UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE PSICOLOGIA

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**Efeito do estresse social sobre o
comportamento materno do hamster Sírio
*(Mesocricetus auratus)***

São Paulo

2010

MARIE ODILE MONIER CHELINI

**Efeito do estresse social sobre o comportamento
materno do hamster Sírio (*Mesocricetus auratus*)**

Tese apresentada ao Instituto de
Psicologia da Universidade de São
Paulo para obtenção do título de
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Orientadora: Profa Dra Emma Otta

Co-Orientador: Prof. Dr. Luciano
Freitas Felício

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*“For the hand that rocks the cradle
Is the hand that rules the world.”*

William Ross Wallace, 1865

RESUMO

RESUMO

CHELINI, M. O. M. **Efeito do estresse social sobre o comportamento materno do hamster Sírio (*Mesocricetus auratus*)**. 2010. 118p. Tese (Doutorado) – Instituto de Psicologia, Universidade de São Paulo, São Paulo, 2010.

A flexibilidade da socialidade presente em muitas espécies sugere que muitos dos traços comportamentais e fisiológicos presentes em grupos sociais complexos possam existir em estado latente em indivíduos de espécies solitárias. Hierarquia de dominância e assimetria reprodutiva são traços característicos de espécies sociais. O hamster Sírio é um excelente modelo para o estudo do estresse social e dos seus efeitos fisiológicos e comportamentais. Na presente pesquisa foram enfocados os efeitos do alojamento em grupo antes e no início da gestação na fêmea de hamster Sírio. Investigou-se se o estresse social produz algum grau de assimetria reprodutiva nesta espécie solitária e se o sucesso reprodutivo e o comportamento materno das fêmeas variam em função do seu status social. Dois experimentos foram realizados envolvendo respectivamente 34 fêmeas de hamster criadas em grupo do desmame até o início do estudo e 76 fêmeas criadas isoladamente. Em cada experimento, um terço dos animais foram alojados em gaiolas individuais, enquanto pares de fêmeas desconhecidas umas das outras eram formados com os outros dois terços. O comportamento dos pares foi observado ao longo de 10 dias para determinar o status social de cada fêmea. Todas as fêmeas foram acasaladas neste período. Quatro dias após o parto, os filhotes foram contados, sexados e pesados e as ninhadas padronizadas a seis filhotes, três machos e três fêmeas. As mães e suas ninhadas foram observadas diariamente durante sessões de 40min e 13 categorias comportamentais foram registradas. No intuito de esclarecer os mecanismos fisiológicos subjacentes à relação entre estresse social e fertilidade, os níveis dos hormônios reprodutivos e dos glicocorticóides foram monitorados ao longo da gestação por métodos não-invasivos, previamente validados, de quantificação dos seus metabólitos nas fezes. Foi demonstrada, pela primeira vez, a adequação de um ensaio imunoenzimático e de um conjunto diagnóstico comercial de radioensaio para a quantificação respectiva dos metabólitos fecais de glicocorticóides em hamsters Sírios dos dois sexos e de testosterona no hamster Sírio macho, mas não da fêmea. As variações das concentrações de metabólitos fecais de progesterona, estrógenos e glicocorticóides, refletiram os perfis séricos descritos na literatura para hamsters gestantes. Os resultados mostram que quando fêmeas de hamster Sírio são alojadas individualmente após o desmame, sua fertilidade não é afetada pelo estresse social. Em contraste, quando as fêmeas são criadas em grupo, o estresse social tanto de isolamento como de subordinação a uma fêmea dominante induz um grau significativo de assimetria reprodutiva, traço característico de espécies sociais que criam seus filhotes em comunidade. A manutenção de hamsters cativos adultos em grupos parece despertar traços comportamentais e fisiológicos presentes em grupos sociais complexos e que se encontram em estado latente nesta espécie solitária. A socialidade parece flexível no hamster e modulada pelas condições ecológicas.

Palavras-chave: Hamsters. Estresse social. Reprodução. Endocrinologia. Metabólitos fecais.

ABSTRACT

ABSTRACT

CHELINI, M. O. M. **Effects of social stress on the maternal behavior of the Syrian hamster (*Mesocricetus auratus*)**. 2010. 118p. Tese (Doutorado) – Instituto de Psicologia, Universidade de São Paulo, São Paulo, 2010.

The flexibility of sociality found in many species suggests that many of the behavioral and physiological mechanisms responsible for highly developed social interactions are present as latent traits, even in species usually considered as solitary. Social hierarchy and reproductive skew are typical features of social species. Syrian hamsters (*Mesocricetus auratus*) are an ideal model for the investigation of social stress and its physiological, neuroendocrine, and behavioral effects. The present research focused on the effects of group-housing prior to mating and on the first days of gestation of the female hamster. We investigated if social stress produces some degree of reproductive skew in this solitary species, and if female reproductive success varies as a function of social rank. Two experiments were carried out. The first one involved 34 females group-raised from weaning until the beginning of the trial and the second one 76 singly raised females. In each case one third of the animals were singly housed. Pairs of unrelative females were formed with the remaining animals. Pair behavior was observed through 10 days for assessment of the social rank of each female. All females were mated during this period. On day 4 post-partum, pups were counted, sexed, and weighed and litters were culled to six, three males and three females. Litters and dams were observed daily through 40-min sessions and 13 behavioral categories recorded. In order to assess the physiological relationship between social stress and fertility, we monitored reproductive hormones and glucocorticoids of solitary and pair-housed females during pregnancy by utilizing recently established non-invasive methods for measuring the respective hormone metabolites in the feces. The suitability of an enzyme immunoassay and of a commercial radioimmunoassay for respective quantification of fecal glucocorticoid metabolites of hamsters of both sexes and fecal testosterone metabolites in the male but not in the female was proven for the first time. The patterns of fecal progesterone, estrogen, and glucocorticoid metabolites were similar to blood profiles reported in the literature for pregnant hamsters. Our results showed that when female hamsters are singly housed from weaning, social stress did not affect their fertility. However, when females are group-raised, both isolation and subordination stress induced a significant reproductive skew, which is a characteristic feature of cooperative breeders. It seems that group-keeping of adult hamsters evokes social physiological and behavioral mechanisms present as latent traits in this solitary species. As in other rodents, sociability seems flexible in the Syrian hamster and our findings support the idea that social interactions may be a function of ecological conditions.

Keywords: Hamsters. Social stress. Reproduction. Endocrinology. Fecal metabolites.

ORGANIZAÇÃO GERAL DA TESE

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Em conformidade com as Diretrizes para apresentação de dissertações e teses da USP e as normas do Programa de Pós-Graduação em Psicologia Experimental do Instituto de Psicologia desta universidade, a presente tese compõe-se, além dos apêndices, de três partes: uma Introdução que situa o trabalho dentro do contexto do conhecimento atual relativo às relações entre organização social e reprodução e apresenta as hipóteses e os objetivos que nortearam sua realização; o Desenvolvimento apresenta de modo sucinto os experimentos realizados, a metodologia adotada em cada caso e o essencial dos resultados obtidos; na Conclusão, por fim, tecem-se algumas considerações sobre possíveis interpretações para esses resultados, verificando-se a confirmação ou não das hipóteses iniciais.

Os quatro Apêndices talvez constituam a parte mais importante deste documento. Cada um deles é um manuscrito expondo, detalhadamente, um ou diversos dos experimentos realizados, seus objetivos, sua metodologia, seus resultados e uma discussão destes resultados. Três deles foram submetidos para publicação em revistas indexadas, encontrando-se em processo de avaliação. Eventuais sugestões quanto a sua forma e conteúdo ainda poderão melhorá-los.

O manuscrito: **“Sex differences in the excretion of fecal glucocorticoid metabolites in the Syrian hamster”** (Apêndice A) foi submetido ao *Journal of Comparative Physiology B*.

O manuscrito: **“Validação de um radioimunoensaio para a quantificação de metabólitos fecais de testosterona no hamster Sírio (*Mesocricetus auratus*)”** (Apêndice B) foi submetido à revista *Pesquisa Veterinária Brasileira*.

O manuscrito: **“Social stress and reproductive success in the female Syrian hamster: endocrine and behavioral correlates”** (Apêndice C) foi submetido à revista *Hormones and Behavior*.

O manuscrito: **“Social behavior in a solitary species”** será submetido ao periódico *Animal Behavior*.

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1 INTRODUÇÃO

1 INTRODUÇÃO

Socialidade significa vida em grupo e se os grupos se formam e se mantêm, é porque, de alguma maneira, trazem para todos os indivíduos envolvidos alguma vantagem genética (Alexander, 1974). O que acontece, porém, quando as condições ambientais obrigam indivíduos de espécies consideradas como solitárias a conviver com co-específicos? Será verdadeira a recíproca e podemos dizer que vida em grupo significa socialidade? A afirmação parece verdadeira para algumas espécies cujo sistema social varia em função das condições ambientais. É o caso, por exemplo, do rato da taquara (*Kannabateomys amblyonyx*) monogâmico em certas regiões do Brasil e poligâmico noutras (Silva; Vieira; Izar, 2008). Do mesmo modo, enquanto *Rhabdomys pumilio*, um roedor africano, vive em grupo numa região semidesértica, tem hábitos solitários nos campos úmidos da África do Sul (Schradin; Pillay, 2005). Ademais, Sachser (1998) mostrou que, em cobaias, a maneira como os indivíduos interagem também é afetada por sua experiência social prévia. Esta flexibilidade da socialidade sugere que muitos dos traços comportamentais e fisiológicos presentes em grupos sociais complexos possam existir em estado latente em indivíduos de espécies solitárias. Um desses traços, conhecido como assimetria reprodutiva (*reproductive skew*), é muito comum nas sociedades animais. Consiste no monopólio da reprodução, total (como no rato-toupeira-pelado, *Heterocephalus glaber* (Clarke; Faulkes, 1998)) ou parcial (como no sagüi de tufo branco (Sousa et al., 2005)) por um indivíduo dominante. É comum observar uma assimetria reprodutiva alta entre as fêmeas de espécies que criam seus filhotes em cooperação, enquanto ela é geralmente baixa quando isso não ocorre (Creel, 2001). *A fortiori*, a ocorrência de assimetria reprodutiva parece muito pouco provável entre as fêmeas de espécies solitárias. Entretanto, ao observar o comportamento de carcajus (*Gulo gulo*), alojados num recinto comunitário, Dalerum, Creel e Hall (2006) não só encontraram nestes carnívoros, solitários em vida livre, níveis de interação social comparáveis àqueles apresentados por muitas espécies que vivem em sociedade, como também falhas reprodutivas provavelmente associadas ao status social, ainda que nenhuma fêmea tenha monopolizado a reprodução.

Estritamente territorial, de hábitos solitários na natureza (Gattermann et al., 2001), hamsters são geralmente alojados em grupos no cativeiro (Mori; Chelini; Couto, 2009). Nestas condições, às quais não têm nenhuma possibilidade de escapar, só lhes restam envolver-se em interações sociais, associadas, entretanto, nos adultos a sintomas de estresse (Gattermann; Weinandy 1996-97). A espécie foi considerada, por isso, excelente modelo para o estudo dos efeitos do estresse social sobre a reprodução (Pratt; Lisk, 1989). De fato, diversos autores relatam o estabelecimento de uma hierarquia social estável entre hamsters alojados em grupo, assim como certo grau de assimetria reprodutiva (Huck et al 1988; Pratt; Lisk, 1989; Pratt; Lisk, 1991) resultando não somente numa maior porcentagem de falhas reprodutivas como também no nascimento de ninhadas com uma maior proporção de fêmeas entre as fêmeas subordinadas. Ainda que suas causas não sejam muito claras, a mesma influência do estresse social sobre a razão sexual da prole foi constatada em outras espécies (Cameron, 2004).

Por outro lado, estudos mais recentes mostram que machos e fêmeas de hamster reagem de modo diferente ao estresse social. Enquanto a condição de derrotado modifica de maneira profunda e persistente o comportamento dos machos, inibindo totalmente a agressão territorial e estimulando a exibição frequente de comportamentos de submissão e defesa, não induz nenhuma alteração durável no comportamento agonístico das fêmeas (Huhman et al., 2003; Taravosh-Lahn; Delville, 2004). Em particular, elas se mostram muito menos propensas do que eles a estabelecer relações claramente polarizadas e estáveis entre dominante e dominada (Fritzsche; Riek; Gattermann, 2000). A ocorrência de supressão da reprodução parece pouco compatível com tais observações.

Potenciais influências endócrinas sobre a agressividade e a submissão foram investigadas com resultados ambíguos. Pratt e Lisk (1991) relataram uma redução significativa das concentrações de progesterona circulante em fêmeas de hamsters expostas a uma situação de subordinação no início da gestação. A causa sugerida para esta redução seria a ativação do eixo hipotálamo-hipófise-adrenal (HPA) pelo estresse social. No entanto, nenhuma medida fisiológica vem confirmar esta ativação. Em contraste, Fritzsche, Riek, e Gattermann (2000) encontraram níveis de progesterona mais altos em fêmeas alojadas em grupo do que em fêmeas mantidas isoladas,

independentemente do seu status social (dominantes ou subordinadas). Fêmeas tratadas com um implante de estradiol ou de testosterona apresentaram menos demonstrações de submissão do que fêmeas tratadas com progesterona ou sem nenhum tratamento hormonal (Faruzzi et al., 2005). O papel desempenhado pelos glicocorticóides de origem adrenal na modulação do comportamento agonístico e seus possíveis efeitos sobre o sucesso reprodutivo da fêmea de hamster ainda não foram bem esclarecidos.

O objetivo desta tese de doutorado foi o estudo dos efeitos do alojamento em grupo antes e no início da gestação na fêmea de hamster SÍRIO. Investigou-se se o estresse social produz algum grau de assimetria reprodutiva nesta espécie solitária e se o sucesso reprodutivo e o comportamento materno das fêmeas variam em função do seu status social. No intuito de esclarecer os mecanismos fisiológicos subjacentes à relação entre estresse social e fertilidade, os níveis dos hormônios reprodutivos e dos glicocorticóides foram monitorados ao longo da gestação por métodos não-invasivos previamente validados de quantificação dos seus metabólitos nas fezes (Chelini et al., 2005; Chelini et al., Apêndice A; Chelini et al., Apêndice B).

As hipóteses testadas foram que:

1) algum grau de assimetria reprodutiva ocorreria em fêmeas alojadas em grupo:

1a) falhas reprodutivas seriam mais frequentes em fêmeas subordinadas do que em fêmeas dominantes ou isoladas;

1b) as fêmeas subordinadas dariam a luz a ninhadas menores do que as fêmeas dominantes ou mantidas isoladas;

1c) as fêmeas subordinadas, mas não as dominantes paririam mais filhotes fêmeas do que machos;

2) a fertilidade das fêmeas dominantes seria similar àquela de fêmeas mantidas isoladas;

3) a frequência de comportamentos de cuidado dos filhotes seria menor entre as fêmeas subordinadas;

4) os perfis endócrinos dos diversos grupos seriam diferentes.

2 DESENVOLVIMENTO

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Para testar as hipóteses acima foram realizados quatro experimentos cujos procedimentos e resultados estão descritos mais detalhadamente nos respectivos apêndices nos quais os resultados são também discutidos com maior profundidade. Todos os procedimentos experimentais foram aprovados pela Comissão de Bioética da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (Certificado nº 796/2005), cujo Biotério forneceu os animais e os abrigou até o fim dos experimentos. Os resultados de todos os experimentos foram analisados com o programa SPSS for Windows 13.0, com o nível de significância fixado em 5%.

A técnica de dosagem de metabólitos de hormônios esteróides nas fezes, bastante usada para animais de vida livre, de difícil manejo, ou de porte muito reduzido como os roedores de laboratório, aparece como uma alternativa não estressante, econômica, capaz, além de poupar os animais, de fornecer dados bastante precisos, justamente por permitir um grande número de medidas num mesmo indivíduo. Porém, para garantir a relevância dos resultados, os ensaios utilizados na dosagem de cada hormônio devem ser rigorosamente validados não somente para cada espécie como para cada sexo de uma mesma espécie (Touma; Palme, 2005). O uso de conjuntos comerciais de radioimunoensaio destinados, respectivamente, à dosagem de estradiol e progesterona em sangue humano, para o monitoramento da atividade ovariana no hamster Sírio foi validada num estudo anterior (Chelini et al., 2005). Dois experimentos visando à validação da quantificação de metabólitos fecais de testosterona e de glicocorticóides nesta espécie foram realizados no âmbito do presente doutorado.

