced PRL concentration and pulses on the increase in P4 concentration in the Bc group can be attributed to an increase in LH as well as to a decrease in PRL. The increase in LH occurred between Hours 1 and 2, and LH pulses were more numerous and prominent in the Bc group. Increased LH encompassed the hours when P4 was increased and PRL was decreased in the Bc group. The increase in LH when PRL was suppressed by Bc is compatible with a negative effect of PRL on LH. Secretion of PRL has been reported to be an inhibitor of LH secretion in several species (MILENKOVIĆ et al., 1994). In humans and rats, high serum concentration of PRL is a cause of reproductive infertility and is related to LH suppression (SARTORIO et al., 2000; COHEN-BECKER; SELMANOFF; WISE; 1986). The lowering of PRL concentration to physiological levels in hyperprolactinemic women by Bc treatment restores the menstrual cycles and LH pulsatility (SARTORIO et al., 2000). The negative effect of PRL on GnRH and LH occurs during suckling in postpartum cows and is modulated primarily by endogenous opioid peptides (MCNEILLY 1980; CHANG;GIMENEZ;HENRICKS, 1981; GREGG et al., 1986; MONTIEL; AHUJA, 2005). In hens, an increase in LH secretion after PRL suppression resulted in greater egg production (REDDY; DAVID; RAJU, 2007). The negative effect of PRL on LH is at least in part mediated by the direct action of PRL on GnRH neurons (GRATTAN et al., 2007). Several studies with pituitary fragments (SMITH, 1978; CHEUNG, 1983) and pituitary stalk sectioning (ANDERSON et al., 1999) have indicated that gonadotrophs and lactotrophs are functionally connected by a PRL paracrine mechanism within the pituitary gland (HORVATH et al., 1977; JIN et al., 1997; TORTONESE et al., 1998).

The confounding effects of an LH increase in determining the effects of reduced PRL on increasing P4 concentration stems from the demonstrated positive effect of LH pulses on P4 fluctuations. Pulses of LH have a role in increasing the P4 concentration rebound after the transient depression in P4 that occurs at the peak of a PGFM pulse. The positive effect of LH on P4 has been shown by synchrony in the peaks of LH pulses and P4 fluctuations (GINTHER et al., 2011c), reduced number and prominence of P4 fluctuations in heifers treated with an inhibitor of LH (GINTHER et al., 2011b), and a less prominent P4 rebound when LH is blocked during simulation of a PGFM pulse by PGF infusion (SHRESTHA et al., 2011). Earlier studies are also consistent with a positive effect of LH on P4 in cattle, including prolongation of luteal life by exogenous LH (DONALDSON;HANSEL, 1965) and the positive effect of LH on P4 production (QUINTAL-FRANCO et al., 1999). Thus, further

study will be needed to separate the potential negative effects of PRL from the known positive effects of LH on P4 concentrations.

Although a luteolytic action of PRL was not supported in previous *in vivo* and *in vitro* studies in cattle (BARTOSIK et al., 1967; SMITH; MCSHAN; CASIDA, 1957; HOFFMAN et al., 1974; BEVERS; DIELEMAN, 1987), the luteolytic action of PGF2α on the CL is well known (MCCRACKEN; CUSTER; LAMSA, 1999). Previous studies established that sequential episodic PGF2α secretion is necessary for complete luteolysis in several species (GINTHER et al., 2009; GINTHER; SIDIQUI; BEG, 2009; SCHRAMM et al., 1983). The reported time required for the completion of luteolysis to 1 ng/mL is at least partly dependent on the prominence of PGF2α secretion as represented by PGFM concentrations (PUGLIESI et al., 2012a; SCHRAMM et al., 1983). An undersized sequential PGFM pulse or absence of a pulse at the appropriate time during luteolytic period may result in an increase in P4 production (resurgence) by the luteal cells (SCHRAMM et al., 1983). The suppressed PGF2α secretion in the current study by FM during luteolysis affected the function of the CL. This was indicated by the greater P4 concentrations at Hours 16 and 24 and overall in the hourly samples in the FM group than in the controls. In addition, the interval from Hour 0 (first treatment) to the end of luteolysis approached being longer in the FM group.

Hypothesis 3— that the temporal relationship between PGFM and PRL pulses during luteolysis represents a positive effect of PGF2α on PRL secretion rather than an effect of PRL on PGF2α— was supported. This was indicated by the decrease in PRL concentrations after PGF2α suppression, whereas PGFM concentrations were maintained after PRL suppression. This is a novel finding in that it has not been demonstrated previously whether PGF2α affects PRL or vice-versa. However, the results are consistent with the recent findings (GINTHER; BEG, 2011b) that PRL concentrations were greater at the peak and at the hour after the peak of a PGFM pulse. In this regard, the temporal relationship between PRL and PGFM was recently described during luteolysis and postluteolysis in heifers (GINTHER; BEG, 2011b). The PRL pulses were more prominent during the last 12 h of luteolysis and the first 12 h of postluteolysis. Pulses of PGFM were more prominent during than before or after luteolysis (GINTHER et al., 2010c). Pulse prominence during different stages of the luteolysis may alter specific roles of PRL. Pulsatile secretion of PGF2α is well-known for its role in luteolysis (MCCRACKEN; CUSTER; LAMSA, 1999), whereas a role for PRL in luteolysis has not been demonstrated *in vivo*; the current results were confounded by an association between PRL suppression and an LH increase. Further studies using inhibition of LH, shorter intervals for CL scanning, and evaluation of other structural CL

characteristics such as blood flow are needed to clarify the effect of circulatory PRL on morphological CL regression.

In conclusion, measurement of CL area was a more efficient method than number of days after ovulation in targeting functional luteolysis in heifers. A single treatment with 16 mg of Bc during luteolysis inhibited PRL concentrations efficiently for 12 h. Treatment with Bc not only depressed PRL but also was temporally associated with increased prominence of LH pulses, a delayed P4 decrease, and delayed completion of luteolysis without an effect on PGF2α secretion. Owing to both PRL decrease and an LH increase in the Bc-treated heifers, the effect of PRL on CL function could not be determined. The FM-inhibited PGFM concentrations were temporally associated with a decrease in PRL concentrations, indicating a positive effect of PGF2α on PRL secretion rather than an effect of PRL on PGF2α.

5 STIMULATORY EFFECT OF PGF2α ON PROLACTIN (PRL) BASED ON EXPERIMENTAL INHIBITION OF EACH HORMONE IN MARES

5.1 Synopsis

During the luteolytic period in mares, the peaks of 65% of pulses of a PGF2α metabolite (PGFM) and pulses of PRL occur at the same hour. It is unknown whether the synchrony reflects an effect of PGF2α on PRL or vice versa. Controls, a flunixin meglumine (FM)-treated group (to inhibit PGF2α), and a bromocriptine-treated group (to inhibit PRL) were used at 14 d postovulation in June and in September (n = 6 mares/group/month; total, 36 mares). Blood samples were collected hourly from just before treatment (Hour 0) to Hour 10. Concentrations of PGFM in the FM group were lower ($P < 0.05$) at Hours 4 to 6 than in the controls in each month, but bromocriptine had no effect on PGFM. Concentrations of PGFM averaged over all groups and within each group did not differ between June and September. Compared to the controls, concentrations of PRL in June were lower ($P < 0.05$) in the FM group at Hours 4 to 8 and in the bromocriptine group at Hours 4 to 10. Concentration of PRL was lower ($P < 0.0001$) in September (0.9 ± 0.05 ng/mL) than in June (3.0 ± 0.3 ng/mL) averaged over groups and was lower ($P < 0.03$) within each group. Results supported the hypothesis that the positive association between PGFM and PRL concentrations in mares represents an effect of PGF2α on PRL rather than an effect of PRL on PGF2α.

5.2 Introduction

Prolactin (PRL) is secreted by lactotropic cells in the anterior pituitary (GREGERSON, 2006) but has been detected in several extrapituitary sites (BERNICHTEIN; TOURAINE; GOFFIN, 2010). Unlike the gonadotropins, the secretion of PRL is controlled by dopamine that binds to receptors on the lactotropic cells, resulting in inhibition of PRL secretory activity (BEN-JONATHAN;HNASKO,2001). As in other species, PRL has been studied in mares for its role in development of the mammary glands and milk secretion (WORTHY et al., 1986). Prolactin has a luteal role in several species (MURPHY;

RAJKUMAR, 1985), but a role in luteal function or structure has not been demonstrated in mares (BECKER;JOHNSON,1990). However, further *in vivo* study is indicated, owing to the presence of receptors for equine PRL in mature luteal tissue (KING et al., 2010a). An important function of PRL in mares involves the seasonality of reproduction (KING et al., 2010b; JOHNSON, 1986; GREGORY et al., 2000; BESOGNET;HANSEN;DAELS, 1995; EVANS et al., 1991; THOMPSON et al., 1986; THOMPSON; HOFFMAN Jr; DEPEW, 1997). Circulatory and pituitary PRL concentrations increase in the summer and decrease in the winter (THOMPSON et al., 1986). During the transitional period between the anovulatory and ovulatory seasons, increasing concentration of PRL accelerates follicle growth (BESOGNET; HANSEN; DAELS, 1995; NEQUIN et al., 1993) and hastens the ovulatory season (THOMPSON; HOFFMAN Jr; DEPEW, 1997; NEQUIN et al., 1993). In mares, estradiol has an apparent positive effect in regulating PRL release, based on a positive correlation between estradiol and PRL concentrations (ROSER et al., 1987), greater PRL concentrations during estrus than during diestrus (WORTHY et al., 1987), interference with opioid inhibition of PRL release by estrogen treatment in ovariectomized mares (AURICH et al., 1995), and increases in both estradiol and PRL concentrations during the ascending portion of a pulse of a metabolite of PGF2α (GINTHER; BEG, 2009).

One to three transient increases in PRL were detected in mares toward the end of luteolysis in blood samples collected daily (WORTHY et al., 1987), every 6 h (KING et al., 2010b), or every 4 h (SHAND et al., 2000) and were described as surges (KING et al., 2010b). More recently, episodic secretion of PRL in mares was described using blood sampling every hour before, during, and after luteolysis (GINTHER et al., 2012b). The PRL pulse-base interval (nadir to nadir) was about 5 h. The interval between adjacent pulses from peak to peak was 12 h. Concentration at a PRL pulse peak was about three-fold greater during and after luteolysis than before luteolysis.

Flunixin meglumine (FM) is a potent nonsteroidal anti-inflammatory drug that blocks prostaglandin synthesis and delays luteolysis in heifers (ODENSVIK; GUSTAFSSON; KINDAHL, 1998). A single FM treatment (2.5 mg/kg body weight) on Day 16 (Day 0 = ovulation) inhibits PGF2α secretion for at least 9 h in heifers, based on concentrations of a PGF2α metabolite (PGFM) (PUGLIESI et al., 2011b). The FM treatment every 8 h reduced the PGFM concentration during both preluteolysis and luteolysis and resulted in increased progesterone (P4) concentration during midluteolysis and a delay in the completion of luteolysis. In mares, systemic treatment with FM has been used to study hormone interrelationships during induction of hemorrhagic anovulatory follicles (CUERVO-

ARANGO; BEG; GINTHER, 2011; GINTHER; CUERVO-ARANGO; BEG, 2011). Treatment with FM after administration of hCG to induce ovulation resulted in a reduction in concentration of PGFM and formation of an hemorrhagic anovulatory follicle (HAF). Apparently, FM has not been used to study the effects of experimentally decreasing PGF2α before or during luteolysis in mares.

The effects of various products on PRL in mares have been described (BECKER; JOHNSON,1990). Dopamine is the major prolactin-inhibiting factor (FREEMAN et al., 2000), and bromocriptine is a dopamine receptor agonist that reduces circulatory concentration of PRL. In women (THORNER et al., 1981) and cattle (BEVERS; DIELEMAN, 1987; WILLIAMS; RAY, 1980), bromocriptine is an efficient PRL inhibitor. Treatment of mares with bromocriptine during late pregnancy reduces PRL and P4 concentration and the incidence of postpartum agalactia (IRELAND et all., 1991). Treatment of mares with bromocriptine during the anovulatory season decreases the concentration of PRL. During the transition from the anovulatory to ovulatory seasons, bromocriptine appeared to delay the growth of the preovulatory follicle but did not affect the time of ovulation (BENNET-WIMBUSH et al., 1998). A low dose of bromocriptine (10 mg) effectively decreases PRL when concentrations are naturally high, but is not effective during the late fall when concentrations are low (BECKER;JOHNSON,1990). Progesterone concentration is reduced by bromocriptine in late-pregnant mares when the placenta is the main source of P4 (IRELAND et al., 1991). However, bromocriptine has not been used during the estrous cycle to determine if PRL has an effect on P4 production by the CL.

Synchrony (at same hour) between the peaks of PGFM and PRL pulses occurs in mares (GINTHER et al., 2012b) and in cattle (GINTHER; BEG, 2011b). The synchrony in mares is about three-fold greater during and after luteolysis than before luteolysis. The frequency of synchrony in mares has been reported to be 65% during the luteolytic period (GINTHER et al., 2012b). It is not clear which hormone stimulates the other or whether other factors are involved in the simultaneous stimulation of pulses of both PRL and PGFM. In cattle, PRL concentrations centralized to a pulse of PGFM were greater at the peak and 1 h after the peak of a PGFM pulse than during other hours of the PGFM pulse (GINTHER; BEG, 2011b). In mares, treatment with PGF2α induces an increase in PRL concentrations (SHAND et al., 2000). These results tentatively indicate but do not adequately demonstrate that PGF2α is directly or indirectly stimulatory to PRL rather than vice versa.

In the current study in mares, flunixin meglumine or bromocriptine were used to decrease the secretion of PGF2α or PRL, respectively, to determine which hormone directly

or indirectly affects the other in the temporal association between the two hormones. The hypothesis was that the positive association between PGFM and PRL concentrations represents an effect of PGF2α on PRL rather than vice versa. The effect of a reduction in PGF2α or PRL on P4, LH, and FSH concentrations and the difference between the mid- and late-ovulatory seasons were also considered.

5.3 Materials and methods

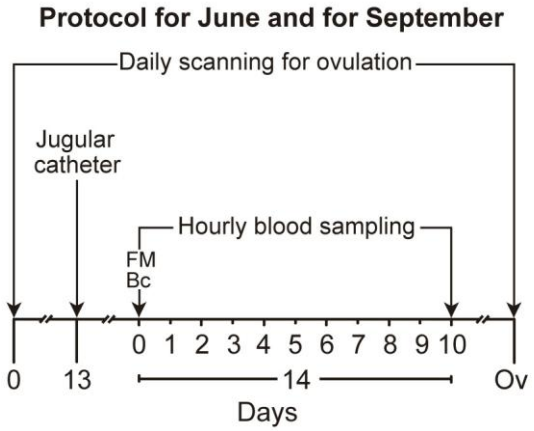
5.3.1 Mares and protocol

Mixed breeds of nonlactating pony mares and apparent pony-horse crosses weighing 350 to 530 kg and aged 4 to 13 yr were used during June and September in the northern temperate zone. Mares were kept under natural light in an open shelter and outdoor paddock and were maintained by free access to water, trace-mineralized salt, and a mixture of alfalfa and grass hay. All mares remained healthy and in good body condition throughout the study. Ultrasonic monitoring for ovulation detection was done daily (GINTHER, 1995). Mares with two CL or an apparent undersized CL (< 2.0 cm^2) on Day 13 were not used. Handling of the mares was in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research.

A total of 18 mares from a herd of 24 were randomized into three groups (n = 6/group) for the month of June (mid-ovulatory season). Eighteen mares were rerandomized from the same 24 mares into three groups (n = 6) for September, near end of ovulatory season (GINTHER, 1992), without regard to the group assignments that were made in June. The three groups were control (no treatment), an FM group (1.7 mg/kg of body weight of flunixin meglumine, i.v.), and a Bc group (32 mg/mare of bromocriptine, i.m.). The protocol is illustrated (Figure. 5.1). A single treatment was given on Day 14 at Hour 0. Day 14 was used because luteolysis is expected to begin on Day 13, 14, or 15, and the interval from beginning to end of luteolysis is about 24 h (GINTHER; HANNAN; BEG, 2011). Mares in the FM group were treated with flunixin meglumine injection (FluMeglumine; Phoenix Pharmaceutical Inc., St Joseph, MO, USA). Mares in the Bc group were treated with bromocriptine (2-bromo-α-ergocryptine methanesulfonate salt, catalog # B2134; Sigma

Aldrich Co, St. Louis MO USA). The bromocriptine was dissolved in absolute ethyl alcohol, and an equal volume of saline was added to make a solution of 3.2 mg/mL.

Figure 5.1 – Diagram of experimental protocol in mares



A single injection of flunixin meglumine (FM) or bromocriptine (Bc) was given on Day 14 postovulation to control, FM, and Bc groups in June and in September (n = 6/group/month) and hourly blood sampling was done as shown. Ov = ovulation.

An indwelling catheter was inserted into a jugular vein on Day 13, and hourly blood samples were collected on Day 14 for 10 h after treatment (Hours 0 to 10) in June and in September. The mares had free access to hay and water between sample collections. Blood samples were collected into heparinized tubes and immediately placed in ice water for 10 min before centrifuging (2000 X *g* for 10 min). The plasma was decanted and immediately stored (–20 °C) until assay.

The mares were assigned, retroactively, to before the luteolytic period (preluteolysis), during the luteolytic period (luteolysis), and after the luteolytic period (postluteolysis) on the basis of P4 concentration during Hours 0 to 10. Mares were assigned to the preluteolytic period on the basis of high P4 concentration (> 5 ng/mL), that did not decrease during the 10 hours, and the transition between preluteolysis and luteolysis did not occur during the 10 hours. The hour of transition between preluteolysis and luteolysis was based on a decrease in P4 concentration at the consecutive peaks of fluctuations as described (GINTHER; BEG, 2011a). Mares that had decreasing P4 throughout the 10 hours or attained the transitional hour during Hours 0 to 10 were assigned to the luteolytic period. Mares with < 1 ng/mL of P4

throughout the 10 hours (GINTHER; HANNAN; BEG, 2011) were assigned to the postluteolytic period.

5.3.2 Hormone end points and assays

In mares, a sampling interval of 1 h has been used to detect PGFM pulses (GINTHER et al., 2008) and PRL pulses (GINTHER et al., 2012b), owing to a mean interval for each hormone of about 5 h between the beginning and ending nadirs during luteolysis. Plasma concentrations of P4, PRL, PGFM, LH, and FSH at 1-h intervals were studied during Hours 0 to 10 and compared among groups. Initially, the hourly concentrations were considered without regard to the location of fluctuations or pulses.

To distinguish between fluctuations and pulses of PRL, PGFM, LH, and FSH, the intra-assay coefficient of variation (CV) was used as described (GINTHER; HANNAN; BEG, 2011; GINTHER et al., 2008). Briefly, a fluctuation was an increase and decrease encompassing at least three hourly values, including nadirs. A fluctuation with a CV at least three times greater than the mean intra-assay CV was defined as a pulse. The peak of an identified pulse was designated as 0 h. The pulses were compared among groups and months. Discrete end points for pulses were number of pulses/10-h; mean concentration for all values in a pulse; and concentrations at the beginning nadir (Nadir 1), peak, ending nadir (Nadir 2), and amplitude (concentrations at peak minus Nadir 1). The interval between pulses was not examined, owing to the limited number of hourly blood-sampling sessions with > 1 pulse. To assess the locations of the peaks of PGFM pulses, data were partitioned into Hours 2 to 5 and Hours 6 to 9, so that the hours with pulse peaks (Hours 2 to 9) were equally divided.

