UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

EFEITO DO USO DE UMA DOSE ADICIONAL DE PROSTAGLANDINA $F_{2\alpha}$ DURANTE O PROTOCOLO DE IATF À BASE DE ESTRADIOL E PROGESTERONA NA FERTILIDADE DE VACAS HOLANDESAS EM LACTAÇÃO EM ANESTRO

FRANCISCO REBÔLO LOPES JUNIOR

Trabalho de apresentado ao programa de Pós-graduação em Zootecnia como parte das exigências para obtenção do título de Mestre.

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉCNICA DE AQUISIÇÃO E TRATAMENTO DA INFORMAÇÃO – SERVIÇO TÉCNICO DE BIBLIOTECA E DOCUMENTAÇÃO - UNESP - FCA - LAGEADO - BOTUCATU (SP)

Lopes Junior, Francisco Rebôlo, 1990-

L864e Efeito do uso de uma dose adicional de prostaglandina f2α durante o protocolo de IATF à base de estradiol e progesterona na fertilidade de vacas holandesas em lactação em anestro / Francisco Rebôlo Lopes Junior.– Botucatu: [s.n.], 2017

75 f.: il., grafs., tabs.

Dissertação(Mestrado)- Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Botucatu, 2017

Orientador: José Eduardo Portela Santos

Coorientador: José Luiz Moraes Vasconcelos

Inclui bibliografia

1. Bovino de Leite – Criação. 2. Reprodução animal – Desempenho. 3. Stress (Fisiologia). 4. Progesterona. 5. Estradiol. I. Santos, José Eduardo Portela. II. Vasconcelos, José Luiz Moraes. III. Universidade Estadual Paulista "Júlio de Mesquita Filho" (Campus de Botucatu). Faculdade de Medicina Veterinária e Zootecnia. IV. Título.

BIOGRAFIA DO AUTOR

Francisco Rebôlo Lopes Junior, nascido no município de Campinas - SP, em 22 de Julho de 1990, filho de Francisco Rebôlo Lopes e Esvani Brait Leite Lopes. No ano de 2008 mudou-se para a cidade de Umuarama no Estado do Paraná onde iniciou seus estudos no curso de medicina veterinária na Universidade Estadual de Maringá (UEM), durante a graduação focou seus estudos e estágios na área de reprodução e produção de bovinos. No ano de 2013 ao finalizar a graduação em medicina veterinária mudou-se para Carmo do Rio Claro, no Estado de Minas Gerais, onde participou de pesquisas na área de desenvolvimento de programas de sincronização do ciclo estral (IATF), sob a orientação do Prof. Dr. José Luiz Moraes Vasconcelos. Em 2015 ingressou no programa de mestrado em zootecnia pela FMVZ-UNESP/Botucatu-SP, no departamento de pós-graduação em produção animal, sob a orientação do Prof. Dr. José Eduardo Portela Santos e co-orientação do Prof. Dr. José Luiz Moraes Vasconcelos. Em agosto de 2015 mudou-se para a cidade de Gainesville, no Estado da Flórida nos Estados Unidos, onde realizou parte de suas pesquisas do mestrado no Department of Animal Science na University of Florida. Durante o mestrado desenvolveu trabalhos na área de fertilidade de vacas de leite e participou de pesquisas nas áreas de saúde e nutrição de vacas de leite em período de transição. Tendo como trabalho para apresentação para obtenção do título de mestre intitulado como: Efeito do uso de uma dose adicional de prostaglandina $F_{2\alpha}$ durante o protocolo de IATF à base de estradiol e progesterona na fertilidade de vacas Holandesas em lactação em anestro.

DEDICATÓRIA

Aos meus pais, Francisco Rebôlo Lopes e Esvani Brait Leite Lopes, pelo amor incondicional e por todo incentivo e esforços que fizeram e fazem para que eu consiga alcançar meus objetivos.

As minhas irmãs, Natália de Freitas Lopes e Marina de Freitas Lopes, por nossa amizade, carinho, amor e por toda ajuda e torcida para meu sucesso.

A toda minha família e amigos.

AGRADECIMENTOS

A Deus, por sempre estar presente em minha vida, me guardando, me dando sabedoria e sempre me mostrando os caminhos onde devo seguir.

Ao Prof. Dr. José Luiz Moraes Vasconcelos pela grande oportunidade ao deixar eu participar do seu grupo de pesquisa, pela amizade, por todos os ensinamentos que levaram ao crescimento pessoal e profissional.

Ao Prof. Dr. José Eduardo Portela Santos pela confiança e oportunidade, que me foi dada ao ter participado de seu grupo de pesquisa, pelos ensinamentos, amizade e cobranças, durante o período de convivência, que ajudaram para meu amadurecimento profissional e pessoal.

Aos Professores Dr. Guilherme de Paula Nogueira, Dr. João Carlos Pinheiro Ferreira e Dr. Ocilon Gomes Sá Filho por participarem da banca do Exame Geral de Qualificação e/ou da Defesa de Dissertação, pelas sugestões e críticas que contribuíram para melhorar a dissertação.

A Universidade Estadual de Maringá, além de proporcionar minha formação profissional, também me deu grandes amigos, João Luiz, Márcio Miranda, Adriano Bach, Thiago Nitta, Darwin Fernandes, Gustavo Alcarria, André Ladeia, Fábio Borges, Heitor Kawase, Fabrício Martini, Fernando Nalin, Gustavo Batista, Isabella Dias, Mariah Vasques e toda a 7ª Turma Vet também!!

Aos companheiros de pós-graduação, professores e estagiários por todo convívio, ensinamentos, companheirismo e pelos momentos de distração nesse período de mestrado: Achilles V. Neto, Adnan Rodrigues, Alan Lorencetti, Alice Brandão, Anderson Kloster, Augusto Madureira, Bárbara Mello, Prof. Bolivar Farias, Camilo Lopera, Carlos Sanches, Diandra Leziér, Eraldo Drago, Everton Carvalho, Prof. Fernanda Paiva (e Ewerton), Giancarlo Negro, Henrique Dias, Izaias Claro Jr., João Pedro, Letícia Sinedino, Lucas

Barbosa, Lucas Giordano, Lucas Menezes, Marcos Pereira, Prof. Maria Lucia Gambarini, Michael Poindexter, Murilo Romulo, Mycon Araújo, Paula Molinari, Rafael Carvalho, Rogério Peres, Roney Zimpel, Thiago Guzella, Tiago Leiva, Wedson Costa e William Ortiz.

A toda ajuda que recebi durante as análises laboratoriais dos experimentos. Obrigado Achilles Vieira-Neto, Estela Araújo, Gessica Franco e Prof. Dr. Maria Lúcia Gambarini e Roney Zimpel.

Ao pessoal da UNESP, por todo período de convívio, amizade e companheirismo: Thiago Nitta, Pedro Victor, Fábio Ximenes, Rodrigo Gennari, Pedro Levy, Fabiana Nunes, todo o pessoal da República Boi na Zona e CONAPEC Jr.

Aos funcionários da sessão de pós-graduação da UNESP – Botucatu: Seila, Ellen e Cláudia; departamento de produção Animal: Renato e Barbosa, pela disposição e prontidão para ajudar

A todos os funcionários (Kino, Joel, Cláudio, Donato, Zé Vitor, Uian, Agnaldo, Joaquim, Todd, Manuel, Erick, Jack, entre outros) e proprietários das fazendas pela ajuda e compreensão da importância da realização dos experimentos.

Ao Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela bolsa fornecida.

A todos meus amigos, e todos que de alguma maneira participaram desta caminhada.

Muito Obrigado a Todos!

SUMÁRIO

LISTA DE ABREVIATURAS

AGNE - Ácido graxo não esterificado

BCS – Body condition score

BEN - Balanço energético negativo

BHBA - β-hidroxibutirato

CIDR - Dispositivo intravaginal de liberação de progesterona

CL - Corpo lúteo

DIM – Days in milk

DPP - Dias pós-parto

E2 - Estradiol

ECC - Escore de condição corporal

eCG - Gonadotrofina coriônica equina

ECP – Cipionato de estradiol

FCM – Fat-corrected milk

FSH - Hormônio folículo estimulante

GH - Hormônio do crescimento

GnRH - Hormônio liberador de gonadotrofinas

hCG - Gonadotrofina coriônica humana

IA - Inseminação artificial

IATF - Inseminação artificial em tempo fixo

IEP - Intervalo entre partos

IFN-τ - Interferon-tau

IGF-1 - Insulin like growth factor 1 (Fator de crescimento semelhante à insulina 1)

IMS - Ingestão de matéria seca

Kg – Quilograma

LH - Hormônio luteinizante

MS - Matéria seca

P/IA - Prenhez por IA

P4 - Progesterona

$PGF_{2\alpha}$ - Prostaglandina $F_{2\alpha}$

TC - Taxas de concepção

TMR – Total mixed ration

TS - Taxa de serviço

US – Ultrassonografia

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CONSIDERAÇÕES INICIAIS

INTRODUÇÃO

O Brasil se destaca no cenário da agropecuária mundial, com um dos maiores rebanhos bovinos do mundo. A bovinocultura leiteira se destaca por ser quinto maior produtor mundial de leite, com 35,2 bilhões de litros/ano e com aproximadamente 23 milhões de vacas em lactação, porém a baixa eficiência produtiva (1.581 litros/vaca/ano - EMBRAPA, 2014) mostra uma das fragilidades no sistema de produção, e a eficiência reprodutiva é um fator limitante para aumentar a produção de leite no Brasil.

Vacas de leite de alta produção apresentam falhas reprodutivas, que são de causas multifatoriais (LUCY, 2001). O atraso no retorno à ciclicidade causa impacto negativo na reprodução, pois há comprometimento para inseminar as vacas imediatamente após o período de espera voluntária (PEV; RHODES et al., 2003). Dependendo do rebanho, a proporção de vacas que não possuem corpus lúteo (CL) aos 60 dias pós-parto pode exceder 40% (WALSH et al., 2007b; SANTOS et al., 2009). Mesmo quando essas vacas são submetidas a programas de sincronização de ovulação apresentam baixa prenhez por inseminação artificial (P/IA; SANTOS et al., 2009; BISINOTTO et al., 2010a; BISINOTTO et al., 2013), e mesmo que se tornem gestantes, existe maior chance de perda de prenhez (SANTOS et al., 2004).

A anovulação no pós-parto é de causas multifatoriais, como exemplo o estado nutricional das vacas (BUTTLER, 2003), hipotálamo refratário ao efeito positivo do estradiol (ALLRICH, 1994), pela liberação de opióide endógeno, resultado do estímulo da mamada ou ordenha (GORDON et al., 1987), perda de escore de condição corporal, ordem de parição (primípara ou multípara), estação de parição, o rebanho e doenças pós-parto (SANTOS et al., 2009; RIBEIRO et al., 2013).

O estresse térmico também é um fator que afeta o desempenho reprodutivo. Temperaturas elevadas podem levar a diminuição de ingestão de matéria seca (IMS; TURNER E TAYLOR, 1983), e como consequência pode levar a vaca a um BEN. O balanço energético negativo pode diminuir a concentração de glicose (JOLLY et al., 1995), que é diretamente ligada a liberação de hormônio luteinizante (LH; BUCHOLTZ et al., 1996), importante para o mecanismo de ovulação. Além disso, vacas que sofrem de estresse térmico tem sua fertilidade comprometida por diversos fatores, como: crescimento do folículo dominante (WILSON et al., 1998), diminuição da esteridogênise (WOLFENSON et al., 1997), da concentração de estradiol periovulatória (WILSON et al., 1998), competência oocitária (TORRES-JUNIOR et al., 2008), redução da taxa de fertilização (SARTORI et al., 2002) e do desenvolvimento embrionário inicial (HANSEN E ARECHIGA, 1999; WOLFENSON et al., 2000; HANSEN et al., 2001).

Sabendo-se que as vacas em anestro têm baixa pulsatilidade da LH (RICHARDS et al., 1989), e que a pulsatilidade da LH é importante para o crescimento do folículo ovulatório, e o diâmetro do folículo está associado positivamente com a produção de estradiol próximo a ovulação e a fertilidade (VASCONCELOS et al. 2001, BRIDGES et al., 2010, JINKS et al., 2013). Assim uma possível causa da baixa fertilidade em vacas em anestro seja relacionada com problemas no desenvolvimento do folículo ovulatório.

Estudos com prostaglandina $F_{2\alpha}$ visando diferentes efeitos, além o da luteólise, mostraram que $PGF_{2\alpha}$ pode atuar na liberação hormônio que é importante para o desenvolvimento folicular. Estudos obtiveram como resultado que a $PGF_{2\alpha}$ pode atuar como estimulador ovulatório em novilhas pré-puberes (LEONARDI et al., 2012; PFEIFER et al., 2014), $PGF_{2\alpha}$ auxiliando na liberação de LH em vacas de corte (RANDEL et al., 1996), em vacas de corte em anestro (CRUZ et al., 1997), ovelhas (CARLSON et al., 1973) e ratos (WARBERG et al., 1976).

Diferentemente de outros estudos, onde foi adicionado uma segunda dose de $PGF_{2\alpha}$ em protocolos de IATF (1 vs. 2 doses) com o objetivo de aumentar a taxa de luteólise e consequentimente melhorar a fertilidade dessas vacas. A adição de uma segunda dose mostrou-se que melhorou a fertilidade em vacas leiteiras (RIBEIRO et al., 2012a), em vacas de corte (KASIMANICKAM et al., 2009) e tendiam a melhorar em novilhas de corte (PETERSON et al., 2011). No entanto, nenhum desses estudos avaliaram ciclicidade dos animais. Porém, Pereira et al., (2015) avaliaram ciclicidade das vacas e mostraram que as vacas leiteiras em anestro que receberam duas injeções de $PGF_{2\alpha}$ durante um programa de sincronização da ovulação foram beneficiadas e tiveram sua fertilidade aumentada quando comparadas com animais que receberam apenas uma injeção de $PGF_{2\alpha}$, sugerindo que a melhora na fertilidade pelo uso da $PGF_{2\alpha}$ não vem apenas da luteólise, que pode ser que a $PGF_{2\alpha}$ desemprenha outras funções na reprodução.

