KATHERINNE MARIA SPERCOSKI

FISIOLOGIA REPRODUTIVA DE LOBOS-GUARÁ (*Chrysocyon brachyurus*, Illiger 1811) E ESTRESSE DE CATIVEIRO: MONITORAMENTO EM LONGO PRAZO DA FUNÇÃO GONADAL E ADRENOCORTICAL

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Orientadora: Prof[a]. Dr[a]. Rosana Nogueira de Morais.
Co-orientador: Prof. Dr. Anderson J. Martino Andrade.

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Após arguir a candidata ***Katherinne Maria Spercoski,*** em relação ao seu trabalho intitulado: "Fisiologia reprodutiva de lobos-guará (*Chrysocyon brachyurus*, illiger 1811) e estresse de cativeiro: monitoramento longitudinal da função gonadal e adrenocortical" **são** de parecer favorável à APROVAÇÃO da acadêmica, habilitando-a ao título de Doutora em Biologia Celular e Molecular, área de concentração Fisiologia.
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Curitiba, 25 de Outubro de 2012

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Coordenador do Programa de Pós-Graduação
em Biologia Celular e Molecular - UFPR
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Alvo Dumbledore

Em Harry Potter e a Câmara Secreta, por J. K. Rowling

RESUMO

O lobo-guará (*Chrysocyon brachyurus*, Illiger 1811) é uma das espécies mais conhecidas de canídeos que habitam as áreas de cerrado. A espécie é classificada pela IUCN – International Union for Conservation of Nature – na categoria "quase ameaçada". Esforços para a conservação de lobos-guará têm sido feitos, visando manter populações de cativeiro viáveis. Infelizmente, a população em cativeiro não é auto-sustentável devido à baixa taxa de prenhez e alta mortalidade neonatal. Especula-se que essa baixa eficiência reprodutiva possa estar associada com distúrbios endócrinos gonadais, como resultado do estresse crônico de cativeiro. Desta forma, nossos objetivos foram gerar dados básicos sobre a função gonadal e adrenocortical de lobos guará cativos em condições normais e submetidos a estimulação adrenocortical aguda e crônica. Foram coletadas amostras de fezes de lobos-guará e os metabólitos de corticóides (MCF), progestágenos (MPF), androgênios (MAF) e estrogênios (MEF) foram extraídos das fezes e quantificados por enzima imunoensaio. No primeiro manuscrito desse estudo, foram caracterizados os padrões de excreção de metabólitos hormonais fecais ao longo do ciclo reprodutivo normal de lobos-guará. Fezes de 9 lobos-guará adultos foram coletadas de 2-5 dias / semana durante período de 5-12 meses. Foram avaliadas as concentrações médias totais obtidas para todos os metabolitos hormonais fecais. As concentrações de todos os metabolitos gonadais fecais apresentaram-se baixas no anestro para ambos os gêneros. Nas fêmeas, durante o proestro observou-se aumento nas concentrações de MEF e MAF. Já as concentrações de MPF tiveram elevação no período peri-ovulatório, mantendo-se elevada durante todo o diestro. Os machos apresentaram aumento nas concentrações de todos os metabólitos gonadais fecais apenas no período referente à lactação e cuidado parental. Em relação à função adrenocortical, as concentrações de metabólitos de corticóides fecais nas fêmeas foram mais altas nos períodos de proestro, lactação e cuidado parental; enquanto que nos machos houve elevação de MCF apenas no período de cuidado parental, quando os mesmos participam do cuidado com os filhotes. No segundo manuscrito desse estudo, foram avaliados os efeitos da estimulação adrenocortical crônica e aguda nas concentrações de metabólitos gonadais fecais. Para melhor entendimento desse manuscrito, o mesmo foi subdividido em três partes, cujos objetivos foram: parte 1) avaliar como a estimulação adrenocortical crônica afeta a função gonadal; parte 2) analisar como a estimulação adrenocortical aguda, por meio da administração de hormônio adrenocorticotrópico (ACTH), afeta a função gonadal; e parte 3) avaliar as funções adrenocortical e gonadal de dois casais cativos de lobos-guará mantidos em recintos menores e com exposição à visitação pública. Nas partes 1 e 3 desse manuscrito, amostras fecais de 5 casais de lobos-guará foram coletadas de 2-5 dias / semana durante 10-21 meses. Na parte 2 foram analisadas amostras fecais de 11 lobos-guará foram coletadas por 3 -7 dias antes até 7-14 dias após os estímulos adrenocorticais agudos, pela administração do ACTH sintético de curta duração. Os resultados obtidos na parte 1 mostraram que machos e fêmeas tiveram evidências endócrinas de estimulação adrenocortical crônica. Os perfis longitudinais para metabólitos gonadais fecais mostraram padrões acíclicos. Na parte 2, o desafio ao ACTH mostrou diminuição nas concentrações de androgênios (MAF) e aumento em estrogênios (MEF) nos machos. Houve correlação positiva ($r^2 = 0,750$, $P < 0,01$) entre as concentrações de MCF e MPF em situações de estímulo agudo em ambos os gêneros. Na parte 3, os perfis

longitudinais de metabólitos de corticóides fecais (MCF) não demonstraram claramente características de estresse crônico nos casais mantidos em recintos menores e sujeitos à visitação pública, no entanto todos os animais apresentaram os elevados níveis de metabólitos gonadais fecais. Em conclusão, o primeiro manuscrito (estudo de caracterização) demonstrou que fêmeas de lobos-guará apresentam flutuações hormonais típicas do ciclo reprodutivo normal de canídeos, enquanto que os machos apresentam aumento nas concentrações de androgênios durante o período de acasalamento e cuidado parental. Aumentos fisiológicos nos corticóides ocorrem na fase de proestro em fêmeas e no período de lactação e cuidado parental em ambos os gêneros. O manuscrito 2 (análise de estresse) demonstrou perda dos padrões hormonais normais durante o ciclo reprodutivo dos animais quando os mesmos estão em condições de hiperestimulação adrenocortical prolongada e, ainda, alterações nas concentrações de metabólitos gonadais fecais nas condições de estimulação adrenocortical aguda, confirmando que tanto a estimulação aguda como, e principalmente, crônica do eixo HHA pode comprometer a eficiência reprodutiva em lobos-guará.

Palavras-chaves: Lobos-guará. Cão doméstico. Metabólitos esteroidais fecais. Ciclo reprodutivo. Função gonadal. Função adrenocortical. Estresse crônico. Estresse agudo. Desafio ao ACTH.

ABSTRACT

The maned wolf (*Chrysocyon brachyurus*, Illiger 1811) is one of the most typical canid species that inhabits the Brazilian grassland areas (known as Cerrado). This species is recognized by the IUCN – International Union for Conservation of Nature - as 'nearly threatened'. Efforts for conservation of maned wolves have been done in order to maintain a viable and self-sustaining captive population. Unfortunately, the captive population is not self-sustained due to low pregnancy success and high neonatal mortality. It is speculated that their low reproductive efficiency may be associated with endocrine gonadal disorders as a result of chronic captivity stress. Thus, our aims were to generate basic data about gonadal and adrenocortical function in captive maned wolves under normal conditions and subjected to chronic and acute adrenocortical stimuli. Fecal samples of captive maned wolves were collected and metabolites of corticoid (FCM), progestagens (FPM), androgens (FAM) and estrogens (FEM) were extracted from feces and quantified by enzyme immunoassay. In the first manuscript of this study, excretion patterns of fecal hormones metabolites throughout normal reproductive cycle in maned wolves were investigated. Feces from 9 adult animals were collected 2–5 days/week for 2-12 months. The overall concentration mean of fecal hormones metabolites was analyzed. Anestrus showed lower concentration of fecal gonadal metabolites in both genders. At proestrus there was an increase on the level of FAM and FEM in female. FPM concentration begins to rise at the periovulatory period, maintaining a high level during diestrus. In males, after the pups' birth, during lactation and parental care period, fecal gonadal metabolites means have a significant increase. Regarding adrenocortical function, fecal corticoid metabolites on females showed higher concentration during proestrus and lactation and parental care period, while in males this elevation was observed only during parental care, as they participate in the care of the pups. In the second manuscript of this study, effects of chronic and acute adrenocortical stimulation on fecal gonadal metabolites were evaluated. To better understand this manuscript it was subdivided in three parts, whose the aims were: part 1) evaluate how a chronic stimulation on adrenocortical activity affects the gonadal function; part 2) analyze how the acute adrenocortical stimulation (by administration of adrenocorticotropic hormone - ACTH) affects the excretion of fecal sex steroid metabolites; and part 3) evaluate fecal adrenocortical and gonadal metabolites concentrations in two couples captive maned wolves kept in smaller enclosures and exposure to public visitation. In parts 1 and 3 of this manuscript, fecal samples of five maned wolf couples were collected 2-5 days/week for 10-21 months. On part 2, fecal samples of 11 maned wolves were collected during 3-7 days before up to 7-14 days after adrenocortical stimuli, by the administration of a short-acting synthetic ACTH. On part 1, male and female demonstrated endocrine evidences of adrenocortical chronic stimulation and gonadal longitudinal profiles showed an acycling pattern. On part 2, the ACTH challenge led to a decrease of androgens and increase of estrogens levels in male. There were positive correlations (r^2=0.750; P<0.01) between FCM and FPM concentration on acute stress for both genders. In part 3, longitudinal profiles did not clearly demonstrate characteristics of chronic stress on couples kept in smaller enclosures subject to public visitation, however all animals presented higher fecal gonadal metabolites levels. In conclusion, the first manuscript (characterization study) demonstrated that female maned wolves present typical hormonal fluctuations of a normal canine reproductive cycle, while male show

increased androgens concentrations during mating and parental care periods. Corticoids normally rise during proestrus in female and lactation / parental care period in both genders. The manuscript 2 (study of stress) demonstrated loss of normal reproductive cycle profiles, when the animals were in conditions of prolonged adrenocortical hyperstimulation; and also, changes on fecal gonadal metabolites concentration in conditions of acute adrenocortical stimulation; confirming that both chronic and acute stimulation of the HPA axis can compromise reproductive efficiency in captive maned wolves.

Keywords: Maned wolves. Fecal steroids metabolites. Reproductive cycle. Gonadal function. Adrenocortical function. Chronic stress. Acute stress. ACTH challenge.

APRESENTAÇÃO

Esta tese está apresentada na forma de dois manuscritos:

- **Characterization of fecal adrenocortical and gonadal metabolites profiles in captive maned wolves (*Chrysocyon brachyurus*) throughout reproductive cycle;**
- **Effects of chronic and acute adrenocortical stimuli on fecal gonadal metabolites concentrations and reproductive cycle in captive maned wolves (*Chrysocyon brachyurus*).**

Nos capítulos 1 e 2 são apresentadas a Introdução geral da tese e a Revisão de Literatura, respectivamente. O capítulo 3 apresenta as Hipóteses e Predições que levaram a execução do presente estudo, sendo que os Objetivos, gerais e específicos, estão apresentados no capítulo 4.

No capítulo 5 os Materiais e metodologia utilizados, resultados, discussão, conclusões e referências encontram-se em cada manuscrito e representam a íntegra desse trabalho.

O capítulo 6 apresenta as Conclusões gerais da tese. As referências referem-se ao conteúdo apresentado na Introdução geral (capítulo 1) e na Revisão de Literatura (capítulo 2).

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LISTA DE ABREVIATURAS

ACTH	Adrenocorticotropic hormone (hormônio adrenocorticotrófico)
ADH	Antidiuretic hormone (hormônio antidiurético)
ANH	Atrial natriuretic hormone (hormônio natriurético atrial)
AZA	American Zoo and Aquarium Association
CBMM	Companhia Brasileira de Metalurgia e Mineração
CITES	Convetion on International Trade in Endangered Species of Wild Fauna and Flora
CL	Corpo lúteo
CRH	Corticotropin release hormone (hormônio liberador de corticotropina)
CV	Coeficient of variation (coeficiente de variação)
D2	Dopamine receptor (receptor de dopamine)
DHEA	Dehidroxiepiandrosterona
EIA	Enzyme immunoassay
FAM	Fecal androgens metabolites
FCM	Fecal corticoids metabolites
FEM	Fecal estrogens metabolites
FPM	Fecal progestagens metabolites
FSH	Follicle stimulating hormone (hormônio folículo estimulante)
GnRH	Gonadotropin releasing hormone (hormônio liberador de gonadotrofina)
HHA	Hipotálamo-hipófise-adrenal
HHG	Hipotálamo-hipófise-gônadas
HPA	Hipotálamo-pituitária-adrenal
HPG	Hipotálamo-pituitária-gônadas
IUCN	International Union for Conservation of Nature
LH	Luteinizing hormone (hormônio luteinizante)
MAF	Metabólitos de androgênios fecais
MCF	Metabólitos de corticoids fecais
MEF	Metabólitos de estrogênios fecais
MMA	Ministério do Meio Ambiente
MPF	Metabólitos de progestágenos fecais

MWSSP	Maned Wolf Species Survival Plan
NRS	Non reproductive season
NT	Near to threatened
PRL	Prolactin (prolactina)
RS	Reproductive season
UC	Unidade de conservação

SUMÁRIO

1 INTRODUÇÃO

O lobo-guará (*Chrysocyon brachyurus*, Illiger 1811) é o maior canídeo sul-americano, ocorrendo nos campos abertos e cerrados da América do Sul (RODRIGUES, 2002). Apesar de sua ampla distribuição, a espécie encontra-se ameaçada de extinção (MMA, 2003; IUCN, 2012), principalmente devido à redução e fragmentação de seu habitat natural, o bioma do cerrado, o qual é considerado um dos mais ameaçados do planeta (MYERS *et al*., 2000). O isolamento da espécie em áreas reduzidas afeta gravemente o lobo-guará, pela sua necessidade de grandes áreas de vida (AZEVEDO,2008; RODRIGUES, 2002), além de, em longo prazo, reduzir a variabilidade genética nestas subpopulações, afetando seu sucesso reprodutivo (DE PAULA *et al*., 2008). Estratégias de conservação têm sido executadas na tentativa de assegurar a sobrevivência desta espécie, incluindo o manejo integrado de populações *in situ* e *ex situ* (PRIMACK e RODRIGUES, 2001; RODDEN *et al*., 1996; DE PAULA *et al*., 2008).

A manutenção de populações viáveis em cativeiro permite a obtenção de informações importantes sobre a biologia da espécie, porém a informação disponível sobre a biologia reprodutiva de lobos-guarás ainda é limitada. Além disto, os dados disponíveis indicam que as populações de cativeiro não são “auto-sustentáveis” (SONGSASEN *et al*., 2006; PRIMACK e RODRIGUES, 2001; VELLOSO *et al*., 1998; CUMMINGS *et al*., 2007) e apresentam baixa eficiência reprodutiva (MAIA e GOUVEIA, 2002; VANSTRELLS e PESSUTI, 2010), a qual pode estar associada a distúrbios endócrinos associados ao estresse crônico de cativeiro. Dados do nosso laboratório demonstraram maior concentração basal de metabólitos de glicocorticóides fecais em lobos-guarás cativos quando comparados a animais de vida livre (SPERCOSKI, 2007).

Sabe-se que a condição de estresse pode alterar a função gonadal em muitas espécies, já que níveis plasmáticos elevados de glicocorticóides podem inibir a secreção do hormônio liberador de gonadotropinas (GnRH) (Ferin, 2006; Berne *et al*., 2004). Além deste efeito direto sobre a adeno-hipófise, estudos evidenciam que a ativação do córtex da adrenal, por meio do desafio com hormônio adrenocorticotrópico (ACTH), também provoca aumento nas concentrações plasmáticas de hormônios sexuais de origem adrenal (MWANZA *et. al*., 2000; CHATDARONG *et al*., 2006; TSUMA *et al*., 1998; HAUNC; HALTMEYER, 1975;

FENSKE, 1997; WILLARD *et al*., 2005; BOLANOS *et al*., 1997; YOSHIDA; NAKAO, 2006; VAN LIER *et al*., 1999; HEDBERG *et al*., 2007; GINEL *et al*., 2012; FRANK *et al*., 2004).

Esse aumento de progestágenos circulantes pode interferir na regulação endócrina do ciclo reprodutivo de fêmeas, já que a ovulação e formação do corpo lúteo para manutenção da gestação dependem de um balanço hormonal muito preciso.

Fêmeas de cães domésticos (FELDMAN e NELSON, 2004) e de lobo-guará (SONGSASEN *et al*., 2006) apresentam perfil hormonal semelhante de controle do ciclo ovariano e, se o aumento de esteróides sexuais adrenais em situações de estresse também for comprovado em canídeos, possivelmente o eixo hipotálamo-hipofisário-gonadal será afetado.

Dados consistentes sobre função gonadal e adrenocortical em lobos-guará ainda são escassos, sendo que questões importantes sobre o impacto da qualidade do ambiente e práticas de manejo sobre a eficiência reprodutiva de lobos-guarás ainda estão em aberto.

2 REVISÃO DE LITERATURA

2.1 LOBO-GUARÁ

A ordem *Carnivora* é formada por 7 famílias, 92 gêneros e 240 espécies de ocorrência mundial (NOWAK, 1991). O lobo-guará, dentro da Ordem *Carnivora*, está inserido na família *Canidae*, que engloba 16 gêneros e 36 espécies, sendo uma única espécie pertencente ao gênero *Chrysocyon*, não havendo ainda subespécies reconhecidas (SHELDON, 1992). O lobo-guará é o maior canídeo da América do Sul (DIETZ, 1984; SONGSASEN *et al*., 2006; AZEVEDO, 2008), medindo entre 95 e 115 cm de comprimento (mais 38 a 50 cm de cauda) e pesando entre 20 e 30 kg (RODRIGUES, 2002). Sua aparência física difere significativamente da de outros canídeos, principalmente devido às pernas longas e magras. Possui orelhas grandes, pêlos longos de coloração laranja–avermelhado na maior parte do corpo, crina negra no dorso, focinho, patas dianteiras e mais da metade distal das patas traseiras de coloração negra (SHELDON, 1992; Rodrigues, 2002) (FIGURA 1).

FIGURA 1: ESPÉCIME DE LOBO-GUARÁ (*Chrysocyon brachyurus*) ADULTO, FÊMEA.
FONTE: Acervo pessoal, Araxá - MG (2011).

A espécie habita áreas de campos abertos, cerrados e matas de capoeira na região central da América do Sul. A área de distribuição cobre cerca de 5 milhões de km^2 em seis países: Argentina, Bolívia, Brasil, Paraguai, Peru e Uruguai (DIETZ, 1984; RODRIGUES, 2002; DE PAULA *et al*., 2008; AZEVEDO, 2009) (FIGURA 2).

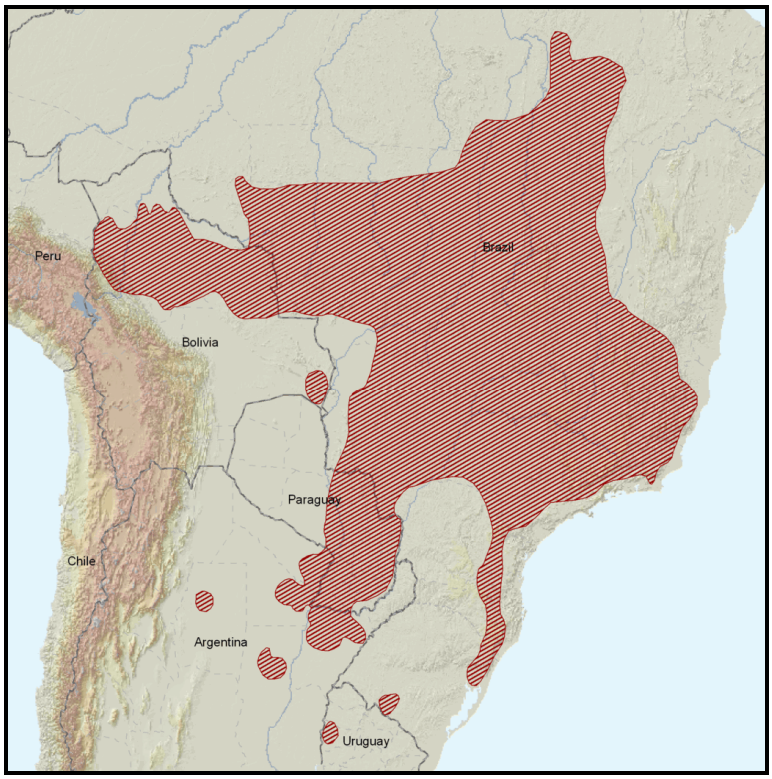

FIGURA 2: MAPA DE DISTRIBUIÇÃO DO LOBO-GUARÁ NA AMÉRICA LATINA. AS ÁREAS HACHURADAS MOSTRAM A OCORRÊNCIA DA ESPÉCIE.
FONTE: IUCN, o*n line* (2012).