2. 1 Experimento 1 (Apêndice A)

O primeiro experimento tinha por objetivo verificar a relevância da quantificação de metabólitos fecais de glicocorticóides (MFG) para o monitoramento da resposta ao estresse de hamsters Sírios machos e fêmeas. Para isso, 10 animais de cada sexo foram submetidos a um teste de desafio com hormônio adrenocorticotrófico (ACTH) que tem por resultado o aumento dos níveis circulantes

de glicocorticóides, enquanto os animais do grupo controle (10 de cada sexo) recebiam uma injeção de solução salina. Todas as fezes excretadas por cada animal foram coletadas de 4 em 4 horas durante 24 h antes das injeções até 48 h depois delas. Os MFG foram quantificados pela técnica de enzimaímunoensaio (EIA) com um anticorpo contra o metabólito 11-oxoetiocolanolona. Os machos apresentaram concentrações basais (concentrações médias das 24 h precedendo as injeções) quase quatro vezes maiores do que as concentrações basais das fêmeas. As concentrações elevaram-se acima do nível basal a partir de 8 h após a administração de ACTH, atingindo seu pico respectivamente 20 h e 28 h após as injeções nos machos e nas fêmeas. Apesar das concentrações absolutas serem muito mais altas nos machos, o aumento relativo (500%) induzido pelo ACTH foi similar nos dois sexos. As diferenças entre os níveis de MFG medidos nos dois sexos estão em conformidade com os resultados de outros estudos sobre a fisiologia da adrenal no hamster. A comparação das respostas do córtex adrenal de machos e fêmeas a um desafio com ACTH forneceu informações inéditas sobre a amplitude e o perfil temporal desta resposta e a excreção de glicocorticóides por hamsters dos dois sexos. Demonstrou-se pela primeira vez que a atividade adrenocortical do hamster Sírio pode ser monitorada através de amostras fecais de modo não-invasivo.

2.1 Experimento 2 (Apêndice 2)

O objetivo do segundo experimento (Apêndice B) foi confirmar a adequação de um conjunto comercial para dosagem de testosterona em sangue humano para a quantificação de metabólitos de testosterona (MFT) nas fezes de hamsters Sírios machos e fêmeas. Para isso, 10 machos foram submetidos a um desafio com um agonista de GnRH para estimular a atividade testicular, elevando os níveis circulantes de testosterona. Cinco fêmeas receberam uma injeção de testosterona enquanto cinco outras receberam uma injeção de solução salina. Amostras de fezes coletadas antes e depois dos procedimentos, assim como amostras fecais de 20 fêmeas gestantes coletadas ao longo da gestação foram analisadas com um conjunto comercial para radioimunoensaio. Um pico de MFT 12 h após a injeção seguido de uma queda abaixo do nível basal comprovou que, nos machos, as alterações nos níveis de MFT refletem

as alterações da concentração de testosterona no sangue. Nestes observou-se um ciclo circadiano das concentrações de MFT com acrofase no início do período claro, correspondendo ao ciclo descrito para as concentrações sanguíneas na literatura, com o atraso devido à metabolização e excreção do hormônio. Nas fêmeas a administração de testosterona exógena provocou uma elevação importante dos níveis de MFT, mas as concentrações medidas ao longo da gestação não refletiram o padrão dos níveis sanguíneos do hormônio endógeno descrito na literatura, o que sugere a ligação ao anticorpo de outras moléculas, talvez andrógenos de origem adrenal, além de metabólitos de testosterona. O conjunto para radioimunoensaio para testosterona em sangue humano mostrou-se, portanto, adequado para o monitoramento da função testicular no hamster macho, mas um ensaio mais específico seria necessário para as fêmeas.

2.3 Experimento 3 (Apêndices C e D)

O terceiro experimento foi delineado para testar as hipóteses enunciadas na introdução. Ele envolveu 34 fêmeas adultas com as quais foram formadas 11 duplas (grupo experimental com estresse social), as 12 fêmeas restantes alojadas isoladamente formando o grupo controle (sem estresse social). O experimento foi desenvolvido em quatro fases sucessivas:

Fase 1: Antes de qualquer procedimento, dois ciclos estrais de cada fêmea foram acompanhados por detecção da descarga pós-ovulatória característica da espécie para confirmação da maturidade sexual e determinação das fases do ciclo de cada fêmea. Uma fêmea foi descartada por não apresentar ciclos estrais regulares.

Fase 2: Formação dos pares e observações comportamentais para determinação da hierarquia de dominância.

Para tal, a cada 4 dias, cada par foi separado por 20 minutos. A seguir, o par era reunido numa caixa limpa no fundo da qual sete pellets de ração foram dispostos, e as fêmeas eram observadas durante 10 minutos, sendo registrado o número de manifestações de comportamento agressivo (ataques, perseguições, mordidas) ou defensivo (esquiva, fuga, posição de submissão) exibido por cada fêmea e de quantos

pellets de ração cada uma conseguia se apossar. A cada sessão, o animal que efetuou o maior número de demonstrações de agressividade (ataques e perseguições) recebeu um ponto e aquele que se apossou do maior número de pellets recebeu também um ponto. Estas sessões de observação foram realizadas entre 15h00 e 17h00, quando os hamsters estavam acordando do sono diurno e retornando à atividade.

As fêmeas isoladas, do grupo controle, receberam igualmente alguns pellets de ração no fundo da caixa a cada quatro dias.

Fase 3: No primeiro estro subsequente à primeira sessão de observação, as fêmeas foram acasaladas com 10 machos de fertilidade comprovada fornecidos pelo mesmo biotério. Para isso, na tarde do estro, cada fêmea ficou alojada com um macho e permaneceu com ele durante 24 horas. As duplas de fêmeas foram reunidas no dia seguinte permanecendo junto por um total de dez dias, até a terceira sessão de observação comportamental. A seguir, todas foram alojadas individualmente até o fim da gestação.

Fase 4: Em razão dos altos índices de canibalismo observados quando as fêmeas de hamster e/ou seus filhotes são perturbados ou manipulados nos primeiros dias após o parto (Da Silva et al. 1989), os filhotes somente foram contados, seu sexo determinado e a mãe e a ninhada pesadas no quarto dia após o parto, por ocasião da primeira troca de caixa. Neste dia, na medida do possível, o número de filhotes em cada ninhada foi reduzido para seis, três machos e três fêmeas.

As observações comportamentais tiveram início no dia 5 e foram efetuadas até o dia 14 após o parto. Cada mãe e sua ninhada foram observadas diariamente durante 40 minutos sempre no período entre 15h00 e 16h00, na sua própria caixa, segundo o protocolo de observação de comportamento materno descrito por Slamberová et al. (2001), adaptado para hamster. Durante cada sessão de 40 minutos, o comportamento de cada mãe foi observado 10 vezes segundo a técnica de animal focal e amostragem por *scan* com intervalos de 4 minutos. Treze categorias comportamentais foram registradas a cada sessão:

- 1) amamentando

- 2) no ninho
- 3) contato corporal com pelo menos um filhote
- 4) contato corporal com pelo menos a metade da ninhada
- 5) lambendo ou limpando pelo menos um filhote
- 6) manipulando a maravalha do ninho
- 7) manipulando maravalha fora do ninho
- 8) comendo
- 9) manipulando alimento
- 10) carregando um filhote
- 11) auto-limpeza
- 12) em posição vertical, cheirando ou mascando a tampa da caixa
- 13) cabeça erguida, farejando

A ocorrência de cada atividade (máximo de 10 vezes em cada sessão) foi quantificada em cada uma das 10 sessões. Os filhotes foram contados a cada sessão para registro de eventuais casos de canibalismo.

O desmame foi realizado no vigésimo primeiro dia após o parto com registro da composição de cada ninhada, do peso da mãe e dos filhotes.

A coleta de amostras fecais começou dez dias antes da formação das duplas e se prolongou até a véspera do parto. As coletas foram realizadas todos os dias às 14h40, colocando cada fêmea numa gaiola de polipropileno limpa, sem cama, até ela defecar, o que ocorria em média após 20 a 30 minutos. Os esteróides fecais foram extraídos por diluição em metanol segundo o protocolo recomendado por Palme (2005).

As dosagens de metabólitos fecais de estrógenos, progesterona e testosterona foram realizadas no Laboratório de Dosagens Hormonais (Registro CNEN 13940) do Departamento de Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da USP sob supervisão do Professor Claudio Alvarenga de Oliveira (Registro CNEN AL 1020). Foram utilizados para tal conjuntos comerciais para radioimunoensaio em fase sólida com tubos revestidos de anti-corpo (Coat-A-Count estradiol, progesterone e testosterone, Siemens, Los Angeles, CA, EUA).

Os metabólitos de glicocorticóides foram dosados no Laboratório de Bioquímica da Universidade de Medicina Veterinária de Viena (Áustria) sob supervisão do Professor Rupert Palme, por meio do recém validado enzimaímunoensaio para o metabólito do cortisol 11-oxoaeticolanolona (Cf. Apêndice A).

Quanto ao comportamento materno, uma Análise de Componentes Principais permitiu selecionar as categorias comportamentais responsáveis por explicar mais de 1% da variância total. A seguir, a análise pelo Modelo Linear Geral para medidas repetidas (rmGLM) revelou um efeito significativo de redução com o tempo da frequência de ocorrência dos comportamentos “no ninho”, “amamentando”, “em contato corporal com pelo menos um filhote” e “em contato corporal com pelo menos a metade da ninhada”, sem efeito de grupo nem de status. A coabitação com uma fêmea desconhecida não afetou, portanto, a frequência dos comportamentos maternos observados.

As variações das concentrações de metabólitos fecais de progesterona, estrógenos e glicocorticóides, mas não de testosterona (Cf. Apêndice B) refletiram os perfis séricos descritos na literatura para hamsters gestantes. As fêmeas dominantes tiveram um maior sucesso reprodutivo do que as subordinadas (90% vs 50%). No entanto, enquanto esperávamos para as fêmeas isoladas do grupo controle uma porcentagem de sucesso próxima da média do biotério, ou seja, entre 80 e 90%, somente 54,5% destas fêmeas levaram a gestação a termo. Não houve diferença entre os diversos grupos quanto ao número médio de filhotes por ninhada, a razão sexual média das ninhadas nem o peso médio dos filhotes. As concentrações de glicocorticóides não foram afetadas nem pelo alojamento (em pares ou individual), nem pelo status social e as poucas diferenças, pontuais, entre os perfis endócrinos dos diversos grupos não são suficientes para justificar a diferença no sucesso reprodutivo. Lembrando que as fêmeas envolvidas neste experimento foram mantidas em grupos de cinco desde o desmame até a idade adulta, quando foram adquiridas, o isolamento social parece ter dificultado a reprodução como fez a subordinação. Esses resultados sugerem que o isolamento social, para animais acostumados a viver em grupo, foi tão perturbador quanto foi a coabitação com um co-específico desconhecido e nos levaram a repetir o experimento em condições levemente diferentes.

2.4 Experimento 4 (Apêndice D)

O objetivo do quarto experimento foi verificar se o estresse social afeta a reprodução de fêmeas de hamster alojadas individualmente desde o desmame (condição social similar àquela encontrada na natureza por Gattermann et al., (2001)) da mesma maneira que afeta a reprodução de fêmeas mantidas em grupo até a idade adulta. Foi realizado com 76 fêmeas (26 duplas e 24 fêmeas isoladas) criadas individualmente desde o desmame. Seu delineamento foi similar ao delineamento do terceiro experimento, porém sem coleta de fezes nem medidas hormonais.

Não houve desta vez diferença entre os grupos quanto ao sucesso reprodutivo, nem ao tamanho ou à razão sexual das ninhadas, nem ao comportamento das mães. A porcentagem de fêmeas isoladas que levaram a gestação ao seu termo tendeu a ser maior neste experimento do que no precedente e, em todos os grupos, o peso médio dos filhotes no desmame foi maior do que no experimento anterior, sem correlação com o peso das mães. Ademais, as fêmeas alojadas em duplas, submetidas, portanto, ao estresse social, deram a luz a filhotes mais pesados.

Os resultados deste experimento sugerem que o estresse social não prejudica o sucesso reprodutivo das fêmeas de hamster Sírio quando elas são alojadas individualmente após o desmame, condição mais próxima do que ocorre na natureza do que o alojamento em grupo geralmente adotado nos biotérios (Mori; Chelini; Couto, 2009). Ao contrário, ele parece estimular respostas fisiológicas que favorecem os filhotes.

A comparação entre os resultados dos Experimentos 3 e 4 (Apêndice D) evidencia a importância das condições de criação das fêmeas antes do experimento. O comportamento de apropriação de alimento, típico de indivíduos dominantes ocorreu menos entre as fêmeas criadas isoladamente do que naquelas criadas em grupo. A supressão da reprodução, outra característica de espécies sociais, somente ocorreu entre as fêmeas criadas em grupo. Estas, por outro lado, apresentaram com menor

frequência do que aquelas criadas isoladas comportamentos de cuidado maternal como amamentação ou cuidados com o ninho e com maior frequência comportamento de cuidado de si mesma, como autolimpeza ou comportamentos alimentares. Isto explica provavelmente, pelo menos em parte, o maior peso ao desmame dos filhotes cujas mães foram criadas isoladas, independentemente de terem sido ou não submetidas ao estresse da coabitação com uma fêmea desconhecida, ou do seu status social. Não podemos descartar totalmente a possibilidade de uma influência da coleta de fezes sobre o comportamento materno. O alto sucesso reprodutivo das fêmeas dominantes no experimento 1 torna, no entanto, esta suposição pouco provável.

3 CONCLUSÃO

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Os estudos de validação realizados no âmbito desta tese de doutorado demonstram, pela primeira vez, a adequação de um ensaio imunoenzimático e de um conjunto diagnóstico comercial de radioimunoensaio para a quantificação respectiva dos metabólitos fecais de glicocorticóides em hamsters Sírios dos dois sexos e de testosterona no hamster Sírio macho. Trata-se de ferramentas não invasivas poderosas para futuros estudos envolvendo tanto a função reprodutiva quanto a resposta ao estresse e o comportamento social deste animal. Estas alternativas humanitárias, práticas e não invasivas à coleta de sangue, difícil numa espécie usada com frequência como modelo animal na pesquisa médica, descortinam a possibilidade de estudos de longo prazo com um número mínimo de animais, garantindo o seu bem-estar. Infelizmente, o radioimunoensaio para testosterona parece ter medido, nas fêmeas, metabólitos de outros esteróides, resultado que confirma, uma vez mais, a necessidade de validar cuidadosamente as técnicas de dosagem de esteróides fecais não somente para cada espécie como para cada sexo de uma mesma espécie.

Retomando as hipóteses iniciais, podemos concluir que

1) algum grau de assimetria reprodutiva ocorre em fêmeas alojadas em pares quando elas estão acostumadas desde o desmame a viver em grupo, mas não em fêmeas que estavam alojadas individualmente desde o desmame:

1a) quando as fêmeas estão alojadas em grupo do desmame à idade adulta, falhas reprodutivas são mais frequentes em fêmeas subordinadas do que em fêmeas dominantes, mas não do que em fêmeas isoladas; a percentagem de falhas reprodutivas independe do tipo de alojamento e do status social quando as fêmeas foram alojadas individualmente do desmame até a idade adulta;

1b) o tamanho das ninhadas independe do tratamento e do status social das fêmeas;

1c) a razão sexual das ninhadas independe do tratamento e do status social das fêmeas;

2) a fertilidade das fêmeas dominantes é similar àquela das fêmeas mantidas isoladas e das fêmeas subordinadas quando todas foram alojadas individualmente após o desmame; quando as fêmeas estão alojadas em grupo do desmame à idade adulta, a fertilidade das fêmeas isoladas tende a ser menor do que aquela das dominantes;

3) quando as fêmeas estão alojadas em grupo do desmame à idade adulta, os perfis de metabólitos fecais de progesterona, estrógenos e glicocorticóides daquelas mantidas isoladas antes da gestação e ao longo dela somente diferem pontualmente dos perfis daquelas que conviveram com uma desconhecida no mesmo período.

3) fêmeas alojadas individualmente desde o desmame e que conviveram com uma desconhecida antes da gestação e no início dela apresentam uma maior frequência de comportamentos dirigidos a sua ninhada do que fêmeas que permaneceram isoladas; quando as fêmeas são alojadas em grupo após o desmame, seu comportamento materno independe delas conviverem ou não com uma desconhecida antes da gestação e no início dela.