Plasma concentrations of PRL were determined by RIA as validated and described for equine plasma in our laboratory (GINTHER; BEG, 2009). The intra- and interassay CV and sensitivity were 7.3%, 4.4%, and 0.16 ng/mL, respectively. Concentrations of PGFM were determined by an ELISA that was developed in our laboratory for bovine plasma (GINTHER et al., 2010) and has been adapted and validated for use in equine plasma (GINTHER; HANNAN; BEG, 2011). The intra- and interassay CV and sensitivity were 8.7%, 15.9%, and 9.0 pg/mL, respectively. Progesterone concentrations were measured by a validated RIA, as described for mare plasma in our laboratory (GINTHER et al., 2005). The intra- and interassay CV and sensitivity were 5.7%, 16.8%, and 0.02 ng/mL, respectively.

Concentrations of LH and FSH (DONADEU;GINTHER, 2002) were determined by RIA as described and validated in our laboratory for equine plasma. The intra- and interassay CV and sensitivity were 13.0%, 1.0%, and 0.25 ng/mL for LH and 12.2%, 2.7%, and 0.86 ng/mL for FSH.

5.3.3 Statistics

Data that were not normally distributed were transformed into natural logarithms or ranks. A two-way factorial analysis for hourly hormone concentrations was done separately for each month. Differences in PGFM and PRL pulses among groups and months were analyzed in a three-way factorial for main effects of group, month, and hour and each of the interactions. The SAS MIXED procedure (Version 9.2; SAS Institute Inc., Cary, NC, USA) was used with a REPEATED statement to account for autocorrelation between sequential measurements. Student's unpaired *t*-test or the Least Significant Difference (LSD) test was used to locate differences among groups within an hour for each of the two months when an interaction was obtained. Differences among hours within a pulse were analyzed by Student's paired *t*-tests. Differences among or within groups for the discrete characteristics were analyzed by ANOVA and LSD test. Chi-square tests of independence or goodness-of-fit were used for frequency data. A probability of $P \leq 0.05$ indicated that a difference was significant. When a probability was $P > 0.05$ to $P \leq 0.1$, approaching significance was inferred. Data are presented as the mean ± SEM, unless otherwise noted.

5.4 Results

On the basis of P4 concentrations during Hours 0 to 10, the number of mares combined for June and September was different ($P < 0.006$) among the luteal periods of preluteolysis (17 mares), luteolysis (16 mares), and postluteolysis (3 mares). The frequency distribution for preluteolysis and luteolysis differed ($P < 0.02$) between months (June: 5 and 11 mares; September: 12 and 5 mares, respectively). The number of mares in preluteolysis, luteolysis, and postluteolysis among groups during June was as follows: controls (3, 3, and 0,

respectively), FM group (2, 3, and 1), and Bc group (0, 5, and 1). The P4 concentration at Hour 0 was lower ($P < 0.005$) in the Bc group (3.4 ± 0.1 ng/mL) than in the controls (7.9 ± 1.1 ng/mL). The number of mares in luteolysis during the hourly blood sampling on Day 14 was greater ($P < 0.02$) for June (11 of 18, 61%) than for September (5 of 18, 28%). The number of mares in preluteolysis was greater ($P < 0.04$) for September (12 of 18, 67%) than for June (5 of 18, 28%). The P4 concentrations were not comparable among groups for June, owing to the confounding from the uneven distribution of luteal stages among groups.

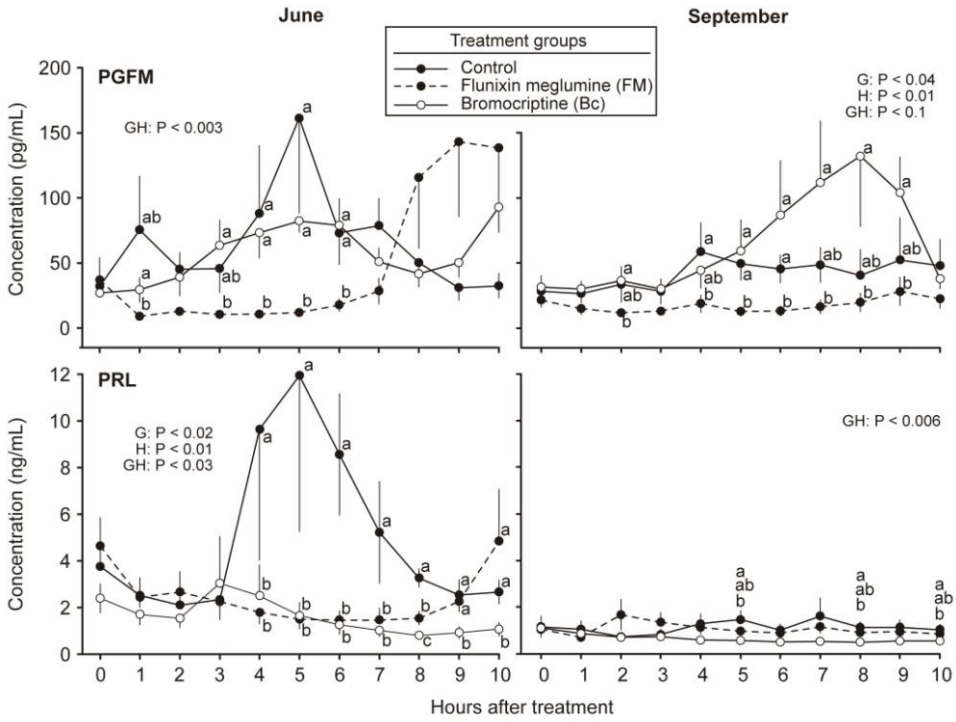
During September, the number of mares in preluteolysis, luteolysis, and postluteolysis was as follows: controls (3, 2, and 1, respectively), FM group (5, 1, and 0), and Bc group (4, 2, and 0). During September, adequate numbers for the comparison of P4 concentrations among groups were available only for mares in preluteolysis; there was no group or hour effect or a group-by-hour interaction (not shown). Combined for mares in preluteolysis for the three groups during September, P4 concentration at Hour 0 (8.9 ± 0.5 ng/mL) was similar to the concentration at Hour 10 (9.2 ± 0.6 ng/mL). The interovulatory interval combined for groups was longer (approached significance, $P < 0.08$) during September (24.5 ± 0.5 d) than during June (23.5 ± 0.5 d). In addition, 1, 1, and 2 mares at Hour 0 in September in the control, FM, and Bc groups, respectively, did not ovulate by Day 30 and began the anovulatory season.

Concentrations of PGFM during June showed only an interaction of group-by-hour (Figure. 5.2). Concentrations were not different between control and Bc groups at any hour. Concentrations were lower ($P < 0.05$) in the FM group than in the controls at Hour 1 and at Hours 4 to 6 and were lower ($P < 0.05$) in the FM group than in the Bc group at Hours 3 to 6. Concentrations of PGFM during September showed significant effects of group and hour and an approaching effect for the interaction. The group effect represented overall concentrations for the control, FM, and Bc groups of 41.8ab ± 5.0 pg/mL, 17.5b ± 1.7 pg/mL, and 64.0a ± 9.0 pg/mL, respectively; means with different superscripts are different ($P < 0.05$). Concentrations were lower in the FM group than in the controls at Hours 4 to 6, but the Bc group had no significant differences from the controls. Concentrations were lower ($P < 0.05$) in the FM group than in the Bc group at Hour 2 and at Hours 5 to 9. Based on paired *t*-tests, PGFM concentrations decreased ($P < 0.05$) in the FM group between Hours 0 and 1 during June and during September. Concentrations did not differ between June and September, averaged over all groups or within each group.

Concentration of PRL during June showed significance for each main effect and the interaction (Figure. 5.2). The main effect of group for June was from overall concentrations

for the control, FM, and BC groups of 5.0a ± 0.9 ng/mL, 2.4b ± 0.3 ng/mL, and 1.6b ± 0.2 ng/mL, respectively. The interaction represented lower ($P < 0.05$) concentration in the FM group than in the controls at Hours 4 to 8, in the Bc group than in the controls at Hours 4 to 10, and in the Bc group than in the FM group at Hours 8 to 10. During September, PRL concentrations showed no main effects but the interaction of group and hour was significant. The interaction represented lower ($P < 0.05$) PRL concentrations in the Bc group than in the controls at Hours 5, 8, and 10. Concentrations of PRL in the FM group were intermediate with no significant differences from the other two groups at any hour. The overall concentration was greater ($P < 0.0001$) in June (3.0 ± 0.3 ng/mL) than in September (0.9 ± 0.05 ng/mL) and was greater during June than September within the controls (5.0 ± 0.04 vs 1.1 ± 0.1 ng/mL, $P < 0.0008$), FM group (2.4 ± 0.3 vs 1.0 ± 0.09 ng/mL, $P < 0.04$), and Bc group (1.6 ± 0.2 vs 0.6 ± 0.04 ng/mL, $P < 0.05$).

Figure 5.2 – Mean ± SEM concentrations of PGFM and PRL in three treatment groups of mares during June and September (n = 6/group/month).



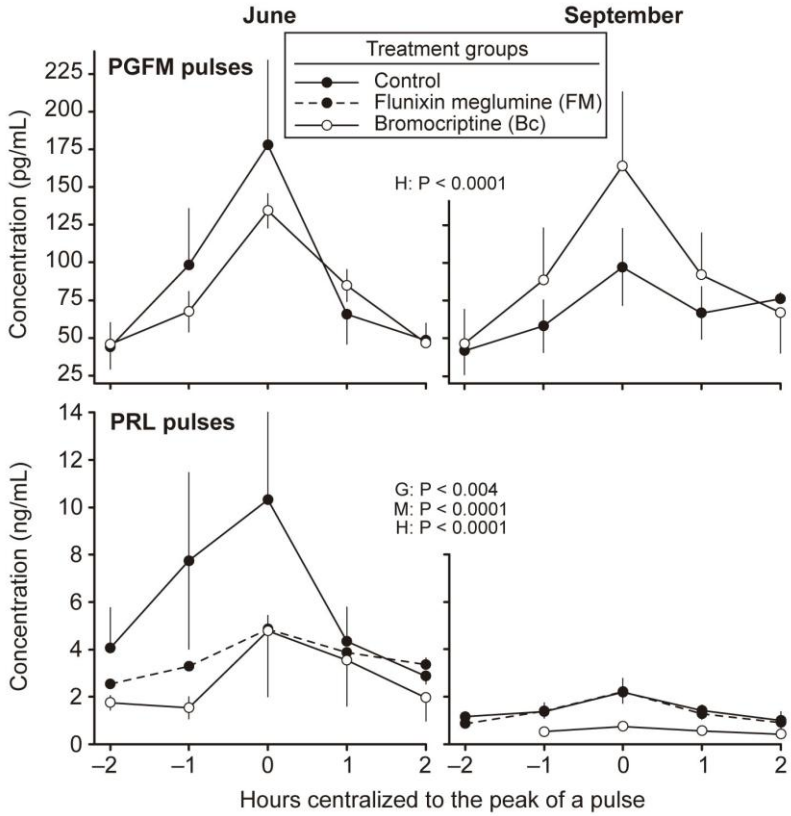
A single treatment (FM, 1.7 mg/kg; Bc, 32 mg/mare) was given on Day 14 postovulation. Probabilities for a main effect of group (G) and hour (H) and the group-by-hour interaction (GH) are shown for PGFM and for PRL for each month. ab, de = means with different superscripts for each hour for June and September, respectively, are different (P < 0.05) among groups.

The number of PGFM pulses/mare with a peak at Hours 2 to 9 in June was lower (P < 0.02) in the FM group (0.8 ± 0.2 pulses) than in the controls (1.5 ± 0.2). The number of pulses/mare during September was not different (P < 0.15) between the FM group (0.7 ± 0.2 pulses) and controls (1.2 ± 0.4). The frequency of PGFM pulses with a peak at Hours 2 to 5 vs Hours 6 to 9 was not different within or between the control and Bc groups. In June, the combined frequency of mares with a PGFM pulse was 8 of 12 at Hours 2 to 5 and 6 of 12 at Hours 6 to 9 and in September was 7 of 12 at Hours 2 to 5 and 7 of 12 at Hours 6 to 9. In addition, the control and Bc groups did not differ in PGFM pulse characteristics (concentrations at peak, nadirs, and amplitude; not shown). In the FM group, the frequency of mares with a PGFM pulse during Hours 2 to 5 (1 of 6 mares) was lower (P < 0.04) than the combined frequency for the control and Bc groups (8 of 12). There were no differences among groups in number of pulses during Hours 6 to 9. The factorial analyses of PGFM pulses considered only the control and Bc groups, owing to the presence of only one (June) or two (September) pulses in the FM group during Hours 2 to 5. The analysis of PGFM pulses in

the control and Bc groups showed only the expected main effect of hour with no main effect of group or month or any interactions.

Pulses of PRL showed main effects of group, month, and hour, but no interactions (Figure. 5.3). The effect of group was from lower ($P < 0.05$) concentration averaged over all values of a pulse for the Bc group (2.0 ± 1.5 ng/mL) than for the controls (4.7 ± 1.0 ng/mL). The FM group was intermediate (2.3 ± 0.2 ng/mL) and was not significantly different from either of the other two groups. The month effect represented greater concentration averaged for all values within a pulse in June (5.0 ± 0.8 ng/mL) than in September (1.3 ± 0.1 ng/mL).

Figure 5.3 – Mean ± SEM concentrations of PGFM and PRL in three treatment groups of mares during June and September (n = 6/group/month).



A single treatment (FM, 1.7 mg/kg; Bc, 32 mg/mare) was given on Day 14 postovulation. Probabilities for a main effect of group (G) and hour (H) and the group-by-hour interaction (GH) are shown for PGFM and for PRL for each month. ab, de = means with different superscripts for each hour for June and September, respectively, are different ($P < 0.05$) among groups.

The number of PRL pulses/mare during the 10 h in June was greater in the controls than in the FM and Bc groups (Table 5.1). The number did not differ among groups during

September. The number in September was lower (approached significance, $P < 0.1$) than in June. The discrete characteristics of the PRL pulses are shown (Table 5.1). Concentrations at the nadirs were lower in the Bc group than in the controls during June and were lower in the Bc group than in the FM group during September. No significant differences in LH or FSH concentrations were detected among groups within each month (not shown).

Table 5.1 – Mean ± SEM characteristics of PRL pulses in three treatment groups during June and September after a single treatment (FM, 1.7 mg/kg; Bc, 32 mg/mare) on Day 14

	Treatment groups		
PRL pulses	Control	Flunixin meglumine (FM)	Bromo-criptine (Bc)
June			
Number of PRL pulses/10h	1.8 ± 0.4a	0.5 ± 0.3b	0.7 ± 0.3b
Concentrations (ng/mL)			
Peak	9.7 ± 4.4	4.9 ± 0.6	4.8 ± 2.8
Nadir 1	2.3 ± 0.3a	3.4 ± 0.3b	1.4 ± 0.3c
Nadir 2	2.3 ± 0.2a	2.5 ± 0.4a	1.0 ± 0.3b
Amplitude	8.0 ± 5.0	1.4 ± 0.8	3.4 ± 2.6
Nadir 1 to Nadir 2 (h)	3.9 ± 0.8	4.7 ± 1.5	5.3 ± 1.1
September			
Number of PRL pulses/10h	1.2 ± 0.3	1.0 ± 0.4	0.5 ± 0.3
Concentrations (ng/mL)			
Peak	3.9 ± 2.0ab	2.2 ± 0.5a	0.8 ± 0.1b
Nadir 1	0.9 ± 0.2ab	0.8 ± 0.1a	0.5 ± 0.1b
Nadir 2	0.8 ± 0.1ab	1.1 ± 0.2a	0.4 ± 0.1b
Amplitude	4.2 ± 2.8	1.2 ± 0.6	0.2 ± 0.1
Nadir 1 to Nadir 2 (h)	4.4 ± 0.6	3.7 ± 0.7	2.7 ± 0.3

ab Means within a row with a different superscript are different ($P < 0.05$).

5.5 Discussion

The uneven distribution of luteal stages among groups precluded consideration of the effect of treatments on P4 concentration. The 6 of 6 mares in the June Bc group and the 3 of 6 in the controls that had begun luteolysis by the hour of treatment (Hour 0) on Day 14 accounted for the lower P4 concentration at Hour 0 in the Bc group than in the controls. The imbalance among groups in number of mares in various luteal stages at Hour 0 in June seemed to be a chance occurrence, considering the strict use of randomization. During September, the number of mares in preluteolysis (12 of 18) throughout the 10 h of hourly sampling was adequate for statistical purposes, but an effect of group on P4 production was not detected.

The difference between June and September in the distribution of the number of mares in preluteolysis vs luteolysis on Day 14 indicated that luteolysis occurred earlier postovulation in June (61% in luteolysis) than in September (28% in luteolysis). However, this interpretation is inconsistent with the reported effect of month during the ovulatory season on length of diestrus and estrus as determined by behavioral studies (GINTHER , 1992). The length of diestrus decreases and the length of estrus increases between the summer months and fall months (GINTHER , 1974). In this regard, the day that P4 decreases to < 1 ng/mL occurs earlier for the last interovulatory interval of the year than for previous intervals (IRVINE; ALEXANDER; MCKINNON, 2000). In the current study, ovulation for 14 of 18 mares following the September treatment occurred between September 24 and October 14. The remaining four mares entered the anovulatory season. On average, therefore, the September group was considered to be close to the end of the ovulatory season. Results of reported behavioral studies on the effect of month and the shortened luteal phase for the last estrous cycle of the ovulatory season seem to contradict the current results of an earlier luteolytic period in June than in September. Further study is indicated.

The similarity between June and September in concentrations of PGFM agrees with a report that PGFM concentration did not differ between summer, mid-ovulatory season, and autumn, August to November or toward the end of the ovulatory season (KING et al., 2010b). However, a failure of secretion of PGF at the appropriate time results in CL persistence or prolonged luteal activity in mares (KING et al., 1990; STABENFELDT; HUGHES, 1987; NEELY et al., 1979). A high incidence of prolonged luteal activity (20 to 25% of mares) occurs in the autumn (KING et al., 2011). In the current study, prolonged luteal activity did

not occur in any of the 18 mares in June or in any of the 18 mares in September, based on P4 concentrations and ultrasonic assessment of the CL. However, the absence of prolonged luteal activity in the current study may have been related to the use of pony or pony-horse crosses rather than horses. The possibility that prolonged luteal activity may be related to the seasonal decline in PRL has been noted but has not been demonstrated (KING et al., 2011). In this regard, prolonged luteal activity did not occur in September in the current study despite a marked reduction in PRL concentration between June and September and a further reduction from bromocriptine treatment in September.

The single treatment with flunixin meglumine (FM) decreased the hourly concentrations of PGFM immediately after treatment during both June and September. Concentrations appeared to remain reduced until Hour 6. The PGFM suppressing effect of FM was demonstrated by the lower concentrations in the FM group than in the controls for Hours 4 to 6 in each month. This is apparently the first report on the hourly responses of PGFM concentrations and PGFM pulses to a single FM treatment before and during the luteolytic period in mares. The effectiveness of FM for 6 h contrasts with the effectiveness for at least 9 h in heifers (PUGLIESI et al., 2011b). However, the dose was lower (1.7 mg/kg) in mares than in heifers (2.5 mg/kg). Based on the location of pulse peaks, FM treatment decreased the number of pulses of PGFM during Hours 2 to 5, but not during Hours 6 to 9. This was shown by detection of only one pulse totaled for the six FM-treated mares during Hours 2 to 5. In contrast, 8 of 12 control and bromocriptine-treated mares had a PGFM pulse during these hours. The effect of FM on PGFM pulses had not been considered previously in mares. In heifers, FM treatment every 4 or 8 h during primarily luteolysis suppressed the prominence and number of PGFM pulses (PUGLIESI et al., 2011b; PINAFFI et al., 2012; PUGLIESI et al., 2012b).