Atualmente o lobo-guará está listado entre as espécies ameaçadas de extinção no Brasil, na categoria Vulnerável (MMA, 2003). Na classificação da International Union for Conservation of Nature (IUCN) encontra-se na categoria próximo de ameaçado (NT – “near to threatened”) (IUCN, 2012) e na classificação do Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) está listado no apêndice 2. Esse apêndice não proíbe o comercio internacional da espécie, entretanto o mesmo é estritamente controlado, numa tentativa de impedir que a espécie possa vir a se tornar ameaçadas de extinção e assim passar ao apêndice 1 (CITES, 2012).

A ameaça mais significativa para espécie é a redução e fragmentação de habitat, porém outras fontes de ameaças, como aumento da mortalidade de

indivíduos por atropelamentos, caça, captura de filhotes pelo comércio ilegal, aumento da incidência de doenças e mortalidade de filhotes devido à interação com cães domésticos também acabam comprometendo, em menor escala, a situação da espécie (RODRIGUES, 2002; DE PAULA *et al*., 2008).

Todos esses fatores servem para reduzir o tamanho populacional, gerando instabilidade demográfica e genética e, consequentemente, aumentando a probabilidade de extinções locais, como têm ocorrido em algumas regiões de campos aberto do Estado do Paraná (comunicação pessoal com Azevedo, F. C.. Coordenadora do Projeto Mamíferos do Cerrado). Estima-se que a atual população brasileira de lobos-guarás em vida livre seja de aproximadamente 20.000 indivíduos, entretanto apesar do número total de indivíduos indicar uma população mínima viável, esses indivíduos estão dispersos, formando subpopulações (DE PAULA *et al*., 2008).

O bioma do cerrado é, desde 2000, considerado como um dos mais ameaçados do mundo (MYERS *et al*., 2000), com cerca de 25% de sua área original preservada (Machado *et al*., 2004). Infelizmente, apenas 2% da área do cerrado encontra-se hoje protegida e manejada em Unidades de Conservação (UC) (KLINK e MACHADO, 2005), sendo que, em sua maioria, essas áreas não possuem tamanho suficiente para manter populações viáveis de grandes predadores, como o lobo-guará (RODRIGUES, 2002).

O lobo-guará é encontrado em quase todas as UCs deste bioma, mas populações viáveis, considerando no mínimo 500 animais, são estimadas em apenas três: Parque Nacional do Araguaia – TO; Complexo Parque Nacional das Nascentes do Rio Parnaíba – Estação Ecológica da Serra Geral do Tocantins - PI e Parque Estadual do Mirador – MA. Desta forma, apenas poucas áreas, isoladas, têm a capacidade de manter populações viáveis de lobos-guará (RODRIGUES, 2002).

A espécie é solitária e territorialista, as áreas de vida são fixas e podem variar de 25 e 132 km^2, um tamanho consideravelmente grande que, em geral, não são ocupadas por outros lobos que não o casal (AZEVEDO, 2008). Na maioria dos casos as limitações das áreas são lugares fisicamente identificáveis como rochas ou estradas, sendo a demarcação feita normalmente com urina e fezes (DIETZ, 1984; AZEVEDO, 2008).

Os machos demarcam inicialmente suas áreas sugerindo um sistema onde o macho determina sua área antes da formação do casal. Novas adições no território

podem ser feitas pelo casal, em períodos de estação reprodutiva (DIETZ, 1984). Tanto pares de machos quanto pares de fêmeas já foram observados em confronto físico direto, demonstrando a natureza territorialista da espécie (Rodrigues, 2002).

A formação do casal normalmente se dá na estação reprodutiva, que inicia no outono (março até julho). A espécie é monoéstrica anual, apresentando características de monogamia facultativa, podendo permanecer com o mesmo par por muito tempo (DIETZ, 1984). A gestação é em média de 65 dias, com a maioria dos nascimentos ocorrendo de maio a setembro, durante a estação seca. O número de filhotes varia, na natureza, de dois a quatro filhotes, os quais permanecem na área de vida da mãe durante aproximadamente um ano, quando começam a dispersar (RODRIGUES, 2002).

Em animais cativos o desmame completo ocorre ao redor de 15 semanas, os filhotes começam a ingerir sólidos regurgitados pelos pais depois de quatro semanas de idade. A maturidade sexual ocorre por volta de um ano, mas normalmente não se reproduzem até o segundo ano. Em cativeiro os lobos-guarás podem viver até 16 anos, mas informações precisas em situação natural são escassas (RODDEN *et al*., 1996).

Sua dieta é considerada onívora, sendo constituída basicamente de frutos e pequenos vertebrados, em proporção aproximada de 50% para cada categoria. A maioria dos estudos indica os frutos de lobeira (*Solanum lycocarpum*) como a categoria alimentar mais freqüente. A lobeira é particularmente importante por estar disponível o ano todo, garantindo suprimento de frutos na estação seca (inverno), quando a maioria das outras espécies não está com frutos (DIETZ, 1984; RODRIGUES, 2002; SILVA; TALAMONI, 2003; QUEIROLO; MOTTA-JUNIOR, 2007). Os animais consumidos por lobos-guarás são na maioria de pequeno a médio porte, como pequenos mamíferos (roedores, lagoformos e marsupiais), répteis, aves, peixes e anfíbios (MORATÓ, 2001; QUEIROLO; MOTTA-JUNIOR, 2007).

2.2 ESTRESSE E MECANISMOS FISIOLÓGICOS DE RESPOSTA AO ESTRESSE

A condição de estresse tem sido, nos últimos anos, amplamente discutida e utilizada para definir o grau de qualidade de vida dos animais, tanto de espécies domésticas como de selvagens (MILLSPAUGH; WASHBURN, 2004; MOSTL; PALME, 2002; SHERIFF *et al*., 2011).

O termo estresse (do Inglês “stress”) foi usado inicialmente na física para traduzir o grau de deformidade de um material quando submetido a um esforço ou tensão. Hans Selye (1907 – 1982) foi o primeiro pesquisador que utilizou este termo na medicina e biologia, para traduzir o esforço de adaptação do organismo frente a mudanças consideradas ameaçadoras ao seu bem estar ou à sua vida (KORTE *et al*., 2005). De forma geral, a palavra tem sido associada com eventos negativos e suas conseqüências são conhecidas como “resposta ao estresse” (MORGAN; TROMBORG, 2007). Entretanto, como um mecanismo fisiológico, a resposta ao estresse *per se* não é deletéria, ao contrário, melhora a capacidade de mobilização energética do organismo, adaptando-o para reagir ou fugir do estímulo estressor (MOSTL; PALME, 2005).

Uma série de eventos neuro-endócrinos estão envolvidos com esta resposta orgânica e dentre os hormônios envolvidos, os mais utilizados como indicadores de estresse têm sido os glicocorticóides (BREUNER; HAHN, 2003; MILLSPAUGH; WASHBURN, 2004; KORTE *et al*., 2005; KEAY *et al*., 2006; MORGAN; TROMBORG, 2007; SHERIFF *et al*., 2011; BUIJS *et al*., 2011).

As glândulas adrenais são órgãos complexos e multifuncionais e que, juntamente com o sistema nervoso autonômico, têm papel-chave nos processos fisiológicos de adaptação a mudanças (YOUNG *et al*., 2004; PALME *et al*., 2005).

A glândula apresenta duas partes estrutural e funcionalmente bem distintas: o córtex, onde são produzidos e secretados hormônios esteroidais importantes, como glico e mineralocorticóides; e a medula, responsável pela síntese e secreção dos hormônios catecolaminérgicos. Entretanto, células do córtex podem estar presentes na medula, ao mesmo tempo em que células medulares podem estar presentes no córtex, permitindo a influência direta de uma região glandular sobre a outra. Essa relação íntima entre o córtex e a medula da adrenal é similar à relação anátomo-funcional entre o sistema nervoso adrenérgico e o eixo hipotálamo–hipófise–adrenal (HHA) (BERNE *et al*., 2004; MCNICOL, 1992).

Nos mecanismos fisiológicos de resposta ao estresse, o estímulo estressor é percebido por diversas áreas do sistema nervoso central, que ativam tanto neurônios adrenérgicos, que secretam adrenalina e noradrenalina; quanto neurônios hipotalâmicos, que secretam os hormônios liberador de corticotrofina (CRH) e antidiurético (ADH). A ativação destes neurônios é mutuamente reforçada, pois a noradrenalina aumenta a liberação de CRH, enquanto este, por sua vez, eleva a

descarga de noradrenalina. (BERNE *et al*., 2004; GUYTON, 2006; MOSTL ; PALME, 2002; SUTHERLAND *et al*., 2009).

A liberação dos hormônios hipotalâmicos CRH e ADH estimula a liberação do hormônio adrenocorticotrófico (ACTH) na hipófise anterior, o qual, por sua vez, estimula a síntese e secreção de glicocorticóides adrenais, elevando seus níveis plasmáticos. Ao mesmo tempo, o estímulo adrenérgico direto sobre a medula provoca a elevação dos níveis plasmáticos de adrenalina e noradrenalina. Juntos, os sistemas elevam a produção de glicose e priorizam a utilização deste substrato para o sistema nervoso, disponibilizando outros substratos metabólicos para os demais tecidos (BERNE *et al*., 2004; GUYTON, 2006).

Esta etapa inicial da resposta ao estresse pode ser chamada de reação de alarme, onde todas as respostas corporais entram em estado de prontidão geral, sem envolvimento específico ou exclusivo de um órgão em particular (BERNE *et al*., 2004; GUYTON, 2006).

Se o estímulo estressor continua por períodos mais longos, sobrevém uma segunda etapa chamada fase de resistência, que se caracteriza pela hiperatividade da glândula adrenal. A adrenalina e o CRH produzem um estado geral de vigilância, atenção focalizada e ativação de comportamento defensivo e/ou agressivo. O CRH inibe a liberação do hormônio do crescimento e de gonadotropinas, podendo inibir a atividade sexual, ao mesmo tempo em que os altos níveis plasmáticos de cortisol podem suprimir a ovulação (BERNE *et al*., 2004; FERIN, 2006). Neste estágio, o organismo começa a ajustar-se aos estímulos, e entra num processo de adaptação para poder suportar a condição por mais tempo.

Caso os estímulos continuem, tornando-se crônicos e repetitivos, as respostas metabólicas adversas tornam-se mais evidentes, podendo ocasionar modificações físicas ou psicológicas como comportamento estereotipado (WURBELL; STAUFFACHER, 1996), fraqueza, perda de peso, tendências anti-sociais, baixa capacidade reprodutiva dentre outras (CHAND; LOVEJOY, 2011; CHARBONNEl *et al*., 2008; CYR; ROMERO, 2007; DALEY *et al*., 2000; DOBSON; SMITH, 2000; FARSTAD, 1998; PEREIRA *et al*., 2006; MCCONNACHIE *et al*., 2012a, 2012b; MOORE; JESSOP, 2003; TURNER *et al*., 2005; YOUNG *et al*., 2006). O organismo entra em estado de exaustão, com queda da capacidade adaptativa e falha nos mecanismos de ajuste e redução das reservas de energia.

Esse estado de exaustão está relacionado com a própria regulação do eixo HHA, cuja principal forma de regulação se dá por meio da retroalimentação negativa. A ativação do eixo aumenta a síntese e secreção de glicocorticóides, que por sua vez, inibem a secreção de CRH hipotalâmico e ACTH hipofisário, diminuindo a atividade do eixo. Os efeitos da retroalimetação negativa, pelos glicocorticóides, na liberação de ACTH também podem ser indiretamente moduladas por meio de informações neurais de outras áreas do sistema nervoso central para os neurônios do CRH no hipotálamo. Além disso, os glicocorticóides ativam o gene que codifica o hormônio natriurético atrial (ANH), que também inibe a liberação basal de CRH e ACTH. A própria regulação na tradução, transcrição e exposição dos receptores de glicocorticóides pode ser modulada na exposição a concentrações elevadas destas substâncias (fenômeno de “down regulation”). Os principais tipos de receptores assim modulados são os receptores genômicos de baixa afinidade (BERNE *et al*., 2004; GUYTON, 2006).

Dessa forma, a ação supressiva dos glicocorticóides pode perdurar mesmo após cessar a exposição a estas moléculas. A hipersecreção crônica de glicocorticóides leva a atrofia funcional do eixo HHA e a sua recuperação completa, após a retirada da influencia supressiva, pode levar até um ano, durante esse tempo a resposta normal da glândula adrenal ao estresse não pode ser assegurada (BERNE *et al*., 2004).

2.3 CARACTERÍSTICAS HORMONAIS DO CICLO OVARIANO DE CADELAS

A fisiologia reprodutiva da fêmea de cão doméstico possui particularidades únicas, distinguindo-se de outras espécies. Normalmente o ciclo ovariano da cadela pode ocorrer de 1 a 3 vezes ao ano, com intervalo de 5 a 12 meses, dependendo da raça, e não apresenta características sazonais (CONCANNON *et al*., 1989; FELDMAN; NELSON, 2004).

Normalmente o ciclo é dividido em quatro fases distintas: proestro, estro, diestro e anestro. O Proestro é o período de crescimento folicular. Ao final do anestro, sinalizações ovarianas e principalmente extra-ovarianas atuam sobre o hipotálamo, aumentando a atividade do eixo hipotálamo-hipófise-gônadas (HHG) (CONCANNON, 2009). O aumentando a concentrações de gonadotrofinas

(hormônios folículo estimulante (FSH) e luteinizante (LH)) atuam sobre os ovários, estimulando o crescimento folicular e, consequentemente, a produção e secreção de estrogênios (CONCANNON, 2009; FELDMAN; NELSON, 2004). Desta forma, hormonalmente o proestro é caracterizado pelo aumento nos níveis plasmáticos de estrogênios que resulta na ocorrência de descarga vaginal sanguinolenta, atração de machos e preparação uterina para possível gestação (FELDMAN; NELSON, 2004).

As concentrações plasmáticas de estrogênios continuam aumentando e alcançam o pico máximo (de até 4,6 vezes o valor dos níveis observados durante o anestro) em 24 – 48 horas antes do pico ovulatório de LH, quando passam a decair (FELDMAN; NELSON, 2004).

Concentrações plasmáticas de progestágenos, por sua vez, são baixas durante quase todo proestro, elevando-se rapidamente nas últimas 24 a 72 horas desta fase, antes do pico ovulatório de LH, sendo essa uma das características marcantes do ciclo ovulatório de cadelas. Esse aumento nas concentrações de progestágenos parece estar associado à prévia luteinização do folículo, antes da ovulação (CONCANNON, 2009; VERSTEGEN-ONCLIN; VERSTEGEN, 2008).

Além disso, também ocorre aumento nos níveis sanguineos de testosterona, que alcançam o pico máximo muito próximo ou ao mesmo tempo que o pico ovulatório de LH (FELDMAN; NELSON, 2004; CONCANNON, 2009).

Sendo assim, o período de transição da fase do proestro para o estro é hormonalmente caracterizado pelo declínio nas concentrações de estrogênios ao mesmo tempo em que os níveis de progestágenos aumentam. Esse balanço hormonal estimula dois importantes eventos: 1) mudanças no comportamento sexual da fêmea, que passa a aceitar a monta pelo macho; 2) esse balanço sinaliza o hipotálamo e a hipófise para liberação do pico ovulatório de LH (FELDMAN; NELSON, 2004).

No estro, as concentrações de estrogênios continuam a decair enquanto que as de progestágenos progressivamente aumentam e se mantém alta durante o restante da fase e todo o diestro (FELDMAN; NELSON, 2004; SONGSASEN; WILDT, 2007).

Hormonalmente, o diestro é caracterizado pela predominância de progestágenos. Após a ovulação, o corpo lúteo (CL) é capaz de sintetizar e secretar progesterona por todo período de gestação e além, em fêmeas não gestantes. Desta forma, outra característica do ciclo reprodutivo de cadelas é a ocorrência de pseudogestação fisiológica, onde o corpo lúteo permanece ativo,

independentemente do fato de ocorrer gestação, pois nessa espécie não há nenhum mecanismo luteolítico conhecido e sendo assim o CL pode permanecer ativo por até 55 – 75 dias (VERSTEGEN-ONCLIN; VERSTEGEN, 2008; FELDMAN; NELSON, 2004; SONGSASEN; WILDT, 2007; CONCANNON, 2009).

Outra característica importante é que durante a gestação não há gonadotrofina placentária, secreção de progesterona placentária ou atividade de aromatase placentária e a produção e secreção de esteróides sexuais são inteiramente de origem ovariana (CONCANNON, 2009).

O anestro é a fase do ciclo reprodutivo onde ocorre a involução uterina, caracterizado pela quiescência de atividade ovariana e redução nas concentrações de progesterona e outros hormônios esteróides ovarianos. Em fêmeas pseudo-gestantes o início dessa fase não é facilmente perceptível, não havendo uma demarcação clínica óbvia (FELDMAN; NELSON, 2004; SONGSASEN; WILDT, 2007).

2.4 ATIVIDADE ADRENOCORTICAL X FUNÇÃO REPRODUTIVA

A função gonadal é regulada pelo eixo hipotálamo-hipófise-gônadas (HHG). Resumidamente, o hipotálamo, por meio da liberação do hormônio liberador de gonadotrofinas (GnRH), comanda a porção hipófise-gonadal do eixo. Vários núcleos hipotalâmicos liberam, de forma pulsátil, GnRH que por sua vez estimula a liberação das gonadotrofinas hormônio folículo estimulante (FSH) e hormônio luteinizante (LH). Essas gonadotrofinas agem nos ovários e testículos, estimulando a atividade gonadal e assim a secreção de esteróides gonadais (progestágenos, estrogênios, androgênios) (BERNE *et al*., 2004; GUYTON, 2006; FERIN, 2006).

A natureza pulsátil desse hormônio é importante sinalizadora para os gonadotropos na produção e secreção de FSH e/ou LH. Desta forma, condições que interfiram na geração dos pulsos de GnRH irão perturbar o eixo HHG. A completa ausência de pulsos resulta em inatividade total do eixo, enquanto que anormalidades menores, tais como, redução da freqüência de pulsos de GnRH, como normalmente ocorre durante situações de estresse, alteram a produção e liberação de

gonadotrofinas, interferindo na função reprodutiva em maior ou menor grau (FERIN, 2006).

Os mecanismos, centrais ou periféricos, pelos quais os estímulos estressores podem interferir com a função reprodutiva normal são inúmeros e intrincados e podem influenciar no eixo HHG em qualquer nível de controle, entretanto acredita-se que o impacto inicial e predominante seja no sistema nervoso central, especificamente sobre o controle da geração de pulsos de GnRH com conseqüência na liberação de LH, já que grande parte dos estímulos estressores conhecidos e estudados atua inibindo a secreção de LH (FERIN, 2006).

Evidências sugerem o papel do hormônio liberador de corticotrofina (CRH), neuropeptideo que controla a atividade do eixo HPA, e do hormônio antidiurético (ADH), outro neuropeptideo que sinergiza com o CRH na ativação do eixo HPA, como elos de ligação entre o aumento da atividade do eixo HPA e a inibição do eixo HPG. Essas evidências foram obtidas por observações de que a neutralização da atividade do CRH endógeno, por utilização de antagonistas dos receptores de CRH, resultava na restauração de atividade reprodutiva normal dos animais durante situações estressantes (FERIN, 2006).