Além do uso da $PGF_{2\alpha}$ como estimulo de liberação de LH que possa com que melhore o desenvolvimento folicular em programas de sincronização, o aumento da duração do protocolo de sincronização também é uma ferramenta que pode levar o aumento do folículo ovulatório e consequentemente a concentração de estradiol e expressão de estro próximo a IA. (VASCONCELOS et al., 2001; PERRY et al., 2014), e vacas que manifestam estro têm melhor fertilidade e maior manutenção da gestação (PEREIRA et al., 2016). Existem algumas estratégias que podem ser utilizadas para aumentar a circulação de estradiol próximo a IA e para melhorar a expressão do estro, como o uso de ECP no final dos protocolos ovsynch (PANCARCI et al., 2002; CERRI et al., 2004), aumentar o intervalo entre a injeção de $PGF_{2\alpha}$ e o momento da IA, proporcionando maior proestro (PEREIRA et al., 2013b) e aumentando o comprimento do protocolo de sincronização (PEREIRA et al., 2014).

Portanto em protocolos de sincronização o estímulo para o desenvolvimento do folículo ovulatário tem grande importância, seja esse estimulo por uso de hormônios onde faça com que esse folículo se desenvolva melhor e mais rápido ou, seja por aumentar a duração do procolo, onde esse folículo tenha mais tempo para se desenvolver. Isso pode levar um aumento da fertilidade das vacas em protocolos de sincronização, otimizando seu uso.

REVISÃO DE LITERATURA

Estresse calórico

O Brasil como um país de clima quente, na parte do território e na maior parte do ano, e vacas leiterias de raças especializadas para alta produção serem animais de climas mais amenos, onde conseguem demonstrar todo seu potencial, o assunto estresse calórico sempre tem muita importância. Estudos mostraram que há uma diferença na fertilidade das vacas de leite em lactação e o período do ano, onde vacas inseminadas em períodos mais quente do ano tem pior fertilidade (INGRAHAM et al., 1974; PEREIRA et al., 2015).

O estresse térmico também acarreta problemas para o desenvolvimento folicular e embrionário. Nessas condições, é observado o comprometimento do crescimento do folículo dominante (WILSON et al., 1998), da esteridogênise (WOLFENSON et al., 1997), da concentração de estradiol periovulatória (WILSON et al., 1998), competência oocitária (TORRES-JUNIOR et al., 2008), redução da taxa de fertilização (SARTORI et al., 2002) e do desenvolvimento embrionário inicial (HANSEN E ARECHIGA, 1999; WOLFENSON et al., 2000; HANSEN et al., 2001).

Em animais em condições de estresse térmico, ocorre aumento do número de folículos médios e grandes durante o crescimento folicular, atenuando a dominância folicular (BADINGA et al., 1993; WILSON et al., 1998), pelo estresse calórico diminuir o tamanho do folículo dominante e acelerar a emergência da segunda onda folicular, pode-se levar um aumento do período de dominância (WOLFENSON et al., 1995). O aumento do período de dominância folicular está relacionado a embrião de pior qualidade (CERRI et al., 2009) e menor fertilidade das vacas de leite (SANTOS et al., 2010a). A co-dominância folicular devido ao estresse térmico também está associada ao aumento do número de folículos que ovulam ao final de programas de sincronização da ovulação (PEREIRA et al., 2015) e ao aumentando na taxa de parto gemelar (RYAN E BOLAND, 1991).

A redução da ingestão de matéria seca é uma estratégia usada pelo organismo para tentar combater o estresse térmico, por diminuir fermentação ruminal, assim diminuindo a produção de calor (TURNER E TAYLOR, 1983). Porém a diminuição da IMS por prologando período leva a vaca a um balanço energético negativo, diminuindo a concentração de insulina, glicose, IGF-1 e aumentando de ácidos graxos não esterificados (AGNE; LUCY et al., 1992; JOLLY et al., 1995). O aumento de concentração circulante de AGNE está associado ao aumento de AGNE no fluido folicular e pior desenvolvimento embrionário (LEROY et al., 2005; LEROY et al., 2008a), e consequentemente uma piora na fertilidade.

Condição anovulatória pós-parto

No período final de gestação há alta concentração de estradiol, causando um feedback negativo sobre o hipotálamo e resultando em mínima liberação de hormônio folículo estimulante (FSH), bloqueando o desenvolvimento folicular no final de gestação (GINTHER et al., 1996a). Assim que ocorre o parto, a concentração de estradiol vai diminuindo e, por volta da primeira semana após o parto, a maioria das vacas apresentam pulsatilidade de FSH e em seguida desenvolvem a primeira onda folicular (GINTHER et al., 1996a). Porém nem todas as vacas ovulam o folículo de primeira onda: apenas 45% delas conseguem ovular, episódio que ocorre por volta de 20 dias pós-parto; em 35% das vacas sofrem atresia folicular e em 20% das vacas ocorrem o desenvolvimento de cisto folicular (BEAM E BUTLER, 1999). Essa ausência de ciclicidade é denominada anovulação (WILTBANK et al. 2002). Esse período de anovulação pode se estender pelos dois primeiros meses de lactação, e em alguns rebanhos podem atingir 40% das vacas no final do período de espera voluntária (WALSH et al., 2007b; SANTOS et al., 2009).

Foram caracterizados três padrões de crescimento folicular em vacas classificadas anovulares (WILTBANK et al. 2002). O primeiro são as vacas com crescimento folicular limitado, onde o diâmetro folicular é inferior do que determina a dominância. Provável que aconteça devido a inadequada pulsatilidade de LH. O segundo padrão são folículos que atingem diâmetro compatível a folículo ovulatório, mas eles perdem sua capacidade de dominância e regridem. Possivelmente devido a uma desestabilização do sistema e fator de crescimento semelhante a insulina (BEAM E BUTLER, 1999; BUTLER 2000; BUTLER 2003). O terceiro padrão são vacas com folículos maiores que 18mm de diâmetro, na ausência de CL, comumente chamado em rebanhos leiteiros de cisto folicular. Provavelmente devido a falta do “feedback” positivo do estradiol na secreção de GnRH e LH. (GÜMEN E WILTBANK, 2002; 2005a).

O atraso da ovulação no pós-parto é de causas multifatoriais, como exemplo o estado nutricional (BUTTLER, 2003), no início de lactação, quando as vacas leiteiras estão em um estado de BEN, prejudicando a liberação de LH, e a ovulação (JOLLY et al., 1995). Também é comum ocorrer um hipotálamo refratário ao estradiol, devido a alta concentração de estradiol no final da gestação (ALLRICH, 1994). Outra causa comum de anovulação no pós-parto ocorre devido ao estímulo da ordenha ou da amamentação (principalmente em vacas de corte), atrasando o retorno da ciclicidade (SHORT et al., 1990). Stevenson et al. (1997) mostraram que o número de vezes que as vacas são ordenhadas influencia no retorno da ciclicidade: em vacas que são ordenhadas 3 vezes ao dia, a ovulação ocorre por volta do dia 26 pós-parto; já as vacas que são ordenhadas 6 vezes demoraram 11 dias a mais para retornarem a ovular. Em vacas amamentando, quando os bezerros são retirados, a ciclicidade retorna em poucos dias essas (WILLIAMS, 1990). Isso ocorre pelo estímulo da mamada ou ordenha, que libera opióides endógenos (β-endorfinas) e inibem a secreção de GnRH, diminuindo a frequência de pulsos de LH (GORDON et al., 1987). A perda de escore de condição corporal, ordem de parição (primípara ou multípara), estação de parição, o rebanho e doenças pós-parto também são fatores que levam ao atraso da ovulação (SANTOS et al., 2009; RIBEIRO et al., 2013). O atraso na atividade cíclica acarreta perdas no desempenho reprodutivo, pois essas vacas não apresentam cio e, quando são submetidas a programa de sincronização de ovulação, apresentam baixa P/IA (SANTOS et al., 2009; BISINOTTO et al., 2010a; BISINOTTO et al., 2013), e maior o risco de perda de prenhez (SANTOS et al., 2004).

Estratégia reprodutiva

Devido aos problemas de fertilidade que vacas anovulares apresentam, estratégias são usadas para minimizar os efeitos adversos, como aumentar o número de dias do período de espera voluntária (PEV). Porém ao tentar aumentar o PEV, Chebel et al., (2006) verificaram que apenas 30% das vacas anovulares aos 49 DPP se tornaram cíclicas aos 62 DPP, mostrando que não houve um benefício efetivo em retorno de ciclicidade dos animais. Para induzir a ovulação, um tratamento com GnRH pode ter eficácia entre 23% a mais de 90% de ovulação, dependendo da fase do ciclo estral que essa vaca estiver (VASCONCELOS et al., 1999). Porém esses tratamentos apenas tentaram eliminar o estado anovular de uma parcela das vacas, mas não deram a chance desses animais serem inseminados. Uma alternativa para isso é o uso de pré-sincronização seguida de sincronização da ovulação e IA, aumentando a chance das vacas iniciarem o protocolo de sincronização com um corpo lúteo (Bello et al., 2006; Chebel et al., 2006). Aproximadamente 30% das vacas pós-parto não tem CL quando se inicia o protocolo de sincronização (STEVENSON et al., 2008; SANTOS et al., 2009; BISINOTTO et al., 2010a), e de 22 a 46% das vacas que são ressincronizadas também não tem CL no início do protocolo (FRICKE et al., 2003; SILVA et al., 2009). Vacas que não estão ciclando tem menor fertilidade (BISINOTTO et al., 2010a; DENICOL et al., 2012) e maior perda de gestação (SANTOS et al., 2004). Sugerindo que a falta de progesterona no desenvolvimento do folículo ovulatório é prejudicial para a fertilidade de vacas de leite. Além da estratégia de pré-sincronização das vacas, para iniciarem o programa reprodutivo com um CL, outro tratamento hormonal também pode ser utilizado em vacas que iniciam o protocolo de sincronização com baixa concentração de progesterona, ou seja, sem CL, é o uso de 2 dispositivos liberadores de progesterona, que causa efeito positivo na fertilidade de vacas leiteiras (BISINOTTO et al., 2013; 2015a).

Desenvolvimento de programas de sincronização da ovulação

O desafio para melhorar o desempenho reprodutivo de vacas em lactação envolve entender os princípios fisiológicos e bioquímicos que controlam a reprodução e o processo de lactação das vacas de leite (THATCHER et al., 2006). Programas reprodutivos vem sendo utilizado para manejo reprodutivo de rebanhos de vacas de corte e leite; e foram evoluindo de acordo com o entendimento dos mecanismos que envolvem o ciclo estral e disponibilidade de fármacos que permitem a manipulação do ciclo estral. Os programas de sincronização têm como objetivo o controle do estro e ovulação e a máxima submissão a IA, a qual permite alta fertilidade em programas de sincronização em tempo fixo, sem a necessidade de observação de cio (PURSLEY et al. 1997). Controlando o período de dominância (CERRI et al., 2009), concentração de progesterona durante o desenvolvimento folicular (BISINOTTO et al., 2010a; 2013) e duração do proestro (PEREIRA et al., 2013b), concentração de progesterona próximo à IA (BRUSVEEN et al., 2009; PEREIRA et al., 2013b), concentração de estradiol próximo à IA (CERRI et al., 2004) e controle da concentração de progesterona após IA (DEMETRIO et al., 2007).

Existem duas abordagens para os desenvolvimentos de protocolos de sincronização do ciclo estral, uma utilizando GnRH + $PGF_{2\alpha}$ e outra utilizando E2 + P4, e no Brasil a forma mais utilizada é a segunda por diversos fatores, um deles é por ser um programa de mesma eficácia, porém com menor custo.

Programas à base de E2 e P4

Esses programas são amplamente disseminados na América do Sul, principalmente no Brasil e Argentina. Entretanto o uso de estrógeno é proibido em países como Estados Unidos, Canadá, Nova Zelândia e países Europeus. Embora doses de estrogênio usado para sincronizar emergência da onda folicular e ovulação só atingem concentrações endógenas semelhantes aos observados em estro ou durante a gestação (BARUSELLI et al., 2012).

Estudos mostraram que uma fonte exógena de progesterona suprime a liberação de LH, altera a função do ovário, impede o estro e a ovulação em bovinos (ADAMS et al, 1992; SAVIO et al., 1993). O estradiol quando administrado na presença de progesterona, causa uma supressão na liberação de FSH e LH, provocando uma regressão dos folículos dependentes desses hormônios, após o E2 ser metabolizado ocorre liberação de FSH e o início do crescimento de uma nova onda folicular (BÓ et al., 1994; BURKE et al., 1996).

O tipo de estradiol e a dose administrada podem interferir no momento da emergência da onda folicular (BURKE et al., 2003). Quando ocorre o aumento da concentração de E2 na ausência de P4, ocorre a liberação de GnRH pelo hipotálamo, estimulando a liberação de LH pela hipófise, acontecendo a ovulação como desfecho (RATHBONE et al., 2001), o mesmo processo que ocorre quando é aplicada uma fonte exógena de E2 ao final do programa de sincronização da ovulação.

Pancarci et al. (2002) compararam duas maneiras de indução de ovulação ao final do protocolo Ovsynch, a maneira tradicional usando GnRH comparando com o uso de cipionato de estradiol (ECP), chamando o programa de Heatsynch (GnRH – 7d – PGF – 1d – ECP – 2d – IA) e como resultado ambos tratamentos apresentaram a mesma P/IA, mostrando que poderia ser usado as duas abordagens para sincronização de vacas de leite em lactação. Vasconcelos et al. (2011) testaram o protocolo Heatsynch (com adição de um CIDR até o momento da PGF) com um protocolo totalmente à base de E2 e P4 (Inserção do CIDR + BE – 7d – PGF – 1d – Retirada de CIDR + ECP – 2d – IA), ou seja, substituindo o GnRH do início do protocolo por uma dose de benzoato de estradiol (BE), com intuito de aparecimento de uma nova emergência de onda folicular, e foi observado que os dois tratamentos obtiveram a mesma fertilidade em vacas de leite. Por outro lado, Melo et al. (2016) ao comparar GnRH e BE no início de programas de sincronização, observaram uma tendência em melhora da fertilidade em vacas tratadas com GnRH.