Sintetizando, nossos resultados mostram que quando fêmeas de hamster Sírio são alojadas individualmente após o desmame, condição que condiz com as características de territorialidade e hábitos solitários da espécie na natureza, sua fertilidade não é afetada pelo estresse social. Estes dados estão em acordo com os achados de Tamashiro, Nguyen e Sakai (2005), segundo os quais entre os roedores, ao contrário dos machos, as fêmeas não apresentam respostas significantes de estresse à derrota social e à submissão. Esta maior habilidade das fêmeas em administrar o estresse social, mesmo repetido, pode representar uma vantagem importante para o seu sucesso reprodutivo. Faz com que elas sejam capazes de garantir a defesa do seu ninho e da sua prole contra eventuais invasores. Ao contrário, pode ser mais vantajoso para um macho poupar sua vida fugindo, do que enfrentar um adversário potencialmente mais forte do que ele.

Em contraste, quando as fêmeas são criadas em grupo e assim permanecem até a idade adulta, o estresse social tanto de isolamento como de subordinação a uma fêmea dominante induz um grau significativo de assimetria reprodutiva, traço característico de espécies sociais que criam seus filhotes em comunidade. A manutenção de hamsters cativos adultos em grupos parece despertar traços comportamentais e fisiológicos presentes em grupos sociais complexos e que se encontram em estado latente nesta espécie solitária. Como em outros roedores (Schradin; Pillay 2005), a socialidade parece flexível no hamster e modulada pelas condições ecológicas.

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APÊNDICE A

APÊNDICE A

Sex differences in the excretion of fecal glucocorticoid metabolites in the Syrian hamster

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Running title: Fecal glucocorticoid metabolites in hamster

Abstract

We verified the relevance of measuring fecal glucocorticoid metabolites (FGM) to assess the stress response of the Syrian hamster. Male and female hamsters (n=10 each) were submitted to an adrenocorticotrophic hormone (ACTH) challenge test, whereas animals in the control group received 0.5 mL of sterile isotonic saline solution. All feces voided by each animal were collected at 4 hours intervals from 24 h before (baseline) until 48 h after injections. FGM were quantified using an 11-oxoetiocholanolone enzyme immunoassay (EIA). Basal concentrations of FGM were almost 4 times higher in males than in females. Following ACTH administration, levels were higher from 8 h onwards, reaching peak concentrations 20 h or 28 h post injection in males and females, respectively. Despite the much higher absolute concentrations present in males, the relative increase (500%) in response to the ACTH stimulation was similar in both sexes. Sex differences in FGM levels are in accordance with results reported by others regarding the hamster adrenal physiology. The comparison of the adrenocortical response of males and females to an ACTH challenge provided new information about the amplitude and the timing of such a response and the excretion of glucocorticoids in both sexes. We demonstrated for the first time in the Syrian hamster that adrenocortical activity can be monitored in fecal samples in a non-invasive way. Our study provides a humane, practical and noninvasive alternative to blood removal, and therefore a powerful tool for stress-related studies in a species frequently used as an animal model in medical research.

Keywords: Hamster; Cortisol; Noninvasive monitoring; Fecal metabolites; ACTH challenge test

Introduction

Since the capture of the first litter in 1930, Syrian hamsters (*Mesocricetus auratus*) have become one of the species mostly used in biological and medical research, due to some unique features these animals present. For instance, hamsters can reproduce very fast and actually present the shortest gestation period (16 days) among all eutherian mammals. Additionally, hamsters are extensively used in cardio-vascular research (Bajusz 1969; Missihoun et al 2009) because their circulatory system shares some similarities with the human system. Moreover, these solitary living and strictly territorial animals (Gattermann et al 2001) have been widely used as models for behavioral and stress-related studies (e.g.: Cain et al 2004; Cordner et al 2004; Gebhardt-Heinrich et al 2007; Taravosh-Lahn and Delville 2004; Touma and Palme 2005; Wommack et al 2004; Zhang et al 2008) for which measuring the activity of the hypothalamic–pituitary–adrenal axis is a basic tool (Möstl and Palme 2002). Classically, such studies have relied on blood sampling and the measurement of serum cortisol or corticosterone, depending upon which glucocorticoid is predominantly secreted in each species (Palme et al 2005). Whereas in the rabbit and most rodents, including the rat and the mouse, corticosterone is well-recognized as the major glucocorticoid, a controversy exists about this point in the hamster. Lehoux et al (1992) and Cloutier et al (1997), for instance, reported that hamsters, as human beings, synthesize more cortisol. In contrast, Ottenweller et al (1985) measured three or four times higher basal concentrations of corticosterone than cortisol. Therefore, these authors suggested that both glucocorticoids should be measured when assessing adrenocortical function in the hamster. Methods for blood removal from laboratory mammals and birds have been reviewed by the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement (1993). The Group was unable to recommend a

preferred route for venepuncture in the gerbil and hamster. Actually, blood collection in hamsters is generally performed by cardiac puncture under general anesthesia or decapitation. Noninvasive methods of quantifying fecal glucocorticoid metabolites are now broadly used with free-ranging and intractable animals (Bosson et al 2009; Charbonnel et al 2008; Franceschini et al 2007; Huber et al 2003; Monclús et al 2009; Rehnus et al 2010; Soto-Gamboa et al 2009; Strauss et al 2007; Thiel et al 2008) as well as with laboratory animals (Bauer et al 2008; Lepschy et al 2007; Nováková et al 2008; Touma et al 2003) to investigate hormone–behavior relationships, as well as questions in the fields of animal welfare, ecology, conservation biology, and biomedicine (Touma and Palme 2005). These techniques avoid effects associated with blood sampling, which can seriously confound the experimental results. They enable frequent sampling of the same animal (and thus longitudinal studies), using individuals as their own controls. Moreover, no animal material is easier to collect than feces, and sampling can be done without disturbing the animals. All these advantages justify the increasing success of the analysis of fecal steroids. However, because the metabolism and the excretion of steroids differ significantly between species, and sometimes even between sexes (Palme et al 1996; Palme et al 2005; Touma et al 2003), these methods must be rigorously validated for each species and sex before application, to demonstrate that the assay technique is able to detect changes in the levels of fecal steroid metabolites related to respective changes of steroid concentrations in the blood (Palme 2005). Despite the large number of studies dealing with FGM measurement in a wide range of animal species, validation experiments have been performed in only a few species (Touma and palme 2005) and never, to our knowledge, in the Syrian hamster. Therefore the objective of this work was 1) to detect whether diurnal changes in endogenous glucocorticoid levels are reflected in FGM concentrations, 2) to examine the effect of an

ACTH challenge test on fecal glucocorticoid metabolite concentrations and 3) to compare such effects in male and female Syrian hamsters.

Materials and methods

Animals and housing conditions

A total of 41 heterogenic, adult (10 to 15 weeks of age), sexually mature Syrian hamsters (20 males and 21 females) were obtained from the Laboratory Animal Facility of the Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil, whose Ethics Committee approved the experimental design. The animals were individually housed in standard propylene cages in the same animal facility under conventional conditions (12:12-h light:dark, lights on at 03:00 h; room temperature: 22 ± 2 °C; 20 changes of air per hour; air pouch filters) according to guidelines from the Institute for Laboratory Animal Research (1996). Specific pelleted food (Nuvilab CR1, Nuvital, Curitiba, Brazil) and filtered bottled tap water were supplied ad libitum. In order to facilitate feces collection, absorbent paper pads were substituted for the traditional wood-shaving bedding.

ACTH challenge test

The ACTH injection was used in ten males and ten females randomly chosen (ACTH groups) to stimulate adrenocortical activity (i.e., to increase circulating glucocorticoid levels). On the second day (13:00 h) of the experiment, these hamsters were injected with 100 µg of synthetic ACTH (Synacthène Retard, 1 mg/ml, Novartis Pharma SAS, Rueil-Malmaison, France) dissolved in 0.5 ml sterile isotonic solution. To investigate the potentially stressful effects of the injection procedure itself on the pattern

of excreted FGM, another 11 females and 10 males (Control groups) received an injection of 0.5 mL sterile isotonic saline solution.

Feces collection

All feces voided by each animal were collected at 4-hour intervals from 24 h before until 48 h after the injections. In this way, we were able to determine baseline concentrations and the normal circadian pattern of FGM in each group and each group was used as its own control. All samples were weighed, immediately identified, and stored in a freezer at -20°C .

Steroid extraction and quantification

Fecal steroids were extracted using the methanol-based procedure described by Palme(2005). Because hamster feces are very dry, lyophilization was not necessary. After the homogenization of each fecal sample, we shook an aliquot of 0.5 g or the whole sample for 20 min on a multivortex with 5 ml of 80% methanol for samples heavier than 0.25 g, 2.5 ml for samples whose weight was between 0.1 and 2.5 g and 1 ml for samples lighter than 0.1 g. The suspension was then centrifuged at 500 g for 10 min. The supernatant was stored at -20°C until assayed. Fecal glucocorticoid metabolites were quantified in an aliquot of the extract (50 μl diluted 1:10) using a group specific 11-oxoetiocholanolone enzyme immunoassay (EIA measuring glucocorticoid metabolites with a $5\beta\text{-}3\alpha\text{-ol-}11\text{-one}$ structure) first described by Möstl et al(2002) and successfully validated for the common hamster (*Cricetus cricetus*) (Franceschini et al 2007). All samples were assayed in duplicate. Concentrations of glucocorticoid metabolites were expressed as nanograms per gram fecal dry matter.

Statistics

Data were analyzed using the software SPSS 13.0 for Windows. As they did not fulfill the assumptions of normality and homogeneity of variance FGM data were log transformed after replacing missing values by linear interpolation. Repeated measure General Linear Model (rmGLM) was used to detect sex, treatment (ACTH or saline), and time effects on these concentrations. We performed Wilcoxon signed ranks tests (Bonferroni corrected) as a post hoc test to compare concentrations measured in different intervals within a sex. Means are given with standard deviations and significance levels for all tests was set at $p < 0.05$ unless otherwise noted.

Results

Baseline levels

The rmGLM analysis of FGM concentrations measured on the day preceding injections showed a strong effect of sex ($F = 288.5$, $p < 0.005$) but no effect of treatment ($F = 0.193$, $p = 0.663$) as between subject factors. In fact, male baseline concentrations (320 ± 16 ng/g feces) were almost four times higher than female ones (89 ± 50 ng/g feces). However, despite individual differences in FGM concentrations (range: 30 to 283 ng/g feces for females; 135 to 605 ng/g feces for males), the homogeneity of the groups was confirmed by similar baseline concentrations among females and males (Females: ACTH 88.6 ± 43.8 ng/g feces; Control: 88.6 ± 49.3 ng/g feces; Males: ACTH: 306 ± 69 ng/g feces; Control: 335 ± 65 ng/g feces). A clear effect of the interaction between sex and time (Wilks' Lambda $F = 11.004$, $p < 0.0005$) indicated that concentrations varied in a different way in females and males. As no effect of time of

day as a within subject factor was detected (Wilks' Lambda $F = 1.207$, $p = 0.328$), no circadian rhythm of FGM excretion was documented by our assay.

Fig. 1 Variation of fecal glucocorticoid metabolites (FGM) over 24 h in male (n= 20; white boxes) and female (n=21; light grey boxes) Syrian hamsters

ACTH challenge test

FGM values (of the 4 h intervals) from the moment of injections onwards were submitted to rmGLM with time as a within subject factor. FGM concentrations changed significantly in males and females in response to the ACTH stimulation, and the time course of FGM excretion differed between the sexes. There were marked time (Wilks' Lambda $F = 25.009$, $p < 0.0005$) and interaction effects (time x sex: Wilks' Lambda $F = 13.108$, $p < 0.0005$; time x treatment: Wilks' Lambda $F = 22.009$, $p < 0.0005$; time x sex x treatment: Wilks' Lambda $F = 2.968$, $p = 0.010$). There were also strong effects of

both, sex ($F = 456$, $p < 0.0005$) and treatment ($F = 142$, $p < 0.0005$) as between subject factors. As found with basal concentrations, absolute values were much higher in males' feces, but the relative increase was quite similar for both sexes ($509 \pm 131\%$).

Fig. 2 Boxplots of fecal glucocorticoid metabolites (FGM; ng/g) of male Syrian hamsters after an injection of adrenocorticotrophic hormone (ACTH; dark grey boxes) or saline solution (Control; white boxes). Respective median baseline values of the ACTH (--●--) and control group (····) at the same time of the day are also shown. Asterisks beside the box indicate significant differences compared to the baseline levels of the same group

Wilcoxon signed ranks test with Bonferroni correction (significance level $p < 0.016$) confirmed that FGM concentrations in males of the ACTH group were significantly higher than the ones measured at the same time on the day prior to injection from 8 h after injection ($Z = -2.66$) onwards, and reached its highest value (median: 1408 ng/g feces) 20 h after administration (Figure 2). It was also from 8 h after

the injection in females that the ACTH group showed significantly higher concentrations compared to the corresponding baseline values ($Z = -2.803$), but the highest concentrations (median: 346 ng/g of feces) were detected only after 28 h (Figure 3). In contrast, FGM were still higher in ACTH males at the end of the sampling period (48 h after injection) than baseline values ($Z = -2.547$). In the female group, FGM had already returned to baseline levels 44 h after injections ($Z = -1.826$, $p = 0.068$).

Fig. 3 Boxplots of fecal glucocorticoid metabolites (FGM; ng/g) of female Syrian hamsters after an injection of adrenocorticotrophic hormone (ACTH; dark grey boxes) or saline solution (Control; light grey boxes). Respective median baseline values of the ACTH (--●--) and control group (····) at the same time of the day are also shown. Asterisks beside the box indicate significant differences compared to the baseline levels of the same group

Following the saline injection no significant (Wilcoxon signed ranks test, Bonferroni corrected $p > 0.016$ for all comparisons between concentrations measured at

the same time intervals on the different days) increase in FGM was found in males. In females only FGM values 32 h after the injection (median: 154 ng/g feces) were significantly higher (Wilcoxon signed ranks test, $Z = -2.934$, $p = 0.003$).

Discussion

Our study describes and successfully validates a non-invasive method for evaluating adrenocortical activity in hamsters by measuring fecal glucocorticoid metabolites (FGM) for the first time. It demonstrates that in hamsters adrenocortical activity is well reflected in concentrations of FGM, measured with an 11-oxoetiocholanolone EIA. This indispensable validation (Touma and Palme 2005), the lack of which some authors already deplored (Gebhardt-Heinrich 2007), provides the basis for a reliable and powerful non-invasive tool for further investigations involving monitoring of hamsters' glucocorticoids. Beyond this, there are other important findings in our study. One of them, which has already been reported for cortisol concentrations in hamster blood samples (Gebhardt-Heinrich 2007; Lucas et al 1999) is the presence of large inter-individual differences regarding baseline FGM concentrations. For example, levels between females varied almost 10-fold during the day prior to the injection. This confirms one more time the importance of longitudinal studies, in which each animal or group is its own control, which is almost impossible in small rodents, if blood is sampled.

In most laboratory rodents, females have larger adrenal glands and greater steroid production than males. This dimorphism is reversed in Syrian hamsters and the adrenal glands of male hamsters are larger and secrete more cortisol than those of the females (Huhman et al 2003; Nikicicz 1984). Our data show that, as expected, this sex difference is reflected in FGM concentrations: Males exhibited four times higher baseline and peak concentrations than females. Glucocorticoid excretion was shown to

differ significantly between sexes in a related species, the Common hamster (*Cricetus cricetus*) (Franceschini 2007). However, when these authors compared FGM concentration before, during and after the reproductive period, they only found significant differences during the reproductive phase, when male FGM levels increased significantly, whereas FGM concentrations in females remained relatively constant throughout the active season. Therefore, they associated higher male levels to competition for females and general high mating effort. Our study was performed in laboratory conditions with constant temperature ($22 \pm 2^{\circ}\text{C}$) and photoperiod (12:12-h light:dark), conditions considered as photostimulatory (Ottenweller 1985) all year around. In fact, our hamsters reproduce all year around and may present high glucocorticoid levels related to reproduction at every time.