Além do CRH e ADH, uma série de outros fatores hormonais e neurais podem modular a secreção pulsátil de GnRH, e como dito anteriormente, podem atuar não apenas sobre a atividade geradora do pulso de GnRH, mas também em qualquer nível de controle do eixo. Sendo assim, não apenas os glicocorticóides, mas os outros hormônios liberados durante a resposta ao estresse podem modular a função reprodutiva do organismo (FERIN, 2006; DOBSON; SMITH, 2000; KORTE *et al*., 2007) (FIGURA 3).

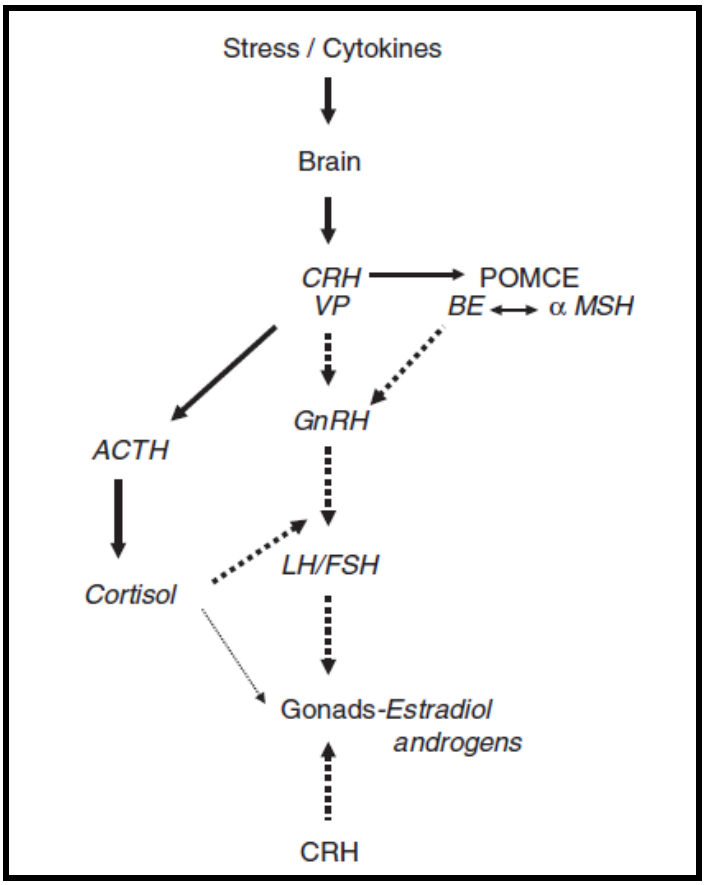

FIGURA 3: ESQUEMA DAS PRINCIPAIS VIAS MEDIADORAS DA RESPOSTA ENDÓCRINA DO EIXO HHG AO ESTÍMULO ESTRESSOR. Linhas continuas: estimulação; linhas tracejadas: inibição. FONTE: Ferin, 2006. Stress and the reproductive system. In: Knobill and Neill's Physiology of Reproduction, 3º Edição, Elsevier, p. 2656.

3. HIPÓTESES E PREDIÇÕES

Nós propomos que, estimulações agudas e/ou crônicas na função adrenocortical de lobos-guará cativos possam desencadear alterações na função gonadal e, com isso, levar a falhas no ciclo reprodutivo dos animais, principalmente em fêmeas. Se essa hipótese for verdadeira, esperamos encontrar, nos animais submetidos a hiperestimulação prolongada (estimulação crônica), alterações nos perfis hormonais de esteróides gonadais (metabólitos de estrogênios, progestágenos e androgênios fecais) que demonstrem / indiquem falhas no ciclo reprodutivo.

Para tanto, devido à escassez de informações acerca da função adrenocortical de lobos-guará, torna-se primeiramente necessário caracterizar o perfil de atividade adrenocortical, por meio da quantificação de metabólitos de corticóides fecais (MCF), normal durante as diferentes fases do ciclo reprodutivo da espécie.

Além disso, em relação ao estímulo adrenocortical agudo, esperamos que, nos animais que foram submetidos ao desafio hormonal por meio da administração do hormônio adrenocorticotrópico (ACTH), ocorram alterações significativas nas concentrações desses mesmos metabólitos fecais (estrogênios, progestágenos e androgênios), sugerindo que o córtex da glândula adrenal também possa secretar hormônios sexuais em resposta ao estímulo pelo ACTH.

4. OBJETIVOS

4.1 Objetivo Geral

O presente estudo tem por objetivo gerar dados básicos sobre a função adrenocortical e gonadal de lobos-guará cativos em condições normais e quando submetidos à hiperestimulação adrenocortical prolongada e aguda.

4.2 Objetivos Específicos

- Caracterizar os perfis de atividade adrenocortical, por meio da quantificação de metabólitos de corticóides fecais, de machos e fêmeas ao longo do ciclo reprodutivo normal da espécie.

- Caracterizar os perfis de atividade gonadal, por meio da quantificação de metabólitos gonadais fecais (estrogênios, progestágenos e androgênios), de machos e fêmeas ao longo do ciclo reprodutivo normal da espécie.

- Comparar os achados por fases do ciclo reprodutivo de todos os metabólitos hormonais fecais em fêmeas, machos e entre os gêneros.

- Avaliar os dados obtidos de todos os metabólitos hormonais fecais com base na estação reprodutiva da espécie.

- Analisar os efeitos da hiperestimulação adrenocortical prolongada nos perfis individuais e nas concentrações médias totais de cada metabólito gonadal fecal.

- Analisar os efeitos da hiperestimulação adrenocortical aguda, por meio da administração do hormônio adrenocorticotrópico (ACTH), nas concentrações de metabólitos gonadais fecais.

- Comparar as possíveis diferenças encontradas entre os gêneros.

- Analisar as funções adrenocortical e gonadal de dois casais de lobos-guará cativos mantidos em recintos menores e sujeitos à visitação pública.

5 MATERIAIS, MÉTODOS E RESULTADOS

5.1 CONSIDERAÇÕES GERAIS

Todo o material, a metodologia e as técnicas empregados, bem como os resultados e a discussão específica de cada um dos dois estudos que compõem esta tese estão descritos nos manuscritos apresentados a seguir (manuscrito 1 e 2). Todos os estudos foram aprovados pela Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA 23075.031103/2012-23) e tiveram licença do Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA (22610-SisBio).

5.2 MANUSCRITO 1

Characterization of fecal adrenocortical and gonadal metabolites profiles in captive maned wolves (*Chrysocyon brachyurus*) throughout normal reproductive cycle.

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ABSTRACT

Patterns of fecal adrenocortical and gonadal hormones metabolites excretion were investigated throughout normal reproductive cycle in captive maned wolves (*Chrysocyon brachyurus*). Fecal samples of 9 adult maned wolves (4 females and 5 males) were collected 2–5 days/week for 4-11 months. Hormone metabolites of estrogens (FEM), progestagens (FPM), androgens (FAM) and corticoid (FCM) were extracted from feces and quantified by enzyme immunoassay. The overall concentration means, including the findings in males, were analyzed considering: 1) the female reproductive phases; and 2) based on the reproductive season of the specie (from February to July). The concentration of fecal gonadal metabolites is lower during the anestrus period for both genders. On females, at the proestrus there is an increase on the FAM and FEM levels, while the FPM concentration begins to rise only at the periovulatory period, maintaining a high level during diestrus and then decaying on lactation and parental care period. On males, the FAM level begins to rise on females' proestrus and periovulatory period and decrease on diestrus phase. After the pups' birth, during lactation and parental care period, means concentration of FAM, FEM and FPM presented significant increase. The pattern of fecal corticoid metabolites excretion shows a higher concentration during proestrus and lactation and parental care period on females. Males show no variation of FCM during most part of the female' reproductive cycle, although a significant increase is observed during parental care, when the males participate in the care of the pups. When data are grouped based on the breeding season, many previously observed differences disappear, mainly in the profiles of androgens and corticoids in males.

KEY WORDS: maned wolf, reproductive cycle, gonadal function, adrenal activity, fecal steroids metabolites, captive breeding.

1. **Introduction**

The maned wolf (*Chrysocyon brachyurus*, Illiger 1811) is one of the most typical canid species that inhabits the Brazilian grassland areas (known as Cerrado) (Dietz 1984). The maned wolf is recognized by the IUCN – World Conservation Union - as a 'nearly threatened' species (IUCN 2012), mainly due to the reduction and fragmentation of its natural habitat for agricultural development and cattle farms (Silveira and Jácomo 2003). The Brazilian Cerrado is comprised of unique fauna and flora, ranking it one of the world's 25 biodiversity hotspots (Myers et al. 2000). Unfortunately the fragmentation of this biome is an ongoing process (Carvalho et. al. 2009) and only 2% of it is government-protected and managed in units of conservation (UC) (Klink and Machado 2005). This reality seriously affects the maned wolves since they demand a significant sized home range (averaging 50.9 km^2/547 ft^2) (Azevedo 2008). Maned Wolves' Population and Habitat Viability Assessment estimated that nearly 20,000 wolves are still in nature, mostly in Brazil (de Paula et. al. 2008), but the exact number of individuals remains unknown and wild populations are increasingly at risk.

Since 1984, in response to the uncertain future of wild populations, the American Zoo and Aquarium Association (AZA) created the Maned Wolf Species Survival Plan (MWSSP) and it had one critical goal: to maintain a viable and self-sustaining captive population (Rodden et. al. 1996). Since then, together with an integrated management of *in situ* populations, efforts for conservation of maned wolves have been done in order to develop policies to optimize captive population management (Maia and Gouveia 2002). Maintaining viable *ex situ* populations is considered an important element against extinction, besides being a great source for researches that try to understand better the biology of this species.

Although the reproduction of captive maned wolves has improved throughout the past few years, this population is still not self-sustained due to low pregnancy success and high neonatal mortality (Songsasen et al. 2006; Maia and Gouveia 2002; Vanstreels and Pessutti 2010). Basic information on gonadal endocrine profiles of captive maned wolves has been reported (Velloso et al. 1998; Songsasen et al. 2006; Costa et al. 2008) and it is speculated that their low reproductive efficiency may be associated with endocrine gonadal disorders as a result of chronic captivity stress (Songsasen et al. 2006; Cummings et al. 2007). Unfortunately, there are few studies in maned wolves involving adrenocortical function and no reports are found describing how the glucocorticoids concentration varies along a normal reproductive cycle, not even in couples who have had reproductive success. Based on this information more studies may be designed to assess the real incidence of chronic stress in captive maned wolves' couples and how the increase of adrenocortical activity is influencing their reproductive cycles.

Therefore, the aims of this study were to characterize fecal adrenocortical and gonadal hormone metabolites profiles in captive male and female maned wolves based on females' normal reproductive cycle and also on the known reproductive season of the species.

2. Methods

2.1. Animals and sample collection

Fecal samples were collected from 9 adult maned wolves (4 females and 5 males; age range 2-9 years) maintained in a Brazilian conservation breeding center (Criadouro Científico de Fauna Silvestre para Fins de Conservação, Companhia

Brasileira de Metalurgia e Mineração, Araxá-MG) (Table 1). During the breeding season (March – June) the feces of females were collected 3 times/week and males' 1-2 times/week. Out of the breeding season (July – February) the samples of females were collected 1-2 times/week and males' 1 time/week. One female was monitored for 2 successive breeding season (16 months) and two couples were monitored during the first semester of the year (4-6 months), totalizing 10 endocrine profiles.

All animals were exposed to natural photoperiod, housed in pairs and were subject to the public. Their management was done according to institution's routine. The size range of the enclosures varied from 2000 to 5000 m^2 (10,760 to 53,820 ft^2) and the wolves' diet was compound of approximately 40% commercial dog food and/or minced beef, 40% fruit and 20% vegetables supplemented with vitamins. Additionally, the wolves received a freshly killed white rat (raised at the institution) and one boiled egg 3 times a week. All the animals had access to fresh water *ad libitum*.

The fecal samples of females and males were differentiated by the presence of seeds and papaya peel (*Carica papaya*) in the males' scats. The institution's handlers offered papaya to the males at one end of the enclosure, over the screen fence, while the females received banana (*Musa spp*.) at the other end. This management ensured that the papaya seeds and peel were eaten only by males.

The samples were collected non-invasively, directly from the enclosures' floor, by morning (7:00-8:00 a.m.), as part of the handling routine, were placed in plastic bags (labeled with date and animal's identification) and stored at -20ºC (-4 ºF) before being transported in ice for analysis.

2.2. Fecal extraction and analysis

2.2.1. Fecal extraction

Fecal extraction and hormone quantification were performed in the Laboratory of Reproductive Physiology, Universidade Federal do Paraná, Curitiba, PR. All reagents (except when specified) were purchased from Sigma-Aldrich (Sigma-Aldrich Brasil Ltda, São Paulo, Brazil) and all solutions prepared with Milli-Q water. Fecal extraction was performed by the methods of Spercoski et. al. (2012) with slight modifications (Anexo 1). Briefly, an aliquot of ~0.5 g of wet, well-mixed, fecal sample was placed in a glass tube containing 5 ml of 80% ethanol:20% distilled water and was vigorously shaken for 30 min using a Multi-Pulse vortexer (Glass-Col, Terre Haute, IN). Each sample was centrifuged (1,000xg, 15 min) and the supernatant was recovered. The mean (± SEM) of extraction efficiency, by the addition of labeled H^{3+}-cortisol, was 86.2 ± 0.5% with a coefficient of variation (CV) of 9.1%.

2.2.2. Fecal enzyme immunoassays (EIA)

Fecal extracts were quantified by enzyme immunoassay (described by Brown et. al. (2004) for fecal estrogens (FEM), progestagens (FPM), androgens (FAM) and corticoids (FCM) metabolites. The polyclonal antiserum used in the study were conjugate estrone (R522-2; 1:20,000 dilution; FEM), pregnane (CL425; 1:10,000 dilution; FPM), testosterone (R156/7; 1:10,000 dilution; FAM) and cortisol (R4866; 1:8,500 dilution; FCM) provided by Coralie Munro (University of California–Davis, Davis, CA).

Validation assays for all hormones had already been described (Songsasen et al. 2006; Spercoski et al. 2012). Intra- and inter assay coefficients of variation were <3 % and <15%, respectively, for all EIAs.

2.3. Data analysis

The overall data of the individuals were combined, divided in different groups for statistical analysis and checked for normality. Parametric data were statistically analyzed using one way ANOVA followed by Tukey' multiple comparison procedure or t-test. Non-parametric data were analyzed using Kruskal-Wallis one way ANOVA on ranks followed by a Dunn's multiple comparison procedure or Mann-Whitney rank sum test. All analysis were considered significant when $P<0.05$.

The results are presented in ng/g of wet feces, except for FPM where the concentration is presented in μg/g.

2.3.1. Oestrus cycle phases

The data, both female and male, was divided in 5 different reproductive cycle phases based on fecal hormone concentrations of the females: 1) anestrus phase – data of samples obtained before the proestrus phase and after diestrus phase for pseudo-pregnant or after lactation and parental care phase for pregnant females; 2) proestrus phase - from the increase of FEM concentration to the day of the FEM peak; 3) peri-ovulatory phase - comprising the 2 days after the FEM peak (late proestrus) and the first 7 days of oestrus. This phase was determined based on the FEM peak that happens simultaneously with an increase on FPM concentration and the FAM peak (Feldman and Nelson 2004; Concannon 2009); 4) diestrus phase – from 17 days after the FEM peak to the following 65 days for pseudo-pregnant or

until one day before parturition for pregnant females; 5) lactation and parental care phase – from the day of parturition to the following 60 days. To better understand this division see figure 2.

Overall data were separated by gender and each fecal gonadal and adrenocortical metabolite was analyzed for differences between the reproductive phases and for differences between genders in each of the reproductive phases.

2.3.2. Reproductive season profiles

The hormonal data of maned wolves were also analyzed based on reproductive season. Data of both genders were divided in two groups: 1) fecal samples obtained during the reproductive season (RS) and 2) fecal samples obtained during non-reproductive season (NRS). All samples collected between February 25 and July 15 (date range from the proestrus onset to the end of the periovulatory period) were considered RS. All fecal gonadal and adrenocortical metabolites were analyzed for differences between RS and NRS and between genders.

3. Results

3.1. Hormone data grouped based on females' reproductive cycle

Overall concentration mean of fecal metabolites (estrogens, FEM; progestagens, FPM; androgens, FAM; corticoids, FCM) of captive maned wolves, for each phase of the reproductive cycle, are shown in figure 1.

3.1.1. Females

FEM concentration was lower during anestrus (4.3 ± 0.3 ng/g; n=82; P<0.05) and rose from proestrus (9.4 ± 0.7 ng/g; n=152) to diestrus (15.7 ± 0.7 ng/g; n=63). During the proestrus period a high frequency of FEM peaks (average 5.0 ± 2.3; mean highest value 23.7 ± 7.3 ng/g) was observed. Similarly, during diestrus the level of FPM (2.6 ± 0.3 μg/g; n=53; P<0.05) was elevated above anestrus (0.3 ± 0.04 μg/g; n=66) and proestrus (0.3 ± 0.02 μg/g; n=130). Lower FAM concentration was found during anestrus (22.9 ± 2.6 ng/g; n=66; P<0.05) compared to proestrus (39.8 ± 3.5 ng/g; n=130) with a progressive return to the values observed at the anestrus phase, during lactation and parental care period (24.4 ± 3.9 ng/g; n=38). As observed in estrogens metabolites, FAM peaks (average 3.3 ± 0.9; mean highest value 138.3 ± 31.0 ng/g) were more frequent during the proestrus phase.

As for fecal adrenocortical patterns, FCM concentration was high during proestrus (190.1 ± 13.5 ng/g; n=90) and lactation / parental care (230.7 ± 35.5 ng/g; n=31; P<0.05) when compared to the anestrus phase (140.2 ± 16.0 ng/g; n=62). At the other periods no significant variation on the level of FCM was observed.

3.1.2. Males

The results of males were also divided and analyzed based on females' reproductive phases, in attempt to verify how the females' hormonal fluctuations through oestrus cycle affect males' hormonal profiles.

The FEM concentration in males was high only during parental care period (12.9 ± 0.6 ng/g; n=31; P<0.05). From proestrus (8.8 ± 0.8 ng/g; n=91) to diestrus (9.3 ± 1.8 ng/g; n=9) no differences were found. The anestrus overall concentration mean

was 5.1 ± 0.6 ng/g (n=57) and it did not show differences when compared to proestrus, periovulatory and diestrus phases.

During parental care FPM presented higher concentration (0.7 ± 0.1 μg/g; n=31; P<0.05) and lower concentration was found during proestrus (0.2 ± 0.01 μg/g; n=91; P<0.05).

Higher concentration of FAM was observed at proestrus (42.7 ± 4.2 ng/g; n=90; P<0.05), periovulatory (58.2 ± 21.2 ng/g; n=6) and parental care phases (47.2 ± 6.0 ng/g; n=31). Anestrus (25.9 ± 2.9 ng/g; n=57) and diestrus (25.4 ± 3.6 ng/g; n=33) levels were significantly low (P<0.05). The fecal corticoid metabolites concentration differed during parental care (229.7 ± 34.0 ng/g; n=31; P<0.05) with a higher level when compared to anestrus (134.1 ± 18.3 ng/g; n=50). From proestrus (135.5 ± 14.0 ng/g; n=87) to diestrus (132.2 ± 18.6 ng/g; n=32) no differences were found.

3.1.3. Gender differences

FEM concentration showed differences in gender during diestrus, when females had higher values (P<0.05) (Fig. 1). Females also showed higher baseline FPM concentration at proestrus, periovulatory, diestrus and lactation / parental care phases (P<0.05). Males presented a higher FAM level at lactation / parental care period (P<0.05).

Overall FCM concentration only showed differences during the proestrus phase, when the values in females were higher than in males (P<0.05).

3.1.4. Proestrus length in young and adult female maned wolves

In this study the onset of the reproductive season of female maned wolves was identified by an increase on the FEM concentration that marked the first day of proestrus. The reproductive season was from February 25 to July 15, the date range from the proestrus' onset to the end of the periovulatory period. The average length of the proestrus was 75 days. Longitudinal profiles of FEM and FPM showed that the ovulatory cycle, marked by an FEM peak simultaneous to an increase on FPM concentration, can occur early in breeding season in adult females (6-7 year-old; Fig 2B) or extend for up to 85-92 days from the onset in younger females (2-3 years-old; Fig 2A).