Com objetivo de continuar avaliando a eficácia de programas de sincronização à base E2/P4, Pereira et al., (2013a) compararam protocolo de sincronização à base E2 e P4 com um programa sem estradiol, 5d-Cosynch (Inserção de CIDR + GnRH – 5d – Retirada de CIDR + PGF – 1d – PGF – 2d – GnRH + IA) e obtiveram como resultado que o tratamento à base de E2/P4 diminuiu a perda de gestação entre 32 a 60d e aumentou a proporção de vacas que manifestaram estro antes da IA, que também está associado com melhor fertilidade (CERRI et al., 2004; BISINOTTO et al., 2013; PEREIRA et al., 2016).

Para aprimoramento dos protocolos à base de E2/P4, manipulações foram realizadas ao longo do tempo, como: momento de aplicação de hormônios, doses de hormônios, tempo de duração do protocolo, entre outras mudanças. Visando melhorar a sincronia da emergência folicular durante o programa de sincronização Monteiro Jr. et al. (2015) testaram diferentes doses de BE no início do programa. Para aumentar o proestro Pereira et al., (2013b) anteciparam a injeção de $PGF_{2\alpha}$, aumentando um dia de proestro, resultando em efeito positivo na fertilidade, resultados que concordam com estudos anteriormente feito em gado de corte (MENEGHETTI et al., 2009; BRIDGES et al., 2010) e também em vacas de leite em sistema de pastejo (RIBEIRO et al., 2012b). O aumento da duração do programa de sincronização em um dia também resultou em aumento do número de animais que expressaram estro e diminuição da perda de gestação entre 32 e 60d (PEREIRA et al., 2014) e expressão de estro é associada a maior prenhez (PEREIRA et al., 2016). Com o objetivo de aumentar progesterona durante o desenvolvimento folicular, o uso de 2 CIDR em vacas sem CL no início do programa de sincronização (PEREIRA et al., 2017). E ao testarem o uso de GnRH no início do programa e uma dose adicional de $PGF_{2\alpha}$ Pereira et al. (2015) obtiveram melhora em fertilidade com o uso desse programa, surpreendentemente também observaram que vacas anovulares (sem CL em duas ultrassonografias ovarianas, com intervalo de 7 dias) e receberam duas doses de $PGF_{2\alpha}$ apresentaram maior P/IA aos 60d comparado com vacas que receberam apenas uma dose [19,4% (19/98) vs. 31,8% (42/132); $P < 0,05$].

Diversos estudos continuam sendo realizados buscando o aprimoramento dos protocolos de sincronização da ovulação, sempre visando o aumento da fertilidade e facilidade em manejo em vacas leiteiras.

Proestro

O ciclo estral em bovinos tem um período de duração de 18 a 24 dias, sendo regulado por hormônios vindo do hipotálamo (GnRH), hipófise (FSH e LH), ovários (P4 e E2) e útero

($PGF_{2\alpha}$). O proestro inicia-se com a regressão funcional do corpo lúteo e é caracterizado pelo aumento da síntese de estradiol pelo folículo pré-ovulatório (WETTERMANN et al., 1972), com início da luteólise e consequentemente diminuição da concentração de progesterona há a maior liberação de LH pela hipófise (KINDER et al., 1996), a medida que o proestro progride e aproxima-se do pico de LH, existe um aumento na frequência de pulsos de LH (IMAKAWA et al., 1986). Esse aumento da pulsatilidade de LH é o fator que leva o folículo ao seu crescimento e desenvolvimento final, onde as concentrações de estradiol no plasma de vacas leiteiras em lactação aumentam de aproximadamente 2,5 pg/mL no dia da luteólise para aproximadamente 8 pg/mL durante o estro (SARTORI et al., 2004), o aumento da concentração de estradiol leva ao início do comportamento sexual, que determina o fim do proestro e início do estro.

Com o objetivo de estudar o efeito da duração do proestro em protocolos de inseminação artificial em tempo fixo (IATF), diversos estudos foram realizados. Em vacas de corte a estratégia em aumentar o perído de proestro de 2 para 4 dias resultaram em maior fertilidade para as vacas de proestro mais longo (PERES et al., 2009; MENEGHETTI et al., 2009), concordando com os achados de Bridges e colaboradores (2010), que observaram que o aumento de 1 dia no proestro das vacas (de 1,25 dias para 2,25 dias) resultaram em uma maior concentração de estradiol nos últimos ~2 dias antes da IA, e consequentemente um aumento no número de vacas gestantes. Não foi diferente quanto a estudos realizados em vacas de leite a pasto, onde, Ribeiro et al., (2012), ao avaliarem duração de proestro observaram que vacas com maior proestro (58h vs. 72h) apresentaram maior concentração de estradiol no momento da IA, e maior P/IA em vacas que foram pré-sincronizadas. Pereira et al., (2013), também encontraram em vacas confinadas com proestro mais longo (2d vs. 3d) maior fetilidade tanto em IATF, como em TETF. O aumento do proestro levou maior número de vacas com conetração de P4 baixa no momento da IA, que é correlacionada com melhor fertilidade (BRUSVEEN et al., 2009).

Estudos mostraram que vacas com proestro mais longo tem maior concentração E2 próximo à IA (BRIDGES et al., 2010; RIBEIRO et al., 2012), e concentração de E2 próximo a IA é correlacionado com melhor fertilidade (JINKS et al., 2013), possivelmente por efeito no transporte espermático (HAWK et al., 1983), maior sobrevivência embrionária e estabelecimento da prenhez (MADSEN et al., 2015). Vacas que demonstram estro tem um concepto de mais longo no dia 19 da gestação, em comparação com vaca que não demonstram estro (DAVOODI et al., 2016), melhor a fertilidade e menor perda de gestação (PEREIRA et al., 2016).

Relação entre prostaglandina $F_{2\alpha}$ e reprodução

As prostaglandinas são derivadas de ciclopentano formados a partir de acidos graxos poli-insaturado, pela maioria dos tecidos dos mamíferos (SAMUELSSON et al., 1978). Têm efeito no transporte do espermatozóide (HAWK, 1983), na ovulação, regressão de corpo lúteo, implantação e mantença da gestação, no parto e vem sendo usada em programas de sincronização do estro, seja sozinha ou em conjunto com progesterona, estradiol ou hormônio liberador de gonodotrofina (WEEMS et al., 2006). Também a prostaglandina E pode atuar em diferenciação de comportamento sexual em ratos (OTTEM et al., 2004).

Para a reprodução, um dos papéis mais importante da $PGF_{2\alpha}$ é atuar na luteólise, definida como a perda de função do CL e sua regressão ou involução (McCRACKEN et al., 1999). A lúteolise se inicia através da liberação pulsátil e intermitente de $PGF_{2\alpha}$ (MANN et al., 2006). Quando é feita aplicação exógena, em uma dose única de $PGF_{2\alpha}$ em bovinos (dinoprost tromethamine ou cloprostenol), o fármaco passa primeiramente pela circulação sistêmica, antes de começar a atuar no CL e induzir a luteólise. As duas formas exógenas de $PGF_{2\alpha}$ são metabolizadas de formas diferentes, o dinoprost tromethamine é metabolizado rapidamente pelo pulmão de maneira similar a $PGF_{2\alpha}$ endógena (BOURNE et al., 1980; McCRACKEN et al., 1999), com meia vida de 7 a 8 minutos (KINDAHL et al., 1976), enquanto o cloprostenol apresenta uma resistência maior ao metabolismo endógeno, metabolizado no fígado e apresenta meia vida de aproximadamente 3 horas (REEVES, 1978). Com o objetivo comparar as bases de prostaglandina (dinoprost tromethamine ou cloprostenol), foi realizado um experimento para avaliar luteólise e prenhez por IA, em protocolos Ovsynch. No estudo vacas que receberam dinoprost tromethamine presentaram maior taxa de luteólise, em relação vacas tratadas com cloprostenol, porém não foi detectada nenhuma diferença em fertilidade das vacas quando comparada as duas bases de prostaglandina $F_{2\alpha}$ (STEVENSON et al., 2010).

Outros estudos que também utilizaram a prostaglandina como ferramenta para reprodução mostraram que a aplicação de $PGF_{2\alpha}$ no pós-parto inicial em vacas de corte anteciparam a ovulação e melhoraram a fertilidade dessas vacas (RANDEL et al., 1988) e também Velez et al., (1991) observaram que vacas que tiveram o útero massageado uma vez no início do pós-parto (29 a 35 dias), apresentaram maiores concentrações de metabólitos de $PGF_{2\alpha}$ (PGFM) sérico, melhor fertilidade e antecipação na prenhez.

Em programas de sincronização da ovulação a $PGF_{2\alpha}$ é usada para promover a luteólise, diminuir nível de progesterona, para que ocorra a sincronização da ovulação. Estudos vem mostrando a importância de uma luteólise completa durante programas de sincronização da ovulação, refletindo em maior fertilidade. Souza et al. (2007) demonstraram que concentrações de P4 > 0,5 ng/mL próximo a IA reduziu significantemente a fertilidade das vacas. Brusveen et al. (2009) observaram que concentrações acima de 0,4 ng/mL já eram prejudiciais para a fertilidade. Entretanto, Pereira et al. (2013) encontraram que em protocolos que usam E2 e P4 para sincronizar a ovulação, a fertilidade pode ser reduzida com concentrações de P4 > 0,09ng/mL próximo ao momento da IA. Um possível motivo pelo qual maior concentração de P4 próximo ao momento da inseminação pode afetar a fertilidade pode ser a piora no ambiente para espermatozoide no trato reprodutivo e transporte do oócito (BRUSVEEN et al., 2009).

Com intuito de aumentar o número de vacas com luteólise e diminuir a concentração de progesterona próximo ao momento da inseminação, experimentos foram realizados usando duas doses de prostaglandina $F_{2\alpha}$ durante programas de sincronização. Brusveen et al. (2009) compararam uma ou duas doses de $PGF_{2\alpha}$ durante o programa de sincronização e observaram que duas doses diminuíram o número de vacas com luteólise incompleta. E outros estudos observaram aumento na fertilidade quando comparada 1 vs. 2 injeções de $PGF_{2\alpha}$, em vacas de leite (RIBEIRO et al., 2012a), em vacas de corte (KASIMANICKAM et al., 2009) e houve tenência de melhorar a fertilidade em novilhas de corte (PETERSON et al., 2011). Pereira et al. (2015) ao testarem a aplicação de 1 vs. 2 doses de $PGF_{2\alpha}$ em protocolos à base de E2 e P4 observaram que em vacas leiteiras sem CL no início do protocolo e 7 dias após (momento da primeira aplicação de $PGF_{2\alpha}$) e que receberam duas aplicações de $PGF_{2\alpha}$ (a primeira aplicação no dia 7 e a segunda no dia 9) apresentaram maior fertilidade, em relação a vacas que receberam apenas uma aplicação de $PGF_{2\alpha}$ [19,4% (19/98) vs. 31,8% (42/132); $P < 0,05$], Os autores não apresentaram justificativa para o observado, mas citaram que a $PGF_{2\alpha}$ pode causar aumento na liberação de LH, e essa é uma possível causa para uma melhora no desenvolvimento folicular final.

Para avaliar efeitos secundários da prostaglandina $F_{2\alpha}$ além da luteólise, outros estudos foram realizados nesse sentido, apresentando resultados positivos. Estudos observaram a atuação de $PGF_{2\alpha}$ como estímulo ovulatório em novilhas de corte pré-púberes, onde os autores também sugeriram que a $PGF_{2\alpha}$ estimula secreção de LH (LEONARDI et al., 2012; PFEIFER et al., 2014), baseando-se em experimento anterior no qual Randel et al. (1996) relataram que a aplicação de $PGF_{2\alpha}$ ajudou na liberação de LH. Também foi notado em outros experimentos que o uso de $PGF_{2\alpha}$ causava liberação de LH em vacas de corte em anestro (CRUZ et al., 1997), em ovelhas (CARLSON et al., 1973) e em ratos (WARBERG et al., 1976). Estudos que mostraram que a prostaglandina pode desempenhar outros papéis na reprodução, e novos estudos devem ser realizados na área, tentando elucidar outras possíveis funções que a prostaglandina $F_{2\alpha}$ tem na reprodução de bovinos. Essa dissertação teve como objetivo continuar os estudos com $PGF_{2\alpha}$ em programas de sincronização da ovulação, testando a hipótese de que o uso de prostaglandina $F_{2\alpha}$ afeta a liberação de LH pré-ovulatória em vacas de leite anovulares. Potencialmente melhorando a resposta a protocolos hormonais e como consequência melhorar a fertilidade de vacas leiteiras.