We were unable to detect in the feces the significant circadian rhythmicity of corticoid secretion reported in male hamsters (Albers 1985; Ottenweller 1987) (we did not find any description of the female circadian rhythm in the literature). However, Ottenweller et al (1987) reported that, whereas plasma corticosterone and cortisol rhythms are present on long days (14:10-h light-dark cycle), only cortisol has a significant rhythm on short days (10:14-h light-dark cycle). Even its amplitude was suppressed compared with the cortisol rhythm on long days. Our animals were housed under an intermediate 12:12-h light-dark photoperiod which may have blunted the amplitude of the diurnal glucocorticoid rhythm. In addition, because fecal hormone metabolite levels reflect the cumulative secretion and excretion of hormones over several hours, unlike blood samples, fecal samples are less affected by the pulsatility of hormone secretion, and fluctuations in the blood plasma are smoothed in the feces (Palme 2005). Similar, a rhythm in FGM levels was absent in guinea-pigs³, but in contrast very expressed in rats and mice (Lepschy et al 2007; Touma et al 2004).

Numerous researchers investigated the adrenal response of the Syrian hamster to a wide range of stressors and to an ACTH challenge test, and various concentrations of cortisol and corticosterone in this species have been published (eg. Gebhardt-Heinrich 2007; Lehoux et al 1992; Lucas et al 1999; Ottenweller 1985). However, as occurred with most rodent models, male hamsters are usually preferred for non reproductive research purposes, because they are less aggressive than females and there is less hormonal interference due to cyclic fluctuations of hormone levels. Therefore, most studies including glucocorticoid measurement are performed with male hamsters and we could not find any record of females' endocrine reaction to an ACTH challenge. Identical treatment applied to both male and female hamsters in our study allows for the first time a reliable assessment of sex differences in adrenal responsiveness and glucocorticoid excretion in the Syrian hamster. A first difference occurred in control animals: whereas the injection procedure caused a transient but significant peak (153 % above level at the same time of the precedent day) in females 32 hours after the injection, no significant response could be measured in the male group. Again, it is possible that the signal of a short rise in circulating glucocorticoid had been dampened in males' feces. It seems, however, that the whole process of capture, immobilization and injection represented only a short mild stress in laboratory animals used to routine handling. This was underlined by results in mice and rats, or guinea pigs (Touma et al 2004; Lepschy et al 2007; Bauer et al 2008), where the injection procedure itself did not cause a significant increase in FGM. In contrast, an ACTH administration induced long lasting increases in our hamsters and peak values were in both sexes about 500% above the baseline. These increases in FGM were lower than those measured for plasma cortisol concentrations by Lehoux et al (1992) after injecting ACTH in male hamsters (approximately 700% above basal level 5 h after the injection) or the 10 fold increase

observed by Gebhardt-Heinrich et al (2007). However, the latter injected 12 male hamsters and decapitated them one by one in ascending time intervals for glucocorticoid measurement, so the real significance of the resulting data may be problematic. Again in males, Ottenweller et al (1985) measured both cortisol and corticosterone blood concentrations following ACTH administration. They reported a significant increase (about 350% above basal levels) in total glucocorticoid concentrations. Interestingly, these authors observed that, although corticosterone and cortisol concentrations increased significantly, the rise in cortisol was greater. Altogether, the comparison of our results with Ottenweller's data suggests that the 5-fold increase in FGM concentrations we detected following ACTH stimulation reflects total variation in plasma glucocorticoids and that our EIA actually detected both cortisol and corticosterone metabolites both in males' and females' feces. This is also in accordance with the characteristics of the antibody used in the 11-oxoetiocholanolone EIA. It measures glucocorticoid metabolites with a 5β - 3α -ol-11-one structure (Möstl et al 2002), which can be derived from both, cortisol or corticosterone. This is an additional advantage of our analytic technique for assessing adrenocortical function in this species, since the measurement of both glucocorticoids is recommended (Ottenweller 1987).

The route of steroid excretion varies among species, as well as among steroids within the same species (Palme et al 1996; Palme et al 2005). We did not find a study about glucocorticoid metabolism and excretion in the Syrian hamster, but Chelini et al (2005) suggested delay times between 5 and 25 hours for the excretion of gonadal steroids. In the present study, the time lag between ACTH injections and the appearance of peak FGM levels was 20 hours (males) or 28 hours (females). These delay times are longer than those found in mice and rats (4 - 12 hours) (Touma et al 2003; Lepschy et al 2007), but comparable to those reported in chinchillas (30 hours) (Ponzio et al 2004). In the

guinea-pig, Bauer et al (2008) observed FGM concentrations reaching peak values between 14 and 20 h after ACTH administration, but, in contrast with these authors, we observed, again a clear difference between sexes in this timing. In males the increase in FGM concentrations peaked (20 h vs 28 h) earlier, but lasted longer (until the end of the experiment) than in females. If these time differences reflect different adrenal responses to ACTH or differences in glucocorticoid metabolism and excretion or more likely both, we are not able to decide, because more accurate investigations such as a radiometabolism study with a characterization of the excreted glucocorticoid metabolites in both sexes of this species is still lacking.

In conclusion, our results show for the first time in the Syrian hamster that adrenocortical activity can be monitored in fecal samples in a non-invasive way. Actually, the satisfactory results generated by our EIA, measuring FGM with a 5β - 3α -ol-11-one structure demonstrated clearly that changes in glucocorticoid concentrations in the blood are well reflected in changes in FGM in both sexes. The difference we observed between male and female FGM levels are in accordance with the sex difference reported by others regarding the hamster adrenal physiology. Therefore, our study provides a humane, practical and noninvasive alternative to blood removal, and therefore a powerful tool for stress-related studies in a species where venipuncture has traditionally been regarded as problematic. This is especially important, as hamsters are used as an animal model for medical research.

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Authors declare no competing interests that might be perceived to influence the results and discussion reported in this manuscript.

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APÊNDICE B

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Validação de um radioimunoensaio para a quantificação de metabólitos fecais de testosterona no hamster Sírio (*Mesocricetus auratus*)²

Marie-Odile M. Chelini^{3*}, Cláudio A. De Oliveira⁴ e Emma Otta²

ABSTRACT.- Chelini M.O.M., Oliveira, C.A. & Otta, E. 2009. [Validation of a radioimmunoassay for the quantification of fecal testosterone metabolites in the Syrian hamster (*Mesocricetus auratus*).] Validação de um radioimunoensaio para a quantificação de metabólitos fecais de testosterona no hamster Sírio (*Mesocricetus auratus*). *Pesquisa Veterinária Brasileira* 00(0):00-00. Departamento de Psicologia Experimental, Instituto de Psicologia, Universidade de São Paulo, Av. Prof. Mello Moraes, 1721 - CEP 05508-030, São Paulo, Brasil.* Autor para correspondência: marodile@usp.br

Besides being models for a variety of human diseases, hamsters have been object of numerous behavioral studies about stress and agonistic behavior. Such studies usually involve hormone change monitoring. The aim of the present study was to confirm the suitability of a commercial kit for human serum testosterone analysis to quantify testosterone metabolites (MFT) in the feces of male and female Syrian hamsters. Ten males were submitted to a GnRH challenge test to stimulate testicular activity and increase circulating testosterone levels. Five females received a testosterone injection and five other females were given a saline injection. Fecal samples collected before and after the procedures, and fecal samples collected from 20 pregnant females through gestation were analyzed for MFT with a commercial radioimmunoassay. The detection of a MFT peak 12 h after GnRH administration followed by a decrease under baseline showed that, in males, changes in the levels of MFT are related to respective changes of testosterone concentrations in the blood. Again in males a circadian cycle of MFT similar to the circadian rhythm described in literature for blood concentrations was detected. The administration of exogenous testosterone caused in females a dramatic peak of MFT. However, the concentrations measured through gestation did not

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reflect the pattern of endogenous testosterone blood levels as described in the literature. Therefore, the use of a radioimmunoassay for human blood testosterone was validated in male Syrian hamster, but a more specific assay would be necessary for female hamsters.

INDEX TERMS: Hamster, testosterone, fecal metabolites, non-invasive monitoring, endocrinology.

RESUMO.- Modelo para o estudo de diversas doenças humanas, o hamster tem sido objeto de numerosos estudos comportamentais e envolvendo estresse e/ou comportamento agonístico que supõem, muitas vezes, o monitoramento das flutuações hormonais nos indivíduos envolvidos. O objetivo do presente trabalho foi confirmar a adequação de um conjunto comercial para dosagem de testosterona em sangue humano para a quantificação de metabólitos de testosterona (MFT) nas fezes de hamsters Sírios machos e fêmeas. Dez machos foram submetidos a um desafio com um agonista de GnRH para estimular a atividade testicular, elevando os níveis circulantes de testosterona. Cinco fêmeas receberam uma injeção de testosterona enquanto cinco outras recebiam uma injeção de solução salina. Amostras de fezes coletadas antes e depois dos procedimentos, assim como amostras fecais de 20 fêmeas gestantes coletadas ao longo da gestação foram analisadas com um conjunto comercial para radioimunoensaio. Um pico de MFT 12 h após a injeção seguido de uma queda abaixo do nível basal comprovou que, nos machos, as alterações nos níveis de MFT refletem as alterações da concentração de testosterona no sangue. Nestes observou-se um ciclo circadiano das concentrações de MFT com acrofase no início do período claro correspondendo ao ciclo descrito para as concentrações sanguíneas na literatura. Nas fêmeas a administração de testosterona exógena provocou uma elevação importante dos níveis de MFT, mas as concentrações medidas ao longo da gestação não refletiram o padrão dos níveis sanguíneos do hormônio endógeno. O conjunto para radioimunoensaio para testosterona em sangue humano mostrou-se adequado para o monitoramento da função testicular no hamster macho, mas um ensaio mais específico seria necessário para as fêmeas.

TERMOS DE INDEXAÇÃO: Hamster, testosterona, metabólitos fecais, monitoramento não invasivo, endocrinologia.

INTRODUÇÃO

A primeira ninhada de hamsters Sírios (*Mesocricetus auratus*) foi capturada em 1930. Desde então, estes roedores conquistaram um lugar de destaque entre as espécies utilizadas para a pesquisa médica e biológica graças a algumas características únicas que os tornam modelo para o estudo de diversas doenças humanas nas áreas de odontologia (Dale et al. 1944), cardiologia (Bajusz 1969, Missihoun et al. 2009), oncologia (Zheng et al. 2009) por exemplo. Ademais, espécie estritamente territorial, de hábitos solitários na natureza (Gattermann et al. 2001), o hamster tem sido objeto de numerosos estudos comportamentais e envolvendo estresse e/ou comportamento agonístico (ex.: Cain et al. 2004, Cordner et al. 2004, Gebhardt-Heinrich et al. 2007, Taravosh-Lahn & Delville 2004, Wommack et al. 2004, Zhang et al. 2008). A investigação dos mecanismos fisiológicos subjacentes ao comportamento supõe muitas vezes o monitoramento das flutuações hormonais nos indivíduos envolvidos. Em se tratando de hormônios esteróides, métodos não invasivos como a quantificação de metabólitos fecais são cada vez mais usados em lugar das tradicionais dosagens sanguíneas, não somente para espécies selvagens de difícil manejo como também em animais de porte muito reduzido como os roedores de laboratório (ex: Chelini et al. 2005, Chelini et al. 2006, Lepschy et al. 2007, Touma et al. 2009). Não exigindo nem anestesia, nem manipulação dos animais, estas técnicas não causam, *per se*, nenhum estresse adicional, podendo ser usadas em animais nos quais uma coleta de sangue poderia causar sérios danos, como fêmeas gestantes ou filhotes. Permitem a realização de estudos longitudinais envolvendo diversos hormônios que o volume de sangue exigido não permitiria levar a cabo pelos métodos convencionais de dosagem sanguínea e nos quais cada animal ou grupo é seu próprio controle. Obtém-se desta forma resultados mais acurados com um número menor de animais em respeito dos princípios humanitários que regem a experimentação animal. No entanto, como o metabolismo e a excreção dos esteróides não somente são mecanismos espécie-específicos, como ainda diferem às vezes entre os sexos dentro de uma mesma espécie (Palme et al. 1996, Palme et al. 2005, Touma et al. 2003), tais técnicas devem ser validadas rigorosamente para cada espécie e cada sexo. O intuito desta validação é demonstrar que os ensaios utilizados têm a capacidade de detectar, nos níveis de metabólitos fecais dos esteróides, alterações que refletem as variações correspondentes, espontâneas ou provocadas, nas concentrações sanguíneas desses esteróides (Palme 2005).

Estudos anteriores já mostraram que é possível monitorar a função ovariana da fêmea de hamster através da quantificação dos metabólitos fecais de estradiol e de progesterona (Chelini et al. 2005), assim como a resposta adrenocortical ao estresse tanto de machos como de fêmeas analisando nas fezes os metabólitos de glicocorticóides (Chelini et al. 2008). Entretanto, não foi possível encontrar na literatura nenhuma validação, para o hamster, da dosagem de metabólitos fecais de testosterona. O objetivo do presente trabalho foi: 1) verificar que as variações diurnas das concentrações séricas de testosterona de hamsters machos se refletem nos níveis de metabólitos fecais de testosterona (MFT) 2) avaliar o efeito da administração de GnRH sobre as concentrações de MFT hamsters machos, 3) verificar o efeito da administração de testosterona exógena sobre as concentrações de MFT de hamsters fêmeas, 4) monitorar as concentrações de MFT de hamsters fêmeas ao longo da gestação, 5) confirmar a adequação de um conjunto comercial para dosagem de testosterona em sangue humano para a quantificação de metabólitos de testosterona nas fezes de hamsters Sírios machos e fêmeas.

MATERIAL E MÉTODOS

Um total de 20 hamsters (10 machos e 10 fêmeas) adultos (100 dias de idade) foi adquirido do Biotério do Departamento de Patologia da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (FMVZ – USP). As salas deste biotério, onde os animais ficaram alojados até o fim do experimento, são submetidas a condições ambientais de 20 trocas de ar por hora, temperatura de $22 \pm 2^{\circ}\text{C}$ e fotoperíodo de 12h de claro por 12h de escuridão com luzes acesas às 03h00. Ração para roedores de laboratório (Nuvilab CR1, Nuvital, Curitiba) e água filtrada foram fornecidas *ad libitum*. Os machos foram alojados individualmente e as fêmeas em dois grupos de cinco. Para facilitar a coleta de amostras fecais, a cama de maravalha foi substituída por papel absorvente substituído a cada coleta. As coletas noturnas foram realizadas sob luz vermelha fraca. Fezes contaminadas por urina não foram utilizadas. O delineamento experimental obedeceu aos princípios éticos para o uso de animais de laboratório adotados pela Sociedade Brasileira de Ciência em Animais de Laboratório e foi aprovado pela Comissão de Bioética da FMVZ – USP (Certificado nº 796/2005).

Os machos foram submetidos a um desafio com um agonista de GnRH para estimular a atividade testicular, elevando os níveis circulantes de testosterona. Cada

animal recebeu uma injeção intramuscular de 0,1ml (equivalente a 2,5 µg) de acetato de gonadorelina (Gestran Plus®, Tecnopec Cons. Com. Repres. Ltda, São Paulo, Brasil). Amostras fecais individuais foram coletadas de 4 em 4 horas 24 h antes da injeção e 48 h depois. Nas fêmeas, uma injeção do mesmo produto teria estimulado a síntese de estradiol a partir da testosterona e não podíamos ter certeza de que, nem os níveis circulantes desta, nem as concentrações dos seus metabólitos fecais iriam elevar-se. Por esta razão, optamos por administrar às fêmeas o próprio hormônio, de modo a garantir a elevação de sua concentração no sangue e provavelmente nas fezes. Assim, cinco fêmeas receberam uma injeção intramuscular de 180 µL (equivalente a 45 mg) de testosterona (Durateston®, Organon, São Paulo, Brasil). Cinco fêmeas formando o grupo controle receberam uma injeção intramuscular de 0,5 mL de solução salina isotônica estéril. A totalidade das fezes produzidas por cada grupo de fêmeas foi coletada diariamente durante quatro dias antes das injeções e quatro dias depois. Todas as injeções foram aplicadas às 12 h, ou seja, três horas antes do apagar das luzes. Paralelamente, para confirmar a relevância fisiológica das medidas para os metabólitos de testosterona endógena, foi coletada diariamente, no fim do período claro, uma amostra fecal de cada uma de 20 fêmeas gestantes, durante seis dias antes do acasalamento e deste até o décimo quinto dia da gestação.

Os esteróides fecais foram extraídos por diluição em metanol segundo o protocolo recomendado por Palme (2005). As amostras foram pesadas e homogeneizadas com metanol 80% num multivortex, durante 20 minutos. A quantidade de metanol foi de 5 mL para as amostras pesando de 0,1 a 0,5 g, e de 1mL para as amostras pesando menos de 0,1g. A seguir os tubos foram centrifugados durante 15 minutos a 500 x g. O sobrenadante foi armazenado a - 20° C até a realização dos ensaios de dosagem.