3.2. Reproductive season

Overall concentration means of fecal gonadal and adrenocortical metabolites, grouped in reproductive and non-reproductive seasons (RS and NRS, respectively) are shown in figure 3.

Female maned wolves in RS presented higher concentrations for all fecal metabolites when compared to the NRS. In males, high concentration was found only for FEM. Unlike the females, the FPM level of males was higher during NRS. The FCM concentration in males did not differ between seasons. There were differences associated with gender during RS in FEM, FPM and FCM concentrations where females had a higher overall mean value. There were no differences between genders during NRS.

4. Discussion

This study characterizes fecal adrenocortical and gonadal hormones metabolites profiles along all reproductive cycle in captive maned wolves, generating baseline data, mainly for corticoids overall concentration, of non-invasive monitoring of this species. It also shows the fluctuations on corticoids levels throughout a reproductive cycle.

The proestrus phase in female maned wolves is marked by increased concentrations and frequency of peaks of fecal estrogens and androgens metabolites. Later, during late proestrus and the periovulatory period, fecal progestagens metabolites concentrations rise. Altogether these hormones profiles identify the increase on activity of the hypothalamic-pituitary-gonadal axis (HPG). These hormones profiles observed on female maned wolves are very similar with the hormonal events that occur on normal domestic dog reproductive cycle (Feldman and Nelson 2004; Songsasen et al. 2006).

In female domestic dog at late anestrus and onset of proestrus, progressive changes in the ovary and, more importantly, in extra-ovarian tissues produce factors that release the hypothalamus and pituitary from suppressive effects and allow an increase of GnRH and LH pulse frequency, which act on the ovaries, promoting follicular development and consequently increased estrogens and androgens levels (Concannon 2009; Rajkovic et a. 2006; Hunzicker-Dunn and Mayo 2006).

Many features of the canine reproductive cycle are unique when compared to other species, including a periovulatory rise in serum progesterone that stays elevated for as long as 100 days in non-pregnant females, due to the absence of luteolytic mechanism; delay in oocyte maturation after ovulation; delay in implantation of the embryo; during pregnancy, there is no placental gonadotrophin, no placental

progesterone secretion or aromatase, and sex steroid production is entirely of ovarian origin (Verstegen-Onclin and Verstegen 2008; Concannon 2009).

Hormone-wise, the normal onset of a canine ovarian cycle is characterized by elevated plasma levels of estrogens that rise from 5-15 pg/mL at late anestrus up to 60-70 pg/mL at late proestrus. The peak of plasma estrogens is reached 24-48 hours before the end of proestrus, marked by an LH surge. Serum progestagens concentration is low (<0.5 ng/mL) throughout most of proestrus, rising on the last 6-3 days of this phase. This raise is a striking feature of the canid ovarian cycle, as mentioned above, and is associated with pre-ovulatory luteinization of follicles, as early as 6-3 days before the LH surge (Verstegen-Onclin and Verstegen 2008; Concannon 2009; Feldman and Nelson 2004).

Plasma concentration of androgens also increases at late proestrus, however in smaller magnitude, reaching higher levels near the LH peak, which occurs in early estrus (Feldman and Nelson 2004). This increase on androgens level is probably associated to estradiol production when androgens are the substrate for the aromatase enzyme complex (Guthermuth et al. 1998; Concannon 2009).

Thus, the periovulatory period (from late proestrus to early estrus) is marked by an increase of plasma progesterone and androgens concentrations associated with a decrease on estrogens levels, reflecting the final maturation process of ovarian follicles. This endocrine pattern of sex steroids at this phase leads to two important events: changes on the female's sexual behavior, which starts to accept mating; and gives a strong feedback to the hypothalamus and pituitary, resulting on the LH surge (Feldman and Nelson 2004).

During diestrus, progestagens plasma concentration continues to grow and remains high throughout the entire phase due to the corpus luteum (CL) activity, for

the start and maintenance of pregnancy. Normally, plasma concentration of estrogens is lower at diestrus, although variations within individual bitches have been reported (Feldman and Nelson 2004).

Fecal progestagens and estrogens metabolites profiles found in this study are similar to serum endocrine profiles reported for canine reproductive cycle (Feldman and Nelson 2004). FPM concentration remains elevated during all pregnancy in maned wolves. Although FEM concentration is higher on diestrus phase, this finding was already reported for fecal metabolites profiles in female domestic dogs (Guthermuth et al. 1998) and individual variations in serum profiles of estrogens on diestrus have also been found (Olson et al. 1982).

It is believed that estrogens may play a role in sustaining corpus luteum (CL) function, but this has not been clearly identified. Luteal regulation in canine diestrus appears to be a complex and dynamic process in which several complementary hormones interplay, such as progesterone, prolactin, LH, relaxin and estrogens (Verstegen-Onclin and Verstegen 2008).

Some authors report that the increase of estradiol levels in pregnant bitches, from 10 days of the onset of diestrus, can simply reflect luteal stimulation by an increased prolactin (PRL) level (Concannon 2009), as it is suggested that estradiol is entirely of ovarian origin by the absence of detectable placental aromatase in domestic dogs (Nishiyama et al. 1999). The two main luteotrophic hormones are LH and PRL, whereas prolactin seems to be the main and essential support for canine CL (Kooistra and Okkens 2002).

Endocrine gonadal profiles showed that the maned wolves' reproductive season can start as early as the end of February and extend up to mid July. The average of proestrus length found in this study is very different from those reported for maned

wolves (Songsasen et al. 2006; Velloso et al. 1998; Rodden et al. 1996) and for domestic dogs (Feldman and Nelson 2004; Concannon 2009). Two endocrine profiles of females wolves that raised pups showed 13-15 days of proestrus length, which is considered normal, however the other three profiles, being one of a female that also conceived, showed prolonged proestrus (range 85-107 days). This phenomenon seems to be associated with age, since the prolonged proestrus profiles are from 2-3-year-old females while the others are from 6-7-year-old females. The individual profiles of those females (see figure 2A) can reveal possible anovulatory cycles where FEM peaks were not coincident with an increase of FPM concentration.

As previously described, female domestic dog's luteinization occurs before ovulation, with rapid and generalized proliferation of luteinizing cells accompanied by an increase of serum progesterone concentration (Feldman and Nelson 2004; Verstegen-Onclin and Verstegen 2008). Irregularities in the LH surge and consequently on the ovulation can occur as a result of abnormal early luteinization. Clinically this can be recognized as false estrus, anovulatory cycles and/or prolonged proestrus (Verstegen-Onclin and Verstegen 2008).

Fecal adrenocortical patterns of female maned wolves show higher fecal corticoid concentration at proestrus and lactation / parental care period. Relative to proestrus, a similar result is reported in female rats when the circadian peak in serum corticoid at proestrus is twice higher than at estrus or metaestrus (diestrus) (Cavigelli et al. 2005).

In maned wolves the females' proestrus marks the onset of the reproductive season and it is possible that this higher concentration is associated with two different factors: 1) increase of adrenocortical activity, due to higher energy demand

for this period (male approximation and breeding); 2) release of corticoids from sex steroid-binding globulins.

It is known that circulating corticoids in females can also bind with gonadal steroid-binding globulins to a considerable extent (Touma and Palme 2005). With the onset of a new ovarian cycle, increases in concentration of estrogens and progestagens can influence the occupancy of plasma gonadal steroid-binding globulins, increasing the serum concentration of free corticoids and their metabolism, thereby causing an increase of fecal metabolites.

As for the increase of adrenocortical activity mentioned above, many studies report that the adrenocortical activity, as well as the reactivity of the hypothalamic-pituitary-adrenal axis (HPA), can be seasonally modulated according to different requirements throughout a life cycle, such as growth, pregnancy and lactation (Touma and Palme 2005; Romero 2002).

So it is more likely that the increase on FCM levels during proestrus is associated with a higher production of sex steroids, while during lactation and parental care it is linked to modulation of adrenocortical activity.

In females, pregnancy itself induces a series of hormonal changes and almost all endocrine glands have their activity increased, especially near the parturition, when corticoid levels rise due to a release of fetal corticosteroids. During the lactation period, the metabolic demand of the females is even higher, and the high levels of corticoids can lead to amino acids, glucose and fatty acids mobilization from the diet, as well as from maternal tissues, for milk production (Feldman and Nelson 2004; Berne et al. 2006).

Fecal gonadal metabolites profiles of male maned wolves show higher concentrations of androgens precisely at the female's proestrus and peri-ovulatory

phase. Behavioral and ecological studies have already indicated that the maned wolf is a seasonal breeder (Dietz 1984; Rodrigues 2002), therefore these results in fecal androgens pattern are in agreement with other studies conducted with male maned wolves that reported higher concentrations of serum testosterone (Maia et al. 2008), better sperm quality and increased testicular size (Teodoro et al. 2012) during the reproductive season.

Seasonal changes in spermatogenesis, morphology of the testis and peripheral serum testosterone concentration have been reported in other canid species, such as the red and blue foxes (Farstad 1998), African wild dogs (Newell-Fugate et al. 2012), raccoon dogs (Qiang et al. 2003), Ethiopian wolves (Van Kesteren et. al. 2012), Iberian wolves (Barja et al. 2008) and coyotes (Minter and DeLiberto 2008).

To cope with the changes of environmental seasonal fluctuation most species exhibit seasonal cycles of physiological adaptation, such as the ability to restrict breeding activity to a specific time of year, meeting the highest energy demanding stages of reproduction (late pregnancy, birth, lactation, weaning of the young) with annual peaks of food availability and more propitious conditions for survival of the young. The annual cycle of photoperiod is the major source of environmental information in control of a great variety of seasonal physiological activities, through a rhythmic secretion of melatonin. It can modulate reproductive activity by regulating the HPG axis so gametogenesis, sexual behavior and pregnancy are timed accordingly (Malpaux 2006).

Another increase on fecal androgens levels occurs during parental care period. At this phase, male maned wolves also show increased levels of estrogens, progestagens and corticoids metabolites. Studies involving both captive and free-living male maned wolves report that the males participate in parental care by

protecting and providing food to their offspring and mates (Dietz 1984; De Melo et al. 2009; Veado 1997, apud De Melo et al. 2009). So it is possible that these changes in the hormonal profiles are associated with male parental behavior.

Among mammalian species, in which fathers contribute to the care of the youngs, distinct relationships exist between hormones and parental behavior; and prolactin has received most attention. Males in a variety of mammalian species experience elevations in prolactin levels during periods in which they care for the young, suggesting that prolactin commonly promotes paternal behavior (Nunes et al. 2001; Ziegler 2000).

It is known that estrogens can regulate prolactin release in mammals, promoting the stimulation of lactotrophs by activating classic nuclear receptors, which leads to an increased expression of prolactin. Estrogens can also modify the responsiveness of lactotrophs to other molecules and at high concentrations (e.g. late proestrus) decrease the density of dopamine receptors (D2). Additionally, estrogens also alter the density of the voltage-dependent calcium channel and electrical excitability of lactotrophs, facilitating the process of exocytosis and increasing both basal activity and responsiveness of these cells (Gregerson 2006).

Therefore it is possible that high levels of estrogens in male maned wolves during parental care leads to a rise on prolactin levels. No studies were found reporting patterns of prolactin levels in maned wolves during this phase.

The increase of estrogens during parental care suggests rise on the aromatase enzyme complex activity. A recent study with ACTH challenge in male maned wolves reveals that adrenocortical stimuli also increase FEM concentration (Spercoski 2012; 2th chapter). This report is in agreement with our findings, since the concentration of fecal corticoids metabolites is also higher for this same reproductive phase.

The rise on corticoid levels observed after the pup's birth can be linked with the important role of these molecules on energy mobilization during costly periods, as already mentioned for female maned wolves. In males it may also be true due to energy costs associated with testosterone levels and territorial defense (Romero 2002; Ketterson et al. 1991; Sands and Crell 2004).

It has been suggested that male maned wolves play an important role in rearing their offspring, but the partnership between male and female was not equal, with the female spending much more time in the nest while the male brings preys and performs territorial marking, other necessary and important roles for the survival of the offspring (De Melo et al. 2009). Thus, higher corticoids and androgens levels during parental care period found in this study are in agreement with behavior observation studies in male maned wolves. Additionally, it is also known that adrenocortical activity can be seasonally modulated in free-ranging maned wolves, when higher levels of FCM were found during spring, when wolf pairs were raising pups (Spercoski et. al. 2012).

Data analysis based on reproductive season also shows that within the season there is an increase on fecal metabolites patterns; however some hormonal fluctuations, such as the rise of androgens during mating, are not possible to be observed due to the way the data is grouped.

In conclusion, female maned wolves present similar hormonal fluctuations of normal canine reproductive cycle. The anestrus phase is marked by lower levels of gonadal hormones, proestrus is characterized by an increased level and frequency of estrogens peaks; during the periovulatory phase there is an increase of progestagens that remains higher until the end of pregnancy. Males show increase of androgens concentration during females' proestrus and periovulatory phases.

Corticoids normally rise during proestrus and lactation / parental care period in females. Finally, after the birth of the pups and during all parental care period the males show increased fecal gonadal and adrenocortical metabolites levels.

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6. Declaration of Interest

The authors have no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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Tables

Table 1 – Maned wolves' couple analyzed, age (years), monitoring period (months) and enclosure area (m^2).

Animal	Couple	Age	Enclosure area	Monitoring period
Male 1	1	2	2000	6
Female 1		2		6
Male 2	2	3	2500	4
Female 2		3		4
Male 3	3	3	2000	11
Female 3		3		11
Male 4	4	6	5000	11
Female 4		7		11 (first breeding season)
Male 5	5	4	5000	4
Female 4		8		5 (second breeding season)

Figures

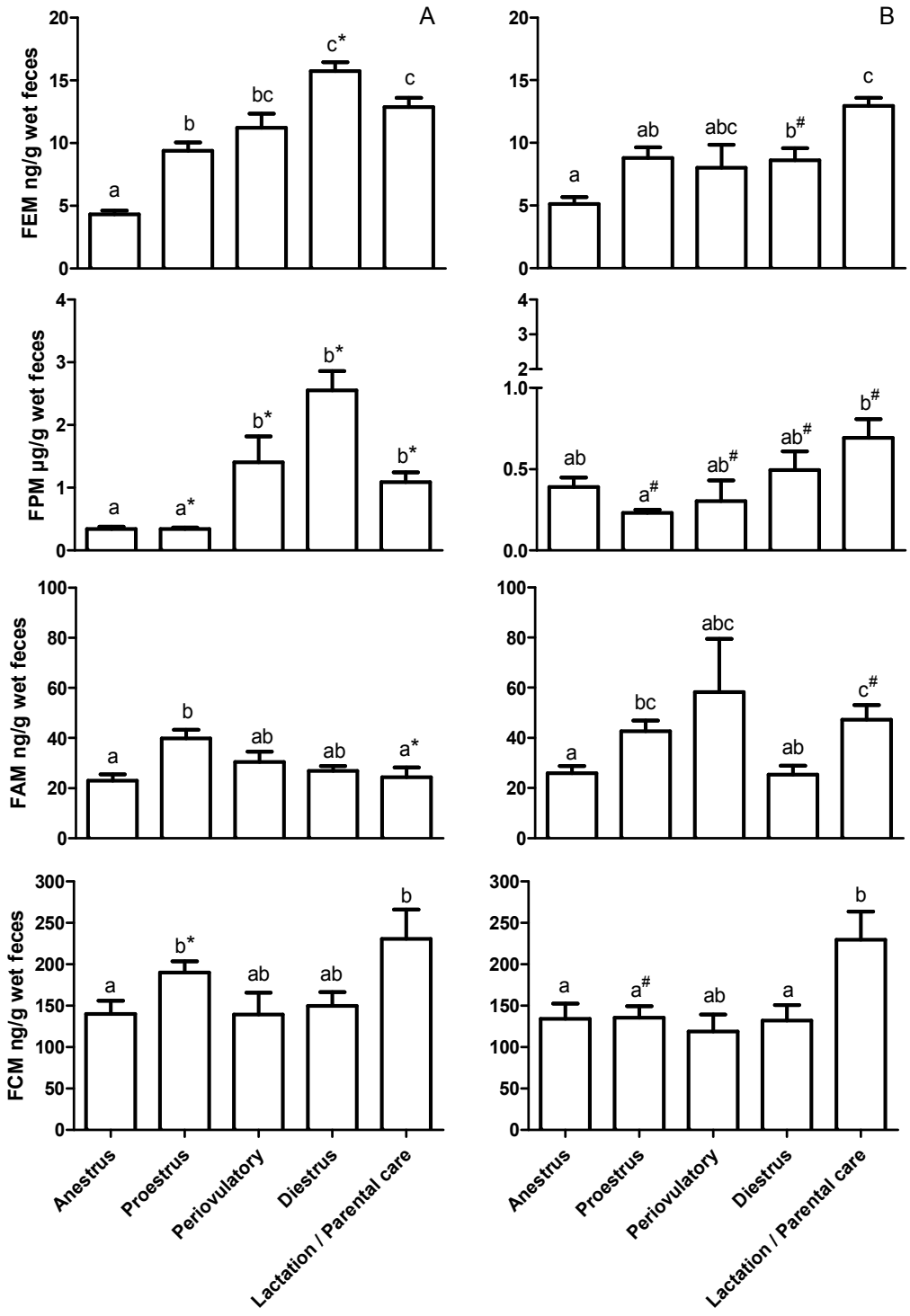

Figure 1 – Overall concentration mean (ng/g and µg/g wet feces; mean ± EPM) of fecal gonadal and adrenocortical metabolites of captive female (A) and male (B) maned wolves throughout reproductive cycle phases. Males' data were also grouped based on females' oestrus cycle. [a,b,c] Different letters within the same graph indicate differences (P<0.05; one way ANOVA, followed by Tukey' multiple comparison procedure and Kruskal-Wallis one way ANOVA on ranks followed by Dunn's multiple

comparison procedure) between phases; *,# indicate differences (P<0.05; non pared t-test and Mann-Whitney rank sum test) between gender for each fecal metabolite.

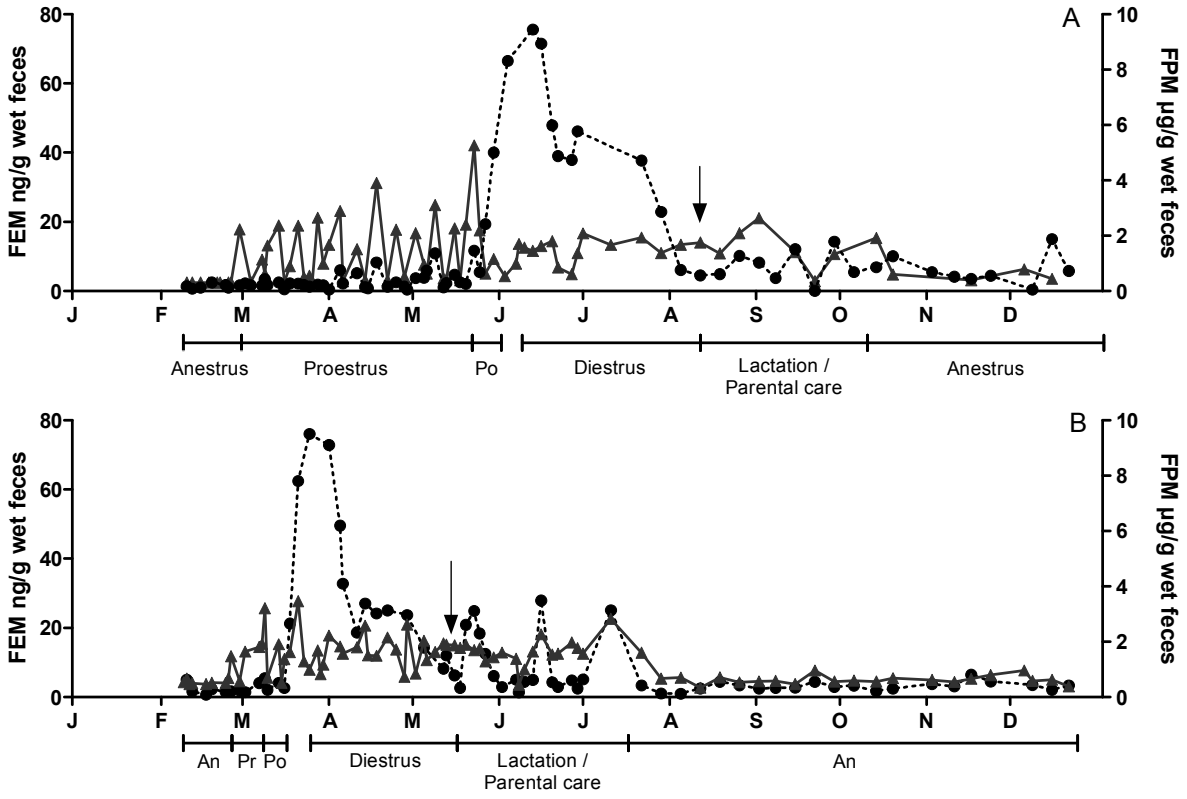

Figure 2 – Longitudinal profiles of FEM (ng/g wet feces; triangles, grey lines) and FPM (µg/g wet feces; circles, black lines) in 3-year-old (A) and 6-year-old (B) female maned wolf. The underlines indicate the reproductive phases: anestrus (An), proestrus (Pr), periovulatory (PO), diestrus and lacation / parental care. The arrows indicate the day pups were born.