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CAPÍTULO 2

ADDITION OF A SECOND DOSE OF PROSTAGLANDIN $F_{2\alpha}$ TO A FIXED-TIME AI PROTOCOL IMPROVES FERTILITY OF ANESTROUS DAIRY COWS

This paper will be submitted to Journal of Dairy Science

ABSTRACT

The objectives of these experiments were to determine the effects of a second prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) dose in an estradiol (E2) and progesterone (P4) based timed AI (TAI) protocol on LH pulsatility, pre-ovulatory follicle characteristics, and pregnancy per AI (P/AI) in anestrous lactating Holstein cows. In experiment 1, 2,011 Holstein cows had their estrous cycles synchronized and ovaries scanned by ultrasound to determine if a corpus luteum (CL) was present at the time of protocol initiation (d-12) and on the day of $PGF_{2\alpha}$ (d-4). Cows without CL on d-12 and d-4 were classified as anestrous (n = 454) and submitted to the following TAI protocol: d -12 or -11: two intravaginal P4 devices and estradiol benzoate (EB); d -4 $PGF_{2\alpha}$ and withdrawal of one P4 device; d -2 estradiol cypionate (ECP) and withdrawal of the second P4 device; on d 0, TAI was performed. On d -4, cows were randomly assigned to one of four treatments: one dose of $PGF_{2\alpha}$ on d -4 and 9 days with a P4 device (1PGF9d, n = 116); two doses of $PGF_{2\alpha}$, the first on d -4 and the second on d -2 and 9 days with a P4 device (2PGF9d, n = 115); one dose of $PGF_{2\alpha}$ on d -4 and 10 days with a P4 device (1PGF10d, n = 111) or two doses of $PGF_{2\alpha}$, the first on d -4 and the second on d -2 and 10 days with a P4 device (2PGF10d, n = 112). Rectal temperature (RT) was measured on d 0 and 7 and cows were classified as RT below (normothermic) or above 39.0^{o}C (hyperthermic). Pregnancy was diagnosed on d 30 and 58 after AI. In experiment 2, 56 Holstein cows had the estrous cycle synchronized to start the timed AI without a CL. All cows were assigned to an E2/P4 based protocol synchronization. On d -11, cows were blocked by milk yield and parity and randomly assigned to 1PGF, 5 mL of saline on d -2; or 2PGF, a second dose of 25 mg of dinoprost on d -2. Blood was sampled from d -11 to 0 and assayed for P4. Jugular catheters were placed and blood was sampled every 15-min from 1 h before to for 6 h after treatments, and every 2 h thereafter for 58 h. Plasma samples were assayed for concentrations of LH and $PGF_{2\alpha}$ metabolite (PGFM). The pre-ovulatory follicle was aspirated on d 0 and fluid assayed for E2 and P4. In experiment 1, protocol using 9 or 10 days with P4 device did not alter P/AI at 30 d (15.8 vs. 18.2%; $P = 0.50$) and 58 d (13.9 vs. 16.2%; $P = 0.51$). 2PGF increased ($P = 0.04$) ovulation after AI in all cows (75.3 vs. 83.1%). Also, in cows with RT ≤ 39.0, 2PGF increased ($P < 0.03$) P/AI on 58d in all inseminated cows (15.7 vs. 30.7%) or only synchronized cows (19.5 vs. 35.1%), but not in cows with RT > 39.0 (all inseminated cows, 10.0 vs. 9.5%; only synchronized cows, 14.8 vs. 12.2%). In experiment 2, 2PGF reduced ($P = 0.05$) the number of LH pulses/6 h (4.5 vs. 3.9 ± 0.2). Relative to treatment, the beginning of LH surge (22.4 vs. 19.3 ± 2.1 h), the hour when the peak of LH surge was detected (29.0 vs. 28.0 ± 1.8 h), and LH peak (4.1 vs. 3.7 ± 0.3 ng/mL) did not differ between 1PGF and 2PGF, but duration of the surge was longer ($P = 0.04$) for 2PGF than 1PGF (13.1 vs. 15.5 ± 0.8 h). Cows in 2PGF had larger ($P = 0.05$) pre-ovulatory follicle diameter (12.3 vs. 14.4 ± 0.8 mm) with greater ($P = 0.02$) estradiol concentration in the follicular fluid in all aspirated follicles (115 vs. 262 ± 39 ng/mL) or in estrogenic follicles (161 vs. 372.8 ± 28 ng/mL). Treatment with a second dose of $PGF_{2\alpha}$ improved P/AI in normothermic anovular cows because of increased ovulation and improved pre-ovulatory follicle characteristics.

Keywords: Dairy cow, $PGF_{2\alpha}$, LH, follicle, estradiol, fertility

INTRODUCTION

Programs for estrous cycle synchronization have been implemented in many parts of the world to control reproduction in dairy herds. During the last decade, timed-AI (TAI) programs have contributed to increased reproductive performance (Bisinotto et al., 2014). These programs have increased AI submission rate, increased pregnancy rate, without reducing fertility when compared with AI following estrus detection or natural service (Santos et al., 2009; Lima et al., 2012). Besides increasing insemination rate and improving reproduction management in dairy herds, these programs provide an opportunity to choose a strategy to improve pregnancy per AI (P/AI) in cohorts of low-fertility cows (Bisinotto et al., 2013).

High producing dairy cows have higher reproductive failures and it seems to be multifactorial (Lucy, 2001). The slow recovery of ovarian activity after parturition is the major impairment to inseminating cows immediately after the end of the voluntary waiting period (VWP; Rhodes et al., 2003). Depending on the dairy herd, the proportion of cows that lack a corpus luteum (CL) at 60 days postpartum may exceed 40% (Walsh et al., 2007; Santos et al., 2009). Even using timed-AI approximately 30% of postpartum cows lack a CL at the beginning of a protocol (Stevenson et al., 2008; Santos et al., 2009; Bisinotto et al., 2010a) and for resynchronization programs it varies between 22 to 46%, depending on the interval from previous insemination (Fricke et al., 2003; Silva et al. 2009). These cows without a CL have a lower chance of becoming pregnant (Bisinotto et al., 2010a; Denicol et al., 2012; Bisinotto et al., 2013) and also increased late embryonic losses (Santos et al., 2004).

Anestrous cows have low LH pulsatility (Richards et al., 1989), which is an important part of proper growth of the ovulatory follicle. Follicle diameter has also been associated with production of estradiol near ovulation and fertility (Vasconcelos et al., 2001; Bridges et al., 2010; Jinks et al., 2013).

Studies using prostaglandin $F_{2\alpha}$ for purposes other than luteolysis, have shown that $PGF_{2\alpha}$ may act as an ovulatory stimulator in prepuberal heifers (Leonardi et al., 2012; Pfeifer et al., 2014), support the release of LH in beef cows (Randel et al., 1996), ewes (Carlson et al., 1973) and rats (Warberg et al., 1976). It has also been observed $PGF_{2\alpha}$ may increase LH release in anestrous beef cows (Cruz et al., 1997). In other studies that had as a proposal to improve luteolysis, using 2 doses of prostaglandin $F_{2\alpha}$ with this objective, improved fertility in dairy cows (Ribeiro et al., 2012a), beef cows (Kasimanickam et al., 2009) and had tendency to improve in beef heifers (Peterson et al., 2011). However, Pereira et al., (2015) surprisingly showed that anestrous dairy cows receiving two injections of prostaglandin $F_{2\alpha}$ during a timed AI program was beneficial resulting in increased fertility compared with animals that received only one injection of prostaglandin $F_{2\alpha}$.

Increasing protocol length is also a way of trying to increase ovulatory follicle diameter and consequently estradiol near to AI. The greater estradiol concentration near AI is correlated with follicular diameter and estrus expression (Vasconcelos et al., 2001; Perry et al., 2005; Perry et al., 2014) and cows display estrus prior to TAI or TET had increased fertility and decreased pregnancy loss (Pereira et al., 2016).

Some strategies may be used to increase the circulating estradiol near to AI and to improve the expression of estrus, such as use of ECP at the end of ovsynch protocols (Pancarci et al., 2002; Cerri et al., 2004), increasing the interval between $PGF_{2\alpha}$ injection and the time of AI, providing a greater proestrus (Pereira et al., 2013b), and increasing the length of the protocol (Pereira et al., 2014).

The hypothesis of the present study was that anestrous lactating dairy cows that receive a second dose of $PGF_{2\alpha}$ will have better fertility compared to cows receiving 1 dose of $PGF_{2\alpha}$ and cows that received a 10d length program have better fertility than cows that received 9d length program. In experiment 1, the objectives were to investigate the effects of one or two doses of $PGF_{2\alpha}$ in lactating dairy cows without a CL during a timed AI program and also to evaluate two protocol lengths supplementing CIDR inserts for 9 or 10 days and the resulting effects on ovarian responses, P/AI, and pregnancy loss. In experiment 2, the objective was to evaluate hormonal release at the end of the TAI program caused by the second dose of $PGF_{2\alpha}$ and its relation with LH pulsatility, LH surge and pre-ovulatory follicle characteristics.

MATERIALS AND METHODS

Experiment 1

All procedures involving animals in this experiment were approved by the Sao Paulo State University regulatory animal research system committee (Protocol number: CEUA-103/2015). And all procedures followed the recommendations of the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 2010).

Cows, Housing and Diets

This study was conducted at 2 commercial dairy farms in Minas Gerais State, Brazil. Weekly cohorts of cows were enrolled during 36 consecutive weeks and all inseminations were performed from January 22th to October 01th, 2014. At the beginning of the experiment (d -12), cows averaged 136±5.87 days in milk (**DIM**), yielding 25.9±0.37 kg of milk/d, with body condition scores (**BCS**) of 2.79±0.02 (in a 1 [emaciated] to 5 [obese] scale [Wildman, 1982]), and lactation number of 2.9±0.13 (Primiparous [=1] n = 133; Multiparous [≥2] n = 321). During the experimental period, the cows were housed in free-stall barns or paddocks with shade and *ad libitum* access to feed in the trough. Cows were fed a TMR based on corn silage and tifton hay as forages with a concentrate based on corn and soybean meal, minerals and vitamins were also added and balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001). Cows were fed and milked twice daily.

Reproductive Management

This experiment used a total of 454 anestrous lactating Holstein cows that were subjected to a progesterone[P4]/estradiol[E2] timed AI program (Figure 1). Every anestrous cow that was past the voluntary waiting period and was healthy was enrolled at the experiment.

The first timed AI program was initiated without pre-synchronization; the cows were enrolled in random day of the estrus cycle. The timed AI program consisted of two intravaginal P4 devices, containing 1.9g of P4 (CIDR®, Zoetis, Sao Paulo, SP, Brazil) and an intramuscular (i.m.) injection of 2.0mg of estradiol benzoate (EB) (Estrogin®, Farmavet, Sao Paulo, SP, Brazil), followed 7 d later by i.m. injection 25mg dinoprost tromethamine ($PGF_{2\alpha}$; Lutalyse®, Zoetis, Sao Paulo, SP, Brazil) and withdrawal of one P4 device and 2 d later these animals were subjected to an i.m. injection of 1mg estradiol cypionate (E.C.P®, Zoetis, Sao Paulo, SP, Brazil) and withdrawal of the second P4 device and AI was performed 2d after the withdrawal of the second P4 device.

The tailheads of cows were painted at the time of the second P4 device removal (d -2) and removal of chalk was used as an indication of estrus. Cows that were detected in estrus after removal of the second P4 device were inseminated in fixed time and remained in the study.

Ultrasonography of the Ovaries and Treatments

All cows in the study had their ovaries evaluated by ultrasonography using ultrasound (US; Mindray, model DP10 VET, equipped with a 7.5 MHz transrectal linear transducer) on study day -12; the ovaries were scanned by ultrasound to determine if a corpus luteum (CL) was present or absent at the protocol initiation (n = 2,011). The cows that had presence of CL at the first ultrasound were not enrolled in the study (n = 1,298). Cows that did not have a CL at first ultrasound were enrolled in the study (n = 713). These animals received two intravaginal P4 devices and an injection i.m. of estradiol benzoate.

A second ultrasound evaluation of the ovaries on d -4 was performed in all cows that enrolled the TAI program. The cows that had presence of CL at the second ultrasound (d -4) left the experiment (n = 259). Only anestrous cows (without CL at d -12 and d -4) continued in the experiment (n = 454), those cows had one CIDR® device removed and received one i.m. injection $PGF_{2\alpha}$.The remaining cows were blocked by parity (primiparous vs. multiparous). Within each block, cows were allocated randomly into one of four treatments (Figure 1.), (**1PGF9d**, n = 116) were treated with CIDR inserts for 9 days and received 1 dose of $PGF_{2\alpha}$ (-4d); (**2PGF9d**, n = 115) were treated with CIDR inserts for 9 days and received 2 doses of $PGF_{2\alpha}$ (-4d and -2d), (**1PGF10d**, n = 111) were treated with CIDR inserts for 10 days and received 1 dose of $PGF_{2\alpha}$ (-4d); (**2PGF10d**, n = 112) were treated with CIDR inserts for 10 days and received 2 doses of $PGF_{2\alpha}$ (-4d and -2d) (Figure 1). The proportions of 1PGF9d, 2PGF9d, 1PGF10d and 2PGF10d cows enrolled in the study were 25.5, 25.3, 24.4, and 24.6% respectively.

On day -2 all cows had the second P4 device removed and received an i.m. injection of estradiol cypionate. The cows that were treated with 2 doses of $PGF_{2\alpha}$ also received the second i.m. injection of $PGF_{2\alpha}$.

The third US was on 0 d, before AI, to measure the diameter of the largest follicle present, which was determined using the average of the horizontal and vertical measurements of the follicle. The fourth ultrasound was seven days after AI, to evaluate presence of CL and synchronization rate to TAI program. Cows were considered to be synchronized if on day 0 there was no CL and on day 7 post AI had the presence of CL.

The size of the follicle was determined with largest horizontal and vertical measurement of the follicle. And the ovulatory follicle was determined by the largest follicle present on the ovary on d 0 that corresponded to an observed CL on 7 d. Cows with follicles < 8mm on d 0 but with a CL on 7 d were defined as “early ovulators” and were not used in analyses of ovulatory follicle diameters.

Blood Sampling and Analyses of Progesterone

Blood samples were collected on -4 d (n = 454), -2 d (n = 365) and 7 d (n = 434) relative to AI by puncture of the median coccygeal vein or artery into commercial 10 mL blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). After bleedings, tubes were placed on ice immediately, transported to the laboratory and centrifuged at 2,500 x g for 15 minutes at room temperature for serum collection. Serum was stored at -20°C for subsequent P4 analysis. The P4 samples were analyzed using the RIA, method described by Pohler et al., (2016).

Pregnancy Diagnosis and Calculation of Reproductive Responses

Pregnancy was diagnosed by transrectal ultrasonography on d 30 after AI. The presence of an amniotic vesicle containing an embryo with heartbeating was used as the determinant of pregnancy. Pregnant cows on d 30 were re-examined for pregnancy by transrectal ultrasonography 4 weeks later, on d 58 of gestation. Pregnancy per AI (P/AI) was calculated by dividing the number of cows diagnosed pregnant at 30 or 58 d after AI by the number of cows receiving AI. Pregnancy loss was calculated as the number of cows that lost a pregnancy between 30 and 58 d after AI divided by the number of cows diagnosed as pregnant on 30 d after AI.

Body Condition Score, Milk Yield and Rectal Temperature

Body condition of all cows was scored (Wildman, 1982) on -12 d, for statistical analysis, BCS was categorized as low (< 2.75) or high (≥ 2.75). Yields of milk were recorded for individual cows once monthly using on-farm milk meters. Within parity group, cows were categorized according to milk production as being above or below the average for statistical analyses.