As dosagens de MFT foram realizadas no Laboratório de Dosagens Hormonais do Departamento de Reprodução Animal da FMVZ-USP com conjuntos comerciais para radioimunoensaio (RIE) em fase sólida com tubos revestidos de anticorpo (Coat-A-Count testosterone®, Siemens, Los Angeles, CA, EUA). Os parâmetros de controle de qualidade dos ensaios são a sensibilidade (menor concentração detectada) e os coeficientes de variação inter e intra-ensaio. Os coeficientes de variação foram todos inferiores a 10%. O limite mínimo médio de detecção foi 1,80 ng/dL. O anticorpo usado é altamente específico. As respectivas porcentagens de reação cruzada com outros esteróides são fornecidas pelo fabricante em

[http://www.medicalsystems.it/MetodicheSiemens/RIA/pitktt-5_siemens.pdf]. Todas as concentrações foram convertidas para ng/g de fezes (ng/g).

Após confirmação da normalidade da distribuição dos dados pelo teste de Kolmogorov-Smirnov e da homogeneidade das variâncias pelo teste de Levene, as concentrações médias dos diversos grupos nos diversos momentos foram comparadas entre si pelo teste *t* para amostras independentes (com correção de Welch no caso de heterogeneidade de variâncias), ou pelo teste *t* para amostras pareadas no caso de médias de um mesmo grupo. O nível de significância foi fixado em 0,05 para todas as comparações. Os resultados são fornecidos na forma de média e desvio padrão. Foram consideradas como valores de pico as concentrações superiores ao valor basal acrescentado de dois desvios padrões. Os resultados foram analisados com ajuda do software SPSS 13.0 para Windows.

RESULTADOS

Entre os machos as concentrações de MFT variaram numa faixa de 4,2 até 206,0 ng/g antes do desafio com GnRH, chegando a 445,0 ng/g num animal em resposta a este. O valor basal, calculado como a média das concentrações medidas nas fezes das 24 horas que precederam a injeção, foi de $65,0 \pm 48,6$ ng/g com a média das concentrações medidas 8 horas antes da injeção (ou seja, no início do período claro, $99,6 \pm 80,3$ ng/g) mais altas do que aquelas medidas 20 h antes dela (no início do período escuro, $48,1 \pm 26,4$ ng/g; teste *t* para amostras pareadas $t = 2,700$, 8gl, $p = 0,027$). Um pico de $175,8 \pm 129,2$ ng/g foi registrado 12 horas após a administração de GnRH, seguido de um decréscimo das concentrações até um valor inferior ao nível basal 8 h mais tarde ($25,3 \pm 23,5$ ng/g; teste *t* para amostras independentes com correção de Welch $t = 2,326$, 12df, $p = 0,0384$). No fim do experimento, 48 h após a injeção, as concentrações ($63,7 \pm 42,4$ ng/g) tinham voltado ao nível basal (Fig. 1).

Figura 1 – Concentrações de metabólitos fecais de testosterona (MFT) em 10 hamsters machos 24 h antes e 48 h depois de uma injeção de GnRH.

A concentração basal nas fêmeas, calculada como a média das concentrações medidas no grupo controle, foi de $15,5 \pm 7,4$ ng/g, valor aproximadamente quatro vezes inferior à concentração basal dos machos (teste *t* para amostras independentes com correção de Welch, $t = 7,844$, 75 gl, $p < 0,0001$). No grupo experimental, a concentração basal foi determinada como média das concentrações medidas nos 5 dias que antecederam a administração de testosterona. Seu valor foi similar àquele do grupo controle ($18,1 \pm 10,6$ ng/g; teste *t* para amostras independentes $t = 0,5384$, 12gl, $p = 0,60$). Um aumento abrupto da concentração de MFT foi registrado no dia seguinte à administração do hormônio ($1811,4$ ng/g) chegando no segundo dia ao valor máximo de $1957,7$ ng/g. A concentração manteve-se muito alta até o final das coletas quatro dias após a injeção. A injeção de solução salina não provocou nenhuma alteração nas concentrações de MFT do grupo controle (concentração média antes da injeção: $17,0 \pm 6,0$ ng/g, depois: $13,5 \pm 9,5$ ng/g; teste *t* para amostras independentes $t = 0,6759$, 7 gl, $p = 0,52$; Fig. 2).

Figura 2 – Variação da concentração de metabólitos fecais em dois grupos de hamsters fêmeas 4 dias antes e 4 dias depois de injeções de testosterona ou de solução salina.

Entre as fêmeas gestantes, as concentrações de MFT variaram entre 8,3 e 33,2 ng/g nos 6 dias que antecederam o acasalamento. A média das concentrações medidas nesses dias foi similar à concentração basal do grupo controle ($18,8 \pm 5,9$ ng/g; teste *t* para amostras independentes, $t = 1,594$, 133 gl, $p = 0,1133$). No entanto, a partir do segundo dia após o acasalamento, foi observado um aumento rápido, ainda que progressivo, destas concentrações. O teste *t* para amostras pareadas confirmou a significância da diferença entre a concentração basal e as concentrações médias detectadas a partir do segundo dia de gestação até a véspera do parto. A concentração média máxima ($82,8 \pm 90,1$ ng/g) foi registrada no oitavo dia da gestação, porém a partir do quinto dia de gestação e até a véspera do parto, a concentração manteve-se em níveis similares ou até superiores ao nível basal dos machos (Fig. 3).

Figura 3 – Concentrações de metabólitos fecais de testosterona (MFT) em 20 hamsters fêmeas prenhes antes e depois do acasalamento.

DISCUSSÃO

O desafio com GnRH demonstrou claramente que a atividade gonadal, no hamster macho, é refletida nas concentrações fecais de metabólitos de testosterona. Da estimulação da síntese de testosterona resultou nas fezes um pico de MFT equivalente a 300% do nível basal. Seguiu-se uma queda de concentração abaixo do nível basal, sinal da inibição da síntese de testosterona pelo efeito de *feedback* negativo. As flutuações importantes das concentrações observadas nas fezes foram também relatadas no sangue, onde são comparáveis àquelas observadas no rato (Siegel 1985). O nível basal de MFT medido no presente estudo foi inferior ao nível basal medido nas fezes de cobaias (variação de 234,5 a 481,2 ng/g; Bauer et al. 2008) ou de camundongos (173 ± 39 ng/g; Billitti et al. 1998). Ademais, no camundongo, uma injeção de hCG elevou em quase 800% a concentração de MFT. Metodologias diferentes de mensuração talvez possam explicar parte dessas diferenças. O mais provável, no entanto é que elas estejam relacionadas com a menor capacidade individual de síntese de testosterona das células de Leydig do hamster quando comparado com outras espécies (Siegel 1985). Em conjunto, esses resultados confirmam a adequação do ensaio para o monitoramento da atividade do eixo hipotálamo-hipófise-gônadas (HPG) do hamster macho. Nossos

resultados fornecem ainda informações novas e importantes sobre o prazo necessário para a metabolização e excreção da testosterona no macho: o sinal do pico de testosterona no sangue apareceu nas fezes produzidas entre oito e 12 horas mais tarde enquanto o pico negativo subsequente era detectado nas fezes secretadas entre 16 e 20 horas após a administração de GnRH. Podemos estimar, portanto como de 8 a 12 horas a duração da metabolização e da excreção da testosterona no hamster macho. As oscilações das concentrações médias medidas nas 24 horas que antecederam a administração de hormônio confirmam esse resultado. De fato, foram medidas, no início do período claro concentrações mais altas do que no início do período escuro, o que sugere um ciclo circadiano de variação da concentração sanguínea de testosterona com acrofase 12 horas antes, ou seja, no início da noite. Este resultado está de pleno acordo com o ritmo circadiano da produção de testosterona relatado na literatura para o hamster macho (Vaughan et al. 1994). Na mesma espécie, prazos indo de 5 a 25 horas foram relatados para metabolização e excreção de esteróides ovarianos (Chelini et al. 2005) e de 20 horas para o aparecimento nas fezes dos machos do sinal da elevação dos níveis de glicocorticóides em resposta a um desafio por ACTH (Chelini et al. 2008).

Os resultados da administração de testosterona nas fêmeas, com um pico de MFT nas fezes excretadas nas 24 horas que seguiram a injeção sugerem que é também possível monitorar as alterações das concentrações sanguíneas deste hormônio usando radioimunoensaio. No entanto, a elevação acima do nível basal dos machos observada nas fêmeas gestantes está em contradição com dados da literatura segundo os quais a concentração sanguínea de testosterona se mantém baixa ao longo da gestação (Soares & Talamantes 1982). Baseada na dose administrada diariamente a hamsters machos por Huppenbauer et al. (2005), a quantidade de hormônio injetada nas fêmeas parece, porém ter elevado a concentração sanguínea muito acima do nível fisiológico. É possível, então que a capacidade das vias normais de metabolização da testosterona nas fêmeas tenha sido ultrapassada, o que pode ter resultado na excreção de metabólitos diferentes ou mesmo do próprio hormônio, causando a elevação medida pelo RIE. As concentrações relativamente altas de MFT medidas nas fêmeas gestantes comparadas com os baixos níveis séricos descritos na literatura sugerem a ligação ao anticorpo de outras moléculas, talvez andrógenos de origem adrenal, além de metabólitos de testosterona. Situações semelhantes foram descritas na cobaia, com ausência de correlação entre concentrações séricas de testosterona e concentrações de MFT medidas por enzima-imunoensaio (Bauer

et al. 2008), e em diversas espécies de primatas (Möhle et al. 2002). Uma análise bioquímica precisa, por HPLC por exemplo, seria necessária para determinar a natureza dos principais metabólitos de testosterona presentes nas fezes da fêmea de hamster e permitir o desenvolvimento de um ensaio adequado.

Em conclusão, o presente estudo demonstra pela primeira vez a adequação de um conjunto diagnóstico comercial para a quantificação dos metabólitos fecais de testosterona e a investigação da atividade do eixo HPG no hamster Sírio macho. Trata-se de uma ferramenta não invasiva de grande utilidade para futuros estudos envolvendo tanto a função reprodutiva quanto o comportamento social deste animal. Esta alternativa ao uso de amostras de sangue descortina a possibilidade de estudos de longo prazo com um número mínimo de animais, garantindo o seu bem-estar. O mesmo ensaio, entretanto, parece ter medido, nas fêmeas, metabólitos de outros esteróides, resultado que confirma, uma vez mais, a necessidade de validar cuidadosamente as técnicas de dosagem de esteróides fecais não somente para cada espécie como para cada sexo de uma mesma espécie.

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Marie-Odile Chelini

4 jan

para jurgen, pvb, Emma, Claudio

Prezado Dr. Döbereiner,

Remetemos em anexo o manuscrito intitulado "Validação de um radioimunoensaio para a quantificação de metabólitos fecais de testosterona no hamster Sírio (*Mesocricetus auratus*)" de autoria de MOM Chelini, CA de Oliveira e E Otta, que estamos submetendo para publicação na Revista Pesquisa Veterinária Brasileira. O estudo demonstra pela primeira vez a adequação de um conjunto diagnóstico comercial para a quantificação dos metabólitos fecais de testosterona e a investigação da atividade do eixo HPG no hamster Sírio macho. Trata-se de uma ferramenta não invasiva de grande utilidade para futuros estudos envolvendo tanto a função reprodutiva quanto o comportamento social deste animal. Esta alternativa ao uso de amostras de sangue descortina a possibilidade de estudos de longo prazo com um número mínimo de animais, garantindo o seu bem-estar. O manuscrito não foi submetido previamente para publicação sob nenhuma forma e foi aprovado por todos os autores. Agradecemos sua atenção e sua consideração.

--

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jurgen dobereiner

5 jan

para Carlos, marodile

Prezado Professor Ambrósio,

Em anexo, envio-lhe o **Trabalho 1822 MF**, adaptado às normas da PVB, solicitando o *peer review*.

Um abraço,

Jürgen Döbereiner

APÊNDICE C

APÊNDICE C**Social stress and reproductive success in the female Syrian hamster: endocrine and behavioral correlates**

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Abstract

In many mammal species, reproduction is not equally shared among the members of a social unit. Reproductive skew seems unlikely in females of solitary species; however, social organization is affected by environmental factors. Solitary in the wild, captive Syrian hamsters (*Mesocricetus auratus*) are generally group housed. We investigated if social stress produces some degree of reproductive skew in this solitary species, and if female reproductive success varies as a function of social rank. In order to assess the physiological relationship between social stress and fertility, we monitored reproductive hormones and glucocorticoids of solitary and pair-housed females during pregnancy by utilizing recently established non-invasive methods for measuring the respective hormone metabolites in the feces. The patterns of fecal progesterone, estrogen, and glucocorticoid metabolites were similar to blood profiles reported in the literature for pregnant hamsters. Dominant females had higher breeding success than subordinated females, but the percentage of reproductive failure was also very high among the singly housed females of our control group. The number of pups per litter, the average sex-ratio in each group, and the mean weight of pups did not differ among groups. Glucocorticoid concentrations were not affected by housing and social-rank and the few differences between the endocrine profiles of singly and pair-housed females do not provide adequate ground for such a difference in breeding success. It is likely that social isolation impaired reproduction as subordination did. Our findings support the idea that social interactions may be a function of ecological conditions.

Keywords: Hamster, social stress, reproductive success, sociobiology, glucocorticoids, estrogen, progesterone, fecal metabolites, non-invasive monitoring, endocrinology.

Introduction

In many mammal species, reproduction is not equally shared among the members of a social unit. This phenomenon, known as reproductive skew, is widely spread among social, cooperative breeding animals. Typically, a dominant individual monopolizes most (e.g. common marmosets (Sousa et al, 2005)), if not all (e.g. naked mole-rats, *Heterocephalus glaber* (Clarke & Faulkes 1998)) of the group's reproductive output.

High reproductive skews are relatively common among females of cooperative breeding species, whereas low skews, if any, occur in females that do not breed cooperatively (Creel, 2001). A fortiori, reproductive skew seems unlikely in females of solitary species. However, social organization is affected by environmental factors such as density or resource distribution. Actually intraspecific variation in social systems related to environmental variability has been observed in a number of species. For example, studies conducted in Southeastern Brazil found typical traits of polygyny in a population of bamboo rats (*Kannabateomys amblyonyx*), while features associated with monogamy were found in a population living in another region (Silva et al, 2008). Similarly, while *Rhabdomys pumilio*, the African striped mouse, lives in group in a semi-desert region, it is solitary in the moist grasslands of South Africa (Schrader & Pillay, 2005). Moreover, Sachser (1998) showed that in guinea-pigs, the way in which individuals interact is also affected by their previous social experiences.

If ecological factors contribute to modulate sociability, we might expect that many of the behavioral and physiological mechanisms responsible for reproductive suppression are present as latent traits, even in species usually considered as solitary. In fact, studying group housed wolverines (*Gulo gulo*), a solitary species in the wild, Dalerum et al (2005) observed levels of social interaction similar to what is found in many obligate group-living species. Although no single females monopolized reproduction, these authors reported reproductive failures probably related to social rank.

A variety of models for the social suppression of reproduction has been described and used to study the evolution of social group formation (Kokko, 2002). The control of reproduction can be exerted either by dominant individuals in a dictatorial way, or by the subordinates restraining their own reproduction in order to avoid eviction from the group. It may also be the object of a constant conflict among group members (Saltzman et al, 2009). In each case, both behavioral and physiological mechanisms may account for the suppression of reproduction. Conventionally, the lower reproductive success of subordinate females has been considered as an effect of subordination stress and related high levels of glucocorticoids. However, in many species, dominant individuals show higher glucocorticoid levels than subordinates (Creel, 2001). In these species, sexual behavior and reproductive hormone levels seem modulated by still unclear non-glucocorticoid-mediated mechanisms.

Solitary in the wild (Gattermann et al, 2001), captive Syrian hamsters (*Mesocricetus auratus*) are generally group housed. In such a condition, without any possibility to escape, they have no choice but engage in social interaction. However, artificial grouping of adult hamsters has been shown to lead to symptoms of stress (Gattermann & Weinandy, 1996-97). For this reason, hamsters have been considered as an ideal model for studying the effects of social stress on reproduction (Pratt & Lisk, 1989). In fact, several authors reported the establishment of a stable social hierarchy in group housed hamsters, as well as some degree of reproduction suppression (Huck et al, 1988; Pratt & Lisk, 1989, 1991). As a result of short interaction periods during gestation, Huck et al (1988) observed a reduction of the fecundity of subordinate female hamsters. In a similar setting, a reduction not only in litter size but also in the percentage of male pups was reported by Pratt and Lisk (1989).