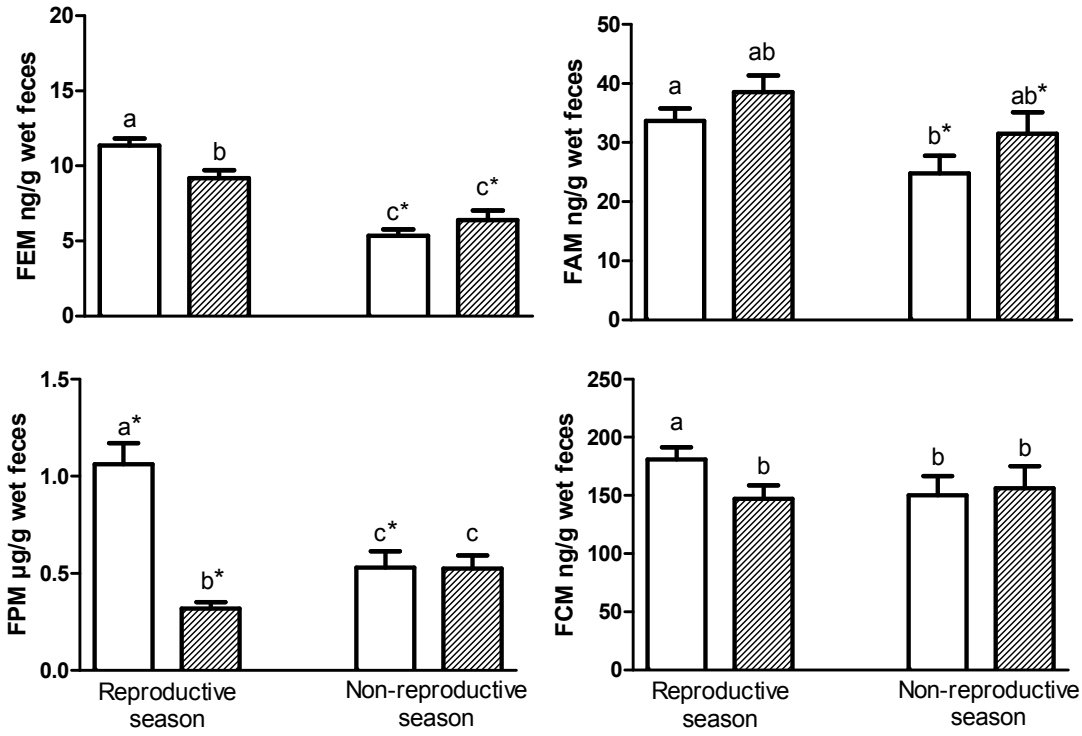

Figure 3 – Overall concentration mean (ng/g and µg/g wet feces; mean ± EPM) of fecal gonadal and adrenocortical metabolites in captive female (smooth columns) and male (diagonal hatched columns) maned wolves during reproductive and non-reproductive seasons. [a,b,c] Different letters within the same graph indicate differences ($P<0.05$; non pared t-test and Mann-Whitney rank sum test) between phases and genders.

5.3 MANUSCRITO 2

Effects of chronic and acute adrenocortical stimuli on fecal gonadal metabolites concentrations and reproductive cycle in captive maned wolves (*Chrysocyon brachyurus*)

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ABSTRACT

Despite conservation efforts, captive maned wolves (*Chrysocyon brachyurus*) still have low reproductive efficiency that may be related to the stressful captive environment. To analyze how chronic or acute stimuli in adrenocortical function can affect hormonal sex concentrations, and consequently, the reproductive cycle in this species, we evaluated three different situations. To better understand our work, this study were subdivided in three parts, whose the aims were: Part 1) evaluate how a chronic stimulation on adrenocortical activity on captive maned wolves affects their gonadal function; Part 2) analyze how the acute adrenocortical stimulation (by administration of adrenocorticotropic hormone - ACTH) on captive maned wolves affects their excretion of fecal sex steroid metabolites; and Part 3) evaluate fecal adrenocortical and gonadal metabolites concentrations in two couples captive maned wolves kept in smaller enclosures and exposure to public visitation. For parts 1 and 3, fecal samples of five couples of captive maned wolves were collected 2-5 days/week during 10-21 months for longitudinal monitoring. For part 2, fecal samples of 11 maned wolves (5 males; 6 females) were collected during 3-7 days before adrenocorticotropic hormone (short-acting synthetic ACTH; Synacthen® Depot; 0.25mg/mL; Novartis) administration and up to 7-14 days after it. Hormone metabolites of corticoid (FCM), progestagens (FPM), androgens (FAM) and estrogens (FEM) were extracted from feces and quantified by enzyme immunoassay. On first part, males were submitted to semen collection procedures, as a part of andrological study conducted in parallel with this work. Males and females demonstrated chronic adrenocortical

stimulation, although the females were not manipulated at any time. Gonadal longitudinal profiles of theses animals, especially females, showed no pattern, with profiles similar to those already reported for acycling female maned wolves. On second part of this study, acute stimuli of adrenocortical gland, induced by ACTH challenge, led to a decrease on androgens and increase on estrogens levels in male maned wolves while in females there was an apparent increase of androgens concentration. No changes were found on progestagens levels in this experiment. On third, with maned wolves couples kept in smaller enclosures (range 516 to 540 m^2/ 5,554 to 5.812 ft^2) and subjected to public exposure (from 2 to 6 days/week, during day time - 8:00 a.m. – 5:00 p.m.), longitudinal profiles of fecal corticoid metabolites presented lower concentrations, while fecal gonadal metabolites levels were higher, mainly for androgens and estrogens. Taken together all parts, this work demonstrated loss of normal reproductive cycle profiles of fecal gonadal metabolites, mainly on females, when occur chronic adrenocortical stimuli and further, the acute stimulation of the HPA axis changed fecal gonadal metabolites concentrations, which can compromise reproductive efficiency in captive maned wolves.

KEY WORDS: maned wolf, adrenocortical stimuli, reproductive cycle, fecal steroids metabolites, ACTH challenge.

1. Introduction

It is known that the reproductive success is poor among captive maned wolves (*Chrysocyon brachyurus*) (Maia and Gouveia 2002; Songsasen et al. 2006; Vanstreels and Pessutti 2010). A recent report based on the Brazilian Studbook for Maned Wolf (BSMW) reveals that the breeding population corresponds to only 15% of the overall captive population and only 36% of those breeding captive animals have successfully bred over history (Vanstreels and Pessutti 2010).

Similarly to what has been observed in North America, the Brazilian captive population presents low reproductive efficiency for two main reasons: high neonatal mortality that was reported in 79% of the animals before one year of age and 42% in the first month of life (Vanstreels and Pessutti 2010); and also low pregnancy rate (Songsasen et al. 2006). Thus, the captive population of maned wolf is considered not self-sustained (Primack and Rodrigues 2001;Vanstreels and Pessuti 2010) and it is believed that this low efficiency can be associated with endocrine disorders associated to chronic captivity stress (Cummings et. al. 2007; Songsasen et al. 2006).

It is known that animals housed in artificial habitats are confronted by a wide range of environmental challenges that lead them to physiological, morphological and behavioral adaptation. While some of these situations can be predictable, others are not. The unpredictable components cause an "emergency state", which results in changes on both endocrine and metabolic systems of the organism (Möstl and Palme 2002).

In an attempt to translate that effort to adaptation, Hans Selye (1907 - 1982) used, for the first time in medicine and biology, the term stress; and since then the word has been associated with adverse events, and their consequences are known as "stress responses" (Korte et al. 2005; Morgan and Tromborg 2007).

A variety of hormones (adrenocorticotropic hormone (ACTH), corticoids, catecholamines, prolactin, etc.) is involved in the stress response. The adrenal glands have a key-role in hormonal reactions to stress as they are involved both in the hypothalamic–pituitary–adrenocortical (HPA) axis and the symphatho-adreno- medullary system (Möstl and Palme 2002). Adverse situations trigger responses of the adrenals, which result in an increase of corticoid and catecholamine secretion. Short-term elevated corticoids concentrations enable animals to escape from life-threatening situations (Möstl and Palme 2002; Sheriff et al. 2011; Young et al. 2004).

The variety of unnatural stressors that captive animals are submitted to may have a strong effect on the function of the HPA axis. Reduced space and restricted movement, forced proximity to humans, reduced feeding opportunities, maintenance in abnormal social groups, exposure to loud or aversive sounds, arousing odors, and other restrictions of behavioral opportunity can lead to a prolonged and sustained corticoid elevation to a sufficient magnitude that can disrupts the homeostatic mechanism of animals, effect known as chronic captivity stress (Morgan and Tromborg 2007).

This prolonged and sustained activation of the HPA axis has large deleterious effects, among others, on the reproductive system, being usually inhibitory, which may increase infertility rates (Ferin, 2006; Mostl and Palme

2002). There are numerous and intricate pathways and mechanisms, central or peripheral, through which stressors may interfere with the normal reproductive function. Most stressors inhibit tonic pulsatile luteinizing hormone (LH) secretion. However, it is still uncertain if this is a result of direct inhibition of the gonadotropin-releasing hormone (GnRH) pulse generator or of change in the sensitivity of the gonadotropes to GnRH stimulation (Ferin, 2006).

In addition to this stressor effect on GnRH release from the brain, many studies report that the activation of the HPA axis can induce secretion of progesterone from the adrenal gland (Mwanza et. al., 2000; Chatdarong et al. 2006; Tsuma et. al. 1998). This increase in circulating progestagens is another factor that may interfere on the endocrine regulation of females' reproductive cycles, since ovulation and corpus luteum formation depend on a very precise hormonal balance. In heifers, an increase on progesterone concentration around the estrus phase promoted alterations on estradiol concentrations with delayed LH surge, which consequently extended the oestrus cycle (Duchens et. al. 1994).

If this increase is also demonstrated in wild canids, such as the maned wolf, which presents a hormonal control of the ovarian cycle similar to domestic dogs (Songsasen et al. 2006; Velloso et al. 1998; Spercoski 2012, 1th chapter), the raise on adrenal sex steroids induced by stress may alter the signaling that promotes the LH surge by the hypothalamus and pituitary.

Endocrine information about the HPA axis activity, through non-invasive methods, already demonstrated higher basal concentration of fecal corticoid metabolites in captive maned wolves when compared to free-living animals

(Spercoski 2007). Nevertheless, important questions on how chronic adrenocortex stimuli on captive can influence the reproductive cycle of maned wolves are still unclear.

Thus, in attempt to answer part of these important questions, this work analyzed and compared, at the same time, adrenocortical and gonadal function on captive non-breeding maned wolves. Our study was conducted in three different situations. To better understand, we divided this study in three parts, whose the aims were: Part 1) evaluate how chronic increased on adrenocortical activity on captive maned wolves affects their gonadal function; Part 2) analyze how the acute adrenocortical stimulation (by administration of adrenocorticotropic hormone - ACTH) on captive maned wolves affects their excretion of fecal sex steroid metabolites; and Part 3) evaluate fecal adrenocortical and gonadal metabolites concentrations in captive non-breeding couples kept in smaller enclosures and subjected to public exposure.

2. Methods

2.1. Animals

Table 1 summarizes information of animals used in this work, in each of the three parts of this study.

On longitudinal studies (first and third parts) were used 10 adult maned wolves (5 females and 5 males; age range 4-10 years), kept in three different Brazilian institutions (Americana Zoo-SP; Ilha Solteira Zoo-SP and the

conservation breeding center Criadouro Científico de Fauna Silvestre para Fins de Conservação da Companhia Brasileira de Metalurgia e Mineração-MG).

The wolves were exposed to natural photoperiod, housed in pairs and were subject to the public. Management was made according to each institution's routine. The size range of enclosures varied from 512 to 5000 m^2 (5,511 to 53,819 ft^2) and the wolves' diet was composed of approximately 40% commercial dogs food and minced beef meat, 40% fruit and 20% vegetables with vitamin supplement and the animals had access to fresh water *ad libitum*.

On second part of this work (ACTH challenge) were used the same maned wolves that were being monitored on first.

2.1.1. Part 1

At the same time that our work was being conducted, other research, about seasonal aspects of reproductive physiology in captive male maned wolves (Teodoro et al. 2012), was performed. The andrological study used the same males that were being longitudinal monitored on this part of this work.

For a period of 13 months the males were submitted to semen collection procedures by the method of digital manipulation of the penis, based on domestic dogs protocols. Briefly, the wolves were handled in their own enclosures, physically restrained using a catch pole and kept in quadruped position. The abdominal region was cleaned before the digital stimulation of the penis. After the semen collection, the animals were release and rewarded with

212 fruit and meat, aiming to condition them in a way they did not relate the
213 procedure to something unpleasant (Teodoro 2011; Teodoro et al. 2012).
214 Semen collection procedures ranged from 38-44 procedures in two males
215 and 15 procedures in a third male, and were performed between April/2010 and
216 May/2011, at a frequency of 1-3 times/ week, but with higher frequency during
217 the breeding season (March - June).
218 The female did not suffer any kind of manipulation and they remained loose
219 in the enclosure, however not kept close during the collection procedure.
220 In attempt to evaluate, at the same time, the adrenocortical and gonadal
221 function of the animals (males and females) this part of the work analyzed two
222 couples during nearly two years (21 months) and one for 9-10 months, totalizing
223 10 annual endocrine profiles. Data were also analyzed based on reproductive
224 season of the species (from February – July; range date obtained for maned
225 wolves breeding pairs, Spercoski 2012 – 1th chapter).
226
227 *2.1.2. Part 2*
228 In attempt to verify the effects of acute adrenocortical stimulation on
229 secretion of sex steroids in maned wolves, was performed the ACTH challenge
230 experiment.
231 The adrenocortical stimulus was made twice using different experimental
232 designs, where the first challenge totalized 21 days of experiment (from 7 days
233 before to 14 days after the stimuli) and the second 10 days (from 3 days before
234 to 7 days after). The difference between the experiments was relative to fecal
235 collection protocols (see bellow, section 2.2) and the stimuli were performed

equally in the two challenges. The first experiment was conducted between December and January in 6 animals (3 females and 3 males) and the second was done eight months after the first on the same animals, except for one male that was submitted to clinical treatment.

The adrenocortical stimulation was performed by an intramuscular injection of analogue synthetic of adrenocorticotropic hormone (tetracosatide acetate) of short-action (Synacthen® Depot; 0.25mg/mL; Novartis) at a dose of an ampoule/animal. The wolves' dose was based on the recommended dose for domestic dogs (0.25 mg/dog; Feldman and Nelson 2004).

The stimulations were conducted by morning (8:00 – 9:00 a.m.). The maned wolves were captured, physically restrained and handled for the administration of the hormone. The entire procedure, from the entrance of the handlers and veterinarian in the enclosures until the release of the animals last for an average of 5-10 minutes. After this, the wolves were observed during the first hour after administration and then every hour until 4:00 p.m. (when the handlers left the institution) in attempt to verify possible side effects.

No animals used in these experiments had clinical signs of adverse reactions and all showed normal behavior after the administration of the hormone and throughout the whole experiment.

2.1.3. Part 3

In attempt to evaluate fecal adrenocortical and gonadal metabolites concentrations in captive maned wolves kept in smaller enclosures and exposure to public visitation, two maned wolf couples from the Americana Zoo –

SP (one couple) and Ilha Solteira Zoo –SP (one couple), not submitted to andrological study, were non-invasively monitored. A longitudinal study was performed during 11 months in one couple and for one year and a half (18 months) in the other, totalizing 6 annual endocrine profiles. Data were also analyzed based on reproductive season following the same criteria used on part 1 of this study.

The animals lived in smaller enclosures (ranging from 516 to 540 m^2 / 5,554 to 5,812 ft^2) when compared to those of the conservation breeding center (from 1000 to 5000m^2 / 10,764 to 53,816 ft^2), and were exposed to public visitation from 2 to 6 days a week, during day time (8:00 a.m. – 5:00 p.m.). Furthermore, in one institution the enclosure of the wolves was located near to enclosures of possible predators of the species.

2.2. Fecal sample collection

For longitudinal monitoring studies (parts 1 and 3) fecal samples were collected by morning, as part of the handling's routine, 3 times/week for females and 2 times/week for males, within the breeding season (from March to June) and 1-2 times/week for females and 1 time/week for males during non-breeding season (from July to February).

Female and male fecal samples were differentiated by the males scats' color (due to the use of green food coloring, used in zoos) or by the presence of papaya seeds and peel (*Carica papaya*) (used in conservation breeding center).

283 At the zoos, the animals were fed separately, while at conservation breeding
284 center the animals received fruits over the enclosures' screen fence before their
285 daily diet. At the same time handlers offered papaya with seeds and peel to the
286 males, while the females received banana (*Musa spp*.) at the other end of the
287 enclosure. Papaya was also used the females' diet, however only the fruit pulp.

288 This dietary management was done in the afternoon prior to the day the
289 feces were collected. The feeding time was always monitored to ensure that
290 both the food coloring and the papaya were eaten only by the males.

291 In the ACTH challenge (part 2), as mentioned above, two protocols of feces
292 collection were performed. On the first, samples were collected once a day, by
293 morning, during 7 days before the challenge and up to 14 days after it, totalizing
294 21 days of experiment. This protocol for sample collection was elaborated
295 based on the information that maned wolves normally defecate once or twice a
296 day (personal communication with others researchers; however after our two
297 experiments this information was documented by Vasconcellos et. al. 2011).
298 However due to the fact that 50% of the animals submitted to challenge did not
299 demonstrate adrenocortex stimuli, a new protocol was established, based on
300 Monfort et. al. (1998).

301 On the second challenge, all feces produced/day were collected during 3
302 days before the challenge and up to 7 days after it, totalizing 10 days of
303 experiment. Before starting the experiment it was observed that the wolves
304 defecate 2-3 times during the day (considering a day from 6:50 a.m. to 4:00
305 p.m.) and in the following morning there was on average of 4 stools / animal in

306 the enclosure. This demonstrated that these maned wolves presented more
307 frequency of defecation that only once or twice a day.

308 Based on this information, the feeding management of the wolves was
309 slightly modified to assure, as much as possible, the differentiation between the
310 male and female feces. The animals started receiving the fruits (papaya with
311 seeds and peel for males and banana for females) in the middle of the morning
312 (10:00 - 10:15 a.m.) and again in the afternoon, before their daily diet (3:00 -
313 3:30 p.m.), as described above.

314 The wolves were observed from 6:50 a.m. to 4:00 p.m. and fecal samples
315 were collected 10-15 minutes after each defecation and immediately stored at -
316 20ºC (-4ºF). The feces produced between 4:00 p.m. and 6:50 a.m. of the next
317 day were collected immediately at the arrival of the handlers in the institution
318 and by 7:30 a.m. the enclosures were all clean.