Hourly concentrations of PRL also were reduced by FM treatment in June but the low concentrations during September were not further reduced. The low concentrations of PRL in the FM group in June returned to pretreatment levels toward the end of the hourly sampling. Thus, the loss of the negative effect of FM on PGFM was associated with a loss of the negative effect on PRL. The effect of month on PRL agrees with previous studies that have shown highest concentration in the summer and lowest concentration in the winter (JOHNSON, 1986; EVANS et al., 1991); concentrations declined from high levels in June to low levels in November (FITZGERALD; DAVISON; MCMANUS, 2000).

Bromocriptine treatment on Day 14 reduced the PRL concentrations as indicated by the concentrations in the hourly samples and by the number and characteristics of PRL pulses.

The reduced concentrations in June extended from Hour 4 to the last sample at Hour 10. Concentrations of PRL were lower in all groups during September compared to June; in the controls the September concentration was 77% lower than the June concentration. Prominence of PRL pulses was also reduced from June to September by bromocriptine. This was shown by the reduced group effect that considered all values in a pulse, by lower concentrations at the nadirs and pulse peak, and by fewer CV-identified pulses during September in the Bc group. Bromocriptine treatment has been given previously to mares during the ovulatory season but without regard to the stage of the estrous cycle (JOHNSON; BECKER, 1987). Treatment suppressed the PRL concentrations in May but not in November when concentrations were naturally low in controls. Pulses of PRL were not evaluated. The effectiveness of bromocriptine in reducing PRL concentration has been demonstrated also during the anovulatory season (BENNETT-WIMBUSH et al., 1998). The reduction in PRL secretion by bromocriptine results from a direct action at the pituitary (MAUERER, 1981) by inhibiting PRL release from pituitary stores (BEN-JONATHAN;HNASKO,2001).

The hypothesis was supported that the positive association between PGFM and PRL concentrations represented an effect of PGF2α on PRL rather than vice versa. Support is from a reduction in both PGFM and PRL in the FM group. Conversely, the reduction in PRL in the bromocriptine group was not associated with a reduction in PGFM. The lowering of the concentrations of PRL within each month by bromocriptine without an effect on PGFM was indicated by no difference in PGFM concentrations between the control and the Bc groups during the 10 h of hourly sampling and no differences in number and characteristics of PGFM pulses. The PRL-reducing effect of bromocriptine confirms the results of previous studies (BENNETT-WIMBUSH et al., 1998; FITZGERALD; DAVISON; MCMANUS, 2000), but the absence of an effect on PGFM concentrations had not been reported previously. Nondetection of an effect of PRL on PGF2α was also supported by the considerable reduction in PRL from June to September averaged for all groups and within each group without an associated decrease in PGFM. The positive effect of PGF2α on PRL in mares is consistent with the report that PGF2α administration increases the secretion of PRL (SHAND et al., 2000). In heifers, bromocriptine treatment on a mean of Day 17 (63% of heifers in luteolysis) reduced the concentrations of PRL but did not affect PGFM (PINAFFI et al., 2012), similar to the present results in mares.

Mares and heifers appear to differ in the LH response to bromocriptine treatment. In heifers, LH concentrations increased during the bromocriptine-induced decrease in PRL (PINAFFI et al., 2012). In the current study and reported studies (BENNETT-WIMBUSH et

al., 1998; BRENDENMUEHL; CROSS, 2000) in mares, an effect of bromocriptine treatment on LH and FSH was not detected.

In conclusion, the control of the temporal relationship between PGF and PRL in mares before and during the luteolytic period was studied. The effects of reducing the concentrations of PGF2α by FM treatment were compared to the effects of reducing the concentrations of PRL by bromocriptine treatment. A single treatment with FM reduced the PGFM concentrations until about 6 h in both June and September, and also reduced the PRL concentrations. Treatment with bromocriptine reduced the concentrations of PRL but did not affect the concentrations of PGFM. Concentrations of PRL in the controls were about 3-fold greater in June than in September, but concentrations and pulses of PGFM were not different between months. Results indicated that the temporally related increases in concentrations of PGFM and PRL reflect an effect of PGF2α on PRL rather than an effect of PRL on PGF2α.

6 OVARIAN AND PGF2ALPHA RESPONSES TO STIMULATION OF ENDOGENOUS PRL PULSES DURING THE ESTROUS CYCLE IN MARES

6.1 Synopsis

The effects of a PRL stimulating substance (sulpiride) on PRL and PGF2α secretion and pulsatility and on luteal and follicular dynamics were studied during the estrous cycle in mares. A control group (Ct, n = 9) and a sulpiride group (Sp, n = 10) were used. Sulpiride (25 mg/mare) was given every 8 h from Day 13 postovulation until the next ovulation. Repeated sulpiride treatment did not appear to maintain PRL concentrations at 12-h intervals beyond Day 14. Therefore, the hypothesis that a long-term increase in PRL altered luteal and follicular end points was not testable. Hourly samples were collected on Day 14 from the hour of a treatment (Hour 0) to Hour 8 for characterization of concentrations and pulses of PRL and a metabolite of PGF2α (PGFM). Concentrations of PRL reached maximum at Hour 4 in the Sp group. The pulses were more prominent ($P < 0.008$) in the Sp group (peak, 19.4 ± 1.9 ng/mL) than in the Ct group (11.5 ± 1.8 ng/mL). Hourly concentrations of PGFM, number and characteristics of PGFM pulses, concentrations of P4, and percentage of CL with color-Doppler signals of blood flow were not affected by the increased PRL. A novel observation was that the peak of a PRL pulse occurred at the same hour or 1 h later than the peak of a PGFM pulse in 8 of 8 PGFM pulses in the Ct group and in 6 of 10 pulses in the Sp group ($P < 0.04$), indicating that sulpiride interfered with the synchrony between PGFM and PRL pulses. The hypothesis that sulpiride treatment during the equine estrous cycle increases the concentrations of PRL and the prominence of PRL pulses was supported.

6.2 Introduction

The luteolytic process consists of both functional and structural regression of the CL. Assay of plasma concentration of progesterone (P4) and transrectal ultrasonic evaluation of the CL and percentage of CL with color-Doppler blood flow signals can be used to evaluate the CL in large animals (GINTHER et al., 2007; GINTHER et al., 2008). In mares, the

average day of transition from preluteolysis to luteolysis is Day 14 (Day 0 = ovulation), based on collection of hourly blood samples (GINTHER; HANNAN; BEG, 2011). Secretion of PGF2α pulses by the endometrium has a fundamental role in luteolysis in mares (GINTHER et al., 2008; GINTHER; HANNAN; BEG, 2011) as in other species (MCCRACKEN; CUSTER; LAMSA, 1999; WEEMS; WEEMS; RANDEL, 2006).

Prolactin (PRL) has luteotropic and luteolytic roles in some species (MURPHY; RAJKUMAR, 1985). In cows, a luteal role for PRL has not been demonstrated, despite the presence of PRL receptors and the expression of PRL mRNA in the CL (SHIBAYA et al., 2006). In mares, specific PRL binding sites are uniformly distributed in the mature CL (KING et al., 2010a), but a luteal role of PRL has not been demonstrated *in vitro* or *in vivo*. Concentrations of PRL are greater during the ovulatory season (summer) than during the anovulatory season (winter) (KING et al., 2010a; AURICH et al., 1995; WORTHY et al., 1987; EVANS et al., 1991; JOHNSON, 1986). Collection of blood samples every 4 h (SHAND et al., 2000) is too infrequent to detect PRL pulses during the estrous cycle in mares, but infrequent collections (4 h to 24 h intervals) have depicted one to three apparent transient increases toward the end of luteolysis and during estrus (KING et al., 2010a; WORTHY et al., 1987; SHAND et al., 2000). On the basis of hourly blood sampling, PRL pulse peaks are greater during and after luteolysis than during preluteolysis in mares (GINTHER et al., 2012b) and heifers (GINTHER et al., 2011b). Synchrony between pulses of PRL and a metabolite of PGF2α (PGFM) is most frequent during late luteolysis and after luteolysis (GINTHER et al., 2012b; GINTHER; BEG, 2011b). However, whether pulses of PGF2α stimulate a pulse of PRL or vice versa and whether other factors are involved in the PGFM/PRL synchrony have not been demonstrated.

Prolactin secretion is regulated by the hypothalamus via a short-loop feedback system (GRATTAN, 2002). The major prolactin inhibiting factor is dopamine and has been reported to decrease PRL secretion both *in vitro* (KOCH; LU; MEITES, 1970) and *in vivo* (MACLEOD; FONTHAM; LEHMEYER, 1970). Sulpiride, a dopamine receptor antagonist, stimulates PRL secretion. In mares, most studies using sulpiride treatments have involved lactation (CHAVATTE-PALMER et al., 2002; GUILLAUME et al., 2003) and the anovulatory and ovulatory seasons (AURICH et al., 1995; BESOGNET; HANSEN; DAELS, 1996; MARI et al., 2009). Increasing the concentrations of PRL by dopamine antagonists stimulates follicle development and hastens the first ovulation of the ovulatory season (BESOGNET; HANSEN; DAELS, 1996; MARI et al., 2009). Although considerable attention has been given to the role of PRL in reproductive seasonality, only limited

information is available on the effects of stimulation during the estrous cycle. Sulpiride increases the PRL concentrations in mares that are in estrus or diestrus (AURICH et al., 1995), but the effect of sulpiride on PRL concentrations during specific days relative to luteal stages or follicular development or the effect of increased PRL on the CL or follicles during the ovulatory season have not been reported. In addition, the effects of sulpiride on the number and characteristics of individual PRL pulses apparently have not been reported for any reproductive status or species.

The presence of PRL receptors in the pituitary (GREGORY et al., 2000) indicates a potential role of PRL on pituitary function. Secretion of PRL has been reported to be an inhibitor of LH secretion in several species (MILENKOVIĆ et al., 1994), especially in rats and humans (SARTORIO et al., 2000; COHEN-BECKER; SELMANOFF; WISE; 1986). The combined effect of PRL and dopamine has an inhibitory effect on LH, but PRL alone does not. Sulpiride treatment did not alter LH concentration in mares during the anovulatory season or during diestrus (AURICH et al., 1995) but the effect of a PRL increase on circulating concentrations of LH and FSH in mares requires further study, especially during the estrous cycle.

The present experiment was done from Day 13 to ovulation in mares to evaluate the following hypotheses: (1) sulpiride treatment during the estrous cycle increases PRL concentrations and the prominence of PRL pulses, (2) an increase in PRL concentration affects PGFM concentration and pulses and P4 concentration, and (3) repeated sulpiride treatment (every 8 h) maintains an increase in PRL and alters structural luteal regression, luteal blood flow, follicle development, and time of ovulation. In addition, the effect of sulpiride treatment on the temporal relationships between PRL and PGFM pulses was determined.

6.3 Materials and Methods

6.3.1 Animals and ultrasound scanning

Mixed breeds of large pony mares and apparent pony-horse crosses (n = 20) averaging 430 kg and aged 4 to 13 yr were used during August in the northern temperate zone. The

mares were housed under natural light in an open shelter, with free access to a mixture of alfalfa and grass hay, water, and trace-mineralized salt. Mares were selected on Day 13with no apparent abnormalities of the reproductive tract or undersized CL (< 3 cm^2), as determined by transrectal ultrasound examinations (GINTHER, 1995). Mares were handled according to the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Agricultural Research.

A duplex B-mode (gray-scale) and pulsed-wave color-Doppler ultrasound scanner (Aloka SSD-3500; Aloka America, Wallingford, CT, USA) equipped with a 7.5 MHz transrectal linear-array transducer was used for ultrasound examinations. Number of follicles ≥ 15 mm and diameter of the largest follicle were determined twice each day beginning on Day 13 (ovulation = Day 0). The area of the CL was measured twice each day at the maximal image plane, using the scanner's still image and tracing function. Blood flow in vessels of the CL was displayed in power-Doppler mode while scanning the entire CL in real time. The percentage of the CL that displayed color signals of blood flow was estimated as described (GINTHER; UTT, 2004; GINTHER , 2007).

6.3.2 Experimental design and treatments

Mares were randomized into a control (Ct) and sulpiride (Sp) group (n = 10/group). From Day 13 until ovulation, mares in the Sp group were treated every 8 h with 25 mg of sulpiride (Rood and Riddle Pharmacy, Lexington, KY). The dose of Sp and interval between treatments were based on a previous report (JOHNSON; BECKER, 1987); the PRL increase from a 25 mg dose was similar to a 100 mg dose, and both doses induced elevated PRL concentration for at least 7 h. Blood samples were collected by venipuncture of a jugular vein every 12 h until ovulation. In addition, samples were collected hourly for 12 h on Day 14 beginning at 2 PM. Beginning sampling at 2 PM was based on a report that all of eight mares began luteolysis between 2 PM and 2 AM (GINTHER; BEG, 2011a). Hourly sampling extended from 3 h before to 8 h after the fifth sulpiride treatment at 8-h intervals. The hour of treatment was designated Hour 0 (Figure. 5.1). Hours 0 to 8 were used for study of concentrations of progesterone (P4) and concentrations and pulses of PRL, PGFM, LH, and FSH. Day 14 was used for the hourly sampling because it is the mean day of the beginning of

luteolysis in this experimental herd (GINTHER; HANNAN; BEG, 2011). The length of luteolysis is 23 h on the basis of hourly blood sampling.

The P4 concentrations at Hours –3 to –1 were used as an aid in determining the luteal stage (preluteolysis, luteolysis, postluteolysis) at Hour 0. The luteal transition from preluteolysis to luteolysis was based on a progressive decrease in P4 at the peaks of P4 fluctuations as described (GINTHER; BEG, 2011a). The period before luteal transition was defined as preluteolysis. The hour of transition was compared between the Ct and Sp groups. The period beginning at the luteal transition and extending to P4 < 1 ng/mL was defined as luteolysis; samples with P4 < 1 ng/mL indicated postluteolysis (GINTHER; HANNAN; BEG, 2011).

The long-term effects of Sp treatment were evaluated at 12-h intervals for P4 concentrations, CL area (cm^2), luteal blood flow, number of follicles ≥ 15 mm, and growth of the largest follicle. In addition, blood samples were collected at 1-h intervals for a 12-h session, and concentrations of P4, PRL, PGFM, LH, and FSH were determined. Fluctuations of PRL, PGFM, LH, and FSH concentrations were evaluated to detect pulses during Hours 0 to 8 as described (GINTHER et al., 2007). When the coefficient of variation (CV) of the values comprising a fluctuation from beginning to ending nadirs was at least three times greater than the mean intra-assay CV, the fluctuation was considered to be a pulse. The adequacy for detecting pulses of PRL and PGFM (GINTHER et al., 2012b) and LH (GINTHER et al., 2011a) by hourly sampling in mares for this approximate stage of the estrous cycle has been reported. The interval from Hour 0 to the peak of a PRL pulse was compared between groups. The synchrony (at same hour) for the peaks of PRL and PGFM pulses was examined for differences between groups. Concentrations of P4 were centralized to the peak of a PRL pulse to determine if the pulses influenced the direction of P4 change within the hours of a PRL pulse.

Discrete end points for both PRL and PGFM pulses were number of pulses/8 h; concentration at the beginning nadir (Nadir 1), peak, ending nadir (Nadir 2), and amplitude (concentrations at peak minus Nadir 2); area under the curve; and interval (elapsed number of hours) from Nadir 1 to Nadir 2.

6.3.3 Hormone assays

Blood samples were collected into heparinized tubes (10 mL) and immediately placed in ice water for 10 min before centrifuging (2000 X g for 10 min). The plasma was decanted and stored (–20 °C) until assay. Plasma concentrations of PRL were determined by an RIA that was validated and described for use in equine plasma in our laboratory (GINTHER; BEG, 2009). The intra-assay CV and sensitivity were 5.8% and 0.03 ng/mL, respectively. Concentrations of PGFM were assayed by ELISA as developed in our laboratory for bovine plasma (GINTHER et al., 2010b) and adapted and validated for use in equine plasma (GINTHER; HANNAN; BEG, 2011). The intra- and interassay CV and sensitivity were 5.1%, 19.9%, and 9.5 pg/mL, respectively. Plasma P4 was measured by a solid-phase RIA kit containing antibody-coated tubes and I^{125}-labeled progesterone (Coat-A-Count Progesterone; Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure has been validated and described for equine plasma in our laboratory (GINTHER et al., 2005). The intra-assay CV and sensitivity were 3.9% and 0.03 ng/mL, respectively. Concentrations of LH and FSH were analyzed by RIA, as described for mare plasma in our laboratory (DONADEU; GINTHER, 2002). The intra-assay CV and sensitivity were 9.9% and 0.23 ng/mL for LH and 14.2% and 1.1 ng/mL for FSH, respectively.

6.3.4 Statistical analyses

Data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were transformed to natural logarithms or ranks. The Dixon test was used for detection and removal of outliers from the statistical analyses. An outlier was detected for two PRL values. Factorial analyses for main effects of group and hour and the hour-by-group interaction was done with SAS PROC MIXED (Version 9.2; SAS Institute Inc., Cary, NC, USA) with a REPEATED statement to minimize autocorrelation between sequential measurements. When the interaction between group and hour was significant or approached significance, differences between groups at each hour were examined by Student's unpaired *t*-tests. The LSD test was used to locate differences among hours within groups or hours. Student's paired *t*-tests were used for selected comparisons of means within

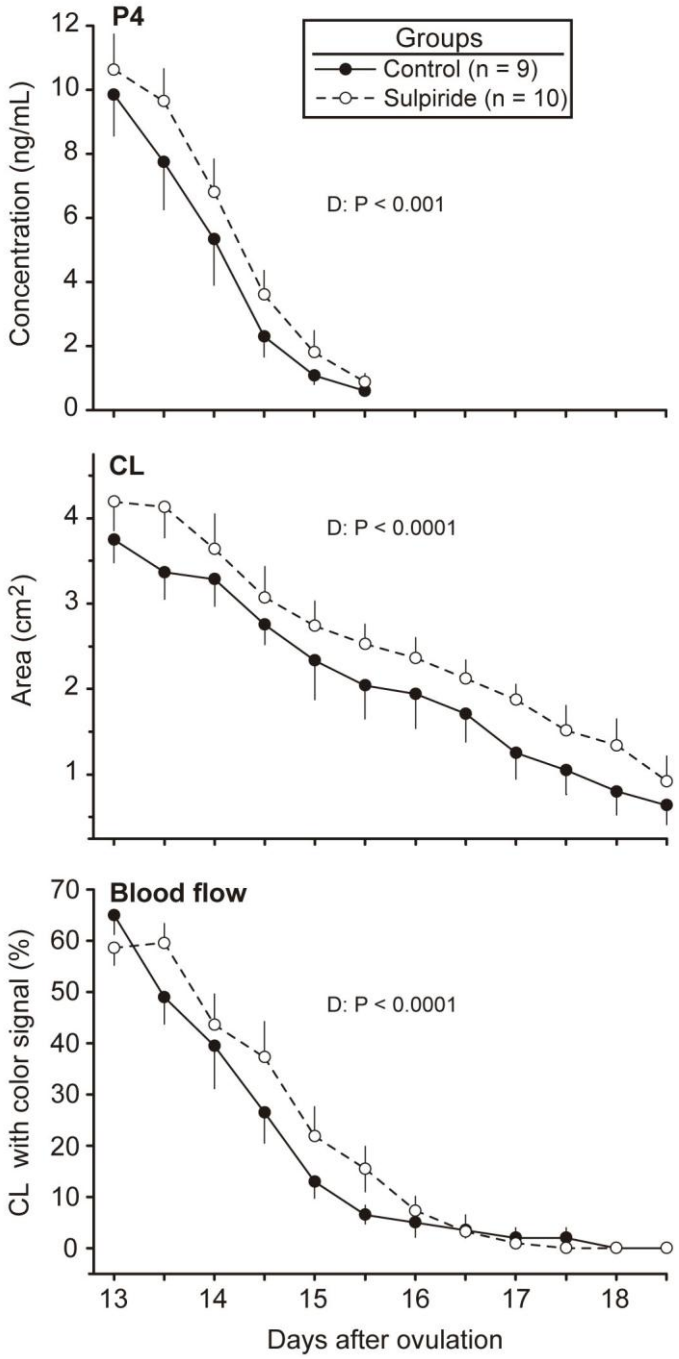
a group. Differences between groups for discrete characteristics were analyzed by ANOVA. Chi square test was used to test independence in the frequency of pulses among groups, and chi square goodness-of-fit was used to test equality (e.g., number of luteal stages within a group). A probability of $P \leq 0.05$ indicated a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise noted.