On the other hand, more recent studies suggested sex differences in the reaction of Syrian hamsters to social stress. Whereas in males social defeat leads to profound and persistent behavioral changes, characterized by a total absence of territorial aggression and by the frequent display of submissive and defensive behaviors, it does not cause any long-lasting

effect on the agonistic behavior of females (Huhman et al, 2003; Taravosh-Lahn & Delville, 2004). Specially, the group-housing-induced dominance hierarchy, which is stable in males, is unstable among females (Fritzsche et al, 2000).

Potential endocrine influences on aggressiveness and submissive behavior have been examined with ambiguous results. Pratt and Lisk (1991) reported a significant reduction in circulating progesterone concentrations in female hamsters exposed to social subordination early in pregnancy. Although they did not provide any physiological measure of the hypothalamic-pituitary-adrenal (HPA) axis activity, they suggested that the stress-related activation of HPA is responsible for this decrease. In contrast, Fritzsche et al (2000) found higher levels of progesterone in both dominant and subordinate group-housed cycling females when compared with individually housed ones. Females treated with an estradiol or testosterone implant displayed less submissive behavior than females receiving progesterone or no hormone (Faruzzi et al, 2005). The exact role of adrenal glucocorticoids in the modulation of agonistic behavior and their potential effects on the reproductive success of female hamsters is still unclear.

The aim of this study was to examine the effects of social grouping before and at the beginning of gestation on the female Syrian hamster. We investigated if social stress produces some degree of reproductive skew in this solitary species, and if female reproductive success varies as a function of social rank. In order to assess the physiological relationship between social stress and fertility, we monitored reproductive hormones and glucocorticoids of solitary and pair-housed females during pregnancy by utilizing recently established non-invasive methods (Chelini et al., 2005; Chelini et al., submitted) for measuring the respective hormone metabolites in the feces.

We hypothesized that: 1) the fertility of the dominant females would be similar to that of the isolated ones; 2) some degree of reproductive skew would occur in pair-housed females; 3) there would be more reproductive failure among subordinated females than among

dominant or isolated ones; 4) subordinate females would deliver smaller litters in comparison to dominant or isolated females; 5) the sex ratio of the subordinate female litters, but not of the dominant ones, would be female biased; 6) the endocrine profile would be different between groups.

Material and Methods

Animals and housing conditions

A total of 34 heterogenic, adult (12 weeks of age) female Syrian hamsters were obtained from the Laboratory Animal Facility of the Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil, whose Ethics Committee approved the experimental design. The animals were housed in standard propylene cages in the same animal facility under conventional conditions (12:12-h light:dark, lights on at 03:00 h; room temperature: 22 ± 2 °C; 20 changes of air per hour; air pouch filters) according to guidelines from the Institute for Laboratory Animal Research (ILAR, 1996). Specific pelleted food (Nuvilab CR1, Nuvital, Curitiba, Brazil) and filtered bottled tap water were supplied *ad libitum*. In order to facilitate feces collection for endocrine analysis, absorbent paper pads were substituted for the traditional wood-shaving bedding.

Procedure

At least two consecutive 4-day cycles per female were monitored before the beginning of the trial by detection of the characteristic post-ovulatory discharge (POD) in the morning following ovulation. One animal was discarded because it was not regularly cycling. Among the other ones we selected 11 pairs of non-relative females (PH = pair housed) with similar body weight (± 5 g). As the expression of aggressive behavior is estrus-cycle dependent, females in a pair had synchronized estrus cycle (Fritzsche et al, 2000, Solomon et al, 2007). Each female was marked with a commercial hair dye in an individually recognizable pattern to facilitate

behavioral recording. These animals remained housed in pairs during 10 days. The 11 resting females, forming the control group (ISOL = isolated), remained housed singly.

To determine the social rank of pair-kept females, the behavior of each pair was observed during three 10-min sessions with four-day intervals between sessions. The procedure was as follow: each pair was separated for 20 min before the routine exchange of cage and then regrouped in a clean cage on the bottom of which seven food pellets were scattered. Aggressive (upright/side offense, chase, bite, attack) and defensive (upright/side defense, flee, full submissive posture) behavior were recorded as well as how much food pellets each animal managed to secure. Huck et al (1988) showed that dominant females successfully removed food from the subordinate females. In each encounter the female which displayed more aggressive behavior and the one that secured more food pellets was scored one point. The female who had the higher score in at least two sessions was considered dominant (DOM) and the other one subordinate (SUB). All sessions were completed during the first 2 h of the dark phase of the LD cycle under dim red light.

On the first estrus (D0) following the first behavioral observation, all females were mated with proven breeders. Each female was housed with a male for 24 h. Afterwards the former female pairs were regrouped until completing 10 days. All the females were then transferred to individual cages and stayed singly housed until parturition (D16). To avoid cannibalism, common in hamsters when females are disturbed after delivery, it was only on day 4 after parturition (D20) that pups were counted and sexed (by measurement of anogenital distance), and dams and litters were weighed.

Collection of fecal samples

To habituate the females to the sampling procedure, feces collection started 10 days before pair forming. It proceeded daily until the day before parturition to avoid cannibalism. Sample collection took place between 14:00 and 15:00 h at the end of the light period. We

transferred each animal into a separate cage until it defecated (approximately 15 to 20 minutes). After defecation, it was immediately returned to its home cage and we collected fecal samples directly from the cage floor.

Steroid analyses

Fecal steroids were extracted using the methanol-based procedure described by Palme (2005). After the homogenization of each fecal sample, we shook an aliquot of 0.5 g (dry weight) or the whole sample for 20 min on a multivortex with 5 ml of 80% methanol for samples heavier than 0.25 g, 2.5 ml for samples whose weight was between 0.1 and 0.25 g and 1 ml for samples lighter than 0.1 g. The suspension was then centrifuged at 500 x g for 10 min. The supernatant was stored at -20°C until assayed.

Fecal cortisol metabolites (FCM) were quantified in an aliquot of the extract (50 µl further diluted 1:10 with assay buffer) using a group specific 11-oxoetiocholanolone enzyme immunoassay (EIA measuring glucocorticoid metabolites with a 5β-3α-ol-11-one structure) successfully validated for the Syrian hamster (Chelini et al^a, submitted). Details of this assay were described by Möstl et al (2002). All samples were assayed in duplicate. Concentrations of glucocorticoid metabolites were expressed as nanograms per gram fecal dry matter. Fecal estrogen (FEM) and progesterone metabolites (FPM) were quantified in 40-fold dilutions of the fecal extracts using commercial solid-phase radioimmunoassays with antibody-coated tubes previously validated for the female Syrian hamster (Chelini, 2005) following the manufacturer's protocol (Coat-A-Count estradiol, and progesterone, Siemens, Los Angeles, CA, EUA). Respective cross-reactivities percentages are provided by the manufacturer (http://www.medicalsystems.it/MetodicheSiemens/RIA/pitke2-8_siemens.pdf and http://www.medicalsystems.it/MetodicheSiemens/RIA/pitkpg-7_siemens.pdf, respectively). Mean sensitivity was 2,14 pg/mL for both the estradiol and progesterone assay. Intra- and interassay coefficients of variation for all assays were <12%.

Statistics

We used chi-square analysis to compare the proportions of breeding success between isolated and pair-housed females and between isolated, dominant and subordinate females. Mean numbers of pups per litter, pup weight and sex-ratio were compared between groups using ANOVA. The effects of time, pregnancy, housing, social status on concentrations of steroid metabolites were assessed using GLM for repeated measures (rmGLM). Correlations between mean hormone concentrations were calculated. Means are given with standard deviations and significance level for all tests was set at $p < 0.05$ unless otherwise noted. We performed statistical analysis with the software SPSS 13.0 for Windows.

Results

Social rank

Pair-housing led to social hierarchy in most pairs, defined in few minutes after pairs were formed in eight of 11 pairs. In six of these eight pairs, agonistic behavior was intense on the first encounter, but only sporadic during the subsequent observation sessions. In two pairs we did not observed any manifestation of aggression, but from the beginning one female secured all the seven food pellets which is a behavior displayed by dominants (Huck et al, 1988). In another pair, a female was very aggressive on the first encounter but did not display any aggressive behavior during the other encounters. In contrast, during all the observation sessions its partner took immediately possession of all the pellets of food, claiming its dominant status without fighting. In one pair, social rank was inverted on the third session with the former subordinate attacking and chasing the former dominant and securing all the food pellets. In the last pair females displayed similar amounts of agonistic and hoarding behavior, so we were not able to derive relative social rank between these individuals (IN = indeterminated).

Reproductive success

Of the 33 mated females, 21 delivered a total of 194 live pups (Table 1). The number of delivered litters was affected by social rank ($\chi^2 = 3.810$, 1df, $p = 0.05$), but not by group-housing ($\chi^2 = 0.589$, 1df, $p = 0.35$). Dominant females had higher breeding success than subordinated females. The number of pups per litter (ISOL x PH, t -test, $t = 0.654$, 19df, $p = 0.52$; ISOL x DOM x SUB, ANOVA, $F = 1.371$, 2df, $p = 0.28$), the average sex-ratio in each group (ISOL x PH, t -test, $t = 0.816$, 19df, $p = 0.42$; ISOL x DOM x SUB, ANOVA, $F = 0.710$, 2df, $p = 0.51$), and the mean weight of pups on day 4 post-partum (ISOL x PH, t -test, $t = 0.752$, 19df, $p = 0.46$; ISOL x DOM x SUB, ANOVA, $F = 0.437$, 2df, $p = 0.65$) did not differ among groups.

Table 1 – Number of delivered litters, total number of live borne pups, mean number of pups per litter, mean weight of pups per litter 4 days after parturition and at weaning and offspring sex-ratio of female Syrian hamsters kept isolated or pair-housed during 10 days before mating and in the beginning of gestation. Housing condition and social rank in the pairs did not affect these results.

	Isolated	Pair-housed	Dominant	Subordinated	Indetermined
n	11	22	10	10	2
Breeding success	6 (54.5%)	15 (68.2%)	9 (90.0%)	5 (50%)	1 (50%)
Total of pups	51	143	81	57	5
Pups per litter	8.5 ± 3.2	9.5 ± 3.3	9.0 ± 3.6	11.4 ± 1.1	5
Weight of pups on day 4 post-partum	5.3 ± 1.2	5.0 ± 0.9	5.0 ± 1.1	4.7 ± 0.6	5.1
Offspring sex-ratio (% of male pups)	39.4 ± 24.5	50.3 ± 16.8	53.3 ± 19.2	44.8 ± 11.3	20.0

Concentrations of fecal cortisol metabolites (FCM)

FCM concentrations varied between individuals and over time within a wide range (6 to 1060 ng/g feces). We observed a significant within-subject effect of time ($F = 4.103$, $p < 0.001$) and of the interaction time x pregnancy ($F = 3.309$, $p = 0.001$) on FCM concentrations. FCM were also affected by pregnancy ($F = 1079.8$, $p < 0.001$) as between-subject factor. Neither housing ($F = 1.128$, $p = 0.30$), nor social rank ($F = 0.693$, $p = 0.51$) had an effect on FCM

in both pregnant and non-pregnant females (housing: $F = 1.021$, $p = 0.34$). In pair-housed females FCM concentration was not affected by pair formation (113 ± 47 ng/g feces on the day of pair formation and 123 ± 62 ng/g feces one day after; paired samples t -test $t = 0.698$, $21df$, $p = 0.49$). Among pair-housed but not singly-housed females, FCM increased significantly as a result of mating (Table 2).

Table 2 – FCM concentrations (mean $\pm$ SD; ng/g feces) before and after pair formation and mating.

	Isolated	Pair-housed	Dominant	Subordinated	Indetermined
n	11	22	10	10	2
Baseline	135 ± 34^a	151 ± 44^a	139 ± 32^a	149 ± 33^a	217 ± 104
Day of pair formation	75 ± 31^b	113 ± 47^b	104 ± 27^b	118 ± 63	132 ± 50
Day after pair formation	83 ± 38^b	123 ± 62^b	105 ± 38	137 ± 87	146 ± 1.4
Day of mating (D0)	119 ± 62	$108 \pm 52^{b,c}$	109 ± 69	112 ± 38^b	87 ± 16
Day after mating (D1)	116 ± 77	215 ± 189^d	193 ± 88	263 ± 261	148 ± 37

(a, b); (c, d) Different letters in the same column indicated significant difference (t -test for paired samples, $p < 0.05$).

On Day 3 after mating FCM concentrations were higher in non-pregnant females than in pregnant ones (204 ± 86 ng/g feces versus 135 ± 46 ng/g feces; t -test for independent samples $t = 2.556$, $p = 0.02$). However, from day 8 after mating until parturition FCM increased significantly in pregnant females while they remained basal in non-pregnant animals (t -test for independent samples $p < 0.05$; Figure 1).

Figure 1 – Boxplot graphs of fecal cortisol metabolites (FCM) in pregnant (grey) and non-pregnant (white) female hamsters from day 6 (d-6) before mating (d 0) to one day before parturition (d 15). * and black bar indicate different FCM concentrations on day 3 and from day 8 to day 15.

Concentrations of fecal estrogen metabolites (FEM)

FEM concentrations varied from 4.6 ng/g feces to 238.8 ng/g feces. The rmGLM analysis showed significant effects of time ($F = 5.481$, $p < 0.001$) and of the interaction time x pregnancy as within subject factors as well as of pregnancy as a between subject factor ($F = 6.071$, $p = 0.02$). Among pregnant females but not non-pregnant ones, FEM concentrations were affected by housing but not by social ranking as between subject factors (housing: $F = 7.588$, $p = 0.014$; social rank: $F = 0.194$, $p = 0.67$). More precisely, it was only on the day before mating (proestrus) that FEM concentrations were different between groups with pair-housed

females levels higher than isolated females ones (46.1 ± 79.2 ng/g feces and 35.7 ± 88.6 ng/g feces respectively; *t*-test for independent samples $t = 3.368$, $p = 0.002$). FEM concentrations of pregnant females increased from day 13 of pregnancy, reaching significantly higher concentration on day 14 if compared to non-pregnant females (75.9 ± 26.1 ng/g feces versus 38.0 ± 16.6 ng/g feces, respectively; *t*-test for independent samples $t = 4.146$, $p < 0.001$). It decreased again on day 15, one day before parturition (Figure 2).

Figure 2 – Boxplot graphs of fecal estrogen metabolites (FEM) in pregnant (grey) and non-pregnant (white) female hamsters from day 6 (d-6) before mating (d 0) to one day before parturition (d 15). * indicate different FEM concentrations on day 14.

Concentrations of fecal progesterone metabolites (FPM)

FPM concentrations varied on a 100-fold range, from 10 ng/g feces to 1605 ng/g feces. There was a significant effect of time ($F = 6.926$, $p < 0.001$) and of the interaction time x pregnancy ($F = 2.834$, $p = 0.005$) as within-subject factors and of pregnancy as a between subject factor ($F = 28,524$, $p < 0.001$) on FPM levels. Figure 3 shows that FPM increased progressively in pregnant females after mating and were significantly higher in comparison with non pregnant females from day 7 to day 15 (t -test for independent samples, $p < 0.05$). Among pregnant females, FPM were affected by housing ($F = 9.371$, $p = 0.007$) but not by social rank ($F = 0.387$, $p = 0.55$) as between subject factors. Specially, pair-housed females showed higher FPM levels than isolated ones on day 11 of pregnancy (502 ± 115 ng/g feces and 329 ± 156 ng/g feces, respectively, t -test for independent samples, $t = 2.696$, $p = 0.015$). The same did not occur among non-pregnant females (housing: $F = 1.295$, $p = 0.29$; social rank: $F = 2.248$, $p = 0.19$). On day 3 after mating FPM concentrations were negatively correlated with FCM concentrations (Pearson coefficient $r = -0.346$, $p = 0.048$).

Figure 3 – Boxplot graphs of fecal progesterone metabolites (FPM) in pregnant (grey) and non-pregnant (white) females hamster from day 6 (d-6) before mating (d 0) to one day before parturition (d 15). FPM concentrations were higher in pregnant females from day 7 until day 15 of pregnancy (black bar).

Discussion

The changes of steroid hormone levels in the blood during the pregnancy of the Syrian hamster have been described a long time ago (Siegel, 1985; Soares & Talamantes, 1982).

However, to our knowledge, it is the first time that the patterns of estrogen, progesterone and glucocorticoid concentrations are monitored through gestation in this species by quantifying their fecal metabolites. Our results are similar to blood profiles reported in the literature.