319 The fecal collection for all experiments was done non-invasively, directly
320 from the enclosure's floor, being placed in plastic bags (labeled with date, hour
321 and animal's identification) and stored at -20ºC (-4ºF) before being transported
322 in ice to analysis. Fecal samples obtained in a 24-hour period, during the ACTH
323 challenge experiment, were pooled before extraction.

324
325

326 **2.3. Fecal extraction and analysis**

327 *2.3.1. Fecal extraction*

328 Fecal extraction and hormone quantification were performed in Laboratório
329 de Fisiologia da Reprodução, Universidade Federal do Paraná, Curitiba,

330 Paraná, Brazil. All reagents (except when specified) were purchased from
331 Sigma-Aldrich (Sigma-Aldrich Brasil Ltda, São Paulo, Brazil) and all solutions
332 prepared with Milli-Q water. Fecal extraction was performed by the methods of
333 Spercoski *et. al*. (2012), with slight modifications (Annex 1, thesis). The maned
334 wolves' fecal sample for the longitudinal monitoring study were extracted wet
335 while the fecal samples of the ACTH challenge experiments were dried (24
336 hours at 70^{o}C) and powdered. Briefly, an aliquot of ~0.5 g of the thawed or
337 powered, well-mixed, fecal sample was placed in a glass tube containing 5 ml of
338 80% ethanol:20% distilled water and vigorously shaken for 30 min using a Multi-
339 Pulse vortexer (Glass-Col, Terre Haute, IN). Each sample was centrifuged
340 (1,000xg, 15 min) and the supernatant recovered. The mean (± SEM) of
341 extraction efficiency, by the addition of labeled H^{3+}-cortisol, was 86.2 ± 0.5%
342 with a coefficient of variation (CV) of 9.1%.
343
344 *2.3.2. Enzyme immunoassays (EIA)*
345 Fecal extracts were quantified by enzyme immunoassay as described by
346 Brown et. al. (2004). The polyclonal antiserum used were pregnane (CL425;
347 1:10,000 dilution; FPM), conjugate estrone (R522-2; 1:20,000 dilution; FEM),
348 testosterone (R156/7; 1:10,000 dilution; FAM) and cortisol (R4866; 1:8,500
349 dilution; FCM), provided by Coralie Munro (University of California–Davis,
350 Davis, CA).
351 Validation assays for all hormones had already been described (Songsasen
352 et al. 2006; Spercoski et al. 2012). Intra- and inter assay coefficients of variation
353 were <3 % and <15%, respectively, for all EIAs.

354 **2.4. Data analysis**

355 The individuals' overall data were divided in different groups that were
356 analyzed and checked for normality. Parametric data were statistically analyzed
357 using one way ANOVA (for comparisons of three or more groups), t-test and
358 paired t-test (for comparisons between two groups). When data did not pass on
359 normality, analysis was performed by Kruskall-Wallis (three or more groups),
360 Mann-Whitney or Wilcoxon tests (two groups), being considered significant
361 when $P<0.05$.

362 For the ACTH challenge profiles, the data were aligned according to the day
363 of the FCM peak, which was considered day zero.

364 Results are presented in ng/g or μg/g (for FPM concentrations) of wet feces
365 on longitudinal monitoring studies and dry feces on the ACTH challenge
366 experiment.

367

368

369 **3. Results**

370 **3.1. Part 1**

371 *3.1.1. Effects of semen collection procedures on adrenocortical function*

372 The acute response of the first semen collection was only observed in one
373 couple, where the increase of FCM concentration post-procedure was 1.8 fold
374 higher in the male and 17.7 fold higher in the female, although the female was
375 not manipulated at any time.

376 Considering the 2-1 days pre-procedure, FCM mean concentration found in
377 males, for the first ten semen collection, was 180.2 ± 45.4 ng/g (n=15). After

that, the pre-procedure mean increased 2.3 fold (423.5 ± 54.38 ng/g; n=55; P<0.05) and remained high until the last semen collection (Fig. 1B). The same result was found in females where the pre-procedure mean for the first ten semen collection was 184.5 ± 43.8 ng/g (n=9) and increased up to 385.4 ± 75.2 ng/g (n=15; P<0.05; Fig. 1A).

Considering post-procedures days, FCM mean concentration for the first ten semen collection was 303.5 ± 56.5 ng/g (n=10) and from the eleventh to 44th was 709.1 ± 143.7 ng/g (n=23), demonstrating an increase (P<0.05) when compared to the pre-procedures, respectively (Fig 1B).

In females, FCM post-procedures mean for the first ten semen collection was 481.6 ± 67.3 ng/g (n=36) and showed a significant increase (P<0.05) compared to pre-procedure mean. However, after ten semen collections the FCM concentration mean did not differ between pre and post procedures (511.6 ± 58.6 ng/g; n=24 on post procedure) (Fig. 1A).

The response amplitude, observed by % increase on the FCM concentration mean relative to those found in pre-procedure, presented great individual variability in males (Fig. 1D) for the first manipulations (range 10 – 723%), but not for the following (range 14 – 74%). In females, the response amplitude was diminished after the eleventh procedure (Fig. 1C).

After the end of the semen collection procedures a decrease on FCM mean concentration was observed from mid June in females (417.7 ± 26.74 ng/g; n=188 and 232.2 ± 24.3 ng/g; n=82, during procedures and 30 days after the last semen collection respectively; P<0.05) and from mid June in males (496.3 ± 43.9 ng/g; n=164 and 353.7 ± 36.3 ng/g; n=121; P<0.05) (Fig. 2).

All these findings show endocrine evidences of chronic adrenocortical stimulation from the tenth procedure up to 30 days after the last semen collection.

Beside this, the wolves showed no behavioral changes. During the entire period of study, the animals were regularly observed in attempt to check the occurrence of any kind of behavioral change, including possible agonistic interactions between the couple after collection and on subsequent days, which did not happen. The wolves, both males and females, had behavioral characteristics of the species including reproductive behavior and, during the monitoring of the second breeding season, they were observed mating when the semen collection procedures were still happening (Teodoro 2011).

3.1.2. Effects of chronic adrenocortical stimuli on fecal gonadal metabolites concentration

The longitudinal profile of fecal adrenocortical and gonadal metabolites concentrations from a representative maned wolf couple, in which the male was submitted to semen collection, are shown in figures 3 and 4, respectively.

Female longitudinal profiles did not show any pattern of reproductive cycle during first year of monitoring. In July of the second year, a month after the FCM concentration had diminished (see Fig.4A), it was possible to notice a possible ovulatory cycle by the increase on FPM levels with a sequent decrease of FEM concentration.

Reproductive season profiles showed that FEM concentration mean of females was higher during NRS (12.9 ± 0.6 ng/g; n=367; $P<0.05$) when

426 compared to RS (10.9 ± 0.5 ng/g; n=342). There were no differences in FEM
427 levels of males (9.2 ± 0.4 ng/g; n=217 and 7.9 ± 0.3 ng/g; n=229, on RS and
428 NRS respectively) (Fig. 5).
429 The FPM concentration mean differed with a higher concentration observed
430 during RS (1.5 ± 0.2 μg/g; n=238; P<0.05) than NRS (1.3 ± 0.1 μg/g; n=232) in
431 females. And as observed for FEM, males did not show differences between
432 seasons for FPM (0.52 ± 0.1 μg/g; n=215 and 0.54 ± 0.1 μg/g; n=228, on RS
433 and NRS respectively).
434 Androgens during NRS were higher in both female (56.8 ± 4.6 ng/g; n=228;
435 P<0.05) and male (42.5 ± 2.8 ng/g; n=229; P<0.05) in comparison to RS (30.1 ±
436 2.2 ng/g; n=229 and 36.9 ± 2.8 ng/g; n=212, in female and male respectively).
437 No differences were founded on FCM concentration mean between RS and
438 NRS females (329.1 ± 23.2 ng/g; n=163 and 331.1 ± 22.5 ng/g; n=226, on RS
439 and NRS respectively) and males (440.4 ± 35.29 ng/g; n=208 and 392.4 ±
440 29.25 ng/g; n=226)
441 There were gender differences between the seasons for FEM and FPM and
442 females had a higher value.
443
444
445 **3.2. Part 2**
446 Out of the 11 maned wolves that were submitted to the challenge, only 2
447 females and 3 males responded. The FCM concentration mean of the fifty days
448 prior to the ACTH challenge presented differences between non-responsive and
449 responsive females, with higher concentrations found in non-responsive

450 animals (938.9 ± 167.5 ng/g; n=17 and 388.4 ± 75.5 ng/g; n=14; P<0.05, for
451 non-responsive and responsive female, respectively) (Fig. 6). In males there
452 were no significant differences between the means (591.4 ± 95.3 ng/g; n=14
453 and 507.5 ± 59.2 ng/g; n=16, for non-responsive and responsive male,
454 respectively).

455 Fecal gonadal and adrenocortical metabolites profiles of the ACTH
456 challenge experiment in captive female and male maned wolves are shown in
457 Fig. 7. Increases on FCM after the challenge were found at 24h in males and
458 48h in females.

459 Male maned wolves showed a decrease of FAM concentrations and
460 increase on FEM levels after the challenge (P<0.05). FPM comparisons did not
461 show differences between pre and post concentrations.

462 The statistical analysis for female maned wolves was not performed due to
463 the low n of animals that responded to the challenge, besides this the two
464 females showed increase of 1.6-1.8 fold on androgens concentrations and 1.2-
465 1.9 fold on progestagens levels. For FEM concentration it was not possible to
466 detect any tendency since an increase was presented by one female while the
467 other presented a decrease after the challenge.

468 Response amplitude, observed by % increase on fecal metabolites
469 concentration on the day of the corticoid peak in relation to the day prior the
470 ACTH challenge for both genders are shown in Fig. 8.

471

472

473

3.3. Part 3

Analysis of fecal adrenocortical and gonadal metabolites concentrations in captive non-breeding couples kept in smaller enclosures and subjected to public exposure showed that longitudinal profiles and analysis by reproductive season did not clearly demonstrate characteristics of chronic adrenocortical stimuli (Figs. 9, 10). Wolves from two institutions showed lower FCM concentration mean during NRS compared to the reported for breeding pairs, and within the breeding season only one female presented similar values to those of breeding females (Spercoski 2012, 1th chapter).

Differently from FCM, all fecal gonadal metabolites were higher when compared with those already reported for maned wolves breeding pairs (Spercoski 2012, 1th chapter) and also from those reported on part 1 of this work. Longitudinal profiles of fecal gonadal metabolites from a couple are shown in Fig. 11.

Analysis by reproductive season showed no difference on the FEM concentration mean (ng/g wet feces) between seasons for both females (20.5 ± 1.1; n=189 and 18.0 ± 1.5; n=90 on RS and NRS respectively) and males (17.8 ± 1.6; n=85 and 18.2 ± 1.4; n=62) (Fig.12).

FPM values (μg/g wet feces), within RS females, were 6.9 ± 0.6 (n=131) and 1.5 ± 0.2 (n=86) for males. During the months out of the season the concentration means were 3.5 ± 0.3 (n=64) and 1.4 ± 0.2 (n=62) in females and males, respectively.

For androgens (ng/g wet feces) 153.8 ± 24.1 (n=86) was found in males and 120.7 ± 11.3 (n=131) in females. The concentration mean out of the season for

498 males and females were 139.9 ± 18.8 (n=62) and 141.9 ± 16.4 (n=64),
499 respectively.

500 There were differences between seasons and genders only on FPM levels
501 mean, where RS showed higher values and, as for genders, males had lower
502 concentration mean during both seasons.

503

504

505 **4. Discussion**

506 This study evaluated the effects of chronic adrenocortical stimuli on the
507 gonadal function of captive maned wolves, showing that a sustained increase
508 on corticoids concentrations in great magnitude and for a prolonged time
509 causes abnormalities on reproductive cycles. It also shows that acute
510 adrenocortical stimuli, accessed by the ACTH challenge, alter fecal gonadal
511 metabolites concentrations in maned wolves.

512 Longitudinal profiles of the couples that were submitted to management
513 change for semen collection showed a persistent increase of adrenocortex
514 activity causing significant alterations on the reproductive cycle profile. FCM
515 concentration rose in both males and females, despite any kind of manipulation
516 on the female, and over the time the animals presented endocrine evidences of
517 chronic adrenocortical stimulation.

518 In canids, most commonly, semen is collected from ejaculates obtained by
519 digital manipulation or electroejaculation. Simple digital manipulation is
520 generally used in domestic dogs and is also reported as a method of semen
521 collection in farm foxes (*Vulpes vulpes* and *Alopex lagopus*), as the males are

522 trained to accept manipulation (Thomassen and Farstad 2009). For larger wild
523 canids, however, this method can be risky and major studies used
524 electroejaculation procedures (Zindl et. al. 2006; Cummings et al. 2007;
525 Johnston et al. 2007). Nevertheless, the digital manipulation method has also
526 been reported in maned wolves (Paula et al. 2002; Mascarenhas et al. 2002).
527 The protocol used for semen collection of monitored males in this study, as
528 mentioned above (see section *2.1.1* in methods), was done by digital
529 manipulation of the penis, based on defined procedures for domestic dogs and
530 was chosen to minimize the impacts of welfare caused by electroejaculation
531 after the procedure (personal communication Teodoro, L.O.).
532 Although the protocol did not require any chemical restraint or further
533 analgesia, the amplitude of the FCM peak in males for the first ten procedures
534 ranged from 2 to 8 folds, similar to those reported in jaguars after the first
535 semen collection using electroejaculation protocols (Morato et al. 2004). The
536 increase of corticoids concentration after electroejaculation has also been
537 reported in rams (Damian and Ungerfeld 2011), bulls (Whitlock et. al. 2012),
538 domestic cats (Carter et. al. 1984), cheetahs (Wildt et. al. 1984), clouded
539 leopards (Howard 1986) and others wild cats (Phillips et al. 1988). However, no
540 reports were found describing the impact of semen collection by digital
541 manipulation on the HPA axis.
542 The acute response observed since the first procedure was somewhat
543 expected, considering that under normal physiological conditions the HPA axis
544 activation occurs, causing increase on corticoids concentrations (Busch and
545 Hayward 2009; Möstl and Palme 2002; Young et al. 2004). This acute stress

546 response aims to promote physiological and behavioral adaptation to the
547 noxious stimulus, or ‘‘stressor”. Thus, the response is generally beneficial to the
548 animals (Sapolsky et. al. 2000).

549 In captivity, this occasional exposure to environmental or handling
550 challenges may be considered common as components of long-term
551 husbandry regimes and can occur in various situations, such as direct stare
552 from a more dominant individual (in the same or in the next enclosure),
553 approach of a human with restraining instruments, changing of enclosures,
554 transporting captive animals, etc. (Morgan and Tromborg 2007).

555 The changes on the animals' management for semen collection altered their
556 routine and it is possible that each of the stressors (capturing, handling and
557 restraining the male) resulted in acute and/or persistent stress responses that,
558 whether mounted simultaneously or sequentially, can result in a state of chronic
559 adrenocortical stimulation (Dickens et. al. 2010). Furthermore, it is suggested
560 that the act of mating itself has positive effects on serum corticoid levels (Lane
561 2006; Veronesi et. al. 2011; Veronesi et. al. 2010) and fecal corticoid
562 metabolites (Sands and Crell 2004) in males.

563 So the acute response elicited by the first procedure, as mentioned above,
564 was already expected, however it was not expected that the procedures would
565 lead to a chronic condition, including the females. During semen collection the
566 males remained calm, allowed the manipulation and had normal ejaculation
567 (Teodoro 2011). In domestic dogs, fear and/or pain prevent males from
568 attaining a complete erection and ejaculating (Kutzler 2005), which was not
569 observed in the wolves.

570 Gonadal longitudinal profiles of theses animals, especially in females,
571 showed no pattern with large hormonal variation of the FEM and FPM
572 concentrations, presenting profiles similar to those already reported for acycling
573 female maned wolves (Songsasen et al. 2006; Velloso et al. 1998) and red
574 wolves (*Canis rufus*) (Walker et. al. 2002). Longitudinal profiles of normal
575 cycling female maned wolves generally show the onset of ovarian activity by an
576 increase of FEM concentration and frequency of peaks after a long anestrus
577 period.

578 The periovulatory phase is characterized by rising FPM levels, due to the
579 previous luteinization of follicles, and remains high for 60-100 days depending
580 whether the female is pregnant or pseudopregnant and, finally the ovarian
581 steroids return to anestrus values (Spercoski 2012, 1th chapter; Songsasen et
582 al. 2006; Velloso et al. 1998). Both longitudinal profiles and reproductive season
583 analysis show higher concentrations of fecal gonadal metabolites in females
584 when compared to breeding animals (Spercoski 2012, 1th chapter).

585 In this study, both acute and chronic HPA axis stimulation led to changes on
586 fecal gonadal metabolites concentrations. Unfortunately, our findings for the
587 ACTH challenge were too little, due to the small number of animals that
588 responded to the adrenocortex stimuli (range 2-3) and the great individual
589 variability observed.

590 Out of the 11 animals that were submitted to the challenge and only five
591 responded (2 females and 3 males). All maned wolves used in the experiment
592 belonged to conservation breeding center, where the males were also been
593 used in the andrological study. To conduct the first experiment, semen

collection was interrupted and the stimulus was made after one month in 3 males and 3 females, and only 1 male and 2 females were responsive. The second challenge was conducted five months after the end of the semen collection procedures in 2 males and 3 females, and only the males responded.

This low number of responsive animals is associated with higher FCM concentration in the days prior the procedure, which is observed in females, where the overall values mean of FCM in non-responsive animals were higher than the responsive ones, within fifteen days prior to the challenge. This was not verified for male maned wolves; however it is possible that all males could have answered, because the supposedly "non-responsive" animals were going through the first experiment, where the collection of fecal samples was performed only once a day and maybe the FCM peak was lost.

Fecal progestagens metabolites did not change concentrations after the adrenocortex stimuli in maned wolves as a response to the ACTH challenge. Increases on serum concentration of progestagens stimulated by exogenous ACTH have been reported in many species, such as rabbits (Haunc and Haltmeyer 1975), guinea pigs (Fenske 1997), bovines (Willard et. al. 2005; Bolanos et al. 1997; Yoshida and Nakao 2006), sows (Tsuma et al. 1998), sheep (Van Lier et al. 1999), horses (Hedberg et al. 2007), domestic cats (Chatdarong et al. 2006) and even in domestic dogs (Ginel et. al. 2012; Frank et. al. 2004).

As mentioned above, our findings are from a very low n and there is great individual variability, so we cannot infer with certainty that after stimulation of the adrenal cortex no changes occur in the concentration of FPM. Despite these

618 results of the ACTH challenge, analysis of longitudinal profiles of FCM and FPM
619 show positive correlation (r2 = 0.750; P <0.05) between the concentration of
620 these hormones during the period of semen collection for both male and female,
621 showing that the activation of the HPA axis increase the FPM concentrations in
622 maned wolves.

623 The origin of these progestagens is probably associated with the process of
624 steroid biosynthesis in the adrenal gland where pregnenolone can be directed
625 to the synthesis of progesterone (via 3b-hydroxysteroid oxidoreductase and
626 $\Delta_{5\rightarrow4}$-3-oxosteroid isomerase) or 17- hydroxypregnenolone (via 17-
627 hydroxylase). Either of these forms can be further be converted to 17-
628 hydroxyprogesterone, which can then be directed toward the production of
629 corticosteroids (Fraser 1992). Thus, the production rate of theses precursors
630 may be greater than the conversion rate for forming corticoids, with a
631 consequent rise in the levels of serum progestagens and also FPM.

632 This data showing rising levels of FPM after induction of acute stress in
633 female maned wolves was of great importance for the characteristics of the
634 canids' reproductive cycle, in which, at the beginning of proestrus, progesterone
635 concentrations are low, rising only at late proestrus, concomitantly with a
636 decline of estrogen levels. The combination of increasing progestagens
637 concentrations and decreasing estrogens levels in late proestrus stimulate two
638 major events: changes in the female sexual behavior, which comes to accept
639 the mount; and the strong positive feedback to the hypothalamus and the
640 pituitary, that results on the LH surge at the beginning of estrus (Feldman and
641 Nelson 2004; Concannon 2009). Thus, changes in progestagens concentrations

642 during this important period, related to acute stressful events, may be able to
643 prevent or alter the normal occurrence of these two events. This way the
644 possibility of failures on the ovulatory process is increased and consequently
645 the female cannot reach reproductive success.