Rectal temperature was measured in every cow using a digital thermometer (Jumbo Display Laboratory Thermometer; Delta Track, CA) at 0 d and 7 d (Figure 1.). In order to categorize the cows, we used the average of the temperatures on days 0 and 7 (Temperature d0 + Temperature d7 / 2), the cows were categorized according to temperature as being without hyperthermia or hyperthermia for statistical analyses. Hyperthermia was defined as rectal temperature ≥39.1°C and normothermic ≤39.0°C, given that 39.1°C is considered as a threshold for hyperthermia in dairy cattle (Berman et al., 1985; West, 2003) and associated with lower pregnancy in lactating dairy cows (Demétrio et al., 2007; Vasconcelos et al., 2006 and 2011a).

Experiment 2

All procedures involving animals in this experiment were approved by the University of Florida regulatory animal research system committee (Protocol number: 201609399). And all procedures followed the recommendations of the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 2010).

Animals, Housing, Diet and Milking

The experiment was conducted in a Dairy Research Unit at the University of Florida during May to July 2016. Weekly cohorts of cows were enrolled during 7 consecutive weeks. Primiparous (n = 8) and multiparous (n = 52) cows were housed separately in free-stall barns equipped with sprinklers and fans. One day before the intensive bleeding, the cows were moved and maintained to a tie-stall barn for approximately 84 hours. The cows received a TMR to meet or exceed the nutrient requirements for a lactating Holstein cow weighing 650 kg of BW and producing 30 kg of 3.5% FCM/d (NRC, 2001). Cows were fed twice and milked twice daily.

Treatments

All cows had their estrous cycles pre-synchronized (Figure 2.) on day -7 with an injection of $PGF_{2\alpha}$ (25mg of dinoprost tromethamine; Lutalyse®, Zoetis Animal Health, New York, NY) and a controlled internal drug-release insert previously used for 5 days (CIDR®; Eazi-Breed CIDR®, Zoetis Animal Health, New York, NY) containing 1.38 g of progesterone. Four days later (-3d) cows received an injection of $PGF_{2\alpha}$ and on -2d another shot of $PGF_{2\alpha}$, for all cows to have their synchronized estrous cycle and complete luteolysis. On 0d the drug-release insert CIDR® was removed, and 2 hours later, another CIDR® (previously used for 5 days) was inserted and an injection of estradiol benzoate (2mg of β-Estradiol 3-benzoate, Sigma-Aldrich Co., USA) was administered. Seven days later (7 d) an injection of $PGF_{2\alpha}$ was given and on day 9, the internal drug-release insert was removed. All cows received an injection of estradiol cypionate (1mg of β-Estradiol 17-cypionate, Sigma-Aldrich Co., USA). The cows were allocated randomly to receive treatments on day 9 as well: One injection of prostaglandin $F_{2\alpha}$, at day 7 (**1PGF**) or two injections of prostaglandin $F_{2\alpha}$, at the days 7 and 9 (**2PGF**), the same approach used for experiment 1.

Ovarian Ultrasonography

All cows in the study had their ovaries evaluated by ultrasonography using an ultrasound (Aloka SSD-500, Aloka Co. Ltd., equipped with a 7.5 MHz transrectal linear transducer). The ultrasound evaluation of the ovaries on experiment on days -7, -3, 0 was performed intended to detect the presence of a CL to determine the cyclic status. Maps of the ovaries were drawn on days -7, -3, 0, 5, 7, 9 and 10 for each individual cow and were used to follow the development of the dominant follicle. Follicles ≥ 5mm of diameter were drawn with their respective size and position in the ovary. All cows that presented a CL on day 0 or after, were excluded from the experiment (n = 4).

Blood Sampling and Analyses of Progesterone

Approximately 10 mL of blood was collected by puncture of the median coccygeal vein or artery utilizing Vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ) with K2 EDTA. Samples were placed immediately in ice, transported to the laboratory, and centrifuged at 2,800 × *g* for 20 minutes for separation of plasma at room temperature. Plasma samples were frozen at −25°C, and later analyzed for concentrations of P4. Blood samples for analysis of P4 concentration were collected on 0 d. These samples were collected before the second CIDR® insertion (2 hours after the first CIDR® was removed) and on days 1, 3, 5, 7, 9, 11. Concentrations of P4 were analyzed in samples by RIA using a commercial kit (Progesterone Coated Tube RIA Kit, MP Biomedicals, Solon, OH). Samples with known concentrations of P4 (1.0 and 4.0 ng/mL) were incorporated into the assay for quality control. Standards for this assay were 0.00, 0.15, 0.50, 1.0, 5.0, 10.0 and 20.0 ng/mL, and the intra- and inter-assay CV were 4.3 and 8.9%, respectively.

Intensive Bleeding Samples and Analyses of Luteinizing Hormone (*LH) and 13,14-Dihydro-15-keto-PGF*2α metabolite *(PGFM)*

All cows underwent an intensive blood collection during days 9 to 11. Briefly, a catheter was inserted into the jugular veins of each cow one day before (day 8) the intensive collection.

Approximately 10 mL of blood was collected by puncture of the jugular vein utilizing 20mL syringes and the blood was injected into tubes with K2 EDTA. Blood was drawn every 15 minutes for 7 hours (-60, -45, -30, -15, 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210, 225, 240, 255, 270, 285, 300, 315, 330, 345, 360 minutes relative to treatment). The blood continued to be collected, but less frequently, at hours 12, 18, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 48, 50, 52, 54, 56, 58 relative to treatment. For PGFM analyses, we utilized samples until 24 hours after treatment and for LH analyses, we utilized all samples.

Samples were placed immediately in ice, transported to the laboratory and centrifuged at 2,800 × *g* for 20 minutes for separation of plasma at room temperature. Plasma samples were frozen at −25°C, and later analyzed for concentrations of LH and PGFM. Concentrations of LH were determined by RIA using USDA-bLH-B-6 for iodination and the reference standard. The primary antibody used was anti-bLH USDA-309-684P NHPP and secondary antibody used was PEL07418 SARGG (Biotek Source, Mass.) Concentrations of LH in unknown samples were estimated from a standard curve (0.08, 0.16, 0.31, 0.63, 1.25, 2.50, 5.0, 10 and 20 ng/mL). Samples from cows in estrus and diestrus were incorporated into the assay for quality control with known concentrations of LH (0.2 and 1.5 ng/mL). Sensitivity of the assay was 0.04 ng/mL and the intra- and inter-assay CV averaged 5.6% and 11.3%, respectively. Concentrations of PGFM were determined by ELISA. The primary antibody was (Rabbit anti-PGFM, gift from Dr. W. W. Thatcher, University of Florida, USA) and secondary antibody used was (Goat anti-Rabbit IgG, Abcam Inc, Boston MA, USA) Concentrations of PGFM in unknown samples were estimated from a standard curve (39, 78, 156, 312, 625, 1250, 2500, 5000 pg/mL). The standards were prepared in flunixin meglumine treated bovine plasma. The intra- and inter-assay CV averaged 9.7% and 23.8%, respectively.

Follicular Fluid Collection and Hormonal Analyses.

The follicular fluid of the dominant follicle was aspirated 44±1h after CIDR® removal and estradiol cypionate injection of the synchronization protocol (approximately day 10.8). A 5.0-MHz convex-array transducer (Aloka SSD-500, Aloka Co. Ltd.) was used to guide the transvaginal (WTA, Sao Paulo, Brazil) placement of the aspiration needle. The 20-gauge single lumen needle and the transducer were mounted in a plastic handle and carefully introduced into the vaginal fornix after the cow received an epidural anesthesia containing 4 mL of 2% lidocaine (Lidocaine HCl 2%, RXV Products, Roanoke, TX). The dominant follicle targeted for aspiration was then localized on the display of the ultrasound, and the needle was inserted carefully into the follicle with placement confirmed by the appearance of the echogenic tip of the needle inside the follicle. The follicular fluid was then slowly aspirated using a 5mL sterile syringe attached to the aspiration line until the follicle completely disappeared on the display. Following collection, the follicular fluid was immediately placed in 3mL microtubes and kept on ice and later transported to the laboratory and centrifuged at 1000 × *g* for 10 minutes at room temperature for separation of follicular fluid and cells. The supernatant resulting from centrifugation was withdrawn and divided into aliquots of 100 µl in cryogenic tubes. Were used 41 follicles from 29 cows (15 cows from 1PGF treatment and 14 cows from 2PGF treatment). After the follicular fluid was processed, it was stored at -80°C until analysis. The follicular fluid was analyzed for concentrations of estradiol by a commercial kit (Estradiol Serum EIA Kit, Arbor Assays, MI, USA) using a

1:500 dilution for the samples of follicular fluid. Samples with known concentrations of estradiol were incorporated into the assay for quality control. All samples were assayed in a single assay and the intra-assay CV was 19.0%.

Concentrations of P4 were analyzed in samples by RIA using a commercial kit (Progesterone Coated Tube RIA Kit, MP Biomedicals, Solon, OH). The samples were diluted using the dilution 1:4 of follicular fluid. The standards for this assay were 5.0, 10.0, 20.0, 40.0, 60.0 and 80.0 ng/mL, and all samples were assayed in a single assay and the intra-assay CV was 7.24%

Were considered estrogenic the follicles that had higher proportion estradiol than progesterone (17/28).

Experimental Design and Statistical Analyses

Experiment 1. Cows lacking a CL on experiment −12 d and - 4 d were assigned to treatments in a randomized block design. Weekly, cows were blocked by parity (primiparous or multiparous) and randomly assigned to either 1PGF9d, 1PGF10d, 2PGF9d and 2PGF10d. Binary responses were analyzed by logistic regression using the GLIMMIX procedure of SAS. Parity (primiparous vs. multiparous), BCS category (≤2.50 or ≥2.75), milk yield category (above or below the mean milk production), service number (first postpartum AI vs. resynchronized AI), rectal temperature category (≤ 39.0 or ≥ 39.1), estrus detection, protocol synchronization, double ovulation, and the respective interactions with treatment were included as covariates. For P/AI and pregnancy loss, sire, technician, and their interactions with treatment were also included in the statistical models. Multivariable logistic models were built and a backward stepwise elimination method was applied, in which covariates were continuously removed when *P* > 0.10. Treatments (1PGF vs. 2PGF + 9d vs. 10d) and the interaction between treatments (PGF*Duration) were forced into the final model in all analyses.

Continuous variables (i.e., lactation number; DIM; BCS; milk production; previous AI number and follicular diameter) were analyzed by MIXED procedure of SAS fitting a normal distribution. Tests for normality of residuals and homogeneity of variances were conducted for each variable using the guided data analysis option of SAS. Back-transformed data using the ilink function of SAS are presented for clarity.

Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered as tendencies.

Experiment 2. The study design is a randomized complete block design. Weekly cohorts of cows were blocked by parity (primiparous or multiparous) and milk production (average between 10 days before enrollment at the experiment) within each block and then randomly assigned to either 1PGF or 2PGF treatment.

Binary responses were analyzed using the GLIMMIX procedure of SAS. Parity (primiparous vs. multiparous), BCS category (≤3.50 or ≥3.75), milk yield category (above or below the mean milk production), percentage of estrogenic follicles and percentage of LH surge. Multivariable logistic models were built and a backward stepwise elimination method was applied, in which covariates were continuously removed when *P* > 0.10. Treatments (1PGF vs. 2PGF) forced into the final model in all analyses.

The MIXED procedure of SAS fitting a normal distribution, was used to analyze the continuous variables (i.e., lactation number; DIM; BCS; milk production; follicular diameter; follicular fluid composition, progesterone concentration, area under curve for PGFM and LH, peak of LH, time for begin, end and time to LH surge, LH pulses). To analyze hormonal concentration in sequential collection the models included the effects of treatment, day of measurement, parity, and interactions between treatment and day of measurement.

Pulses of LH have been characterized in cows with blood sampling every 15 minutes, LH pulses was defined as an increase in LH 2 CV intra-assay above the baseline (overall within-cow mean of LH concentrations of the first 7 hours). At least 3 points were necessary to characterize peak (nadir 1, peak, nadir 2). The begin of the LH surge was defined when the concentration of LH reached a value equal to or greater than baseline and continued to increase until reaching the maximum concentration. Timing of LH surge was defined as the moment in which the LH surge occurred (maximum concentration), and the end of the LH surge was defined when the concentration of LH returned to basal levels.

Duration of the LH surge was determined as the time interval from the onset of the LH surge (baseline concentration before the increase in LH concentration induced by estradiol) to its first return to baseline concentrations. Duration of the LH surge was determined as the time interval from the onset of the LH surge until return to baseline concentrations.

Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered as tendencies.

RESULTS

Experiment 1

The proportion of cows classified as anestrous [without CL at initiation of protocol (-12 d) and Prostaglandin $F_{2\alpha}$ (-4 d)] was 22.58% (n = 454). The cows were classified as cyclic when they had at least one CL in one or both ultrasounds when the ovaries were scanned by ultrasound (days -12 and -4). Cows without CL at -12 d and with CL at the -4 d were 12.88% (n = 259); cows with CL at the -12 d and without CL at the -4 d were 18.49% (n = 372) and cows with CL in both days were 46.04% (n = 926). All cows included in the experiment (n = 454) had no visible CL at the beginning of the study (-12 d) and day of first injection of Prostaglandin $F_{2\alpha}$ (-4 d). The treatments PGF and protocol length did not differ between the groups 1PGF9d, 1PGF10d, 2PGF9d and 2PGF10d for lactation number (2.9 ± 0.2 vs. 2.9 ± 0.2 vs. 2.7 ± 0.2 vs. 3.1 ± 0.2), DIM (122.8 ± 10.9 vs. 112.9 ± 11.3 vs. 120.5 ± 10.5 vs. 116.9 ± 11.9 d), milk production (25.6 ± 0.8 vs. 26.8 ± 0.8 vs. 26.4 ± 0.8 vs. 26.8 ± 0.9 Kg/day), BCS (2.8 ± 0.1 vs. 2.7 ± 0.1 vs. 2.7 ± 0.1 vs. 2.8 ± 0.1) and previous AI number (3.6 ± 0.3 vs. 2.9 ± 0.3 vs. 3.1 ± 0.3 vs. 3.2 ± 0.3). There was also an effect between service number and milk production ($P < 0.01$), with cows that received the first AI postpartum produced more milk than resynchronized cows.