6.4 Results

Probabilities for a group effect, hour effect, and group-by-hour interaction for the factorial analyses are given in the figures and the probabilities for differences in discrete end points are given in the table or text. One mare in the Ct group had a persistent CL until Day 70 and was excluded from the analyses, leaving nine mares in the Ct group and 10 mares in the Sp group. On the basis of P4 concentrations, the number of mares in the three luteal stages at the hour of treatment (Hour 0) on Day 14 was not different between groups (preluteolysis, 3 and 5 for Ct and Sp groups, respectively; luteolysis, 5 and 4; postluteolysis, 1 and 1). Seven of eight mares that were in preluteolysis at Hour 0 were in luteal transition between preluteolysis and luteolysis at Hours 0, 1, or 2. Four mares began postluteolysis during Hours 2 to 6.

Concentrations of P4 showed a significant day effect but not a group effect or group-by-day interaction for samples taken every 12 h on Days 13.0 to 15.5 (Figure. 6.1). The day effect was from a gradual concentration decrease averaged for the two groups. The number of days from ovulation to the first P4 sample with a concentration of < 1.0 ng/mL (end of luteolysis) was not different between the Ct group (14.8 ± 0.2 d) and the Sp group (15.1 ± 0.2 d). The end of luteolysis to ovulation was not different between the Ct group (7.1 ± 0.5 d) and the Sp group (6.7 ± 0.4 d). The luteal area (cm^2) and percentage of CL with power-Doppler blood-flow signals showed a day effect for Days 13.0 to 18.5 but not a group effect or an interaction (Figure. 6.1).

Figure 6.1 – Mean ± SEM concentrations of P4, area (cm^2) of CL, and percentage of CL with power-Doppler signals of blood flow in controls and sulpiride-treated mares.

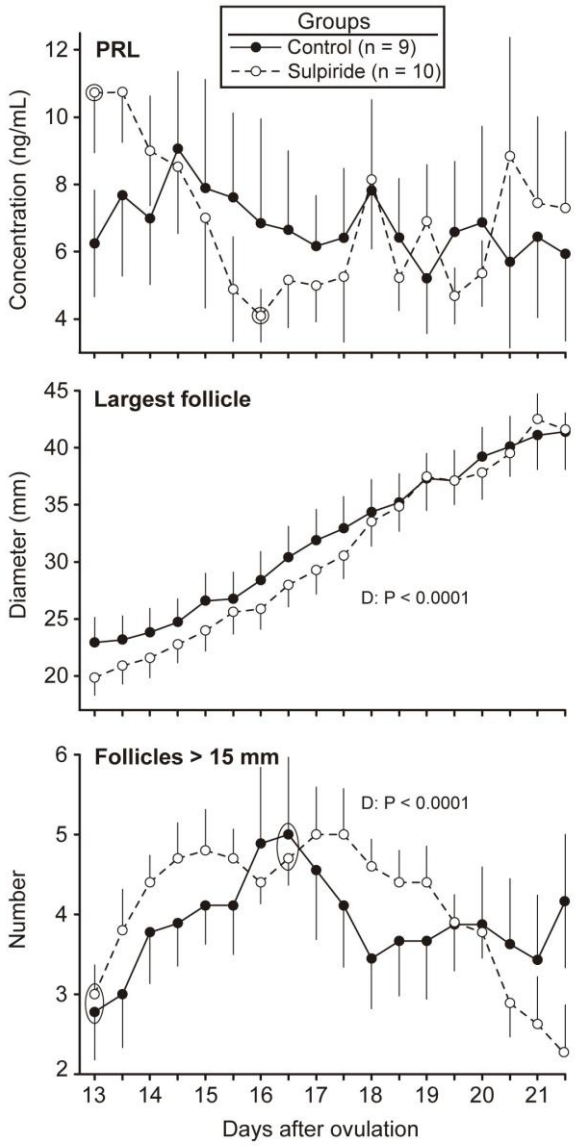


Sulpiride treatment was given every 8 h from Day 13 until ovulation. For each end point, only the day effect (D) was significant.

Concentrations of PRL for Days 13.0 to 21.5 (Figure. 6.2) or for the six days before the posttreatment ovulation (not shown) showed no differences for the factorial analyses (group, day, interaction). Based on unpaired *t*-tests, the only PRL difference between groups

within a day occurred on Day 13.0; PRL was greater ($P < 0.04$) in the Sp group than in the Ct group. The only significant continuing change between days within each group was a decrease ($P < 0.003$) between Days 13.0 and 16.0 in the Sp group. The diameter of the largest follicle for Days 13 to 21.5 showed only a day effect from increasing diameter over days (Figure. 6.2).

Figure 6.2 – Mean ± SEM concentrations of PRL, diameter of largest follicle, and number of follicles ≥ 15 mm in controls and sulpiride-treated mares.

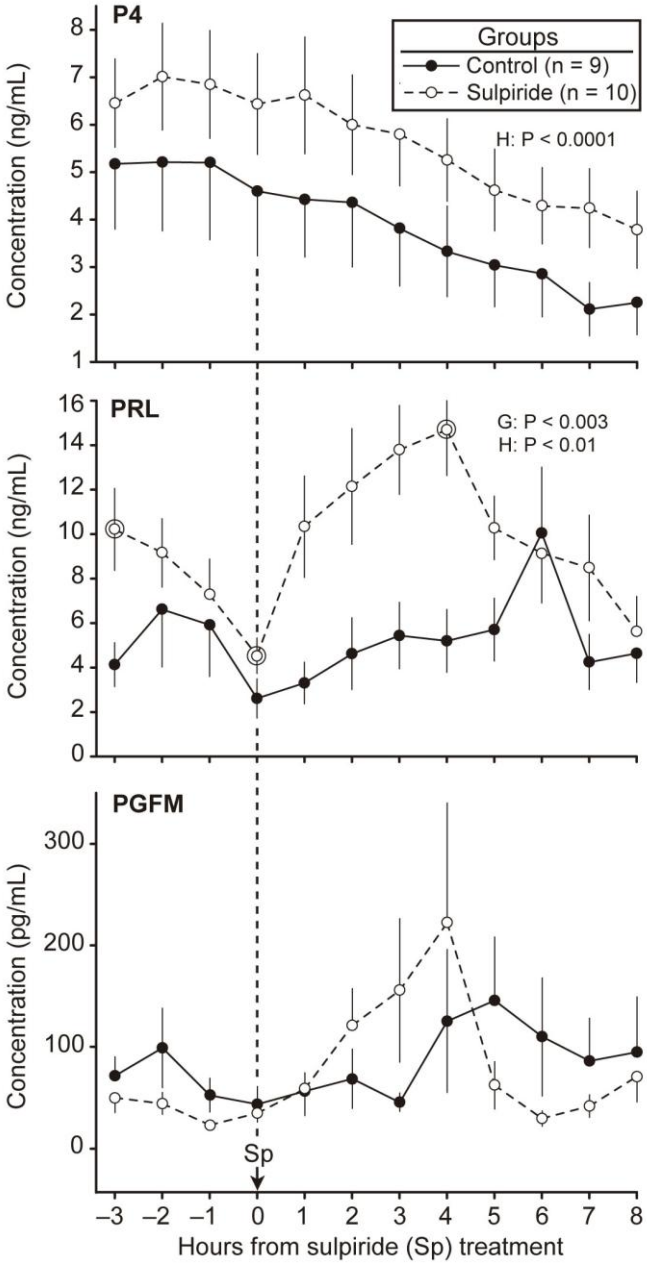


Sulpiride treatment was given every 8 h from Day 13 until ovulation. The first treatment on Day 13 was given 5 h before the blood sample or follicle measurements.. When each group was analyzed separately for PRL, there was a day effect for the sulpiride group ($P < 0.0001$) but not for the control group. Only the day effect (D) was significant for follicles. A circle around a PRL mean in the sulpiride group and around the means for number of follicles ≥ 15 mm combined for both groups indicate a change ($P < 0.05$) in direction, based on paired t-tests.

The number of follicles ≥ 15 mm from Day 13.0 to 21.5 also showed only a day effect. The day effect represented an increase ($P < 0.002$) in number averaged over the two groups between days 13.0 and 16.5 and then a decrease until Day 21.5 ($P < 0.006$). The interovulatory interval was not different between the Ct group (22.1 ± 0.6 d) and the Sp group (22.0 ± 0.5 d). There were no hemorrhagic anovulatory follicles (GINTHER et al., 2006) in either group.

Hourly concentration of P4 between Hours –3 and 8 on Day 14 had only an hour effect from decreasing concentrations in each group (Figure. 6.3). The hourly P4 concentrations did not change during Hours –3 to 0 in either group. The hourly concentrations of PRL during Hours –3 to 8 on Day 14.0 had significant main effects of group and hour, but no interaction (Figure. 6.3). The group effect was from greater overall concentration in the Sp group (10.1 ± 0.5 ng/mL) than the Ct group (5.0 ± 0.7 ng/mL). When each group was considered separately, an hour effect was found only for the Sp group ($P < 0.0001$). Hourly PRL concentrations decreased ($P < 0.0001$) between Hours –3 and 0 in the Sp group. An increase ($P < 0.003$) occurred between Hours 0 and 1, reached maximum at Hour 4, and then decreased so that the concentration at Hour 8 was not significantly different from the concentration at Hour 0; the difference between Hours 0 and 7 approached significance ($P < 0.06$). Concentrations of PGFM did not show an effect of hour or group or an interaction (Figure. 6.3). The concentrations of LH or FSH during Hours 0 to 8 did not differ for the main effects or for the interaction (not shown).

Figure 6.3 – Mean ± SEM concentrations of P4, PRL, and PGFM every hour from 3 h before to 8 h after treatment in controls and sulpiride-treated mares.

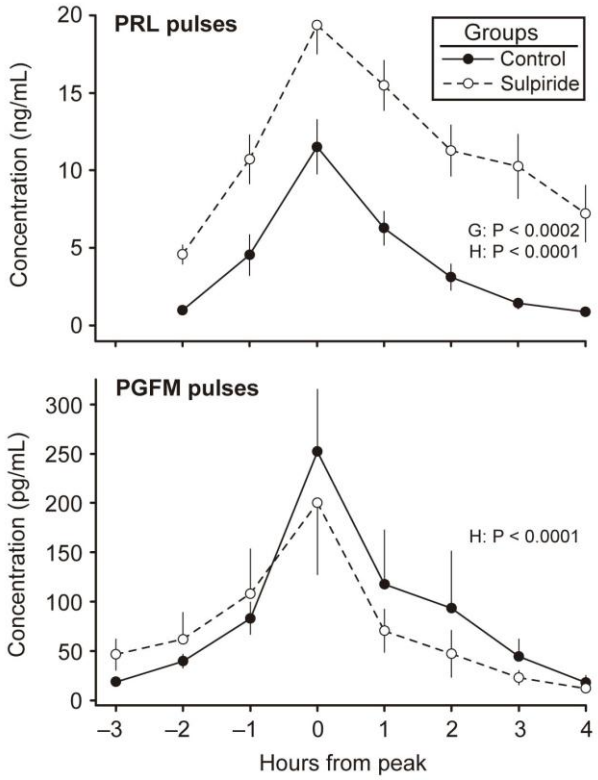


Significant effects from factorial analyses are indicated for the main effect of hour (H) and group (G); there were no interactions. There was an hour effect for PRL in the sulpiride group ($P < 0.001$) but not for the control group. A circle around a mean for PRL concentration indicates a change ($P < 0.05$) in direction.

Prolactin pulses during Hours 0 to 8 showed a greater overall concentration (group effect) in the Sp group (11.8 ± 0.8 ng/mL) than in the Ct group (5.1 ± 0.7 ng/mL; Figure. 6.4). There was no group-by-hour interaction. Discrete characteristics of PRL and PGFM pulses during Hours 0 to 8 are shown (Table 6.1). Concentrations of PRL were significantly greater in the Sp group than in the Ct group for the peak, Nadir 2, and amplitude; concentration at

Nadir 1 approached being greater and area under the curve was greater in the Sp group. The number of pulses/8 h and the interval from Nadir 1 to Nadir 2 were not different between groups (Table 6.1). The interval from Hour 0 to the peak of a PRL pulse was shorter ($P < 0.05$) for the Sp group (3.0 ± 0.4 h) than for the Ct group (4.3 ± 0.6 h). More PRL pulse peaks tended to occur during Hours 2 to 4 (8 pulses) than during Hours 5 to 7 (1 pulse) in the Sp group ($P < 0.08$). The eight versus one pulse in the Sp group differs ($P < 0.02$) from equality. The number was similar between Hours 2 to 4 (5 pulses) and Hours 5 to 7 (5 pulses) in the Ct group.

Figure 6.4 – Mean ± SEM concentrations during pulses of PRL and PGFM in controls and sulpiride-treated mares.



The hour effect (H) for each pulse was expected, owing to centralization to the pulse peaks. A group effect (G) was found only for the PRL pulses.

Pulses of LH and FSH were not detected during Hours 0 to 8 by the CV method. The number and characteristics of PGFM pulses did not differ between groups (Figure 6.4, Table 6.1). The number of PGFM pulses/8 h (1.6 ± 0.2) was greater ($P < 0.002$) than the number of PRL pulses (1.0 ± 0.2) for the Sp group. The number of PGFM pulses did not differ from the

number of PRL pulses in the Ct group. The interval from a PGFM peak to the closest PRL peak was longer (approached significance, $P < 0.08$) in the Sp group (1.0 ± 0.3 h) than in the Ct group (0.4 ± 0.2 h). The peak of a PRL pulse occurred at the same hour or 1 h later than the peak of a PGFM pulse in 8 of 8 PGFM pulses in the Ct group and 6 of 10 pulses in the Sp group ($P < 0.04$).

Figure 6.1 – Mean ± SEM characteristics of PRL and PGFM pulses during 8 h of hourly sampling after treatment in a control group (n = 9 mares) and a sulpiride group (n = 10 mares).

	PRL			PGFM		
Pulse end points	Control	Sulpiride	Prob.	Control	Sulpiride	Prob.
Number of pulses/8h	1.0 ± 0.2	1.0 ± 0.2	NS	1.2 ± 0.1	1.6 ± 0.2	NS
Concentrations (PRL, ng/mL; PGFM, pg/mL)						
Nadir 1[a]	3.3 ± 1.2	4.9 ± 0.7	P < 0.06	16.3 ± 2.1	21.7 ± 3.4	NS
Peak	11.5 ± 1.8	19.4 ± 1.9	P < 0.008	252.1 ± 63.0	200.4 ± 73.0	NS
Nadir 2[a]	2.4 ± 1.4	6.8 ± 1.9	P < 0.04	40.0 ± 18.1	27.3 ± 10.5	NS
Amplitude[b]	8.2 ± 2.1	13.9 ± 2.2	P < 0.009	240.1 ± 69.4	178.7 ± 71.3	NS
AUC (ng/mL/h)	17.4 ± 3.5	44.0 ± 6.2	P < 0.003	448.2 ± 160.6	363.9 ± 156.2	NS
Pulse base (h)[c]	5.2 ± 1.1	6.0 ± 0.6	NS	5.4 ± 1.0	5.1 ± 0.6	NS

[a] Nadir 1 is at the beginning of a pulse and Nadir 2 is at the end of a pulse.
[b] Concentration at peak minus at Nadir 1.
[c] Interval between Nadirs 1 and 2.
AUC = Area under curve
NS = Not significant

6.5 Discussion

The luteal transition between preluteolysis and luteolysis in mares is manifested within 1 h, based on hourly determination of P4 concentration (GINTHER; BEG, 2011a); luteal transition occurred between 2 PM and 2 AM in all of eight mares, and luteolysis encompassed 23 h. The 12-h of hourly blood sampling in the current study began at 2 PM with the expectation that the number of mares in luteolysis would be greater than for 2 AM to 2 PM. In the current study, 16 of 19 (84%) mares were in, began, or ended luteolysis during the 12-h of hourly sampling at 2 PM to 2 AM; 1 and 2 mares were in preluteolysis and

postluteolysis, respectively, during Hours 0 to 8. In addition, 7 of 8 mares that were in preluteolysis at 2 PM began luteolysis at 6, 7, or 8 PM. These findings support the report that luteal transition occurs with greater frequency between 2 PM and 2 AM than between 2 AM and 2 PM (GINTHER; BEG, 2011a). This favored the occurrence of luteolytic period at a more uniform time relative to treatment than if the hour of luteal transition was equally distributed among 24 h.

Hypothesis 1 —— that sulpiride treatment during the estrous cycle increases PRL concentration and the prominence of PRL pulses —— was supported. The support for PRL secretion was shown in the hourly samples collected on Day 14 from Hour 0 (treatment hour) to Hour 8 by the following: (1) PRL concentrations averaged over hours were greater in the Sp group and (2) PRL concentrations in the Sp group began to increase immediately to a maximum at Hour 4 and then decreased, so that Hour 6 was the last hour with concentration greater than the concentration at treatment. The duration of the sulpiride effect seemed similar to what was previously reported for the 25 mg dose during the estrous cycle (JOHNSON; BECKER, 1987). In the previous experiment, the sulpiride was given as a single bolus treatment, and the PRL concentrations were maximum at the first blood sample 15 min after treatment and then gradually decreased. In contrast, in the current study, the Sp group received four treatments at 8-h intervals before the treatment at Hour 0. Thus, it appears that a single sulpiride treatment induces an immediate PRL increase, whereas treatment after previous exposure to sulpiride induces a gradual increase over several hours. The comparison between the two reports suggests that a single treatment causes an immediate release of PRL from pituitary stores, whereas sequential treatments require additional time for synthesis of PRL. In the current study, PRL concentrations were greater in the Sp group than in the Ct group at Hour 0 and even greater at Hour –3; these elevated concentrations are attributable to the residual concentrations from previous treatments at 8-h intervals. Although no differences were found in the factorial analysis of PRL concentration at 12-h intervals in the two groups, a decrease occurred on the basis of a paired *t*-test between Days 13.0 and 16.0 in the Sp group. The concentration was greater on Day 13.0 in the Sp group than in the Ct group, and is attributable to the sulpiride treatment 5 h before the Day 13.0 blood sample.