646 For androgens the acute effects of the ACTH show decrease on FAM
647 concentration in males and an apparent increase in females. Decrease on
648 plasma testosterone after acute stress, by the ACTH challenge or after
649 electroejaculation procedures, has already been reported for bulls (Jonhson et.
650 al. 1982; Welsh and Jonhson 1981), rams (Damián and Ungerfeld 2011), rats
651 (Mann et. al. 1987) and domestic cats (Carter et. al. 1984).

652 The most biologically important androgen synthesized in the testis is the
653 testosterone, which is produced by interstitial Leydig cells under the control of
654 LH, which is under control of the GnRH pulse generator. So conditions that
655 interfere with the function of the GnRH pulse generator will disrupt the HPG
656 axis. Most studies show significant decreases in plasma LH by increases in
657 ACTH plasma levels following acute stress. And major evidence suggests that
658 this decrease in tonic pulsatile LH release by stressors is primarily related to an
659 inhibitory action on the hypothalamic GnRH pulse generator (Jeong and Kaiser
660 2006). Another study reported that ACTH could indirectly reduce the sensitivity
661 of the testis to LH, resulting in lower testosterone release (Mann et. al. 1987).
662 Finally, this decrease on androgens can also happen due to the conversion of
663 testosterone in estrogens by an increase on the aromatase enzyme complex
664 activity.

In vitro studies have shown that the aromatase activity can be stimulated by corticoids, that induce the expression of components of the enzyme complex, and by the ACTH, which besides stimulating the release of estrogen precursors, can also directly signal the adipose tissue to increase aromatase activity (Mendelson et. al. 1984; Simpson et al. 1981).

Therefore, lower FAM concentration after the challenge may be due to reduced plasma levels of LH or by a possible decrease on sensitivity to gonadotropin by the testis or an increase on aromatase activity or by the association of all of these physiological events.

Although the results in females only suggest increased FAM concentration after acute stress stimuli, it is possible that this increase is related to a higher secretion of adrenal androgens and then, of FAM. Increases on DHEA serum concentration stimulated by exogenous ACTH have been reported in others species, such as red squirrels (Boonstra et al. 2008) and asian elephants (Yon et al. 2007).

It is known that the ACTH also regulates the production and secretion of adrenal androgens, thereby the ACTH stimulus of the zone fasciculata cells to increase secretion of corticoids also acts on cells of the zona reticularis increasing the secretion of DHEA (Vermeulen and Rubens 1992).

For estrogens, male maned wolves also presented increased FEM levels after the challenge. In females, the effect was not clear due to the great individual variability. A study on quantification of adrenal secretion rates of sex steroids in male domestic dogs reports that during basal conditions the adrenal produces larger amounts of androgens and estrogens, with

secretion rates 20 – 50.000 fold less than that of cortisol (Santen et. al. 1980) and additionally, as also mentioned, corticoids and ACTH can possibly activate the aromatase enzyme complex.

Therefore, this raise can be associated with increased adrenal secretion rates of estrogens and/or androgens or with possible aromatase activation, and maybe even by the association of these events.

Although the experiment in females was not conclusive, the analysis of longitudinal profiles of FCM and FEM of animals monitored on part 1 of this work show positive correlation ($r2 = 0.600$; $P < 0.05$) between the concentration of these hormones during the period of semen collection for both females and males, suggesting that the activation of the HPA axis increases FEM concentration in females.

Considering the couples that belong to the Zoos, although we cannot infer that the animals were under chronic stress, it is possible that the lower levels found, especially in one of the females, already demonstrate the beginning of a state of exhaustion, a condition in which the adrenocortex, after a long time of hyperstimulation, begins to fail regulation mechanisms of the HPA axis, starting to suppress the release of corticoids (Berne et. al 2004).

The regulation system of the HPA axis happens through negative feedback, where the activation of the axis increases synthesis and secretion of glucocorticoids, which then inhibit the hypothalamic secretion of corticotrophin release hormone (CRH) and of ACTH by the pituitary, decreasing the activity of the axis. The effects of negative feedback on the pituitary by corticoids may also be indirectly modulated by neural information to CRH neurons released in the

713 hypothalamus. Furthermore, corticoids activate the expression of atrial
714 natriuretic hormone (ANH), which also inhibits the basal release of CRH and
715 ACTH. The exposure to high concentrations of corticoids also causes down
716 regulation of its own classic receptor (genomic low affinity receptors) (Berne et
717 al. 2004).

718 Thus, the suppressive action of corticoids can last even after the exposure
719 to these molecules is ceased. The effects of corticoids upon an animal in an
720 exhaustion state lead to functional atrophy of the HPA axis and its full recovery
721 can take up to a year, during which the normal response to stress cannot be
722 assured (Berne et. al 2004).

723 The speculation that animals could be going under exhaustion could also
724 explain the high concentration of fecal gonadal metabolites, considering all
725 changes on sex hormones previously described.

726 On the other hand, it is also possible that these findings of low FCM level
727 and high concentration of fecal gonadal metabolites in all animals are related to
728 technical problems during the procedures of quantification of fecal metabolites,
729 from the collection of fecal samples.

730 An important factor that may have occurred is the delay in collecting the
731 fecal samples. In personal communication with the staff members responsible
732 for each institution, it was reported that the collections were made in the
733 morning, though the exact time has not been determined. Thus, the feces could
734 have been exposed to the environment for up to 19 hours (considering the
735 animal has defecated shortly after the handlers work shift - 5:00 pm - and the
736 collections were only made at 12: 00pm the following day).

737 After defecation, several factors, such as temperature, humidity, and other
738 environmental conditions may influence on the concentration of steroids
739 metabolites, mainly FCM. Additionally, microorganisms can alter these
740 molecules by enzymatic cleavage (Millspaugh and Washburn 2004; Palme
741 2005; Touma and Palme 2005).

742 The metabolism of corticoids generally includes seven different types of
743 reactions generating different groups of metabolites. Corticoid with α-ketol or
744 glycol side chains at C-17 (e.g. cortisol, cortisone) can undergo side-chain
745 cleavage forming 17-oxosteroids groups. The 11β-hydroxyandrostenedione, a
746 17-oxosteroid, is the product of cortisol side-chain cleavage, a type of
747 androstane that can be futher metabolized (Brownie 1992; Möstl and Palme
748 2002; Möstl et al 2005); and it is possible that these metabolites can cross-react
749 with the antiserum used for androgens quantification in the study, for the
750 similarity between the immunoreactive portion of these molecules.

751 Therefore, bacterial enzymes can alter all fecal metabolites after a
752 prolonged exposure to fecal samples, diminishing the concentration of FCM and
753 increasing the concentration of molecules which can be “read” as gonadal
754 metabolites in the sample of these animals; however, this is only a speculation.
755 The best way to investigate the metabolism and excretion pattern of steroids is
756 by using radiolabeled (14C/3H) hormones, although this approach is not always
757 possible due to economic and/or welfare restrictions, mainly when involving wild
758 animals (Möstl et al. 2005).

759 Another possibility, possibly simpler, is by exposing fresh fecal samples
760 (aliquots) to the environment, for a determined period of time and then

761 quantifying the metabolites to check if any change in fecal metabolites occurs.
762 Futures experiments will investigate this in maned wolves' fecal samples.

763 In conclusion, this study demonstrates for the first time the loss of normal
764 reproductive cycle profiles of fecal gonadal metabolites on animals exposed to
765 chronic adrenocortical stimuli, confirming that chronic stimulation of the HPA
766 axis can leads to lower reproductive efficiency in captive maned wolves. We
767 have shown that fecal progestagens and estrogens levels increase under acute
768 stimulation of the HPA axis in both genders, while androgens metabolites
769 decrease in males.

770 It's important to note the importance of andrological studies for generating
771 basic information that may be useful to future management of captive maned
772 wolves, however clarify that even the use of low invasive techniques for semen
773 collection, such as digital manipulation, the HPA axis can positively activate.
774 And considering the acute effects on fecal gonadal metabolites concentrations,
775 these stressful events can also lead to low reproductive efficiency, by increasing
776 the possibility of failures on the ovulatory process or disrupting sexual behavior
777 in the female depending on the reproductive stage in which their occur.

778 This study provided important information that help better understanding the
779 consequences and some mechanisms whereby chronically elevated corticoids
780 levels can compromise reproduction performance of captive maned wolves and
781 possibly free-living animals, since it is known that free-living maned wolves that
782 inhabit farmland regions, where fragmentation and conversion of natural areas
783 is the most apparent, also present high FCM concentration (Spercoski et al.
784 2012). Altogether, our current findings and a growing number of publications

785 from other researchers can be useful to improve the management of both
786 captive and free-living populations aiming the conservation of such a
787 charismatic species.
788
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798
799 **6. Declaration of Interest**
800 The authors have no conflicts of interest. The authors alone are
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802
803 **7. References**
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Tables

Table 1 – Brazilian institutions, animals, age (years), enclosure area (m^2), monitoring period (months) and part of the study which animals were used.

Institution	Animal	Age	Enclosure area	Monitoring period	Part
Americana Zoo – SP	Male 1	9	516	17	3
	Female 1	4		17	3
Ilha Solteira Zoo – SP	Male 2	9	540	12	3
	Female 2	9		12	3
CBMM conservation breeding center– MG	Male 3	5	5000	21	1 and 2
	Female 3	3		21	1 and 2
	Male 4	3	2500	21	1 and 2
	Female 4	6		21	1 and 2
	Male 5	3	2000	10	1 and 2
	Female 5	10		9	1 and 2

Figures

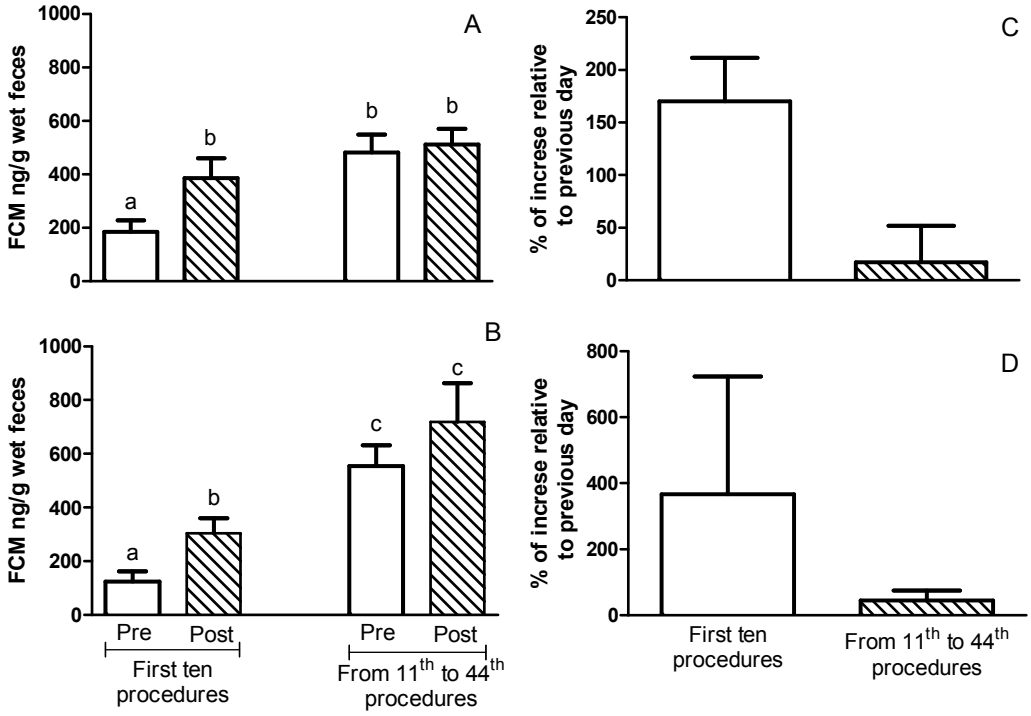


Figure 1 – Graphs on left show FCM concentration mean (ng/g wet feces; mean ± EPM) pre (smooth columns) and post (hatched columns) semen collection for the first ten and from the eleventh to 44th procedures in female (A) and male (B) captive maned wolves. Graphs on right show % increase of FCM concentration mean in relation to the pre procedure mean found in female (C) and male (D). [a,b,c] Different letters indicate differences ($P<0.05$; non pared t-test and Mann-Whitney rank sum test) between groups.

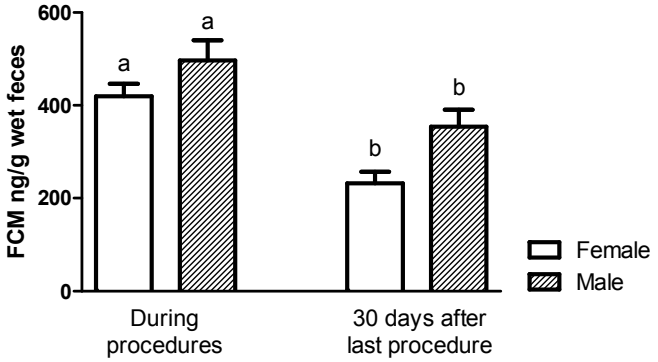


Figure 2 – FCM concentration mean (ng/g wet feces; mean ± EPM) during the period of semen collection and from 30 days after the last procedures to end of the monitoring period in female (smooth columns) and male (diagonal hatched columns) captive maned wolves. [a,b] Different letters indicate differences ($P<0.05$; non pared t-test and Mann-Whitney rank sum test) between groups.

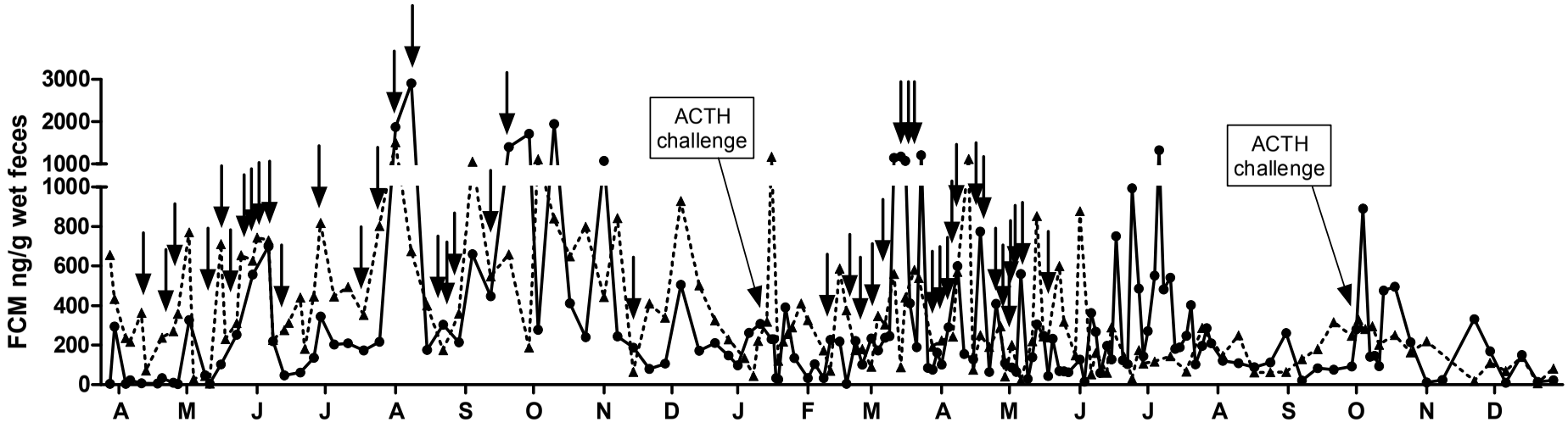
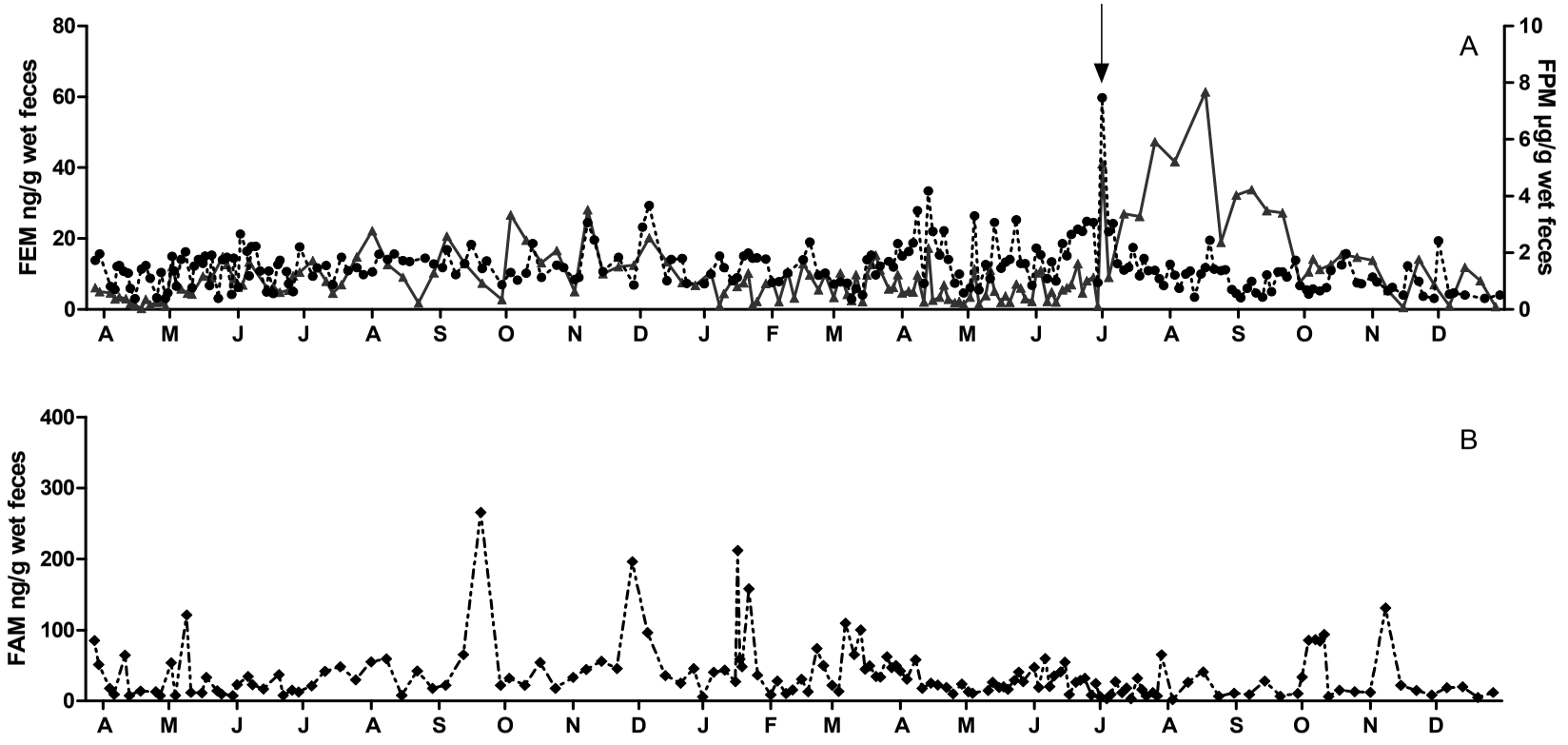


Figure 3 – Longitudinal profile of FCM concentration (ng/g wet feces) of representative female (triangles; discontinuous lines) and male (circles; solid line) captive maned wolves in which the male was submitted to semen collection. Black arrows indicate the days of procedures; ACTH challenge experiments are indicated on the graph.