Siegel (1985) reported an increase of blood cortisol concentration during the second half of the gestation, reaching 500% of baseline levels at the end of the period. The timing of the change was the same in our study but with a lower intensity: One day before parturition, the mean FCM concentration was approximately twice as high as the basal value. Such a difference

may result from the method used: Fecal hormone metabolites, unlike blood levels reflect the cumulative secretion and excretion of hormones over several hours, fluctuations in the blood plasma are smoothed in the feces (Touma and Palme, 2005). The changes in FPM and FEM concentrations in pregnant females were also consistent with the changes reported in the blood: FPM started rising from day 3 after mating and were higher than in non-pregnant animals from day 8 to day 15. Our sampling schedule and the time delay of excreted hormone metabolites hindered the detection of both the dramatic peak that should have occurred one day before delivery and the abrupt decrease on the day of parturition. The changes in FEM levels, rising during the second week of pregnancy and reaching a transient peak two days before delivery, also reflected the profile described in literature for blood estradiol. These results confirm one more time the validity of non-invasive methods to assess endocrine status.

Our results showed that FCM concentrations did not change as a result of pair formation despite the agonistic behavior observed in most pairs immediately after it. As previously explained, it is possible that the signal of a short rise in circulating glucocorticoids had been dampened in the feces, or that we missed it as a result of our collection schedule. However, FCM concentrations were similar in isolated, dominant and subordinated females. This result is in contrast with the finding of Jian-Xu Zhang et al (2008) of a significant increase in cortisol concentration as a result of cohabitation. It is worth noting that, although agonistic behavior was relatively intense in most pairs on the first encounter of the females, we observed only very few attacks (one attack in six of nine pairs in which social rank was clear) and almost no submissive or defensive behaviors on the subsequent encounters. This result is consistent with the solitary habit of female hamsters in the wild. Submission may have a very high cost for females: besides severe wounding, it means losing its food hoard, its litter and may be even its burrow. On the other hand, males have less to lose displaying submissive behavior than fighting with a bigger or more aggressive male. Actually in our study, in six of nine female pairs, the only dominant behavior displayed on the third encounter was securing

more food-pellets. The importance of hoarding behavior for the successful reproduction of female hamsters was demonstrated by Huck et al (1988). In conditions of food restriction, dominant females removed food from the subordinate ones, and reproduced more successfully even than singly housed females.

Overall, of the 33 females that we mated, only 21 (63.6%) gave birth. The 90% reproductive success of dominant females was similar to the percentage usually reached in our animal facility (Chelini et al^b, submitted). In contrast, only 50% of the subordinated females delivered live pups. This result is in accordance with data of previous studies (Huck et al, 1988; Pratt & Lisk, 1991). However, in contrast with these authors, we did not find any difference between pair-housed and isolated females regarding the number of pups per litter, the litter sex-ratio, or the mean weight of the pups. Curiously, the index of breeding success (54%) of isolated females was very low when compared to the percentage (more than 90%) usually reached in our animal facility. Therefore, it seems that reproductive failure was not related to housing and was an all-or-nothing issue. Although fecal sampling is absolutely non-invasive, our procedure involved some handling that might have disturbed the females. However, the high breeding success (90%) of dominant females contradicts such a supposition.

As the glucocorticoid profile was similar in our three groups, we could not infer that stress level was higher in one of them, or that a glucocorticoid-related mechanism was responsible for reproductive failure. The sole indicator of a role for glucocorticoids in reproductive failure was a higher FCM concentration on day 3 after mating in females that did not give birth, associated with a negative correlation between FCM and FPM on that day. Nepomnaschy et al (2006) reported that, in women, pregnancies characterized by increased maternal cortisol during the first 3 weeks after conception (placentation period) were more likely to result in spontaneous abortion. After implantation the embryo signaling results in increasing progesterone levels and decreased risk of miscarriage. As embryo implantation

occurs in hamsters on day 4 of pregnancy, it is likely that the high FCM on day 3 were related to the reproductive failure. If so, the question whether it was a cause or an effect cannot be answered by our data. The increased production of cortisol may indicate a stressful environment and may have signaled poor reproductive conditions in which the cost of reproductive failure would be lower than the cost of a pregnancy with diminished chances of success. The negative correlation found between FCM and FPM one day before implantation suggest that cortisol could have affected the production of luteal progesterone and the maintenance of the pregnancy. However, we cannot discard the possibility that the increase in cortisol concentration was a consequence and not the cause of pregnancy loss. In the same way, the reason for higher concentrations of estrogens in pair-housed females on the day before mating is not clear. It may be related to the low level of submissive behavior displayed by subordinate females (Faruzzi et al, 2005). In sheep, estrogens attenuate glucocorticoid responses to stress (Komesaroff et al, 1998). We did not observe such an effect. Despite their higher FEM levels on the precedent day, mating induced a significant FCM increase in pair-housed females and not in singly-housed ones. No relationship between FCM levels on D0 or D1 and reproductive success was found. Independently of the group, all females were in estrus and receptive to male on the mating day, and this encounter with an unknown male did not seem stressful for singly-housed females. It is possible that the previous cohabitation experience had sensibilized the females to social interaction and had enhanced their adrenal response to social-stress.

Pair-housing related changes in progesterone levels were also reported by several authors but in an inconsistent way. Whereas Pratt and Lisk (1991) reported a significant reduction in circulating progesterone concentrations in female hamsters exposed to social subordination early in pregnancy, Jian-Xu Zhang et al (2008) measured similar progesterone concentrations in singly and pair-housed females, and Fritzsche et al (2000) found higher levels of progesterone in both dominant and subordinate group-housed cycling females when

compared with individually housed ones. Our data support the findings of the latter with higher FPM levels in pair-housed pregnant females in the second half of pregnancy. Again, the physiological meaning of this difference is not clear. The percentage of reproductive failure was similar in subordinated (pair-housed) and isolated females. Moreover, at this time of pregnancy most of the circulating progesterone is produced by the placenta (Siegel, 1985). Therefore, the higher concentration in pair-housed females is not likely to result from a higher number of corpora lutea and does not seem associated with higher fertility.

Our behavioral data confirm that adult hamsters, as well as wolverines, another solitary species (Dalerum et al, 2006), are able to interact socially with other adults of the same sex and to establish social hierarchies when kept in groups. Social environment seems to have determined reproductive failure in subordinate females, which is another social feature of group-living species. Reproduction was not monopolized by dominant females, and the mean number of pups per litter, the litter sex-ratio, and the weight of the pups did not differ between our groups. However, only 50% of the subordinated females reproduced successfully. Huck et al (1988) showed the fundamental importance of hoarding for successful reproduction in hamsters even when food is available *ad libitum*. In our experiment, food was supplied *ad libitum* in the cage feeder, but only a few pellets were available for hoarding and dominant females secured most of them. The conventional explanation that high glucocorticoid levels related with subordination stress may constitute the proximal mechanism of reproductive failure in subordinate females does not fit our results as we did not find social-rank-related differences in FCM concentrations. Moreover, the percentage of reproductive failure was also very high among the singly housed females of our control group. This is perhaps the most intriguing point of our results. All females were normally cycling and all of them were mated with proven breeders. Thus, all of them were theoretically capable of becoming pregnant. Isolated females had the same possibility of hoarding than dominant ones and did not suffer any kind of aggression or intimidation. In fact, FCM concentrations were not affected by

housing and the few differences between the endocrine profiles of singly and pair-housed females do not provide adequate ground for such a difference in breeding success.

Hamsters in our animal facility are kept in groups of five animals of the same sex from weaning and so were our females when we received them. When adult, two or three females from the same group are mated with an unfamiliar male and remain together until few days before parturition. Unlike this normal mating procedure, our control animals were isolated from the beginning of the experiment, approximately two weeks before mating. In fact, it seems that social isolation impaired their reproduction as subordination did in pair-housed females, although no clear physiological mechanism had been demonstrated. Singly housing elevated the anxiety levels of laboratory rodents and primates and affect their response to certain stressors (Olsson & Westlund, 2007). In young rats social isolation induces behavioural, morphological and neurochemical abnormalities (Kone & Porkess, 2008). Because, Syrian hamsters are exclusively solitary in the wild, as confirmed by the investigation of Gattermann et al (2001) in Syria, we hypothesized that cohabitation would be stressful and isolation would not. However, our results suggest that social isolation was as disturbing for animals used to group-living as cohabitation with an unknown conspecific.

In conclusion, we found evidence for some features typically associated with solitary species, but also some others found in group-living species. It is likely that captivity and group-housing until adult age wake up latent physiological and behavioral mechanisms of social interaction. As in other rodents (Schrader & Pillay, 2005), sociability seems flexible in the Syrian hamster and our findings support the idea that social interactions may be a function of ecological conditions.

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APÊNDICE D

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Social behavior in a solitary species

Chelini, MOM, Otta, E.

Abstract

Intraspecific variation in the pattern of social interactions and the resulting relationships among individuals has been observed in several species associated with environmental variability. Then, we might expect that many of the behavioral and physiological mechanisms responsible for highly developed social interactions are present as latent traits, even in species usually considered as solitary. The main objective of this study was to examine the effects of previous social experience on the response of female Syrian hamsters to social stress. We conducted two experiments to investigate if cohabitation with an unknown female before and at the beginning of gestation affected in a different way the fertility and maternal behavior of females that were singly or group-housed from weaning. Cohabitation with an unrelative conspecific induced a dominant-subordinate relationship in both experiments independently of previous housing conditions, but singly raised females were more reluctant in displaying hoarding behavior than group raised animals. When females were group-housed from weaning, the proportion of reproductive failure was, as expected, higher among subordinate females than among dominant ones, and was also high in control females. Among singly raised females, reproductive success was not related with social status. The number of pups per litter, and the litter sex-ratio were not affected by prior housing conditions, cohabitation or social rank. However, females group-housed immediately after weaning displayed higher frequencies of

maternal behaviors, and their pups were heavier at weaning. Group-housing captive adult hamsters seems to evoke some behavioral and physiological features that can be found in complex social groups and are present as latent traits in this solitary species.

Introduction

Sociality means group-living, and in general, groups form and persist because group-living confers net fitness advantages to individuals living in association with conspecifics (Alexander, 1974). It is likely, thus, that group-living induces the evolution of social behavior. Does that mean that social interaction cannot be found in solitary species? A possible definition for solitary species is a species whose individual's general activity is not synchronized, which does not imply that they do not maintain social relationships (Kappeler and van Schaik, 2002). Females of some species of mouse lemurs (*Microcebus spp*), for example, are solitary but associate opportunistically into more or less stable sleeping groups (Dammhahn and Kappeler 2009). Intraspecific variation in the pattern of social interactions and the resulting relationships among individuals has also been observed in several species associated with environmental variability. One example is the African striped mouse, (*Rhabdomys pumilio*). While this rodent is solitary in the moist grasslands of South Africa, multiple adults of both sexes share the same nest and the same territory in a more arid region (Schradin and Pillay, 2005). If ecological factors contribute to modulate sociability, we might expect that many of the behavioral and physiological mechanisms responsible for highly developed social interactions are present as latent traits, even in species usually considered as solitary. In fact, studying group housed wolverines (*Gulo gulo*), a

solitary species in the wild, Dalerum et al (2006) observed levels of social interaction similar to what is found in many obligate group-living species.

Reproductive skew, an unequal distribution of reproduction, is a common feature among social, cooperative breeding animals. Frequently, in such species only one or a few dominant individuals successfully reproduce. In mammals, the most known and radical example is the naked mole-rat, *Heterocephalus glaber*, in which only one female and one to three males breed (Clarke and Faulkes 1998). In other species, such as the common marmoset (*Callithrix jacchus*), the reproductive skew is lower as subordinate females may or may not escape reproductive suppression (Sousa et al, 2005). High or low, reproductive skew is widely spread among social, cooperative breeding animals (Creel, 2001), but a priori, it seems unlikely in females of solitary species. Nevertheless, the finding of Dalerum et al (2006) of socially induced reproductive failures in female wolverines suggests that group-housing of solitary animals may evoke some social behaviors without any genetic or phylogenetic influence.

Despite its popularity as a pet and its widely spread use in medical and biological research, data concerning the ecology of the Syrian hamster in the wild are scarce. It was only in 2001 that the social organization of the species, traditionally regarded as solitary, was confirmed by the observations in the field of Gattermann et al. (2001) who never found more than one adult per burrow. Moreover, the closest distance between occupied hamster burrows was 118 m. In animal facilities, however, hamsters are generally kept in isosexual groups from weaning until adult age (Mori, Chelini & Couto, 2009). Even in such conditions, grouping adult hamsters results in

symptoms of stress (Gattermann and Weinandy, 1996-97). Therefore, hamsters have been considered as an ideal model for the investigation of the effects of social stress (Pratt and Lisk, 1989). Exposure to short periods of social subordination was shown to induce implantation failures and fetal resorption in pregnant hamsters (Huck et al, 1988) resulting in the delivery of smaller and more female-biased litters by subordinated females (Pratt and Lisk, 1989, 1991). In fact, although adaptive value of producing sons under social stress is not known, socially stressed animals typically produce more sons than daughters (Cameron, 2004). More recent studies also reported the quick establishment of dominance hierarchy as a result of group-housing hamsters of the same sex, but with clear sex differences. The rank order seems less stable in female groups than in male ones (Fritzsche et al, 2000). In the same way, social stress induces in male, but not in female hamsters a long-lasting inhibition of agonistic behavior (Huhman et al, 2003; Taravosh-Lahn and Delville, 2004). Previous housing conditions were shown to affect the agonistic behavior of both sexes with increasing periods of social isolation associated with increased aggression (Payne, 1973; Wise, 1974). Therefore, it is possible that, as in guinea-pigs (Sachser, 1998), the way in which hamsters interact is also affected by their previous social experiences.

The main objective of this study was to examine the effects of previous housing conditions on the response of female Syrian hamsters to social stress. We conducted two experiments to investigate if cohabitation with an unknown female before and at the beginning of gestation affected in a different way the fertility and maternal behavior of females that were singly or group-housed from weaning. We hypothesized that when females are group-housed from weaning, cohabitation with an unknown female evokes the establishment of a dominance hierarchy and some degree of

reproductive skew in this solitary species. Therefore: 1) there would be more reproductive failure among subordinate females than among dominant or isolated ones ; 2) subordinate females would deliver smaller litters in comparison to dominant or isolated females; 3) the sex ratio of the subordinate female litters, but not of the dominant ones, would be female biased; 4) the frequency of mother-pups interactions would be lower in subordinated dams; 5) all these features would not be found, or only to a lesser extent, in females singly-housed from weaning until the beginning of the experiment.

Material and methods

Subjects

Subjects were adult (12 weeks of age) female Syrian hamsters (*Mesocricetus auratus*) weighing in average 140 g, that were born and raised in the Laboratory Animal Facility of the Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil, whose Ethics Committee approved the experimental design . The animals were housed in standard propylene cages in the same animal facility under conventional conditions (12:12-h light:dark, lights on at 03:00 h; room temperature: 22 ± 2 °C; 20 changes of air per hour; air pouch filters) according to guidelines from the Institute for Laboratory Animal Research (ILAR, 1996). Specific pelleted food (Nuvilab CR1, Nuvital, Curitiba, Brazil) and filtered bottled tap water were supplied ad libitum. Normal cycling was insured by detection of the characteristic post-ovulatory discharge in the morning following ovulation.

Experiment 1 (Ex1)

Among 34 females kept in isosexual groups from weaning, we selected 11 pairs of non-relative females (PH1 = pair housed experiment 1) with similar body weight ($\pm$ 5g). As the expression of aggressive behavior is estrus-cycle dependent, females in a pair had synchronized estrus cycle (Fritzsche et al, 2000, Solomon et al, 2007). Each female was marked with a commercial hair dye in an individually recognizable pattern to facilitate behavioral recording. These animals remained housed in pairs during 10 days. One animal was discarded because it was not regularly cycling. The 11 resting females, forming the control group (ISOL1 = isolated experiment 1), remained singly housed.

To determine the social rank of pair-kept females, the behavior of each pair was observed during three 10-min sessions with 4-day intervals between sessions. The procedure was as follow: each pair was separated for 20 min before the routine exchange of cage and then regrouped in a clean cage on the bottom of which seven food pellets were scattered. Aggressive (upright/side offense, chase, bite, attack) and defensive (upright/side defense, flee, full submissive posture) behavior were recorded as well as how much food pellets each animal managed to secure. Huck et al (1988) showed that dominant females successfully removed food from the subordinate females. In each encounter the female which displayed more aggressive behavior and the one that secured more food pellets was scored one point. The female who had the higher score in at least two sessions was considered dominant (DOM1) and the other one subordinate (SUB1). All sessions were completed during the first 2 h of the dark phase of the LD cycle under dim red light.