The increase in the prominence of PRL pulses by sulpiride treatment is a novel finding in that previous sulpiride studies did not consider the pulsatility of PRL. The pulsatility of PRL in nontreated animals was demonstrated only recently in mares (GINTHER et al., 2012b) and heifers (GINTHER; BEG, 2011b). The greater prominence of PRL pulses in the Sp group was indicated by the greater means of all values in a pulse; by the greater

concentrations at the peak, Nadir 2, and amplitude; and by the greater area under the curve of a PRL pulse. The number of PRL pulses/8 h did not differ between Ct and Sp groups, but the distribution of the pulses was different. In the Ct group, pulses were distributed evenly between Hours 2 to 4 and Hours 5 to 7. In the Sp group, all pulses but one occurred during Hours 2 to 4. The decrease in number of pulses during Hours 5 to 7 likely reflected the length of the 5-h interpulse interval in heifers (GINTHER; BEG, 2011b).

Hypothesis 2 —— that an increase in PRL concentration affects PGFM concentration and pulses and P4 concentration —— was not supported. The hourly concentrations (Hours 0 to 8) of PGFM and the number and characteristics of PGFM pulses were not different between groups, despite the increased concentration and prominence of PRL pulses. The absence of PGFM stimulation during the PRL increase suggested that the synchrony between PGFM and PRL pulses (GINTHER et al., 2012b) represents a positive effect of PGF2α on PRL rather than an effect of PRL on PGF2α. A novel observation was the greater synchrony of PGFM and PRL pulses in the Ct group than in the Sp group. This was indicated by the occurrence of a PRL pulse peak on the hour of or after a PGFM pulse peak in all PGFM pulses in the Ct group, compared to 60% of pulses in the Sp group. That is, the greater prominence of PRL pulses in the Sp group partially interfered with the PGFM/PRL synchrony.

Progesterone concentration did not differ between groups during the hourly sampling on Day 14 in that the factorial analyses showed only day effect with no group effect or interaction. Furthermore, unpaired *t*-tests did not indicate a significant difference between groups for any day or hour. In addition, the day of the end of luteolysis (P4, < 1 ng/mL) was not different between groups. In addition, P4 concentration did not increase during a pulse of PRL. This result also failed to show a luteotropic effect of a PRL pulse of P4 in mares, unlike the positive intrapulse effect of an LH pulse on P4 in heifers (GINTHER et al., 2011a). Despite the presence of PRL receptors in the CL of mares (KING et al., 2010a), the current *in vivo* study did not demonstrate either a positive or a negative effect of PRL on the corpus luteum or P4 production.

Hypothesis 3 —— that repeated sulpiride treatment (every 8 h) maintains an increase in PRL and alters structural luteal regression, luteal blood flow, follicle development, and time of ovulation —— was not supported. The luteal end points decreased after Day 13 as expected during luteolysis, but with no effect of group or a group-by-hour interaction. The number of follicles > 15 mm, the diameter of the largest follicle, the length of the interval from the end of luteolysis to ovulation, and the length of the interovulatory interval were not altered by

sulpiride. The gradual increase in diameter of the largest follicle until Day 21.5 and the increase in number of follicles ≥ 15 mm between Days 13.0 and 16.5 followed by a decrease in number averaged over the two groups were consistent with the emergence and characteristics of the ovulatory follicular wave (JACOB et al., 2009). That is, there was no indication that sulpiride treatment altered the development of the ovulatory follicular wave. However, the CL and follicle portion of the hypothesis was not adequately tested, owing to the apparent absence of a PRL response to repeated sulpiride treatment. On the first day (Day 13) in the current study, the Sp treatment greatly increased PRL secretion, but the PRL concentrations gradually diminished at 12-h intervals despite each successive treatment. In this regard, a PRL increase might have been detected if hourly post treatment blood samples were taken after Day 14. This hypothesis therefore requires further consideration in a future study during the estrous cycle. The reduced response to repeated Sp treatments was also observed in an experiment during the anovulatory season (ZAGRAJCZUK et al., 2010); mares were treated daily and PRL decreased markedly from the first to the sixth day of treatment. During the transition from anovulatory to ovulatory seasons, sulpiride treatments (once or twice a day) maintained elevated PRL for 25 to 58 days (BESOGNET; HANSEN; DAELS, 1996; MARI et al., 2009; ZAGRAJCZUK et al., 2010). However, the doses were 8 to 20 times greater than the current dose.

The sulpiride treatment did not alter the hourly concentrations of LH and FSH, and pulses of these gonadotropins were not detected during Hours 0 to 8 in either group. In previous studies during the anovulatory season, sulpiride treatment did not alter the concentrations of LH (AURICH et al., 1995) or the concentrations of either LH or FSH (ZAGRAJCZUK et al., 2010). These studies have indicated that increasing the concentrations of PRL in mares does not affect the circulatory concentrations of gonadotropins on either Day 14 of the estrous cycle or during the anovulatory season.

In conclusion, treatment of mares with 25 mg of sulpiride on Day 14 increased the hourly concentration of PRL from the hour of treatment (Hour 0) until Hour 4. Concentrations then decreased and by Hour 7 were no longer greater than the concentration at Hour 0. During Hours 0 to 8, the concentrations of PGFM, P4, LH, and FSH were not altered by the increased PRL concentrations. Pulses of PRL occurred earlier in the sulpiride-treated group, and the synchrony between pulses of PGFM and PRL was partially disrupted. The increased PRL did not affect PGFM concentrations or pulses, indicating that the synchrony between PGFM and PRL pulses was not from an effect of PRL on PGFM. Repeated sulpiride treatment every 8 h from Day 13 to ovulation resulted in a diminishing PRL response. Blood sampling at 12-h

intervals did not detect a PRL increase after Day 13 and the CL area (cm^2) and blood flow, follicle development, and the interovulatory interval were not altered.

7 DISCUSSÃO GERAL

A presente dissertação teve como objetivo elucidar o papel da PRL em eventos reprodutivos nas espécies equina e bovina, com enfoque sobre a luteólise. Seguindo uma linha de raciocínio, cada trabalho testou hipóteses interligadas, encontrou achados inesperados, e elaborou novas hipóteses para futuros experimentos. Por meio da técnica não invasiva da ultrassonografia transretal, orgãos reprodutivos foram acessados e avaliados não-invasivamente tanto em relação à morfologia quanto à vascularização, utilizando-se dos modos B e Doppler, respectivamente. Além disso, foram feitas análises hormonais diversas comparando temporalmente as mudanças hormonais com alterações morfológicas e vasculares dos órgãos reprodutivos.

Pela primeira vez em éguas, foram descritos detalhadamente os pulsos de PRL durante as 24 h finais da pré-luteólise, por todo o período luteolítico, e 24 h iniciais da pós-luteólise (Capítulo 3). Além disso, foram analisados pulsos de PGFM, sendo observada uma interessante relação temporal entre PRL e PGFM. Pulsos de PRL apresentaram uma duração média de 5 h, intervalo entre pulsos de 7 h e intervalo entre picos de 12 h. Ritmicidade foi encontrada tanto em pulsos PRL quanto de PGFM em 67% e 89% das éguas, respectivamente. A proeminência dos pulsos de PRL e PGFM foi maior durante os períodos luteolítico e pós-luteolítico se comparados com o período pré-luteolítico, sendo a proeminência semelhante para os períodos luteolítico e pós-luteolítico. O aumento da proeminência dos pulsos de PRL e PGFM nos períodos luteolítico e pós luteolítico é sugestiva de uma relação tanto com o declínio ou baixa concentração de P4 quanto com o aumento ou alta concentração de estradiol.

A relação temporal entre os pulsos de PRL e PGFM foi indicada pela concentração máxima da PRL ocorrendo durante o pico dos pulsos de PGFM, sincronia entre picos de pulsos de PRL e PGFM, e a curta duração do intervalo entre o pico de PRL e PGFM não sincrônicos. No momento e após o pico do pulso de PGFM, mas não antes do pico, a concentração de PRL apresentou-se mais elevada. A sincronia entre os picos de PRL e PGFM foi maior nos períodos luteolítico e pós-luteolítico do que no período pré-luteolítico, ocorrendo na mesma hora em 50 e 65% das associações temporais. No período pré-luteolítico, somente 17% dos picos de PRL ocorreram na mesma hora do pico PGFM, enquanto que no período pós-luteolítico, em todas as associações temporais entre PRL/PGFM, os picos de PRL ocorreram na mesma hora ou 1 h após os picos de PGFM. A ocorrência do pico de PRL após

o pico de PGFM e o atraso de 1 a 2 h no declínio da PRL após um pico espontâneo de PGFM (GINTHER; BEG, 2009) são sugestivos de um efeito estimulador da PGF2α sobre a PRL.

Em novilhas, a fim detectar um possível papel da PRL sobre o processo de luteólise, foi feita a supressão da PRL no momento em que era detectado um primeiro sinal da regressão morfológica do CL (Capítulo 4). Esse trabalho foi o primeiro a utilizar o fármaco bromocriptina (Bc: agonista dopaminérgico) durante a luteólise em novilhas. Embora a Bc tenha um conhecido efeito inibidor da PRL (BEVERS; DIELEMAN, 1987; WILLIAMS; RAY, 1980; MILLS; LEMENAGER; HORSTMAN, 1989), a dose mínima efetiva para o período luteolítico não havia sido estabelecida, sendo necessário um experimento prévio de titulação para detectar a dose mínima e período médio de ação do fármaco. Em novilhas durante fase final de luteólise e periovulatória, a dose mínima de Bc para inibição da secreção de PRL foi de 16 mg, sendo a supressão feita a partir de 2 horas e mantida por até 12 horas. Entre 5 e 8 horas após o tratamento detectou-se um aumento da concentração de PRL, entretanto esse não foi maior do que a concentração anterior ao tratamento.

Para realizar o estudo durante o processo luteolítico foi necessário estabelecer um método para detecção imediata da ocorrência da luteólise. A avaliação da concentração de P4 mostrou-se inviável, devido á grande variação da concentração de P4 no transcorrer da luteólise e o longo intervalo para a realização da análise hormonal. Desse modo, foi proposta a mensuração da área do CL por ultrasonografia transretal a cada 8 h com início no dia 14 pós-ovulação (Dia 14). No momento em que a área do CL se reduziu em 12,5% da área do Dia 14 foram detectados 63% dos animais em luteólise, monstrando-se um grande avanço se comparado com a escolha de um dia do ciclo estral. O maior número de animais em luteólise se estabeleceu no Dia 16, entretanto este valor foi de apenas 24 %.

O tratamento com Bc foi eficaz na inibição da PRL desde a hora 1 à 12 após o tratamento. A proeminência dos pulsos de PRL apresentou-se reduzida tanto com relação ao pico quanto aos nadires. Do mesmo modo que a Bc, os três tratamentos com Flunixina Meglumina (FM) em intervalos de 4 horas inibiu de forma eficaz a secreção de PGF2α durante as 12 horas de coletas de sangue, suprimindo todos os pulsos de PGFM.

Suportou-se a hipótese de um efeito da PRL sobre a luteólise após a inibição da PRL. Embora não tenha sido observado efeito da inibição da PRL sobre a regressão morfológica do CL, ocorreu um retardo na queda de P4, compatível com um caráter luteolítico da PRL. Entretanto, durante a inibição da PRL ocorreu um aumento da proeminência dos pulsos de LH, sendo o efeito da PRL confundido pela conhecida ação positiva do LH sobre a P4. O efeito positivo do LH sobre a P4 foi revisado (GINTHER et al., 2011c; GINTHER et al.,

2011b; DONALDSON; HANSEL, 1965; QUINTAL-FRANCO et al., 1999). O LH tem a papel de aumentar a concentração da P4 após a depressão transitória causada pelo pico do pulso de PGF2α. Pulsos de LH e flutuações de P4 são sincrônicos, sendo que a inibição da secreção de LH resulta na supressão das flutuações de P4. Além disso, LH exógeno tem a capacidade de prolongar a atividade do CL e aumentar a secreção de P4.

O retardo na queda de P4 pode estar relacionado tanto com a redução da concentração de PRL quanto com o aumento da proeminência dos pulsos de LH. Desse modo, O aumento do LH após a inibição da PRL é compatível com um efeito negativo da PRL sobre o LH. A PRL foi anteriormente descrita como inibidora da secreção de LH em diversas espécies, sendo um exemplo disso os casos de infertilidade em mulheres e ratos com hiperprolactinemia. Em aves, baixas concentrações de PRL estão associadas à elevação dos níveis de LH, resultando em um aumento da produção de ovos. Entretanto, em bovinos o envolvimento de peptídeos opióides endógenos é mais suportado do que um efeito da PRL sobre a inibição de LH. Tanto em bovinos como em outras espécies, gonadotrofos e lactotrofos apresentam-se conectados funcionalmente, demonstrando um controle parácrino da PRL sobre o LH na pituitária. Esse controle aparenta ser feito, pelo menos em parte, diretamente sobre os neurônios de GnRH, o que pode estar presente em bovinos.

A inibição da secreção de PGF2α, um conhecido agente luteolítico, resultou em uma maior concentração média de P4 e um atraso na finalização da luteólise se comparada com o grupo controle. Tal resultado era esperado baseando-se em achados prévios sobre a PGF2α. A secreção episódica sequencial de PGF2α é necessária para a ocorrência da luteólise em várias espécies (GINTHER et al., 2009; GINTHER; SIDIQUI; BEG, 2009; SCHRAMM et al., 1983). Em períodos apropriados durante a luteólise, pulsos de PGF2α pequenos ou ausentes podem resultar em um aumento da produção de P4 por células luteais (SCHRAMM et al., 1983). A duração da luteólise é pelo menos em parte dependente da proeminência da secreção de PGF2α (PUGLIESI et al., 2012a; SCHRAMM et al., 1983).

A concentração de PRL decaiu após a inbibição da PGF2α, enquanto que a PGFM não foi afetada pela inibição da PRL, comprovando um efeito positivo da PGF2α sobre a PRL. Esse achado foi inédito e comprova a hipótese de que a PGF2α seria o hormônio estimulador da sincronia entre PGFM/PRL. A diferença nos padrões de secreção de PRL e o PGFM sugerem papéis específicos destes hormônios em diferentes estágios da luteólise. Pulsos de PRL são mais proeminentes durante as 12h finais da luteólise e 12h iniciais da pós-luteólise; enquanto que os pulsos de PGFM são mais proeminentes durante a luteólise se comparados com pulsos da pré ou pós-luteólise (GINTHER et al., 2010c).

Do mesmo modo que em vacas, a hipótese de um efeito estimulador da PGF2α sobre a PRL foi testado em éguas (Capítulo 5). Para estudar a interferência sazonal, o experimento foi realizado em duas etapas, no verão (Junho) e no outono (Setembro), utilizando-se de inibidores da PRL (Bromocriptina) e PGF2α (Flunixina Meglumina) no Dia 14 pós-ovulação. O grupo utilizado no outono estava, em média, bem próximo da estação anovulatória (22% das éguas não exibiram ciclicidade após o experimento). No momento do tratamento, 61% das éguas no verão e 28% das éguas no outono estavam em luteólise, indicando uma ocorrência mais antecipada da luteólise no verão. Contradizendo o achado, estudos anteriores (GINTHER , 1992; GINTHER , 1974; IRVINE; ALEXANDER; MCKINNON, 2000) mostraram um prolongamento do estro e encurtamento do diestro se comparado com ciclos da estação ovulatória. Mais estudos devem ser feitos para comprovar o achado.

Não houve difereça na secreção de PGF2α no verão ou outono, como já reportado (KING et al., 2010b), nem a ocorrência de CL persistente. Uma falha na secreção de PGF2α em período apropriado resulta em uma atividade mais prolongada do CL (KING et al., 1990; STABENFELDT; HUGHES, 1987; NEELY et al., 1979). Durante a transição da estação ovulatória para a anovulatória há uma alta incidência (20-25%) de CL persistente, associada a baixas concentrações de PRL (KING et al., 2011). Apesar da marcante redução nos níveis de PRL no outono pela bromocriptina, não houve ocorrência de CL persistente no presente estudo. Entretanto, a utilização de pôneis e pôneis cruzados ao invés de éguas, pode ter sido a causa da ausência de animais com CL persistente.

O tratamento com FM nas éguas foi eficaz em reduzir a secreção de PGF2α por 6 h, em uma dose de 1,7 mg/kg. O número de pulsos de PGFM foi menor entre as horas 2 a 5 do que 6 a 9 após o tratamento com FM; essa diferença não ocorreu após o tratamento com bromocriptina. Durante o verão (concentração de PRL mais elevadas), mas não no outono, a PRL também foi suprimida pelo tratamento com FM. A concentração de PRL retornou a níveis anteriores ao tratamento no fim das amostras de hora em hora, semelhante à perda do efeito negativo da FM sobre o PGFM. O presente estudo é, aparentemente, o primeiro a descrever mudanças na concentração e pulsos de PGFM e PRL em resposta a um único tratamento com FM durante os períodos pré-luteolítico e luteolítico em éguas.

A Bc reduziu tanto a concentração média durante as 10 h e durante cada pulso, quanto o número de pulsos de PRL. No verão, quando as concentrações de PRL apresentavam-se naturalmente mais altas, a supressão feita pela Bc se deu a partir de 4 h após o tratamento até o fim da amostragem (10 h após o tratamento). Pulsos de PRL durante o verão, quando comparado com o inverno, apresentaram concentração e proeminência maiores em todos os

grupos. No grupo controle, a concentração de PRL apresentou-se 77% mais elevada no verão quando comparado com o inverno

Suportou-se a hipótese em éguas de que a PGF2α seria o fator estimulador na relação temporal entre PGFM/PRL, semelhante ao observado em vacas. Houve uma redução tanto do PGFM quanto de PRL após inibição de PGF2α com FM; enquanto que a inibição de PRL com Bc não causou alteração na concentração de PGFM. Além disso, a marcante redução das concentrações de PRL no inverno não afetou as concentrações de PGFM, confirmando a neutralidade da PRL sobre a PGF2α. Com relação aos níveis de LH e FSH, não houve efeito da redução dos níveis de PRL. O poder estimulador da PGF2α sobre a PRL havia sido reportado anteriormente pela admnistração de PGF2α exógena em éguas (SHAND et al., 2000). Em novilhas, a redução dos níveis de PRL não afetou a concentração de PGFM, mas resultou em um aumento da proeminência dos pulsos de LH (PINAFFI et al., 2012; GINTHER et al., 2012c).

Além de estudar o papel da PRL durante o processo luteolítico, foi feito o estudo do papel da PRL sobre a foliculogênese e ovulação em éguas (Capítulo 6). A idéia surgiu de estudos prévios em que, durante a transição da estação anovulatória para a ovulatória, o aumento da concentração de PRL por antagonistas de dopamina (sulpirida ou domperidona) estimularam o desenvolvimento folicular e antecipam a primeira ovulação (BESOGNET; HANSEN; DAELS, 1996; MARI et al., 2009). Para o presente experimento foi escolhida a sulpirida (Sp), por estimular a secreção de PRL tanto em éguas em estro quanto em diestro (AURICH et al., 1995).

A parte do estudo voltada para o efeito do tratamento com Sp sobre a luteólise foi feito a partir de amostras de sangue de hora em hora coletadas entre 2 da tarde e 2 da manhã do Dia 14 pós-ovulação. A escolha do período se deve aos resultados de um experimento prévio em que (GINTHER; BEG, 2011a): a transição entre pré-luteólise e luteólise se manifestou em 1 h, ocorreu em média no Dia 14, e em 100% das éguas se deu entre 2 da tarde e 2 da manhã. No presente estudo, 84% (16 de 19) das éguas estavam em, iniciaram ou terminaram a luteólise durante as coletas de sangue de hora em hora; uma égua estava em pré-luteólise e duas éguas em pós-luteólise. Além disso, 88% (7 de 8) das éguas em pré-luteólise às 2 da tarde iniciaram a luteólise as 6, 7 e 8 da tarde. Esses achados suportam a informação de que a transição da pré-luteólise para luteólise ocorre com maior frequência entre 2 da tarde e 2 da manhã (GINTHER; BEG, 2011a). Essa característica de espécie equina favoreceu uma uniformidade de ocorrência da transição em relação ao tratamento, se comparado com igual distribuição da transição ao longo das 24 h do dia.