1146

1147 **Figure 4** – Longitudinal profile of fecal progestagens (µg/g wet feces; triangles, grey solid line) and estrogens (ng/g wet feces; circles, black
1148 discontinuous line) of representative female (A) and fecal androgens (ng/g wet feces; losanges, black discontinuous line) of representative male (B)
1149 captive maned wolves during andrological study. Black arrow indicates possible ovulatory cycle.
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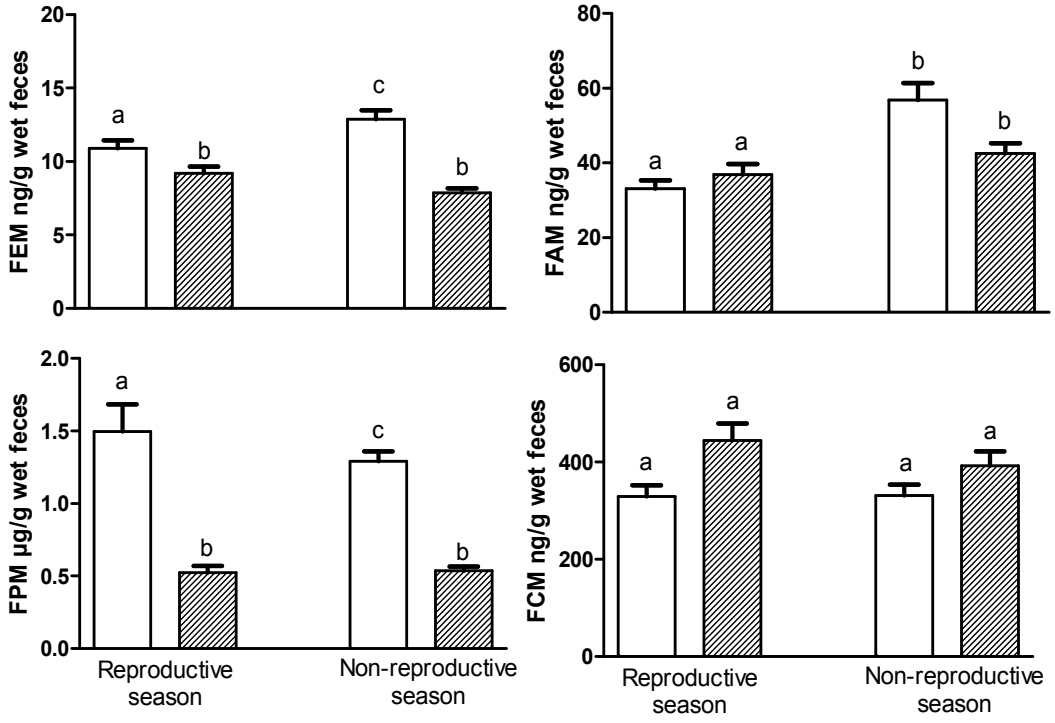

Figure 5 – Overall mean concentration (ng/g and µg/g wet feces; mean ± EPM) of fecal gonadal and adrenocortical metabolites in captive female (smooth columns) and male (diagonal hatched columns) maned wolves during reproductive and non-reproductive seasons. [a,b,c] Different letters within the same graph indicate differences (P<0.05; non pared t-test and Mann-Whitney rank sum test) between seasons and genders.

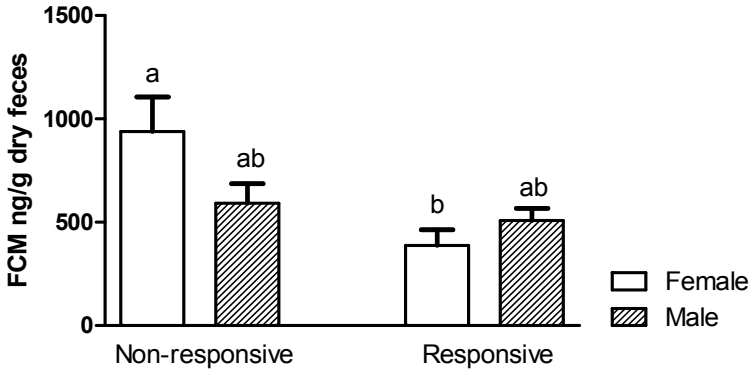

Figure 6 – Pre-challenge FCM concentration means (ng/g dry feces; mean ± EPM) in responsive and non-responsive maned wolves to the ACTH stimuli. Females: smooth columns; males: diagonal hatched columns. [a,b] Different letters within the same graph indicate differences (P<0.05; non pared t-test and Mann-Whitney rank sum test) between groups and genders.

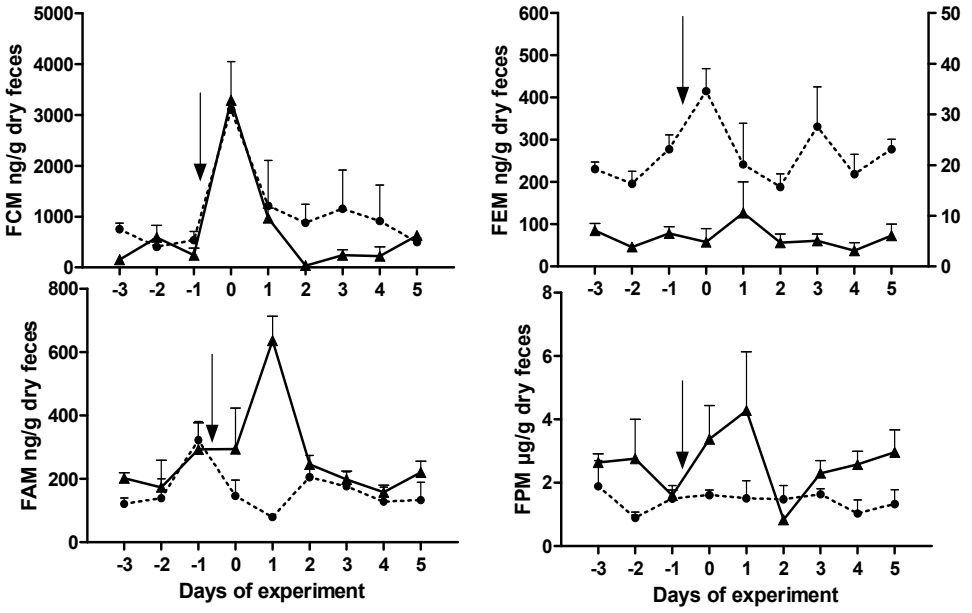

Figure 7 – Profiles of adrenocortical and fecal gonadal metabolites concentrations (ng/g wet feces, except for FPM shown in µg/g) in captive maned wolves submitted to the ACTH challenge experiment. Females: triangles, solid line; males: circles, dashed line. Black arrows indicate the administration of ACTH. Note: on two Y axis graphs the right axis shows values of males.

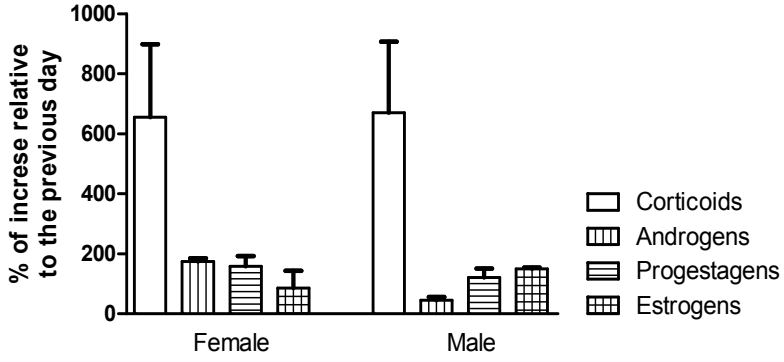

Figure 8 - % increase on fecal corticoid (smooth columns), androgens (vertical hatched columns), progestagens (horizontal hatched columns) and estrogens (crosshatch grid columns) metabolites concentrations in captive maned wolves on the day of the corticoid peak in relation to the day prior the ACTH challenge.

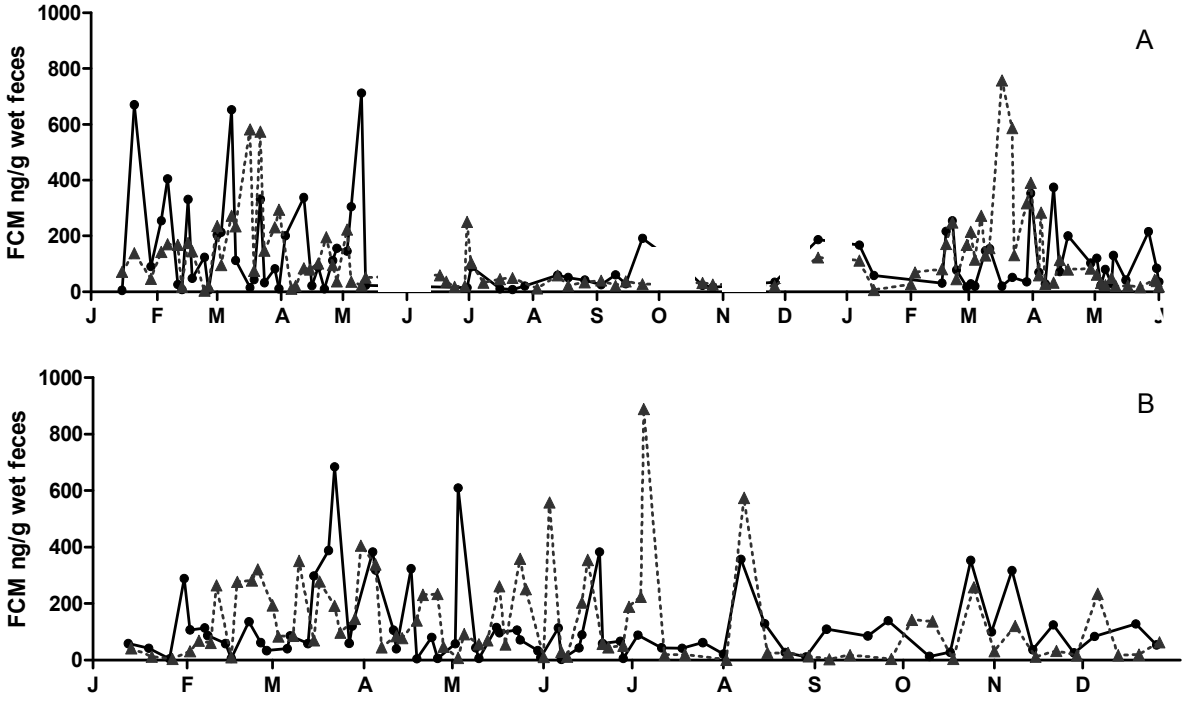

Figure 9 – Longitudinal profile of FCM concentration (ng/g wet feces) in female (triangles; discontinuous lines) and male (circles; solid line) captive maned wolves from Americana (A) and Ilha Solteira (B) Zoos.

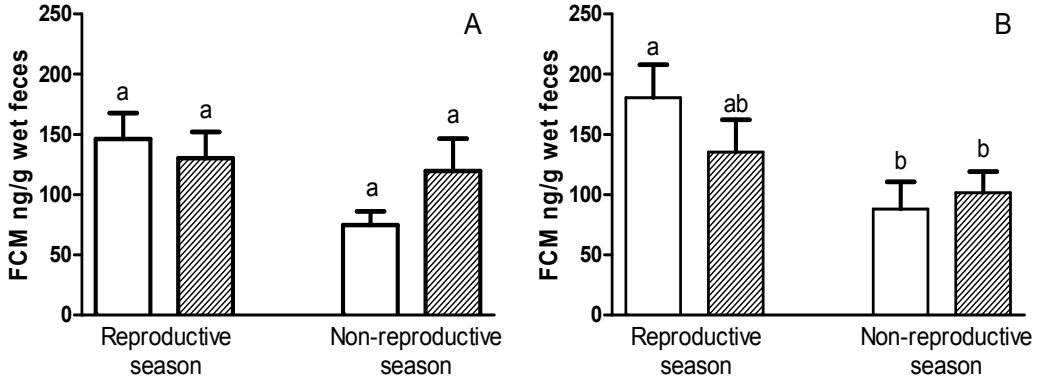

Figure 10 – FCM concentration mean (ng/g wet feces; mean ± EPM) in female (smooth columns) and male (diagonal hatched columns) captive maned wolves from Americana (A) and Ilha Solteira (B) Zoos during reproductive and non-reproductive seasons. [a,b] Different letters within the same graph indicate differences ($P<0.05$) between seasons and genders.

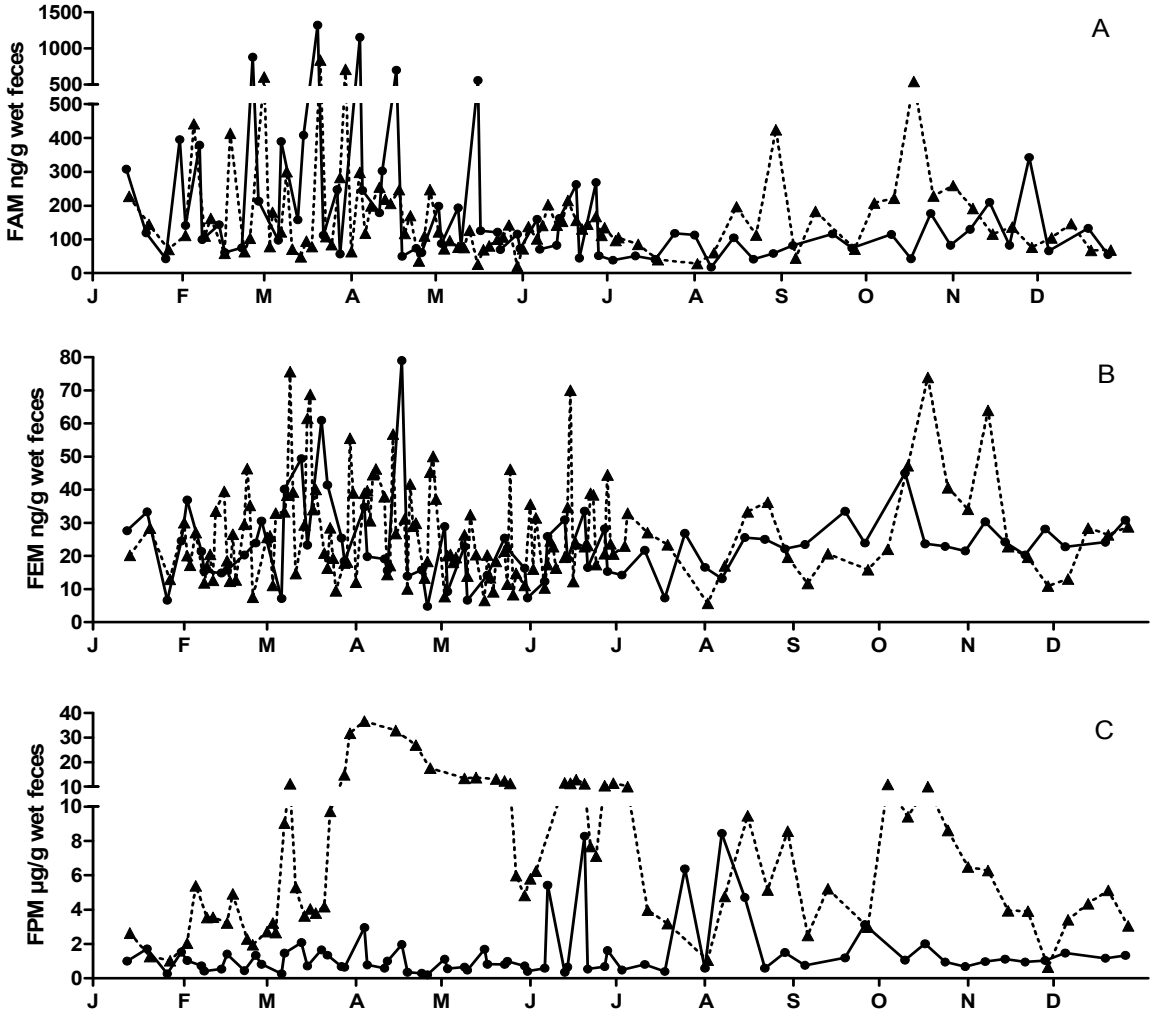

Figure 11 – Longitudinal profile of fecal androgens (A; ng/g wet feces), estrogens (B; ng/g wet feces) and progestagens (C; μg/g wet feces) concentrations from a female (triangles, discontinuous line) and male (circles, solid line) captive maned wolves.

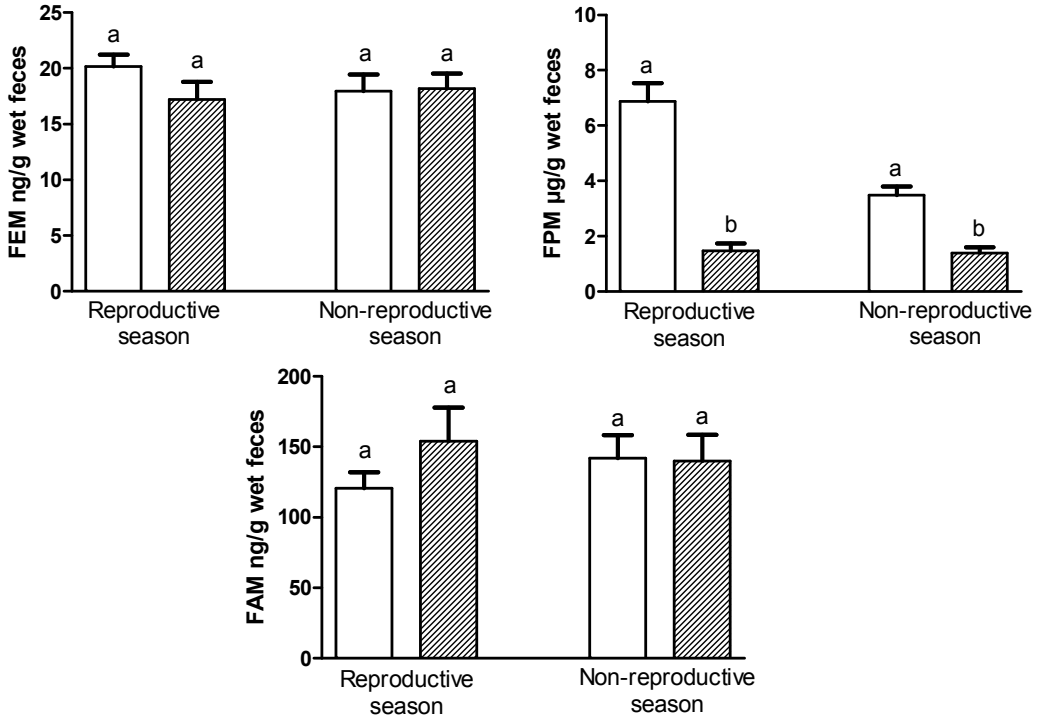

Figure 12 – Concentration mean (ng/g and µg/g wet feces; mean ± EPM) of fecal gonadal metabolites in female (white columns) and male (grey columns) captive maned wolves during reproductive and non-reproductive seasons. [a,b] Different letters within the same graph indicate differences ($P<0.05$) between seasons and genders.

6. CONCLUSÕES

Com base nas condições experimentais aqui descritas e nos resultados obtidos, pode-se concluir que:

- As fêmeas de lobo-guará apresentam variações hormonais típicas do ciclo ovariano de cadelas. A fase de anestro é marcada por reduzidas concentrações nos metabólitos gonadais fecais. O proestro é caracterizado pelo aumento nas concentrações e freqüência de picos de estrogênios. No período peri-ovulatório ocorre aumento nas concentrações de metabólitos de progestágenos fecais que se mantém altas até o final da gestação.
- Os machos de lobo-guará apresentam aumento nas concentrações de metabólitos de androgênios fecais durante a fase de proestro e período peri-ovulatório das fêmeas.
- As concentrações de corticóides aumentam normalmente durante o proestro e fase de lactação e cuidado parental nas fêmeas.
- Após o nascimento dos filhotes os machos apresentam aumento nas concentrações de todos os metabólitos gonadais e corticóides fecais.
- O perfil normal de metabólitos gonadais fecais foi alterado devido à estimulação crônica do eixo hipotálamo-hipófise-adrenal, mostrando que o estresse crônico ocasiona baixa eficiência reprodutiva.
- O estímulo adrenocortical agudo promovido pela administração de ACTH aumentou as concentrações de metabólitos de progestágenos e estrogênios fecais em fêmeas de lobos-guará. Nos machos os androgênios fecais foram reduzidos pelo estímulo.

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