Follicle Diameter at AI, Expression of Estrus and Synchronization Protocol

No difference was observed between PGF treatments (13.40 ± 0.30mm vs. 13.31 ± 0.30mm; $P = 0.82$) and protocol length (13.45 ± 0.30mm vs. 13.26 ± 0.30mm; $P = 0.62$) in average ovulatory follicle size at the time of AI in cows that had their estrous cycle synchronized.

The overall proportion of cows detected in estrus on the day before or day of AI was affected by treatment. Cows submitted to the 10 day length protocol with CIDR® tended to show more estrus (9d = 76.8% vs. 10d = 85.2%; $P = 0.06$; Table 1). Also, cows with high BCS showed more estrus than cows with low BCS (92.44% vs. 61.03%; $P < 0.001$) and cows without hyperthermia during the first 7 days after AI than cows with hyperthermia (85.67% vs. 76.22%; $P = 0.02$).

There was an effect on percentage of cows that had their estrous cycle synchronized (Table 1). Cows that had a longer length protocol had greater synchronization (9d = 74% vs. 10d = 84%; $P = 0.01$) and cows that received two injections of $PGF_{2\alpha}$ also had greater synchronization (1PGF = 75.3% vs. 2PGF = 83.1%; $P = 0.04$) (Table 2). Also, cows with high BCS increased estrous cycle synchronization than cows with low BCS (83.65% vs. 67.02%; $P < 0.001$) and cows without hyperthermia during the first 7 days after AI than cows with hyperthermia (85.5% vs. 71.83%; $P < 0.001$). However double ovulation did not differ between treatments PGF ($P = 0.73$) and protocol length ($P = 0.85$; Table 1).

Pregnancy per AI and Pregnancy Loss

Pregnancy per AI was affected by hyperthermia during the first 7 days after AI. Cows that suffered hyperthermia had lower P/AI at 30d and 58d (30d = 11.6% vs. 24.2%; $P = 0.0005$; 58d = 9.8% vs. 22.3%; $P = 0.0004$). There was no effect of parity on P/IA at 58d (Primiparous vs. Multiparous), number of AI (First AI vs. Resynchronized) and BCS (≤ 2.5 vs. ≥ 2.75). However, cows with above average (25.9 Kg/day) milk production had a tendency ($P = 0.10$) to have higher P/AI at 58 d compared to cows that were below average milk production.

Pregnancy per AI on 30 d and 58 d after insemination did not differ between treatments in all inseminated cows and only cows that had their estrous cycle synchronized (Table 1). Also, cows receiving 1 or 2 doses of prostaglandin $F_{2\alpha}$ the P/AI on 30 d and 58 d did not differ between treatments in all inseminated cows and only cows that had their estrous cycle synchronized (Table 2). Cows that received a protocol of 9 or 10 days length protocol in all inseminated cows (30d: 15.78% vs. 18.23%; *P* = 0.50; 58d: 13.90% vs. 16.17%; *P* = 0.51) and only in synchronized cows (30d: 23.52% vs. 23.93%; *P* = 0.93; 58d: 19.15% vs. 19.40%; *P* = 0.95).

Clearly many cows suffered from hyperthermia in this experiment with 61.5% (n = 454) of cows having elevated rectal temperature (≥39.1°C) during the first 7 days after AI. Cows with rectal temperature ≥39.1°C had lower P/AI at 30 d and 58 d after AI compared with cows with temperature ≤39.0°C (30d = 11.58% vs. 24.19%; *P* = 0.0005 and 58d = 9.78% vs. 22.32%; *P* = 0.0004).

When the effect of treatment was evaluated only on cows that did not suffer from hyperthermia during first 7 days after AI, the treatment 2PGF improved P/AI on 30d (18.2% vs. 31.4%; *P* = 0.04) and 58d (15.7% vs. 30.7%; *P* = 0.02) to compared treatment using 1PGF, and had no effect between treatments when cows suffered hyperthermia (Table 3).

Also, the longer length protocol tended to improve the P/AI in cows that did not suffer from hyperthermia at 30d (20.2% vs. 31.8%; *P* = 0.09), but not at 58d (19.4% vs. 24.8%; *P* = 0.40) and cows that suffered hyperthermia had no effect on P/AI on 30d (13.2% vs. 10.7%; *P* = 0.52) and 58d (9.3% vs. 9.5%; *P* = 0.94).

The treatment 2PGF continued to improve pregnancy when it was evaluated in cows that had their estrous cycle synchronized and did not suffer the effects of hyperthermia during 7 first days P/AI on 30 d (24.6 vs. 39.3%; *P* = 0.05) and 58 d (19.5 vs. 35.1%; *P* = 0.03; Table 3).

Pregnancy loss between 30 d and 58 d of gestation did not differ between PGF treatments (*P* = 0.15) and protocol length (*P* = 0.99; Tables 1; 2; 3).

Experiment 2

The treatments 1PGF and 2PGF did not differ for lactation number (2.7 ±0.3 vs. 2.6 ± 0.2; *P* = 0.85), milk production (20.41 ± 1.07 vs. 20.30 Kg/day ± 1.07; *P* = 0.82), BCS (3.89 ± 0.09 vs. 3.84 ± 0.09; *P* = 0.70), and DIM (335.8 ± 20.4 vs. 357.1 ± 20.7 d; *P* = 0.47).

As expected, 2PGF group increased (*P* < 0.001) concentrations of PGFM in plasma (47.0 vs. 702.8 ± 25.1 pg/mL) starting immediately after treatment during until 6 h after treatment, also had greater (P < 0.001) AUC (1548 vs. 570605 pg/mL/Hour, 1PGF and 2PGF respectively. Also, as expected, the both groups had had subluteal concentrations of P4 between d-11 and d0 (1PGF = 0.59 vs. 2 PGF = 0.45 ± 0.06 ng/mL).

Concentration, Pulsatility and Peak of LH

Mean responses and ranges in values for each characteristic are summarized for 56 cows studied (Table 5). Higher concentration of LH was observed in the treatment 1PGF (0.57 vs. 0.45 ng/mL; P < 0.01). There was no difference in the proportion of cows that had pre-ovulatory peaks of LH between treatments (89.3 vs. 93%; *P* = 0.64). Also no difference was observed between the interval from CIDR® removal and ECP injection to the begin of LH surge between 1PGF and 2PGF groups (22.4 ± 2.1 vs. 19.3 ± 2.0 h; *P* = 0.27) or time to LH surge (29 ± 1.8 vs. 28 ± 1.8 h; *P* = 0.65) and time to the end of LH surge (35.5 ± 1.7 vs. 34.8 ± 1.7 h; *P* = 0.73), however the treatment using 2 PGF had a longer duration of the LH surge (13.1 ± 0.8 vs. 15.5 ± 0.8 h; *P* = 0.04), but the peak of pre-ovulatory concentration of LH (4.1 ± 0.29 vs. 3.7 ± 0.29 ng/mL; *P* = 0.33) was similar between treatments. Surprisingly, treatment using two dose of $PGF_{2\alpha}$ decreased the amount of LH pulses during the first 6 hours after removal of the CIDR® device (4.5 ± 0.24 vs. 3.9 ± 0.24 pulses/6h; P = 0.05) and had greater proportion of pulses per hour (0.75 ± 0.04 vs. 0.65 ± 0.04 pulse/hour; P = 0.05).

Dominant Follicle Diameter and Fluid Follicular Hormonal Profile

Diameter of the all dominant follicle one day before the follicular aspiration, the last measurement, were greater for 2PGF than 1PGF treatment (12.3 ± 0.8 vs. 14.4 ± 0.8mm; P = 0.05), the concentration of estradiol in the follicular fluid were greater (115.4 ± 22.9 vs. 262.3 ± 55.8 ng/mL; P = 0.02) for 2PGF treatment compared with 1PGF treatment, and the progesterone was similar between groups (335.1 ± 82.3 ng/mL vs. 291.6 ± 88.4 ng/mL; P = 0.72).When were analyzed only estrogenic follicles, the diameter did not differ (12.4 ± 1.1 vs. 13.7 ± 0.8mm; P = 0.44), however the estradiol concentration remains higher in 2PGF group (160.6 ± 29.6 vs. 371.8 ± 27.9 ng/mL; P < 0.01) and progesterone tended to be higher in 2PGF group (73.9 ± 39.8 vs. 179.2 ± 37.5 ng/mL; P = 0.07) and the estradiol to progesterone ratio did not differ (4.93 vs. 7.54; P = 0.52) between treatments.

DISCUSSION

These experiments evaluated whether there would be an effect on fertility to TAI protocol in anestrous dairy cows adding a second injection of $PGF_{2\alpha}$ and a longer length an E_2/P_4 based timed-AI protocol. In experiment 1, even the longest protocol (10 day CIDR) which has been reported to increase estrus expression and synchronize cows at the end of the protocol was not enough to improve cow fertility. However, an effect was observed in $PGF_{2\alpha}$ treated cows, but was not observed the same effect on cows that received a longer length TAI protocol. The magnitude of the response to treatment using two $PGF_{2\alpha}$ doses was quite impressive in cows that had not hyperthermia, with 15 percentage points or almost 100% improvement in fertility (15.7% to 30.7% P/IA at 58 d) relative to the cows bred by TAI that received only one $PGF_{2\alpha}$ dose. In contrast, cows suffering hyperthermia had no effects from adding a second $PGF_{2\alpha}$ dose, according to other studies reporting lower fertility in cows with hyperthermia post AI (Vasconcelos et al., 2006; Demétrio et al., 2007; Vasconcelos et al., 2011a). There are many factors and consequences that increase body temparture has in lactacting dairy cows such as reducing follicular growth (Wilson et al., 1998), compromising steroidogenesis (Wolfenson et al., 1997), altering production of steroids by growing follicles (Wolfenson et al., 1995; Wolfenson et al., 1997; Wilson et al., 1998), oocyte competence (de S Torres-Junior et al., 2008; Rispoli et al., 2013) reducing fertilization rate (Sartori et al., 2002a) and early embryo development (Hansen and Arechiga, 1999; Wolfenson et al., 2000; Hansen et al., 2001). Thus, it appears that there is a dramatic effect of the second $PGF_{2\alpha}$ dose in addition to luteolysis. It was showed in experiment 2 that is likely that effect of the second $PGF_{2\alpha}$ dose increasing LH surge, improved ovulatory follicle and probably improved the proestrus.

Anestrous cows have decreased LH pulsatility (Richards et al., 1989), LH release is important for the intiating cyclicity after calving (Gümen e Wiltbank, 2002), and plays a critical role for fertility, such as follicular growth, which is associated with estradiol production and fertility (Vasconcelos et al., 2001; Bridges et al., 2010; Jinks et al., 2013). Anestrous cows do not display estrus and when bred to TAI protocols have decreased fertility (Santos et al., 2009; Bisinotto et al., 2010a; Bisinotto et al., 2013). In addition, even when these cows conceive to TAI, there is an increase chance of pregnancy loss (Santos et al., 2004). However, when these cows are synchronized in TAI protocols, approximately 30% of the dairy cows lack a CL at the start of the protocol (Stevenson et al., 2008; Santos et al., 2009; Bisinotto et al., 2010a) and 22 to 46% in resynchronized cows (Fricke et al., 2003; Silva et al. 2009).

Several studies document the importance of adequate circulating progesterone during the development of the ovulatory follicle (Bisinotto et al., 2010a; Bisinotto et al., 2013). Cows that have a CL at time of $PGF_{2\alpha}$ have increased fertility to TAI protocols basis of GnRH/$PGF_{2\alpha}$ (Bisinotto et al., 2010b) and E_2/P_4 (Pereira et al., 2013a). However, some authors have shown that, in addition to being important follicular development under high concentration of progesterone, it is also extremely important that progesterone is already in very low concentration (≤ 0.09 ng/mL) at AI, so that the cows have good fertility (Souza et al., 2007; Brusveen et al., 2009; Pereira et al., 2013b).

Aiming to increase luteolysis and decrease the concentration of progesterone close to AI and improve the fertility, many studies used 2 doses of $PGF_{2\alpha}$ during the TAI protocol. Two $PGF_{2\alpha}$ doses improved the fertility in dairy cows (Ribeiro et al., 2012a), beef cows (Kasimanickam et al., 2009) and beef heifers (Peterson et al., 2011), but none of these studies evaluated the cyclicity of these animals. In contrast, Pereira et al. (2015) evaluated the cyclicity and observed that anestrous cows receiving two doses of $PGF_{2\alpha}$ had better fertility than cows receiving only one dose [1PGF = 19,4% (19/98) vs. 2PGF = 32,1% (25/78); *P* = 0,05], agreeing with the main result in experiment 1, when two $PGF_{2\alpha}$ doses increased P/AI at 30 d (1PGF = 18.2 vs. 2PGF = 31.4%; *P* = 0.04) and 58 d (1PGF = 15.7 vs. 2PGF = 30.7%; *P* = 0.02) in cows that did not suffer with hyperthermia during the first seven days after AI.

Many studies show that prostaglandin may have other effects than luteolysis, mainly related to LH release and ovulatory stimulus. In the experiment 1, cows that received 2 doses of $PGF_{2\alpha}$ had greater synchronization to the protocol, that is ovulated more (1PGF = 75.3% vs. 2PGF = 83.1%; P = 0.04). As found in beef cattle, acting as an inducer of ovulation (Leonardi et al., 2012; Pfeifer et al., 2014), suggesting that $PGF_{2\alpha}$ could be a potential LH release mechanism (Carlson et al., 1973; Randel et al 1996; Cruz et al., 1997).

To evaluate only the effects of prostaglandin $F_{2\alpha}$ on fertility of anestrous dairy cows, analysis was performed where only evaluating synchronized cows to the protocol, that is, excluding the effect of ovulation to a TAI protocol in fertility, having exclusively response of prostaglandin and fertility. The results continued to show a positive effect of using two injections of prostaglandin $F_{2\alpha}$ in cows that did not suffer the effects of hyperthermia at the P/AI on 30d (24.6 vs. 39.3%; P = 0.05) and 58d (19.5 vs. 35.1%; P = 0.03).