O resultado das análises de PRL a cada hora no dia 14 pós-ovulação desde o tratamento (Hora 0) até a Hora 8 mostrou que: (1) a concentração média de PRL foi maior no grupo tratado com Sp e (2) o aumento da concentração de PRL após o tratamento com Sp foi imediato, atingindo um valor máximo na Hora 4 seguido de um decréscimo, sendo que a Hora 6 foi a última hora com valores de concentração de PRL maiores do que no momento do tratamento. Desse modo, a hipótese de que o tratamento com Sp durante o ciclo estral aumenta a concentração de PRL e proeminência dos pulsos de PRL foi suportada. A duração do efeito da Sp foi similar a resultados prévios para 25 mg durante o ciclo estral (JOHNSON; BECKER, 1987), embora não tenha sido observado um aumento imediato (15 min), mas sim gradual. Diferentemente, o presente estudo avaliou mudanças na concentração de PRL após quatro administrações sucessivas de PRL (a cada 8 h), sugerindo que sugestivo que a administração de um único bolus de Sp induz um aumento imediato na PRL, enquanto que após exposição prévia o aumento da PRL induzido pela Sp torna-se gradual. Uma hipótese sugerida seria que um único tratamento causa uma liberação imediata de PRL dos estoques de PRL, enquanto que tratamentos sequenciais requerem um tempo adicional para síntese de PRL. Nas amostras a cada 12 h, apesar de não terem sido encontradas diferenças entre os dois grupos na análise fatorial da PRL, houve um declínio entre os Dias 13 e 16, baseado em um teste-*t* pareado. No Dia 13 a concentração de PRL foi maior no grupo tratado com Sp do que no grupo controle, resultado do tratamento 5 h antes da coleta da amostra de sangue.

O aumento da proeminência dos pulsos de PRL por tratamento com Sp é um achado novo, sendo o presente experimento o primeiro a considerar a pulsatilidade da PRL. A maior proeminência dos pulsos de PRL foi indicada pela média mais elevada em todos os valores de um pulso; pela maior concentração no pico, Nadir 2, e amplitude; e pela maior área abaixo da curva de um pulso de PRL. O número de pulsos de PRL/8h não diferiu entre os grupos controle e Sp, mas a distribuição dos pulsos diferiu. No grupo controle, pulsos foram distribuídos uniformemente entre as Horas 2 a 4 e Horas 5 a 7. No grupo Sp, todos os pulsos exceto um ocorreu durante as Horas 2 a 4. A diminuição do número de pulsos entre as Horas 5 e 7 possivelmente reflete a duração de 5 h de intervalo entre pulso em novilhas (GINTHER; BEG, 2011b).

Mais uma vez, como observado anteriormente tanto em éguas quanto em novilhas, foi demonstrado que a PRL não tem efeito sobre a secreção de PGFM e P4. Não houve estimulação da PGFM durante o aumento na concentração de PRL, suportanto que a PGF2α seria o estimulador da sincronia entre pulsos de PRL e PGFM (GINTHER et al., 2012b). Além disso, foi observada uma perda da sincronia entre PRL e PGFM no grupo tratado com

Sp. No grupo controle, todos os picos de PRL ocorreram na hora ou após o pico de PGFM, sendo que no grupo tratado com Sp, isso ocorreu em somente em 60% dos picos de PRL. Esse resultado sugere que o tratamento com Sp remeteu a uma estimulação da secreção de PRL, a qual era anteriormente feita por pulsos de PGF2α, ocasionando uma quebra da sincronia entre pulsos de PRL e PGFM. Não foi detectado efeito da estimulação da PRL sobre a secreção de P4 ou LH. Não foi suportado o possível papel da PRL sobre o CL, apesar da presença de receptores para PRL no CL de éguas (KING et al., 2010a).

Apesar da tentativa, a concentração de PRL não se manteve elevada durante todo o experimento, impossibilitando testar a hipótese de um efeito da estimulação da secreção de PRL sobre mudanças estruturais e vasculares do CL, desenvolvimento vascular, e momento da ovulação. Houve uma aparente ausência de resposta da secreção de PRL a sucessivos tratamento com Sp. A redução da resposta à Sp foi observada em experimento anterior com tratamentos diários durante a estação ovulatória (ZAGRAJCZUK et al., 2010), sendo notada uma marcante queda da concentração de PRL do primeiro ao sexto dia de tratamento. Experimentos prévios que demonstraram uma manutenção dos níveis elevados de PRL por 25 a 58 dias utilizando doses de 8 a 20 vezes mais altas do que o presente experimento (BESOGNET; HANSEN; DAELS, 1996; MARI et al., 2009; ZAGRAJCZUK et al., 2010).

Não houve efeito da estimulação dos níveis de PRL sobre LH e FSH, baseando-se nas amostras coletadas a cada hora. Semelhantemente, após tratamento com Sp, experimentos prévios não detectaram mudanças na concentração de LH (AURICH et al., 1995) ou LH e FSH (ZAGRAJCZUK et al., 2010). Desse modo, o papel da estimulação da PRL sobre o acelerado desenvolvimento folicular e antecipação da ovulação em éguas tratadas com Sp durante a transição da estação anovulatória para a ovulatória (BESOGNET; HANSEN; DAELS, 1996; MARI et al., 2009) ainda continua desconhecido e necessita de mais estudos.

8 CONCLUSÕES

A sequência de cinco trabalhos nos forneceu achados inéditos sobre a dinâmica hormonal durante o processo luteolítico nas espécies equina e bovina. Mais estudos são necessários para maiores detalhamentos sobre o papel da PRL no processo de luteólise. Importantes informações sobre o processo luteolítico e interrelações hormonais nesse período foram encontradas ao final desta dissertação. As principais conclusões obtidas em equinos e bovinos são:

8.1 Equinos

1- Na fase final de diestro, pulsos de PRL apresentam 5 h de duração, intervalo entre pulsos de 7h e intervalo entre picos 12 h.

2- Pulsos de PRL são mais proeminentes durante o período luteolítico e pós-luteolítico do que durante o período pré-luteolítico.

3- O aumento da concentração de PRL durante as horas de um pulso de PGFM demonstraram uma associação temporal entre a secreção de PRL e PGF2α, em especial após o período pré-luteolítico.

4- Um único tratamento com FM reduziu a concentração de PGFM por até 6 h, tanto no verão quanto no outono, reduzindo também as concentrações de PRL.

5- O tratamento com bromocriptina reduziu as concentrações de PRL, mas não afetou as concentrações de PGFM.

6- Concentrações de PRL foram aproximadamente três vezes mais elevadas no verão do que no outono, mas as concentrações e pulsos de PGFM não diferiram entre as estações.

7- O tratamento de éguas com 25 mg de sulpirida no Dia 14 aumenta a concentração de PRL desde a hora do tratamento até 4 h após (Hora 4), ocorrendo um posterior decréscimo, atingindo níveis semelhantes ao anterior ao tratamento na Hora 7.

8- O aumento da PRL não afeta a concentração de P4, LH e FSH.

9- Em éguas tratadas com sulpirida, pulsos de PRL ocorrem antes e a sincronia entre pulsos de PGFM e PRL é parcialmente perdida.

10- O aumento da PRL não afetou a concentração ou pulsos de PGFM, indicando que a sincronia entre pulsos de PGFM e PRL não são originárias de um efeito estimulador da PRL sobre a PGF2α.

11- Os resultados indicam que a associação temporal entre os aumentos de PGFM e PRL representam um efeito da PGF2α na PRL ao invés de um efeito da PRL na PGF2α.

12- Tratamentos repetidos com sulpirida a cada 8 h, iniciando no Dia 13 até o momento da ovulação, resultam numa diminuição da resposta da hipófise na secreção de PRL.

13- Coletas de sangue a cada 12 h não detectaram aumentos na concentração de PRL por tratamentos frequentes com sulpirida após o Dia 13, sendo que os efeitos da PRL na área (cm^2) e fluxo sanguíneo (%) do CL, foliculogênese e intervalo entre ovulações não puderam ser testados.

8.2 Bovinos

1- A mensuração da área do CL foi um método mais eficiente do que o número de dias após a ovulação para detectar a luteólise funcional em novilhas.

2- Um único tratamento com 16 mg de bromocriptina durante a luteólise inibiu concentrações de PRL eficientemente por 12 h.

3- O tratamento com bromocriptina não apenas reduziu as concentrações de PRL, mas também se associou temporalmente a aumentos na proeminência de pulsos de LH, atraso na queda de P4 e atraso na conclusão da luteólise, sem efeitos na secreção de PGF2α.

4- Devido ao aumento do LH concomitante à depressão na PRL, não foi possível determinar o efeito da PRL na função do CL.

5- A inibição da PGFM com flunixina meglumina foi temporalmente associada com uma queda na concentração de PRL, indicando um efeito positivo da PGF2α na secreção de PRL, ao invés de um efeito da PRL na PGF2α.

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1 INTRAOVARIAN RELATIONSHIP OF FUTURE OVULATORY FOLLICLE AND CORPUS LUTEUM AND EFFECT OF A PROLACTIN INHIBITOR (BROMOCRIPTINE) ON VASCULAR PERFUSION AND FUNCTION OF THE CORPUS LUTEUM IN HEIFERS

1.1 Synopsis

The effect of location of the CL and future ovulatory follicle in the same versus opposite ovaries and the effect of bromocriptine on progesterone (P4) concentrations were studied in controls (n = 15) and bromocriptine-treated heifers (n = 15). The interval from Day 16 to the end of luteolysis (P4 < 1 ng/ml) was less in control heifers with the CL and future ovulatory follicle in the same ovary compared to opposite ovaries. The bromocriptine (16 mg/heifer) was given every 8 h from Days 16 to 19 (Day 0 = ovulation). A blood sample was taken before each treatment. Prolactin (PRL) was reduced to the level of assay sensitivity from the second treatment on Day 16 through Day 21. A bromocriptine-induced P4 concentration decrease occurred in heifers that were in preluteolysis on Day 16 but not in heifers that had already begun luteolysis. The first eight heifers in each group were also examined every 8 h by color-Doppler ultrasound to determine the resistance index (RI) in an artery at the periphery of the corpus luteum (CL). The P4 concentration was lower and RI was greater (lower vascular perfusion) in the bromocriptine group than in the controls 24 h before the end of luteolysis when the CL and follicle were in opposite ovaries. Presumably, the observed bromocriptine-induced decrease in P4 was exerted through a reduction in PRL, which tentatively indicated a luteotropic role of PRL in heifers. Results supported the following hypotheses: (1) the location of the CL and the future ovulatory follicle in the same ovary is associated with an earlier occurrence of luteolysis and (2) bromocriptine has an effect on the CL.

1.2 Introduction

Within a few days after ovulation, the corpus luteum (CL) develops peripheral networks or wreaths of arterial, venous, and lymphatic vessels (Hunter RHF, 2003). The timely functional demands upon the CL necessitate rapid development and regression of the extensive luteal vascular system. The vascular dynamics of the ovary containing the CL have been studied in cattle, using the noninvasive technique of transrectal color-Doppler ultrasonography (GINTHER, 2007). The percentage of luteal area with color-Doppler signals of blood flow increases in parallel with the increase in CL dimensions and plasma progesterone concentrations (GINTHER, 2007; ACOSTA et al., 2003). The hemodynamics of luteal blood flow is a consideration in studies that involve luteal maintenance and regression. Increases and decreases in luteal blood flow are associated temporally with individual pulses of a PGF2α metabolite (PGFM) during spontaneous luteolysis (GINTHER et al., 2007). Blood flow increases during the ascending portion of a PGFM pulse, remains elevated for 2 h after the PGFM peak, and then decreases.

A decrease and rebound in progesterone (P4) and an LH pulse occur during the hours of each PGFM pulse and are associated with elevated luteal blood flow in cattle (GINTHER et al., 2012a). Inhibition of LH and infusion of PGF2α to simulate a PGFM pulse have indicated that the P4 rebound is enhanced by the LH pulse (GINTHER et al., 2011b). The relationships of luteal blood flow to luteal function and regression and to LH concentrations have been reviewed (GINTHER et al., 2012a).

Prolactin (PRL) is another hormone that may have an effect on the CL. The biochemical characteristics of PRL and regulation of PRL secretion (GREGERSON, 2006) and the role of PRL in the regulation of the CL in various species (Stouffer RL, 2006; MCNEILLY, 1980) have been reviewed. Prolactin is a constituent of the luteotropic complex in rodents and carnivores (MURPHY; RAJKUMAR, 1985), may be part of the luteotropic complex in swine (CIERESZKO et al., 2002), and may (KANN; DENAMUR, 1974) or may not (LOUW et al., 1974) have a luteal role in sheep. In cattle, binding sites for PRL (POINDEXTER et al., 1979) and messenger RNA for PRL ligand and for PRL receptors (SHIBAYA et al., 2008) have been demonstrated in the CL. Protein for PRL is immunohistochemically detectable in the endothelial cells and smooth muscle cells of the intraluteal arterioles (SHIBAYA et al., 2008). In addition, the CL produces PRL and antiangiogenic PRL fragments, which may mediate angioregression during luteolysis (ERDMAN et al., 2007). Prolactin as well as LH augments P4 production in vitro in perfused luteal ovaries from cattle (BARTOSIK et al., 1967). However, despite considerable in vitro support consistent with a role of PRL in the function or structure of the bovine CL, there

apparently has been no in vivo research support that PRL is either luteotropic or luteolytic in cattle.

Bromocriptine is a dopamine receptor agonist and is a potent PRL inhibitor that is used to treat hyperprolactinemia in women (THORNER et al., 1981) and is an efficient PRL inhibitor in cattle (BEVERS; DIELEMAN, 1987; WILLIAMS; RAY, 1980; PINAFFI et al., 2012). Administration of bromocriptine twice each day during the estrous cycle did not affect concentration of P4 or length of the luteal phase (BEVERS; DIELEMAN, 1987). In a recent study, treatment with bromocriptine during luteolysis resulted in a decrease in PRL and an increase in LH and P4 within 2 h (PINAFFI et al., 2012). The LH increase obscured the effects of reduced PRL on the increasing P4, owing to the previously demonstrated positive effect of LH pulses on P4 fluctuations (GINTHER et al., 2011b; SHRESTHA et al., 2011; GINTHER et al., 2011c).

Preliminary observations [unpublished] in heifers indicated, unexpectedly, that the length of the interovulatory interval was shorter when the CL and future ovulatory follicle were in the same ovary (20.4 ± 0.7 d) than when the two structures were in opposite ovaries (22.5 ± 0.7 d). If confirmed, studies of the luteal phase and luteolytic mechanism should consider the potential confounding effects of the locations of the CL and future ovulatory follicle.

The current experiment in cattle was done to test two hypotheses. Hypothesis 1 was that the location of the CL and the future ovulatory follicle in the same ovary is associated with an earlier occurrence of luteolysis. Hypothesis 2 was that bromocriptine has an effect on the CL and ovulatory follicle. The relationships of P4 concentration to luteal and ovarian hemodynamics and the effect of bromocriptine on luteal blood flow were also considered.

1.3 Materials and Methods

1.3.1 Animals and management

Dairy heifers (Holsteins) aged 16 to 20 mo and weighing 400 to 500 kg were used. Only natural estrous cycles were used, without induced luteolysis or induced or synchronized ovulations. Management of heifers, including housing and feeding (GINTHER et al., 2010c;

PALHAO et al., 2009), transrectal ultrasonic monitoring of follicles and CL, and ultrasonic ovulation detection (GINTHER, 1998) have been described. If more than one CL was present or the single CL was undersized (< 2.5 cm2) on Day 15 (Day 0 = ovulation), the heifer was not used. Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research.

1.3.2 Protocol

The heifers were assigned randomly to controls (Ct group; n = 15) and to bromocriptine treatment (Bc group; n = 15). The concentrations of progesterone were determined for all heifers, but concentrations of PRL and LH also were determined for the first 8 of 15 heifers in each group. In addition, the first eight heifers in each group were studied for the effect of location of the CL and future ovulatory follicle in the same versus opposite ovaries on CL function and vascular perfusion.

Bromocriptine (2-bromo-α-ergocriptine methanesulfonate salt, catalog # B2134; Sigma Aldrich Co, St. Louis, MO) was dissolved in absolute ethyl alcohol, and an equal volume of saline was added to make a solution of 3.2 mg/ml. The bromocriptine treatment was given on Days 16 through 19 every 8 h at 8 AM, 4 PM, and 12 AM (midnight). For intervals that used a day scale, 8 AM, 4 PM, and 12 AM for Day 16, for example, were designated Day 16.0, 16.3, and Day 16.7, respectively. The dose at each treatment was 16 mg/heifer and was based on a titration study (PINAFFI et al., 2012). Blood samples were collected every 8 h immediately before each treatment for P4, LH, and PRL assays. The P4 concentration at the first sample on Day 16.0 was affected by the luteal phase (preluteolytic or luteolytic) and by locations of the CL and follicle in the same or opposite ovaries. Therefore, the subgroup designations of Ct-same, Ct-opposite, Bc-same, and Bc-opposite were used to indicate the treatment group and the location of the CL and future ovulatory follicle in the same ovary or opposite ovaries. Data were centralized to the first 8-h sample that indicated the end of luteolysis (P4 < 1 ng/ml) and was designated Hour 0 (GINTHER et al., 2010c; MANN; LAMMING, 2006). A more accurate determination of the beginning and end of luteolysis would have required hourly sampling (GINTHER, 2012a) and was not attempted. Centralizing to P4 < 1 ng/ml was done so that all heifers were at a common 8-h interval in the luteolytic process.

For the first eight heifers in each group, the CL ovary was examined by gray-scale and color-Doppler ultrasound every 8 h for determining the luteal area (cm2) and vascular end points. The heifers were sedated lightly with 14 mg/heifer (i.m.) of xylazine hydrochloride (AnaSed Injection, Akorn Inc., Decatur, IL) to facilitate luteal blood-flow percentage estimates and spectral analysis for resistance index (RI) of arterial blood flow. Xylazine sedation reportedly produces hemodynamic effects when assessed in a major artery (e.g., internal iliac) but does not affect local vascular perfusion in the ovaries (ARAUJO et al., 2009). A duplex B-mode (gray-scale) and pulsed-wave color-Doppler ultrasound instrument (Aloka SSD 3500; Aloka American, Wallingford, CT) equipped with a linear-array 7.5 MHz transducer was used.

The CL area (cm2) was determined in B-mode at the maximal still-image plane, using the scanner's tracing function. Power-Doppler mode was used for estimation of luteal blood flow. The percentage of CL with blood-flow color signals was estimated from the real-time sequential two-dimensional planes of the entire CL as described (GINTHER, 2007; GINTHER et al., 2007). The subjective estimation of percentage of CL with blood-flow signals as an end point in cattle has been described and validated for CL by independent operators and objectively by using colored pixels in still images (GINTHER, 2007; GINTHER et al., 2007; ARAUJO et al., 2009). In addition, the percentage estimate in the current study was supported by a positive correlation ($r = 0.9$, $P < 0.0001$) between the in vivo percentage estimate for 45 evaluations in four heifers and counting the percentage of pixels with color-Doppler signals on film (GINTHER, 2007). Spectral Doppler examinations for RI were done by placing the sample cursor or gate (1-mm wide) on the most prominent color-flow signal at the periphery of the CL (RI-ovary) and on the most prominent signal in the ovarian pedicle within 1 cm of the CL ovary (RI-pedicle). If the largest follicle and CL were in apposition, the examination was done at the nonapposed periphery of the CL. A lower RI indicates greater arterial blood flow to the tissues upstream from the RI determination [2]. The ultrasound and P4 end points were considered after partitioning the heifers into control and bromocriptine groups and into location of the CL and the future ovulatory follicle in the same or opposite ovaries.