On the first estrus (D0) following the first behavioral observation, all females were mated with proven breeders. Each female was housed with a male for 24 h. Afterwards the former female pairs were regrouped until completing 10 days. All the females were then transferred to individual cages and stayed singly housed until parturition (D16). To avoid cannibalism, common in hamsters when females are disturbed after delivery, it was only on day 4 after parturition (D20) that pups were counted and sexed (by measurement of anogenital distance), and dams and litters were weighed.

A protocol adapted from Slamberová et al (2001) was used for assessing maternal behavior. Maternal and non-maternal activities of the lactating mothers were recorded daily from day 4 to day 14 post-partum (D20-D29) in 40-min experimental sessions, which were carried out between 15:00 and 16:00 h, in the beginning of the dark period, under dim red light. During each session each dam and its litter were observed 10 times at 4-min intervals and 13 types of activities displayed by the mother were recorded. During each observation “1” was given if a behavior occurred and “0” if not. The recorded activities were as follow: 1) nursing, 2) in the nest, 3) mother in contact with at least one pup, 4) mother in contact with more than half of the litter, 5) mother licking or grooming any of her pups, 6) mother manipulating nest shavings, 7) mother manipulating non-nest shavings, 8) mother eating, 9) mother manipulating food, 10) mother carrying a pup, 11) mother self-grooming, 12) mother rearing, sniffing or chewing the cage lid, 13) mother sniffing with head raised.

Experiment 2

Experiment 2 (Ex2) was carried out with 76 females singly raised from weaning. Fifty-two females formed 26 pairs (pair-housed, PH2) and 24 females remained isolated (ISOL2). Otherwise, the experimental procedures were identical as for experiment 1.

Statistics

We used chi-square analysis to compare the proportions of breeding success between isolated and pair-housed females and between isolated, dominant and subordinate females of each group. Mean numbers of pups per litter, pup weight and sex-ratio were compared among groups using ANOVA or *t*-test. The effects of time, previous housing conditions, cohabitation, and social status on the frequencies of maternal activities were assessed using GLM for repeated measures (rmGLM). Behavioral data were submitted to a Principal Component Analysis (PCA) to assess what percentage of the total variance each behavior explained. Behaviors that explained less than 1% of the total variance were excluded from the analysis. Means are given with standard deviations, and significance level for all tests was set at $p < 0.05$ unless otherwise noted. We conducted statistical analysis with the software SPSS 13.0 for Windows.

Results

Social ranking

In both experiments, pair-housing induced in most pairs the establishment of a dominance hierarchy defined on the first encounter. However, in one pair in each experiment, we were not able to determine the relative social rank of the females

because they displayed similar amounts of agonistic and hoarding behavior (IND = indeterminate). In one pair (9.1%) in Ex1, and in seven pairs (26.9%) in Ex2 social rank inverted through the sessions with the former subordinate attacking and chasing the former dominant. These percentages were not significantly different ($\chi^2 = 1,450$, 1df, $p = 0,228$). In contrast, the proportion of females displaying hoarding behavior was lower in Ex2 than in Ex1 (1st encounter: 0.0% vs 45.4%, $\chi^2 = 13.665$, 1df, $p = 0.000$; 3^d encounter: 53.8% vs 90.9%, $\chi^2 = 9.805$, 1df, $p = 0.002$).

Reproductive success

Reproductive success was higher among dominant females than among subordinate ones in Ex1 (90% and 50% respectively) but there was no difference between groups regarding reproductive success in Ex2. The reproductive success of isolated females tended to be higher in Ex2 (83% in Ex2 vs 54% in Ex1). In Ex2 4 days old pups born from pair-housed females were heavier than pups born from isolated dams (5.4 ± 1.4 g vs 4.6 ± 1.4 g,). In all groups, pups were heavier at weaning in Ex2 than in Ex1 (t -test for independent samples, $p < 0,05$ in all cases). Table 1 shows that there were no significant differences among groups and between experiments in each group in mean number of pups per litter, mean sex-ratio of the litters, and number of pups that died during lactation.

Table 1 - Reproductive data of female Syrian hamsters kept isolated or pair-housed during 10 days before mating and in the beginning of gestation

Group	ISOL		PH		DOM		SUB		IND	
Experiment	Ex1 n=11	Ex2 n=24	Ex1 n=22	Ex2 n=52	Ex1 n=10	Ex2 n=25	Ex1 n=10	Ex2 n=25	Ex1 n=2	Ex2 n=2
Breeding success (%)	6 ^a (54%)	20 ^b (83%)	15 (68%)	35 (67%)	9 ^v (90%)	18 (72%)	5 ^u (50%)	16 (64%)	1 (50%)	1 (50%)
Pups per litter	8.5 ± 3.2	9.4 ± 1.9	9.5 ± 3.3	9.0 ± 4.0	9.0 ± 3.6	8.3 ± 4.6	11.4 ± 1.1	9.7 ± 3.3	5	10
Male pups for litter (%)	39.4 ± 24.5	47.0 ± 14.3	50.3 ± 16.8	46.8 ± 15.8	53.3 ± 19.2	48.4 ± 15.1	44.8 ± 11.3	46.4 ± 16.8	20.0	30.0
Pup weight on day 4 pp (g)	5.3 ± 1.2	4.6 ^x ± 1.4 ^e	4.9 ± 0.9	5.4 ^y ± 1.4 ^f	5.0 ± 1.1	5.2 ± 1.4	4.7 ± 0.6	5.6 ± 1.5	5.1	6.19
Pup weight on day 21 pp (g)	38.5 ^c ± 8.8	43.5 ^d ± 3.1	39.3 ^c ± 6.8	45.3 ^d ± 3.4	39.6 ^c ± 7.2	45.3 ^d ± 3.5	37.5 ^c ± 7.2	45.5 ^d ± 3.5	43.7	43.0

^{a,b} The reproductive success of isolated females tended to be higher in Ex2 ($\chi^2 = 3.272$, 1df, $p = 0.070$).

^{u,v} Dominant females reproduce more successfully than subordinate females in Ex1 ($\chi^2 = 3.810$, 1df, $p = 0.05$).

^{x,y} Pair-housed females delivered heavier pups than isolated females in Ex2 (t -test for independent samples, $t = 2.105$, 49df, $p = 0.04$).

^{c,d} Different superscripts in the same line indicate significant differences (t -test for independent samples, $p < 0,05$ in all cases)

Maternal behavior

The mean frequencies of maternal activities in the different groups and conditions are shown in Table 2.

Table 2 – Mean frequencies of maternal activities of females Syrian hamsters during lactation

Group	ISOL		PH		DOM		SUB		ALL	
Experiment	Ex1 n=6	Ex2 n=20	Ex1 n=15	Ex2 n=35	Ex1 n=9	Ex2 n=18	Ex1 n=5	Ex2 n=16	Ex1 n=21	Ex2 n=55
Nursing	6.63 ± 1.20	7.41 ± 0.96	6.62 ± 1.15	7.64 ± 0.92	6.42 ± 1.23	7.70 ± 0.96	6.90 ± 1.17	7.72 ± 0.73	6.63 ^a ± 1.13	7.56 ^b ± 0.93
In the nest	6.57 ± 1.48	7.40 ± 0.99	6.51 ± 1.10	7.65 ± 0.93	6.30 ± 1.24	7.68 ± 1.02	6.76 ± 0.97	7.75 ± 0.71	6.52 ^a ± 1.18	7.55 ^b ± 0.95
In contact with at least one pup	0.50 ± 0.56	0.35 ± 0.27	0.27 ± 0.26	0.30 ± 0.28	0.32 ± 0.30	0.35 ± 0.31	0.22 ± 0.16	0.24 ± 0.25	0.33 ± 0.34	0.32 ± 0.27
In contact with more than half of the litter	6.63 ± 1.48	7.39 ± 0.94	6.54 ± 1.13	7.66 ± 0.87	6.34 ± 1.25	7.73 ± 0.95	6.72 ± 1.09	7.71 ± 0.68	6.57 ^a ± 1.20	7.55 ^b ± 0.90
Licking or grooming a pup	1.60 ± 0.48	1.35 ± 0.57	1.82 ± 0.90	1.36 ± 0.51	1.77 ± 0.72	1.37 ± 0.50	1.50 ± 0.76	1.37 ± 0.54	1.76 ^a ± 0.80	1.36 ^b ± 0.53
Manipulating nest shavings	0.48 ± 0.26	0.68 ± 0.40	0.51 ± 0.28	0.80 ± 0.34	0.53 ± 0.27	0.83 ± 0.30	0.52 ± 0.31	0.76 ± 0.38	0.50 ^a ± 0.27	0.76 ^b ± 0.36
Manipulating non-nest shavings	0.30 ± 0.25	0.54 ± 0.27	0.15 ± 0.15	0.60 ± 0.33	0.18 ± 0.16	0.58 ± 0.30	0.04 ± 0.05	0.63 ± 0.37	0.19 ^a ± 0.19	0.58 ^b ± 0.31
Eating	0.72 ± 0.85	0.61 ± 0.37	0.94 ± 0.63	0.57 ± 0.29	1.14 ± 0.73	0.62 ± 0.32	0.64 ± 0.27	0.51 ± 0.26	0.88 ^a ± 0.68	0.58 ^b ± 0.32
Manipulating food	0.18 ± 0.27	0.22 ± 0.25	0.21 ± 0.17	0.16 ± 0.19	0.14 ± 0.07	0.15 ± 0.20	0.28 ± 0.23	0.18 ± 0.19	0.20 ± 0.15	0.18 ± 0.22
Carrying a pup	0.33 ± 0.14	0.13 ± 0.14	0.14 ± 0.12	0.13 ± 0.15	0.19 ± 0.13	0.08 ± 0.10	0.06 ± 0.09	0.17 ± 0.16	0.19 ± 0.15	0.13 ± 0.14
Self-grooming	0.98 ± 0.43	0.84 ± 0.44	1.13 ± 0.66	0.66 ± 0.30	1.02 ± 0.70	0.69 ± 0.35	1.26 ± 0.71	0.63 ± 0.27	1.09 ^a ± 0.60	0.73 ^b ± 0.37
Rearing sniffing or chewing cage lid	1.38 ± 0.87	0.99 ± 0.70	1.78 ± 1.06	0.83 ± 0.61	1.79 ± 0.75	0.93 ± 0.64	1.96 ± 1.61	0.63 ± 0.39	1.67 ^a ± 1.01	0.89 ^b ± 0.64
Sniffing with head raised	1.77 ± 0.87	1.34 ± 0.42	1.39 ± 0.64	1.36 ± 0.60	1.30 ± 0.51	1.25 ± 0.44	1.64 ± 0.89	1.38 ± 0.68	1.50 ± 0.71	1.35 ± 0.54

^{a,b} Different superscripts in the same line indicate significant differences (t-test for independent samples, $p < 0,05$ in all cases)

PC analysis showed that the behaviors “nursing” and “in the nest” explained together 54% of the total variance in Ex1 and 51% in Ex2. In contrast, “mother self-grooming”,

“mother rearing, sniffing or chewing the cage lid”, and “mother sniffing with head raised” in Ex1 and “mother rearing, sniffing or chewing the cage lid”, and “mother sniffing with head raised” in Ex2 explained less than 1% of the total variance. Therefore these behaviors were excluded from the comparative analysis.

A first rmGLM analysis performed on the whole set of significant behaviors of both experiments showed a significant main effect of time and a significant interaction effect time x previous housing as within subject factors on mother's behavioral pattern through lactation (Time: Wilk's Lambda = 0.463, $F_{117/4027} = 3.732$, $p = 0.000$; Time x Prior housing: Wilk's Lambda = 1.679, $F_{117/4027} = 1.679$, 117df, $p = 0.000$). Maternal behavior was also affected by prior housing as a between subject factor (Wilk's Lambda = 0.348, $F_{13/19} = 7.056$, 13df, $p = 0.000$). As expected, the frequencies of typically maternal behaviors such as “nursing”, “in the nest”, “mother in contact with at least one pup”, “mother in contact with more than half of the litter” decreased through lactation. The frequencies of “nursing”, “in the nest”, “mother in contact with more than half of the litter”, “mother manipulating nest shavings”, “mother manipulating non-nest shavings” behaviors were higher among the females of Ex2. In contrast, “mother licking or grooming any of her pups”, “mother eating”, “mother carrying a pup”, “mother self-grooming”, and “mother rearing, sniffing or chewing the cage lid” were more frequent in Ex1.

The rmGLM analysis was repeated for each experiment and confirms the effect of time as within subject factor (Ex1: Wilk's Lambda = 0.240, $F_{117/1000} = 1.798$, 117df, $p = 0.000$; Ex2: Wilk's Lambda = 0.365, $F_{117/2547} = 3.146$, 169df, $p = 0.000$). However, we did not find any effect of pair-housing nor social rank as between subjects factors on any of the maternal activities included in the comparative analysis.

Discussion

Cohabitation with an unrelative conspecific induced a dominant-subordinate relationship in both experiments independently of previous housing conditions. However, the establishment of such a hierarchy involved some differences. Although not statistically different, possibly due to the relatively low numbers of pairs, the proportion of pairs in which an inversion of status occurred was threefold higher among singly raised females. Moreover, these females were more reluctant in displaying hoarding behavior than group raised animals. It was only on the second observation session that some singly raised hamsters were seen securing food pellets, and even on the third encounter, hoarding was significantly less frequent among them than among group raised females. Huck et al (1988) showed the strong relationship of hoarding with dominance and how important it is for the successful reproduction of female hamsters. Our results suggest that cohabitation resulted in a better established social ranking in group raised females than in singly raised ones.

When females were group-housed from weaning, the proportion of reproductive failure was, as expected, higher among subordinate females than among dominant ones. This result is in accordance with data of previous studies (Huck et al, 1988; Pratt and Lisk, 1991). However, among singly raised females, reproductive success was not related with social status. Moreover, unexpectedly, whereas the 90% reproductive success of dominant females in Ex1 was in the range of the percentage usually reached in our animal facility (Chelini et al^b, submitted), only 54% of the isolated females delivered pups. In Ex2, in contrast, more than 80% of isolated females reproduced successfully, a normal proportion for a control group. It is likely that when

females are used to be group-housed, both isolation and subordination impair their reproductive function. The anxiogenicity of social isolation had been previously reported in laboratory rodents (Olsson and Westlund, 2007), but not in the hamster. Nevertheless, when isolation is their normal condition, hamster reproduction is not affected by cohabitation with an unknown female or by subordination. The number of pups per litter, and the litter sex-ratio were not affected by prior housing conditions, cohabitation or social rank. Actually the sole significant difference related to social stress was in the mean weight of the pups on day 4 post-partum in Ex2: whether they were dominant or subordinate, singly raised females pair-housed during 10 days with an unknown female gave birth to heavier pups than females that remained isolated.

Another interesting difference was found in all groups in the mean weight of pups at weaning, higher in Ex2 than in Ex1. Obviously, if the pups' weight on day 4 post-partum was similar in both experiments, some difference in the care they received during lactation should explain the different increase in their weight. Our behavioral data confirm this assumption: the frequency of litter-directed behaviors, including "nursing", was approximately 10% higher in females singly-raised from weaning and they displayed less self-directed or non-maternal behaviors than group-raised females.

In summary, our results show that previous housing conditions are a determinant issue. In fact, when female Syrian hamsters remain singly-housed after weaning, which is a condition in accordance with the solitary social organization of the species in the wild, social stress does not affect their fertility. Tamashiro, Nguyen e Sakai (2005) reported that social hierarchies are less stressful for rodent females than

for males. In fact, in a solitary species, female submission may have a very high cost. It means losing its food hoard, its litter and may be even its burrow without any reward. As further encounters with the same potentially dominant female are not likely, active defense is probably a better strategy than submission. On the other hand, it may not be worth for a male to fight and risk its life confronting a stronger opponent.

When females are group-housed immediately after weaning and remained so until adult age, social stress both of isolation and subordination induces a significant degree of reproductive skew, which is a characteristic trait of cooperative breeders. We also found in these females a significant reduction of maternal care resulting in smaller pups at weaning. Group-housing captive adult hamsters seems to evoke some behavioral and physiological features that can be found in complex social groups and are present as latent traits in this solitary species.

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