Other studies suggest that $PGF_{2\alpha}$ may act in to increases LH release in beef cows (Randel et al., 1996), ewes (Carlson et al., 1973) and rats (Warberg et al., 1976), also in anestrous beef cows (Cruz et al., 1997). However, unexpectedly and different from reported in the literature, in experiment 2, the treatment using two doses of $PGF_{2\alpha}$ decreased (P = 0.05) LH pulsatility (4.5 ± 0.24 vs. 3.9 ± 0.24 pulses/6h), and did not increase LH peak (4.1 ± 0.29 ng/mL vs. 3.7 ± 0.29 ng/mL; P = 0.33). Though, cows treated with 2 doses of $PGF_{2\alpha}$ had longer (P = 0.04) duration of the LH surge (13.1 ± 0.8 h vs. 15.5 ± 0.8 h), which is critical in the final development of the ovulatory follicle, where quantity of LH receptor (LHR) is associated with LH circulating (Luo et al., 2011), the LH increases the ability of granulosa cells to produce pregnolone, which is an important regulator of steroidogenesis in pre-ovulatory follicles in cattle (Fortune, 1986). This refers in parts to the findings of experiment 2, because cows of the group treated with 2 doses of $PGF_{2\alpha}$ had higher (P = 0.02) estradiol concentrations in the follicular fluid (115.4 ± 22.9 vs. 262.3 ± 55.8 ng/mL). This increase in the concentration of intrafollicular estradiol can also occur by some other mechanism independent of prostaglandin, such as a direct action of prostaglandin in the theca and granulosa cells, where it drives estradiol synthesis (Armstrong, 1981).

The use of two prostaglandin $F_{2\alpha}$ doses may have improved the proestrus of these cows, due to the fact that the follicle size and intrafollicular estradiol increased. Follicle diameter has been associated with P/AI in several studies. For example, ovulation of a small follicle is associated with reduced P/AI and reduced serum E_2 concentration (Vasconcelos et al., 2001). Lopes et al., (2007) also observed that the mean diameter of the pre-ovulatory follicle was significantly larger in cows subsequently diagnosed pregnant compared with non-pregnant cows (14.5 ± 0.4 vs. 15.8 ± 0.3mm; $P = 0.01$), and follicle size was directly related to E_2 concentrations on day of AI with larger pre-ovulatory follicles associated with greater pre-ovulatory E_2 production ($P < 0.001$). Higher estradiol concentration near AI is associated with higher fertility (Lopes et al., 2007; Jinks et al., 2013).

These circulating concentrations of estradiol may result in a uterine environment more favorable to embryo development. The sperm transport through the female reproductive tract is optimized at estrus or when females are under the influence of estrogen (Hawk, 1983), cows with greater E_2 concentration near to AI were more likely to yield a fertilized embryo than an unfertilized oocyte (Jinks et al., 2013), and had greater embryonic survival and pregnancy establishment (Madsen et al., 2015). In addition cows exhibiting estrus have been shown to have a longer conceptus length compared to no estrus cows on day 19 of gestation (Davoodi et al., 2016) and improved fertility (Pereira et al., 2016).

It is not entirely clear how prostaglandin $F_{2\alpha}$ can enhance the fertility in anestrous cows, but in the literature there are some indications that there are a possible mechanism would be that prostaglandin supports. The increase in the follicular estradiol concentration may have occurred due to the greater quantity of time that the cows treated with two $PGF_{2\alpha}$ doses were exposed to high concentration of LH (for having a longer LH surge), having improved follicular development, or can happen by any other steroidogenic mechanism that prostaglandin may to be more intimately connected and still needs to be studied more.

The fact is that different experiments when studying prostaglandin in the reproduction of cows only used the approach of luteolysis (Kasimanickam et al., 2009; Peterson et al., 2011; Ribeiro et al., 2012a). However, in current study, it has been shown that prostaglandin $F_{2\alpha}$ can go beyond luteolyse and develop other functions in reproduction, and more studies are needed to discover its other functions.

CONCLUSION

In experiment 1, extending the length of the timed-AI protocol by 1d from 11 to 12 days, despite improving the percentage of cows in estrus before AI and estrous cycle synchronization, had no effect on fertility. The 2PGF treatment had greater proportion of cows with estrous cycle synchronized and the cows that were not suffering with hyperthermia during the first 7 days after AI, the treatment 2PGF improve fertility of all inseminated cows and at the cows that had their estrus cycle synchronized those cows compared 1PGF treatment. In experiment 2, cows receiving 2 doses of prostaglandin $F_{2\alpha}$ had a higher follicular diameter, and higher estradiol concentration in follicular fluid. Thus, we speculate that the improved P/AI resulted from an improvement of proestrus of the cows that received 2 doses of prostaglandin $F_{2\alpha}$, because had benefit to follicles and possibly increased E_2 production near the time of AI, which have a positive effect on fertilization, later embryonic development, and subsequent pregnancy maintenance. More studies need to be done to better understand the use of prostaglandin $F_{2\alpha}$ in anestrous cows.

ACKNOWLEDGEMENTS

The authors thank the owner and staff of Sanmariana and Marisan dairies (Alpinópolis-MG, Brazil) and staff of Dairy Research Unit (University of Florida, Gainesville, FL, USA) for the use of their cows and facilities. We acknowledge Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for suporting F. R. Lopes Jr.

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Table 1. Effects of the number of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection (1PGF, injection on day -4 and 2PGF, injections on days -4 and -2) and protocol length (9 vs. 10 days with CIDR) of the timed AI program on fertility responses in lactating dairy cows subjected to the P4/E2 timed AI program.

	1 PGF		2 PGF		***P*** [4] =		
Item	**9d**	**10d**	**9d**	**10d**	**PGF**	**Dur**	**PGFxDur**
Expression of estrus[1;2], % (n)	74.4 (116)	87.8(111)	79.1 (115)	82.3 (112)	0.77	0.06	0.18
Estrous cycle synchronization[1], % (n)	66.9 (116)	82.1 (111)	80.1 (115)	85.8 (112)	0.04	0.01	0.38
Double ovulation[1], % (n)	20.3(113)	17.3 (106)	19.2 (112)	20.9 (106)	0.73	0.85	0.53
Pregnant[1], % (n)							
Day 30	12.3 (116)	18.1 (111)	20.1 (115)	18.3 (112)	0.24	0.50	0.26
Day 58	10.9 (116)	14.5 (111)	17.6 (115)	17.9 (112)	0.14	0.51	0.57
Pregnancy loss[1], % (n)	12.9 (16)	19.3 (21)	8.4 (23)	5.4 (24)	0.15	0.99	0.55
Pregnant[3], % (n)							
Day 30	20.2 (77)	23.3 (88)	27.2 (88)	24.6 (95)	0.37	0.93	0.54
Day 58	16.4 (77)	17.8 (88)	22.2 (88)	21.2 (95)	0.29	0.95	0.78
Pregnancy loss[3], % (n)	12.9 (16)	19.3 (21)	8.4 (23)	5.4 (24)	0.15	0.99	0.55

[1]All inseminated cows.
[2]Evaluated based on removal of tail chalk at timed AI.
[3]Cows with no corpus luteum on d 0 and 1 or more visible corpus luteum on d 7 were considered synchronized and were used in this analysis.
[4]PGF = effect of the amount of dose of $PGF_{2\alpha}$; Dur = effect of duration of the days with CIDR; PGF x Dur = interaction between PGF and Dur.

Table 2. Effects of treatments on expression of estrus, diameter at ovulatory follicle, estrous cycle synchronization, double ovulation and fertility responses in anestrous lactating dairy cows subjected to the one (day -4) or two doses of $PGF_{2\alpha}$ (days -4 and -2) to the P4/E2 timed AI program.

	Treatment		
Item	**1 PGF**	**2 PGF**	***P* =**
Expression of estrus[1,2], % (n)	77.6 (227)	83.2 (227)	0.15
Ovulatory follicle on d 0[1], mm	13.40±0.3	13.31±0.3	0.82
Estrous cycle synchronization[1], % (n)	75.3 (227)	83.1 (227)	0.04
Double ovulation[1], % (n)	18.8 (219)	20.0 (218)	0.74
Pregnant[1], % (n)			
Day 30	15.0 (227)	19.2 (227)	0.24
Day 58	12.6 (227)	17.8(227)	0.14
Pregnancy loss[1], % (n)	15.8 (37)	6.7 (47)	0.15
Pregnant[3], % (n)			
Day 30	21.7 (165)	25.9 (183)	0.37
Day 58	17.1 (165)	21.7 (183)	0.29
Pregnancy loss[3], % (n)	15.8 (37)	6.7 (47)	0.15

[1]All inseminated cows.
[2]Evaluated based on removal of tail chalk at timed AI.
[3]Cows with no corpus luteum on d 0 and 1 or more visible corpus luteum on d 7 were considered synchronized and were used in this analysis.

Table 3. Effect of doses of Prostaglandin $F_{2\alpha}$ on the estrous cycle synchronization and fertility responses after TAI program on according to the average of rectal temperature between days 0 and 7.

	≥ 39.1°C		**≤ 39.0°C**		***P*** [3] **=**		
	1 PGF	**2 PGF**	**1 PGF**	**2 PGF**	**Temp**	**PGF**	**PGF x Temp**
Estrous cycle synchronization[1], % (n)	65.8[b] (135)	76.6[a] (144)	83.3 (92)	86.9 (83)	<0.01	0.04	0.65
Pregnant[1], % (n)							
Day 30	12.2 (135)	11.0 (144)	18.2[b] (92)	31.4[a] (83)	<0.01	0.24	0.10
Day 58	10.0 (135)	9.5 (144)	15.7[b] (92)	30.7[a] (83)	<0.01	0.14	0.09
Pregnancy loss[1], % (n)	19.3 (18)	10.1 (18)	12.9 (19)	4.4 (29)	0.38	0.15	0.8
Pregnant[2], % (n)							
Day 30	19.1 (90)	15.9 (110)	24.6[b] (75)	39.3[a] (73)	<0.01	0.37	0.08
Day 58	14.9 (90)	12.4 (110)	19.5[b] (75)	35.1[a] (73)	<0.01	0.29	0.07
Pregnancy loss[2], % (n)	19.3 (18)	10.1 (18)	12.9 (19)	4.4 (29)	0.38	0.15	0.8

[1] All inseminated cows.
[2] Cows with no corpus luteum on d 0 and 1 or more visible corpus luteum on d 7 were considered synchronized and were used in this analysis.
[3] Temp = Average of the temperatures on days 0 and 7; PGF = effect of the amount of dose of $PGF_{2\alpha}$; PGF x Temp = interaction between PGF and Temp.
[a,b] Values with different superscripts differed ($P \leq 0.05$).

Table 4. Effect of treatment with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in lactating dairy cows subjected to the P4/E2 timed AI program on the follicle diameter, composition of the follicular and characteristics of pulses and pre-ovulatory LH surges.

	Treatment		
	1 PGF	**2 PGF**	***P* =**
Follicles Characteristics			
All follicles			
Diameter of dominant follicle, mm, (n)	12.3±0.8 (15)	14.4±0.8 (13)	0.05
Estradiol, ng/mL, (n)	115.4±22.9 (15)	262.3±55.8 (13)	0.02
Progesterone, ng/mL, (n)	335.1±82.3 (15)	291.6 ± 88.4(13)	0.72
Estrogenic Follicles			
Diameter of dominant follicle, mm, (n)	12.4±1.1 (8)	13.7±0.8 (9)	0.44
Estradiol, ng/mL, (n)	160.6±29.6 (8)	371.8±27.9 (9)	< 0.01
Progesterone, ng/mL, (n)	73.9±39.8 (8)	179.2±37.5 (9)	0.07
LH Release Characteristics			
Number of LH pulses/6h, (n)	4.5 ± 0.2 (28)	3.9 ± 0.2 (28)	0.05
Incidence of LH surge, %, (n)	89.3 (28)	93 (28)	0.64
Time from ECP to begin of LH surge, h, (n)	22.4 ± 2.1 (25)	19.3 ± 2.0 (26)	0.27
Time from ECP to LH surge, h, (n)	29.0 ± 1.8 (25)	28.0 ± 1.8 (26)	0.65
Time from ECP to end of LH surge, h, (n)	35.5 ± 1.7 (25)	34.8 ± 1.7 (26)	0.73
Duration of LH surge, h, (n)	13.1 ± 0.8 (25)	15.5 ± 0.7 (26)	0.04
Peak magnitude of LH surge, ng/mL, (n)	4.1 ± 0.3 (25)	3.7 ± 0.3 (26)	0.33
Area under curve in LH surge, (ng/mL)hour, (n)	29.14 ± 2.4 (25)	29.41 ± 2.4 (26)	0.94