Hormone assays

Blood samples were collected into heparinized tubes and immediately placed into ice water for 10 min before centrifuging (2000 X g for 10 min). The plasma was decanted and stored (−20 ºC) until assay. The assays have been validated and described for bovine plasma in our laboratory for the following hormones and procedures: P4, solid-phase Coat-A-Count

RIA (GINTHER et al., 2007); LH, RIA (PALHAO et al., 2009; HANNAN et al., 2010); and PRL, RIA (SHRESTHA et al., 2010). The intra- and interassay and sensitivity, respectively, were as follows: P4, 5.2%, 1.6%, and 0.03 ng/ml; LH, 8.8%, 4.6%, and 0.02 ng/ml; PRL, 9.5%, 3.2%, and 0.22 ng/ml.

1.3.3 Statistical analyses

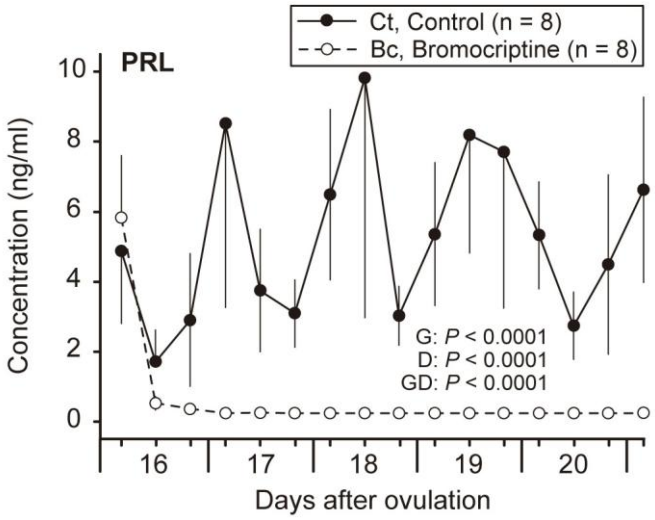
Data that were not normally distributed were transformed to natural logarithms or ranks. The statistical analyses were done by using SAS PROC MIXED (Version 9.2; SAS Institute Inc., Cary, NC, USA) with a REPEATED statement to minimize autocorrelation between sequential measurements. Differences between groups at each examination at 8-h intervals were determined by Student's unpaired *t*-tests. The least significant difference was used to locate differences among hours within groups and Student's paired *t*-tests were used for selected comparisons and for discrete characteristics. Chi square or Fisher's exact test was used for comparison of frequency data among groups. Pearson correlation analysis was used for the comparison between the real-time estimate and the pixel analysis of percentage of CL with blood-flow signals. A probability of $P \leq 0.05$ indicated a difference was significant, and a probability of $P > 0.05$ to ≤ 0.1 indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

1.4 Results

Concentrations of PRL at 8-h intervals for the first eight heifers in each group involved significant effects of group, day, and a group-by-day interaction (Figure. 1); the probabilities for the factorial analyses are given in the figures. The interaction reflected no difference between groups at the first sample on Day 16.0 and a lower ($P < 0.05$) concentration thereafter in the Bc group than in the controls. A reduction in PRL concentration to 0.23 or 0.24 ng/ml occurred by the first sample 8 h after treatment in 67% of heifers and by the next sample in the remaining heifers. The reduced PRL concentration in

each heifer in the Bc group was at the assay sensitivity level consistently from Day 16.7 to Day 20.7.

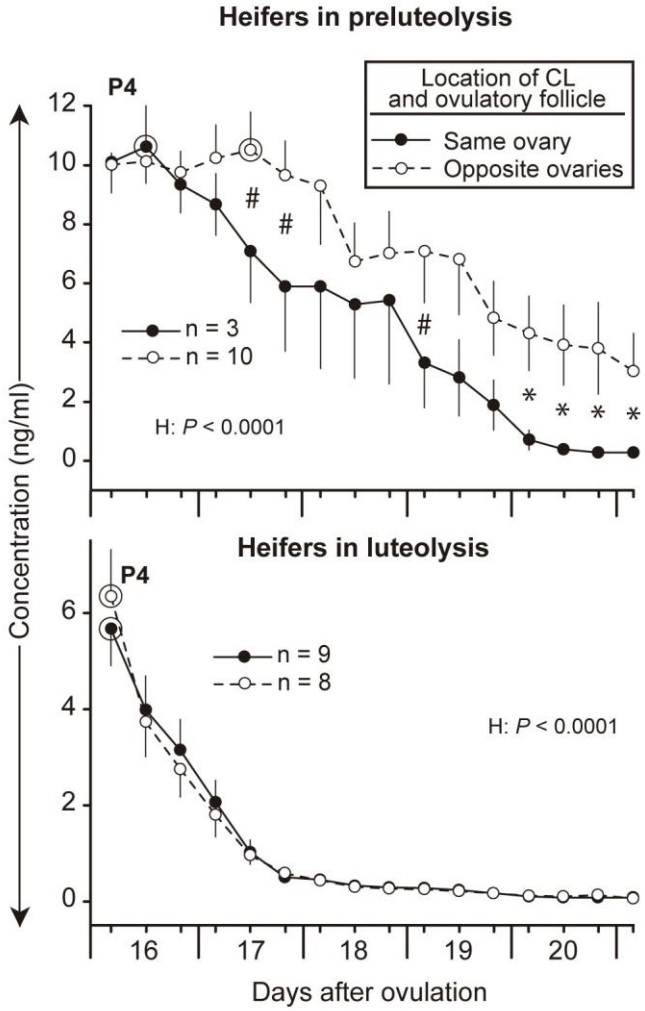
Figure 1 – Mean ± SEM concentrations of PRL in control heifers and in heifers treated with bromocriptine every 8 h from Day 16.0 until Day 19.7.



Concentrations in the Bc group remained at the level of assay sensitivity from Day 17.0 until Day 21.0. Probabilities for main effects (G, group; D, day) and the interaction (GD) that were significant are shown.

For the 15 heifers in the control (Ct) group, the P4 concentration before treatment on Day 16.0, the length of the interval from Day 16.0 to P4 < 1 ng/ml, and the interval between ovulations were significantly less in the heifers with the CL and future ovulatory follicle in same ovary than in opposite ovaries (Table 1). The interval from the first sample with a P4 concentration of < 1 ng/ml to ovulation was not affected by location of the two structures. Eight of 15 control heifers were in luteolysis at the first blood sample on Day 16.0, and more ($P < 0.02$) heifers were in luteolysis when the CL and follicle were in the same ovary (5 of 5) than in opposite ovaries (3 of 10). The number of heifers in luteolysis at the first treatment was not different between the Bc group (10 of 15) and the Ct group (8 of 15). For the 15 heifers in the Bc group, the length of the interval from Day 16.0 to ovulation and the interval between ovulations were not different between the same and opposite locations of CL and future ovulatory follicle. However, when CL and follicle were in the same ovaries, the length of the interval from Day 16.0 to a P4 concentration of < 1 ng/ml was longer ($P < 0.05$) in the Bc group (2.2 ± 0.4 d) than in the Ct group (0.9 ± 0.2 d).

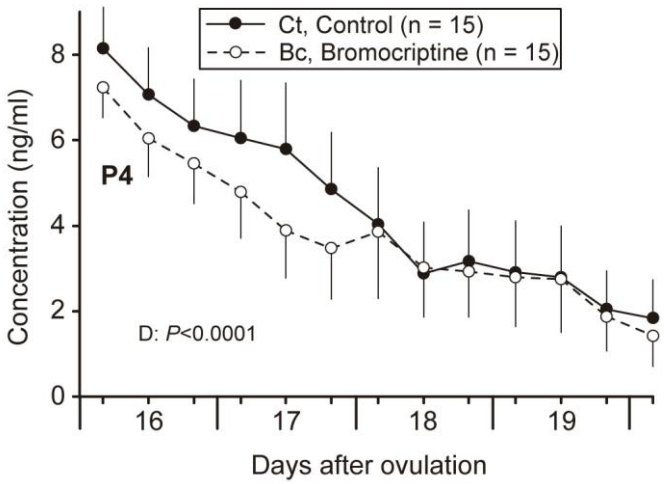
Figure 2 – Mean ± SEM concentrations of P4 from Days 16.0 to 21.0 when the location of the CL and future ovulatory follicle were in the same or opposite ovaries for heifers that were in preluteolysis (upper panel) or luteolysis at the first sample on Day 16.0.



The probability for a main effect of hour (H) is shown. An asterisk (* $P < 0.05$) or hatch mark (# $P < 0.1$) indicate the probability of a difference between locations at the indicated time. A circle around a mean indicates the first sample that was followed by a significant progressive decline in P4 concentration.

Concentrations of P4 beginning on Day 16.0 for the same and opposite CL/follicle locations during preluteolysis and during luteolysis had only the expected main effect of hour for each luteal phase (Fig. 2). Although an interaction of location and hour at 8-h intervals was not obtained during the preluteolytic phase, *t*-tests indicated concentrations were less ($P < 0.05$) when the two structures were in the same ovary on Day 20, and approached being less ($P < 0.1$) during Days 17 and 19. The concentration of P4 began to decrease ($P < 0.02$) on Day 16.3 when the CL and follicle were in the same ovary and on Day 17.3 ($P < 0.04$) when in opposite ovaries. No differences in P4 concentration between the same and opposite locations were found during luteolysis.

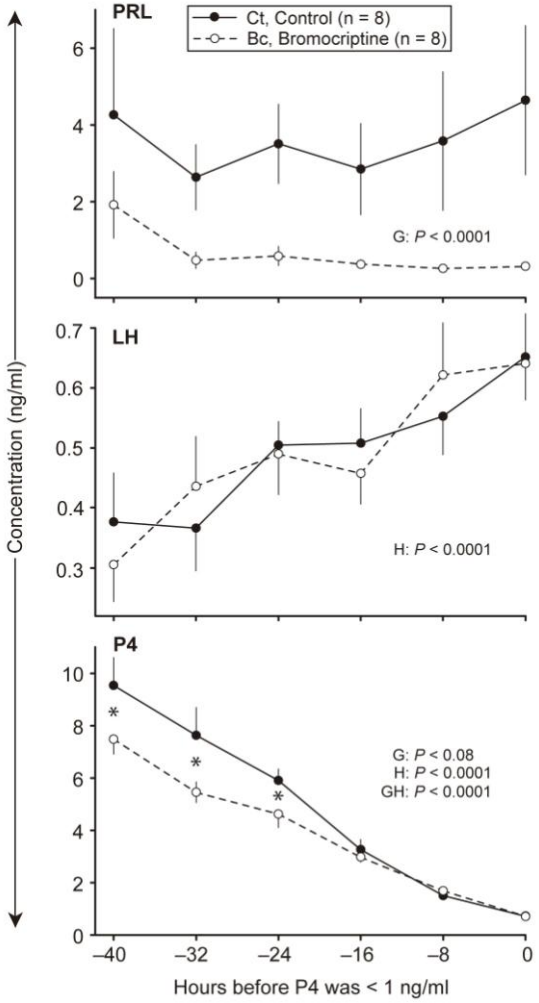
Figure 3 – Mean ± SEM concentrations of P4 for control (Ct) and bromocriptine-treated (Bc) groups, without regard to luteal phase at the first sample on Day 16.0 or to the location of the CL and future ovulatory follicle in the same versus opposite ovaries.



Probability of an effect of day (D) is shown. There was no main effect of group or an interaction of group and day nor were there any differences between groups within a day by unpaired *t*-tests.

Concentrations of P4 decreased over Days 16.0 to 20.0 but the effect of group (control and bromocriptine-treated) and the group-by-hour interaction did not approach significance (Fig. 3). There were no differences between groups within each hour when *t*-tests were used. However, there was a disparity among the four subgroups (Ct-same, Ct-opposite, Bc-same, Bc-opposite) in P4 concentration on Day 16.0. Therefore, the 8-h increment at the end of luteolysis (P4 < 1 ng/ml) was used as the reference point (Hour 0), and data were analyzed, retroactively. Only the group effect was significant for PRL concentrations over Hours –40 to 0 (Fig. 4); concentrations were greater in the Ct group (3.6 ± 0.6 ng/ml) than in the Bc group (0.6 ± 0.2 ng/ml). Only the hour effect was significant for LH, owing to a progressive increase combined for the two groups between Hours –40 to 0. For P4 concentration, the hour effect and the interaction of group and hour were significant. The interaction was primarily from lower ($P < 0.05$) concentration in the Bc group than in the Ct group at Hours – 40, –32, and –24, but not during hours –16 to 0.

Figure 4 – Mean ± SEM concentrations of PRL, LH, and P4 in control (Ct) and bromocriptine-treated (Bc) groups retroactively from the end of luteolysis (P4 < 1 ng/ml).

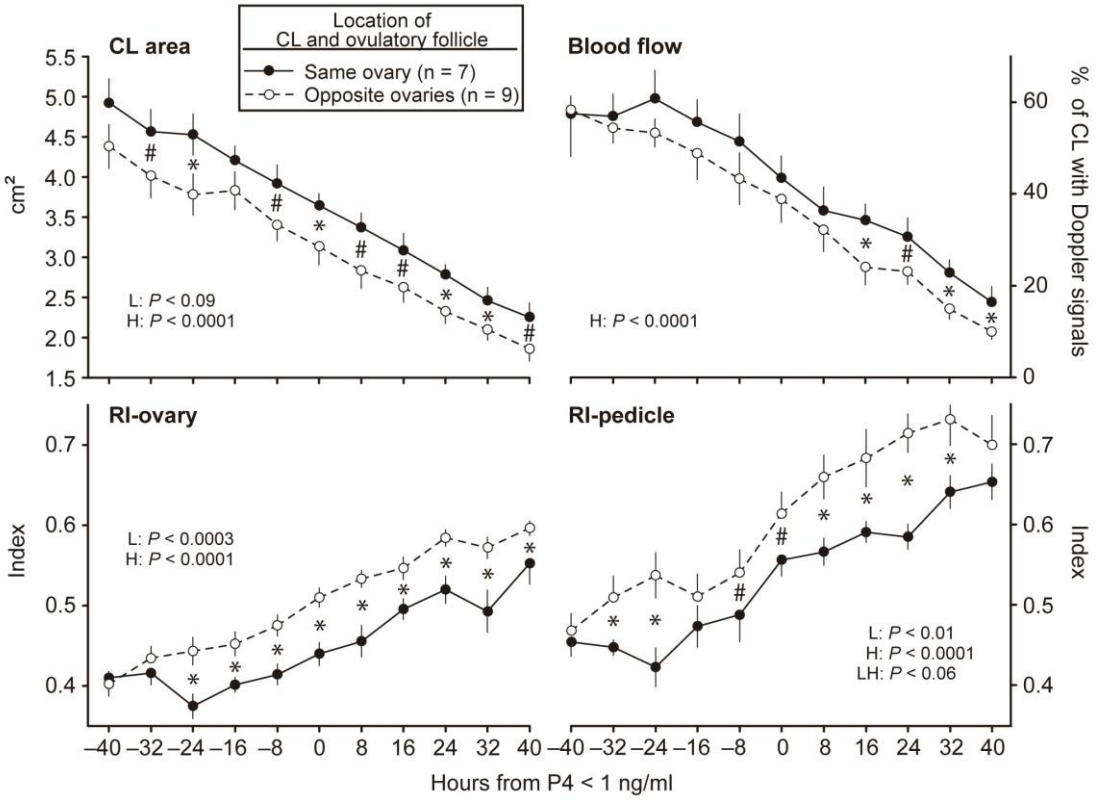


Probabilities for main effects (G, group; H, hour) and the interaction (GH) that were significant or approached significance are shown. An asterisk (*) indicates an hour of a difference ($P < 0.05$) between groups.

The effects of CL/follicle location (CL and future ovulatory follicle in the same or opposite ovaries) on luteal area (cm2) and luteal vascular perfusion retroactive from the hour at the end of luteolysis are shown for Hours –40 to 40 (Fig. 5). The effect of CL/follicle location on CL area approached significance from a greater area for the same versus opposite locations (3.5 ± 0.1 cm2 vs. 3.1 ± 0.1 cm2). The location effect was significant for RI-ovary and for RI-pedicle. For each site of RI determination, the RI was lower when CL/follicle location was in same ovary than in opposite ovaries (RI-ovary, 0.46 ± 0.01 vs. 0.50 ± 0.01; RI-pedicle, 0.54 ± 0.01 vs. 0.60 ± 0.01). The interaction of location and hour was not significant for CL area, percentage of CL with blood-flow Doppler signals, RI-ovary, and RI-

pedicle, but the hours of a difference between groups based on *t*-tests are shown on a preliminary basis (Fig. 5).

Figure 5 – Mean ± SEM area (cm2) of CL and vascular perfusion end points when the CL was located in the same or opposite ovaries as the future ovulatory follicle. Data are centered on the hour of the end of luteolysis (P4, < 1 ng/ml).

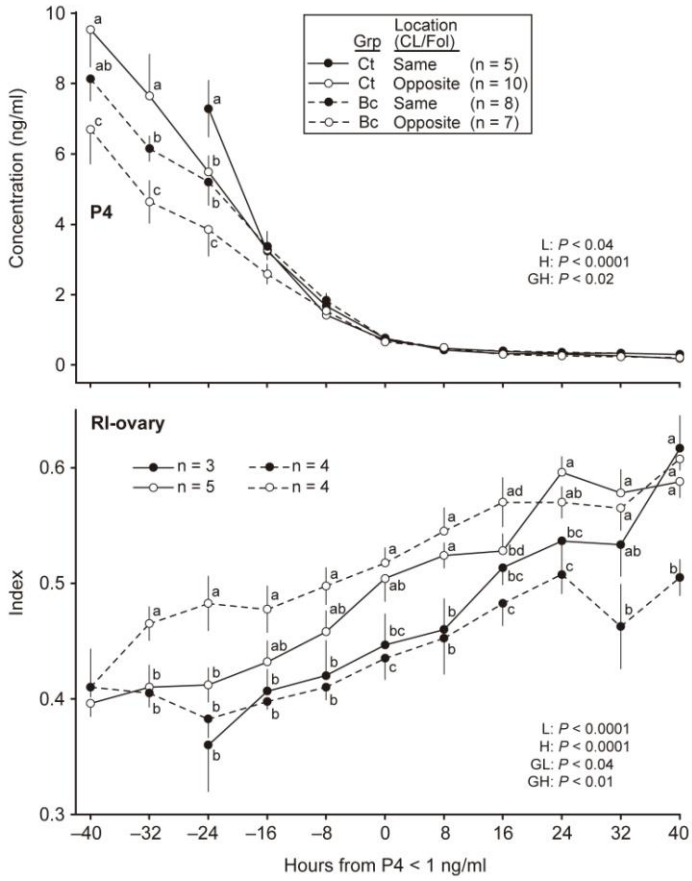


Probabilities for the main effects of location (L) and hour (H) and the interaction of location and hour (LH) are shown for each end point. An asterisk (*; $P < 0.05$) or hatch mark (#; $P < 0.1$) indicate the probability of a difference between locations at the indicated hour.

The P4 concentration in the four subgroups for Hours –40 to 40 is shown for all heifers, and the index for RI-ovary is shown for the first eight heifers in each group (Fig. 6). The analyses were limited by inadequate number of heifers in the Ct-same subgroup, and therefore the factorial analysis excluded Hours –40 and –32. For P4, the main effect of location was significant for Hours –24 to 0 and reflected greater ($P < 0.05$) concentration when the two structures were in the same ovary (2.8 ± 0.3 ng/ml) than in opposite ovaries (2.4 ± 0.2 ng/ml). A significant group-by-hour interaction was primarily from lower ($P < 0.05$) P4 concentration in the Bc group than in the Ct group at Hour –24 but not at Hours –16, –8 and 0. Although the effect of CL/follicle location or interactions that involved location for Hours –

24 to 0 did not approach significance, P4 concentration at Hour –24 was greater for location in the same ovary than opposite ovaries for each group. Bromocriptine reduced the P4 concentration compared to controls for each CL/follicle location (same vs. opposite ovaries). Concentration of P4 was available for the opposite location in each group for Hours –40 and –32, and for each hour the concentrations were different ($P < 0.05$) among subgroups as shown.

Figure 6 – Mean ± SEM concentrations of P4 and resistance index (RI) for an artery at the periphery of the CL before P4 reached < 1 ng/ml (Hour 0) in control (Ct) and bromocriptine-treated (Bc) heifers.

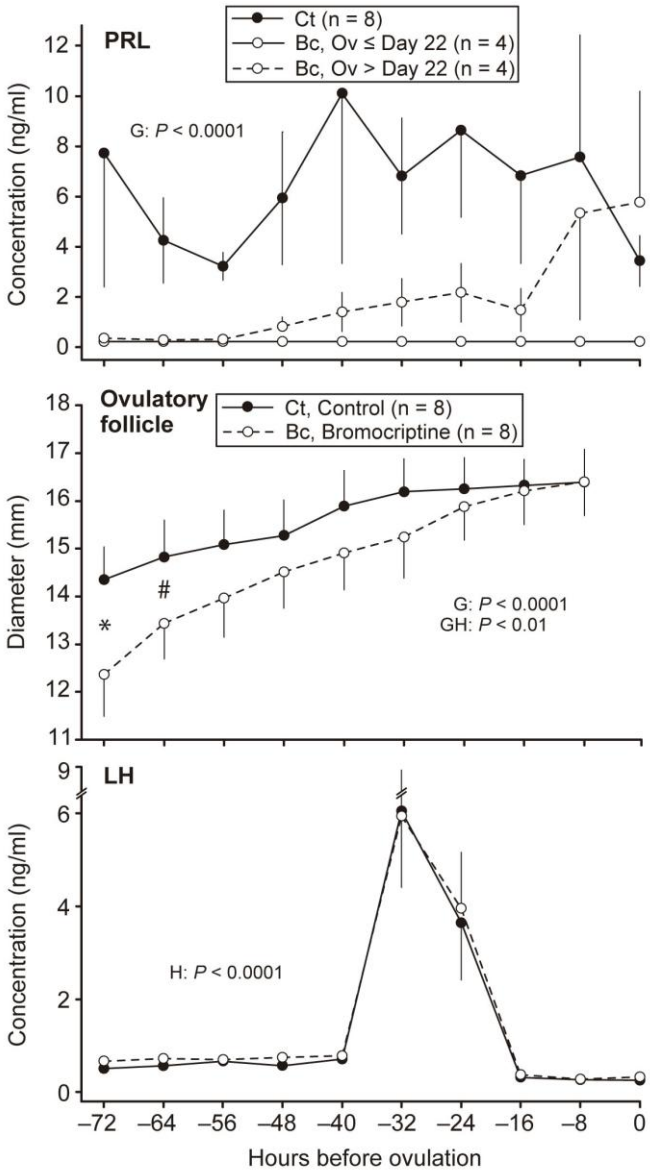


Heifers are separated by treatment group and location of the CL and future ovulatory follicle in the same or opposite ovaries. A three-way factorial analysis for group, location, and hour and their interactions was done for Hours –40 to 0 for P4 and Hours –40 to 40 for RI-ovary. Probabilities that were significant for a main effect of CL/follicle location (L) and hour (H) and interactions of group by hour (GH) and group by location (GL) are shown. abcd = means with a different letter at a given hour are different ($P < 0.05$).

The RI-ovary during Hours –40 to 40 had significant main effects of location and hour and interactions of group by location and group by hour (Fig. 6). The main effect of location was from a lower RI when the CL and follicle were in the same ovary (0.46 ± 0.01) than in

opposite ovaries (0.52 ± 0.01). The interactions reflected variation in differences among groups and subgroups at each hour as shown.

Figure 7 – Mean ± SEM concentrations of PRL, diameter of ovulatory follicle, and concentrations of LH for control (Ct) and bromocriptine-treated (Bc) groups.



For PRL, the Bc group is separated into heifers that ovulated on or before Day 22 and heifers that ovulated after Day 22. Probabilities for an effect of group (G), hour (H), or a group-by-hour (GH) interaction are shown for each panel

Concentrations of P4 for 56 h after the beginning of postluteolysis were not different between the Ct and Bc groups (not shown). The concentrations decreased to a mean of 0.1

ng/ml at Hour 64, the hour when the first heifers (n = 3) ovulated. The first eight heifers in each of the Ct and Bc groups were used to examine the effect of bromocriptine during the preovulatory period beginning 72 h before ovulation. The treated heifers were partitioned into those that ovulated before compared to after Day 22. All heifers that ovulated before Day 22 (mean, Day 20.7) maintained the PRL concentration of 0.23 or 0.24 ng/ml (Fig. 7). The group effect before ovulation was significant and represented average concentrations as follows: Ct, 6.4a ± 1.1 ng/ml; Bc, ovulated before 22 d, 0.2b ± 0.0 ng/ml; Bc, ovulated after 22 d, 2.0c ± 0.6 ng/ml. Concentrations in the Bc-treated heifers that ovulated after 22 d increased ($P < 0.05$) before ovulation. The diameter of the future ovulatory follicle for –72 to –8 h before ovulation showed an interaction of hour and group. Diameter was less ($P < 0.05$) in the Bc group at –72 h and approached being less at –64 h. There were no differences between groups in diameter at the subsequent preovulatory measurements. Concentrations of LH for –72 to 0 h relative to ovulation had only a significant effect of hour.

1.5 Discussion

Hypothesis 1 —— location of the CL and future ovulatory follicle in the same ovary is associated with an earlier occurrence of luteolysis —— was supported. When the CL and the follicle were in the same ovary compared to opposite ovaries in the 15 controls, support for the hypothesis was: (1) lower P4 concentration on Day 16.0, (2) more heifers in luteolysis than in preluteolysis on Day 16.0, (3) earlier completion of luteolysis for heifers that were in preluteolysis on Day 16.0, (4) shorter interval from Day 16.0 to the end of luteolysis, and (5) shorter interovulatory interval. Combined for the Ct and Bc groups, the heifers that were in preluteolysis on Day 16.0 began a P4 decrease a day earlier when the two structures were in the same ovary compared to opposite ovaries. The greater P4 concentration 24 h before the end of luteolysis in the Ct-same subgroup than in the Ct-opposite subgroup was a reflection of more efficient luteolysis. That is, a more rapid decrease in P4 resulted in greater P4 concentration 24 h before the end of luteolysis.

An effect of CL/follicle location in same ovary compared to opposite ovaries also affected structure and hemodynamics of the CL in the 16 heifers that were examined by color-Doppler ultrasound. The lower RI for an artery at the periphery of the CL and separately for an artery in the ovarian pedicle of the ovary containing both the CL and future ovulatory

follicle indicated greater vascular perfusion upstream from the point of RI determination (GINTHER, 2007) than when the CL and follicle were in opposite ovaries. The greater percentage of luteal area with color-Doppler signals of blood flow for Hours –24 to 40 also indicated greater luteal vascular activity when the two structures were in the same ovary but only by *t*-tests and not by the factorial analysis. In conclusion, the ovary with both the CL and future ovulatory follicle compared to the ovary with only the CL was associated with a more efficient reduction in P4 concentration (functional luteolysis) and, based on spectral analyses, greater luteal vascular perfusion (lower RI).

Earlier luteolysis when both structures were in the same ovary may have resulted from greater exposure of the CL to the luteolysin (PGF2α) from the endometrium (GINTHER 2012a), owing to the increased vascular perfusion of the CL. The apparent paradox of less reduction in CL area during the greater decrease in P4 when CL and follicle were in the same ovary was equivocal in that the differences in CL area were found by *t*-tests at various hours and not by the factorial analysis. If the effect on CL area is substantiated in further studies, it would indicate that the more efficient functional luteolysis when the CL and follicle are in the same ovary is not matched by an equally more efficient structural luteolysis.

It is not known if there is a relationship between the novel finding of greater CL vascular perfusion and earlier P4 reduction when the CL and future ovulatory follicle were in the same ovary in cattle and reports that the CL ovary contains more follicular fluid and larger follicles than the opposite ovary in ewes (DUFOUR et al., 1972). In cattle, reports on interovarian relationships between CL and follicles during the estrous cycle have been contradictory (PIERSON et al., 1987; IRELAND et al. 1979; STAINGMILLER et al., 1982; MATTON et al., 1981; GINTHER et al., 1989; PURWANTARA et al., 2006). In women, the CL has been reported to have a local negative effect on follicles during the luteal phase (FUKUDA et al., 1997). Results of these reported studies and the current study demonstrate that one type of structure (CL or follicle) in an ovary can have an intraovarian effect on another type of structure (follicle or CL). It has been reported that either intraovarian or local extraovarian pathways could be involved in local CL stimulation of follicles (FOGWELL et al., 1977). The extraovarian pathway presumably would involve transfer of a CL substance in the ipsilateral ovarian pedicle from the ovarian venous and lymphatic vessels into the adjacent ovarian arteries, similar to the unilateral pathway between the uterine horn and ovary (GINTHER et al., 2012a). In the current study, the future ovulatory follicle may have produced a substance (e.g., estradiol) that had a stimulatory effect on the CL through an intraovarian or local extraovarian pathway. Another interpretation based on the current results

is that the extensive vasculature of one structure altered the intra- and extraovarian hemodynamics, so that the vascularity of the other structure also increased. Further study of the local extraovarian phenomenon is needed, especially by including vascular perfusion determination of both the CL ovary and non-CL ovary.

The greater stimulation of the arterial supply to the CL when the CL is in the same ovary as the future ovulatory follicle can be a misleading factor in studies of luteal mechanisms. Confounding may occur especially when the number of animals in each experimental group is inadequate for separating animals with different CL/follicle relationships. In the current study, the number of heifers (n = 30) was amenable to subgrouping for study of the effects of both bromocriptine and CL/follicle location on P4 concentration.

The effectiveness of bromocriptine treatment on Days 16 through 19 in reducing PRL concentrations was demonstrated by the consistent PRL reduction at 8-h intervals to the level of assay sensitivity until Day 21.3 in each of the eight bromocriptine-treated heifers with PRL determination. The PRL suppression in the Bc group to the level of assay sensitivity encompassed at least 40 h before P4 concentration decreased to < 1 ng/ml in 12 of 15 heifers. A single treatment at the same dose, with a similar weight and type of heifer, and at a similar day postovulation, resulted in a reduction in PRL for at least 12 h but with a partial increase between 7 and 8 h (PINAFFI et al., 2012).

Concentration of LH for the 40 h before the end of luteolysis increased progressively and similarly between the control and bromocriptine-treated heifers. However, a reported single bromocriptine treatment increased the LH concentration during 2 to 5 h posttreatment and may have been responsible for a reported P4 increase during the induced PRL decrease (PINAFFI et al., 2012). It is likely that a similar transient increase in LH would not have been detected at 8-h intervals in the current study. A luteotropic effect of an undetected increase in LH was not likely a factor in the current study in that the bromocriptine decreased rather than increased the P4 concentrations.

Hypothesis 2—— bromocriptine has an effect on the CL and ovulatory follicle—— was supported for an effect on CL function (P4 production) but not for an effect on the preovulatory follicle or ovulation. Support for the effect on the CL was the lower P4 concentration in the Bc group than in the Ct group at Hours –40 to –24 before the P4 decreased to < 1 ng/ml. The negative effect of bromocriptine may have been expressed through interference with a luteotropic effect of PRL. This conclusion represents the first in vivo report of a luteotropic role of PRL in cattle and is consistent with the results of in vitro

studies (see *Introduction*). However, a luteotropic role must be accepted with reservation in that the hypothesis did not indicate whether the bromocriptine effect on the CL is positive or negative. The postulated luteotropic effect of PRL apparently is exerted before or early in the luteolytic process. This was indicated by bromocriptine-induced P4 reduction in heifers that were in preluteolysis but not in luteolysis on Day 16.0 and by reduced P4 concentration at 32 and 24 h and not at 16 and 8 h before the end of luteolysis.

A significant effect of bromocriptine on P4 concentration was not detected by comparison of the Ct and Bc groups, either by a factorial analysis or by *t*-tests, without considering other factors. That is, the effect of bromocriptine was masked by differences in luteal stage among individuals on Day 16 and by the intraovarian location relationships between the CL and future ovulatory follicle. Centralizing the data to a P4 decrease to < 1 ng/ml (Hour 0) provided a luteal reference that was similar for all heifers, unlike the differences at Day 16.0. Using both treatment group and location of CL/follicle as factors in the analyses helped clarify the roles of the two factors during Hours –40 to 0. Bromocriptine treatment, presumably though the reduction in PRL, reduced the P4 concentration when the two structures were in either the same ovary or opposite ovaries, but the decrease during Hours –40 to –24 was greater when the two structures were in opposite ovaries.

The lower P4 concentration at Hours –32 and –24 from bromocriptine treatment, when the CL and follicle were in opposite ovaries, was associated with greater RI in an artery at the periphery of the CL, indicating reduced luteal vascular perfusion. These results indicated that the apparent luteotropic effect was exerted in the luteal arterioles, at least in part. This interpretation is consistent with the in vitro report that protein of PRL is immunohistochemically detectable in the smooth muscle cells of the intraluteal arterioles in cattle (SHIBAYA et al., 2008). Although bromocriptine reduced the P4 concentration when the CL and follicle were in either the same or opposite ovaries, the effect on RI differed between groups only when the two structures were in opposite ovaries. The bromocriptine RI effect when the CL and follicle were in opposite ovaries occurred at Hour –24 but not at Hours –16 to 24. When CL and follicle were in the same ovary, RI was not affected by treatment during the later portion of luteolysis and early portion of postluteolysis. During this time, perfusion was greater in both the Ct-same and Bc-same subgroups than in the Bc-opposite subgroup.

The decrease in P4 during postluteolysis involved a continued reduction over a period of 56 h (2.3 d) after the first decrease to < 1 ng/ml. The lowest concentration (0.1 ng/ml) was reached in both the control and bromocriptine groups at 64 h or the hour when ovulations

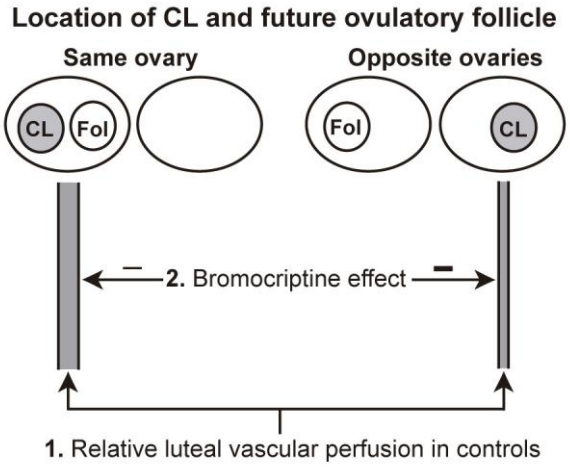
began to occur. Bromocriptine treatment did not alter the P4 decrease during postluteolysis, but for each group the greater vascular perfusion when the CL and follicle were in the same ovary than in opposite ovaries continued until at least Hour 8. Apparently, the location of the CL and follicle continued to play a role in the luteal vascular perfusion during postluteolysis but not on P4 concentration. However, confirmation or clarification is needed. After Hour 8, the effects of CL/follicle location and bromocriptine treatment on RI became complex. The vascularity and growth of the ovulatory follicle to about maximum preovulatory diameter likely complicated the assessment of luteal blood flow when CL and follicle were in the same ovary. Blood-flow Doppler signals increase in the wall of the bovine preovulatory follicle for several hours after the peak of the LH surge and again for several hours before ovulation (SIDDIQUI et al., 2010).

The continued PRL suppression until 1.3 d after the last bromocriptine treatment on Day 19.7 allowed consideration of the effect of bromocriptine on growth of the preovulatory follicle and ovulation in addition to its effect on the CL. An absence of an effect of treatment or CL/follicle location on the interval from the end of luteolysis (P4 < 1 ng/ml) to ovulation (about 3 d) indicated that length of the follicular phase was unaffected. On this basis, the increased length of the interovulatory interval in the Bc group can be attributed to the later defined termination of the luteolytic period. The finding that the increase in diameter of the future ovulatory follicle was more rapid in the Bc group than in the controls was based on a lower diameter 72 h before ovulation without a diameter difference at the 8 h before ovulation. However, this did not appear to represent a direct effect of bromocriptine. Instead, the lower diameter at –72 h may have represented more heifers with three-wave cycles in the Bc group than in the controls, but the number of waves was not determined. An increase in number of three-wave cycles occurs in nontreated heifers with a longer luteal life span (GINTHER et al., 1989) and in heifers with a prolonged luteal phase from inhibition of LH (GINTHER et al., 2011b). Thus, it cannot be concluded that bromocriptine had a direct effect on the preovulatory follicle.

The decrease in PRL to baseline from bromocriptine treatment apparently did not affect ovulation, considering that the PRL decrease encompassed the time of ovulation in four heifers. Previous studies in ewes (PICAZO et al., 2000; MCNEILLY; LAND 1979) and the current study in heifers have not found a direct effect of bromocriptine on the preovulatory follicle, the LH preovulatory surge, or the ovulatory process in ruminants. In a study with daily sampling in cattle, PRL concentrations were low during diestrus and increased during the 3 d before estrus (SWANSON; HAFS; MORROW, 1972). Sampling at 2-h intervals

indicated that PRL concentrations increased 68% during estrus in heifers (RZEPKOWSKI et al., 1982). In the current study, concentrations of PRL in the controls appeared to increase during the 2 d before ovulation but not significantly.

Figure 8 – Diagrammatic illustration of results for bromocriptine effect on luteal vasculature.



(1) The location of the CL and future ovulatory follicle in the same ovary in controls was associated with greater luteal vascular perfusion than when the two structures were in opposite ovaries. (2) Treatment with bromocriptine had a greater effect (bolder negative sign) on reducing the luteal vascular perfusion and reducing the P4 production when the two structures were in opposite ovaries than in the same ovary.

In conclusion, the luteolytic period occurred earlier and luteolysis was more efficient in nontreated heifers when the CL and future ovulatory follicle were in the same ovary than when the two structures were in opposite ovaries (Fig. 8). This intraovarian relationship was indicted by a greater percentage decrease in P4 and earlier completion of luteolysis. Spectral Doppler study indicated greater luteal and ovarian vascular perfusion when both structures were in the same ovary. Bromocriptine treatment every 8 h reduced the PRL concentrations to the level of assay sensitivity and induced lower P4 concentrations 40 to 24 h before the end of luteolysis. Bromocriptine had a greater effect on reducing vascular perfusion of the CL and P4 production when the CL was in the ovary opposite to the ovary with the future ovulatory follicle than when the two structures were in the same ovary. Results indicated an intraovarian effect between the CL and ovulatory follicle and tentatively indicated that PRL may have a luteotropic effect in heifer.

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