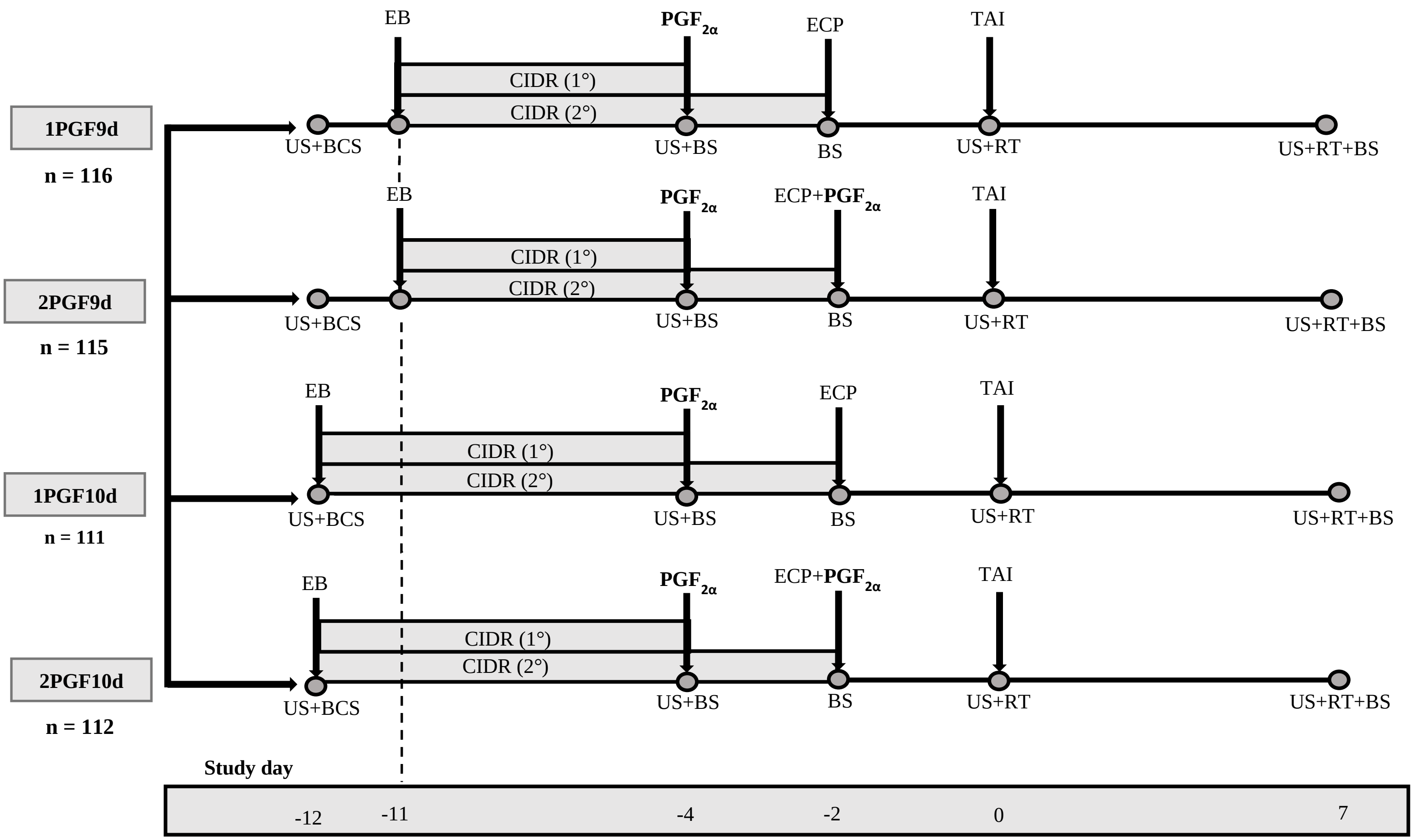

Figure 1. Diagram of activities. Study day 0 is the day of insemination. Treatments were: 1PGF9d = 9 days with progesterone (P4) device and one injection of $PGF_{2\alpha}$; 2PGF9d = 9 days with P4 device and two injections of $PGF_{2\alpha}$; 1PGF10d = 10 days with P4 device and one injection of $PGF_{2\alpha}$; 2PGF10d = 10 days with P4 device and two injections of $PGF_{2\alpha}$. TAI = timed artificial insemination; BCS = body condition scoring; BS = blood sampling; US = ovarian ultrasonography; RT = rectal temperature.

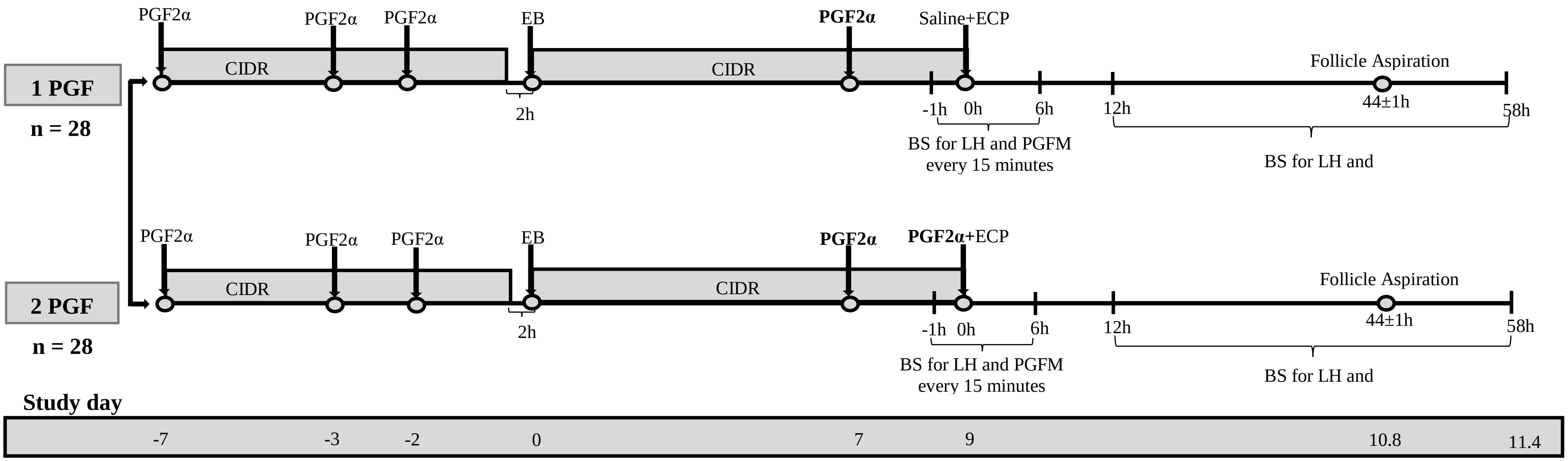

Figure 2. Diagram of activities. Study day 10.8 is the day of follicle aspiration. Treatments were: 1PGF = 7 days pre-synchronization for CL lysis and 9 days with progesterone (P4) device and one injection of $PGF_{2\alpha}$; 2PGF = 7 days pre-synchronization for CL lysis and 9 days with progesterone (P4) device and two injection of $PGF_{2\alpha.}$ BCS = body condition scoring on day -7; BS = blood sampling; US = ovarian ultrasonography on days -7; -3; 0; 5; 7; 9; 10; Intensive blood sampling on days 9 - 11.4 (-1h – 6h, every 15 minutes; 12h, 18h, 24h – 58h every 2 hours).

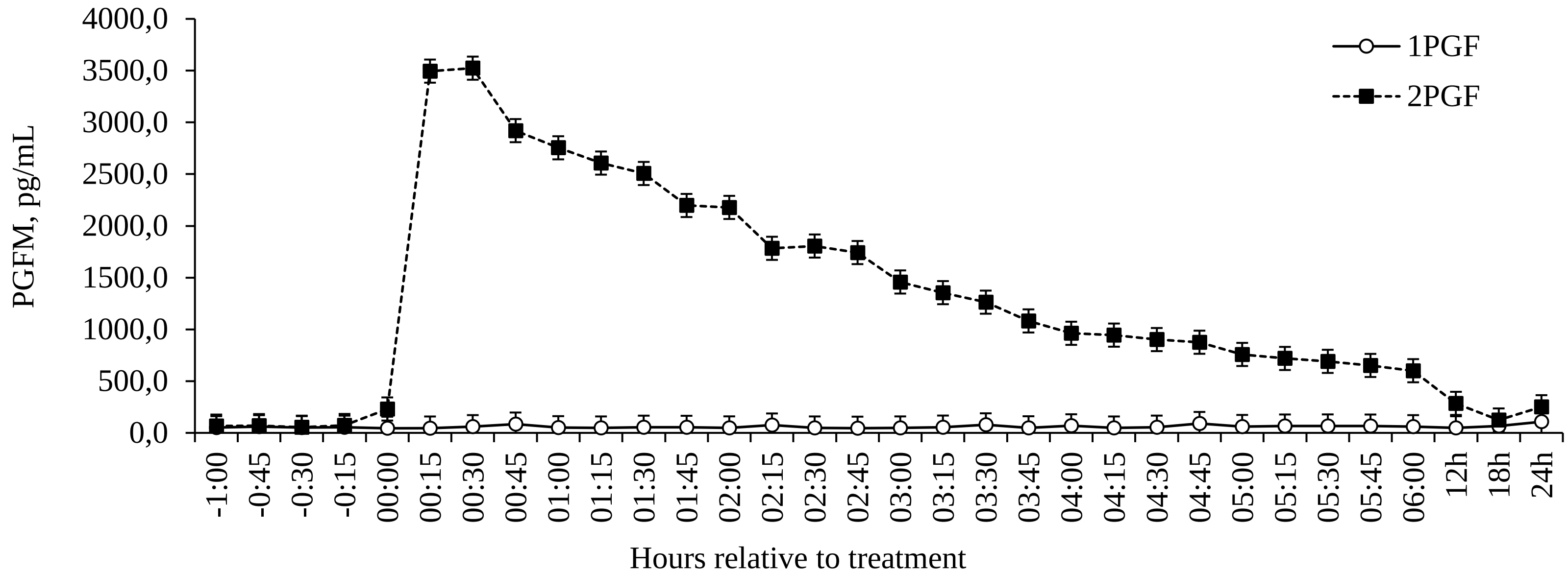

Figura 3. Effect of PGFM release in response to an injection of 25mg of dinoprost tromethamine ($PGF_{2\alpha}$), during E2/P4 timed-AI protocol. . Treatments: 1PGF received saline injection on 0:00 hour; 2PGF received second dose of $PGF_{2\alpha}$. on 0:00 hour. Blood was sampled for PGFM every 15 min from −1 to 6 min relative to $PGF_{2\alpha}$ injection and at 12, 18 and 24 hours. Concentrations of PGFM from d 0 to 6 hour averaged 47.0 vs. 702.8 ± 25.1 pg/mL for 1PGF and 2PGF, respectively Effects of treatment ($P = 0.001$), hour ($P < 0.001$), and interaction between treatment and hour($P < 0.001$).

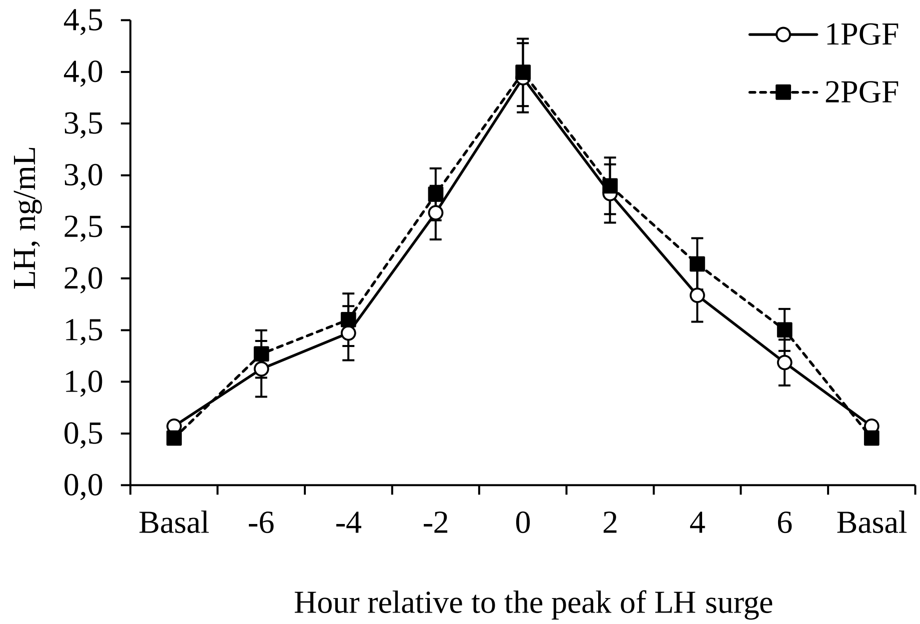

Figure 4. Pre-ovulatory surge of LH in response to an E2/P4 timed-AI protocol. Treatments: 1PGF received saline injection on 9 d; 2PGF received second dose of $PGF_{2\alpha.}$ on 9 d. The peak of LH (4.1 vs. 3.7 ± 0.3 ng/mL) and AUC [29.1 vs. 29.4 ± 2.4 (ng/mL)hour] were similar between treatments for 1PGF and 2PGF, respectively.

CAPÍTULO 3

CONSIDERAÇÕES GERAIS E IMPLICAÇÕES

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Existem rebanhos onde aproximadamente 40% das vacas ainda não retornaram sua ciclicidade aos 60 dias pós-parto, levando a fazenda à uma menor taxa de inseminação logo após ao PEV, pois essas vacas não são visualizadas em cio e apenas através do uso da IA convencional elas não podem ser inseminadas. Com o desenvolvimento da IATF, todas as vacas podem ser inseminadas, independentemente de sua ciclicidade, porém vacas que estão em anestro tem menor chance de se tornarem gestantes e quando gestantes tem maior chance de perda de gestação, ou seja, essas vacas tem pior fertilidade. Dessa forma, melhorar a fertilidade nesses animais é de grande valia para as fazendas leiteiras.

O estudo confirmou dados da literatura relacionados à hipertermia das vacas e baixa fertilidade, mostrando que além da preocupação em melhorar os programas reprodutivos os técnicos que trabalham na área também precisam se preocupar com conforto térmico das vacas.

O uso de um protocolo mais longo (10 dias com CIDR) proporcionou mais animais que expressaram estro e mais animais que sincronizaram o ciclo estral, entretanto não foi observado maior número de vacas gestantes, portanto esse protocolo pode ser indicado para vacas receptoras de embrião, onde o uso desse protocolo levará ao maior aproveitamento do protocolo, ou seja, mais vacas receberão um embrião ao fim do protocolo, e consequentemente melhorará a taxa de serviço da fazenda. O uso de 2 doses de $PGF_{2\alpha}$ foi benéfica nas vacas que não sofreram com hipertermia nos primeiros 7 dias após a IA, esse protocolo melhorou em aproximadamente 15 pontos percentuais a prenhez em animais que receberam a segunda dose, um aumento expressivo, sugerindo que em propriedades que tem um controle adequado do conforto térmico das vacas e/ou em épocas mais frias do ano, o uso desse protocolo melhorará o desempenho reprodutivo das vacas.

Programas reprodutivos que melhoram a fertilidade das vacas tem vantagens em: diminuição de mão-de-obra nas propriedades, diminuição de descarte involuntário de vacas, aumento do número de novilhas para reposição ou venda e são associados a aumento da produção leiteira, entre outros benefícios.

Com os resultados obtidos nos experimentos dessa dissertação é notório a necessidade de mais pesquisas sobre o uso de $PGF_{2\alpha}$, de descobrir e/ou elucidar suas funções em folículo e esteroidogênese.