



UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL  
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE  
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA: FISILOGIA

**INFLUÊNCIA DE DIFERENTES AMBIENTES PÓS-NATAIS SOBRE O  
DESENVOLVIMENTO E RESPOSTAS COMPORTAMENTAIS E  
NEUROENDÓCRINAS DE RATOS ADULTOS.**

**NATALIA URIARTE**

**PORTO ALEGRE, 2008**

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DESENVOLVIMENTO E RESPOSTAS COMPORTAMENTAIS E  
NEUROENDÓCRINAS DE RATOS ADULTOS.**

Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Fisiologia, da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do Título de Doutor em Ciências Biológicas: Fisiologia.

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**PORTO ALEGRE, 2008**

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## RESUMO

A experiência durante os períodos iniciais da vida dos animais é de fundamental importância para a formação das ligações sociais e o estabelecimento da relação do indivíduo com o ambiente. A mãe e os irmãos dentro do ninho constituem a fonte mais importante de estimulação sensorial para os filhotes de rato recém-nascidos, a qual é crucial para a organização de respostas comportamentais e endócrinas durante as etapas precoces do desenvolvimento.

O objetivo da presente tese foi estudar a influência do comportamento maternal e do ambiente social sobre o desenvolvimento comportamental, respostas endócrinas e função reprodutiva de ratos, utilizando duas abordagens experimentais que pretendem se aproximar às condições naturais.

Os resultados mostraram que o comportamento maternal é uma conduta flexível e modificável por variáveis fisiológicas ou ambientais. Os diferentes ambientes maternos ou sociais decorrentes dessas variações modificaram a experiência precoce que receberam os filhotes, provocando mudanças na sua reatividade emocional durante a idade adulta. Também mostramos que essas variações alteram a responsividade ao estresse e a função reprodutiva em forma sexualmente dimórfica. Estes resultados evidenciam a importância da experiência precoce como moduladora das respostas comportamentais e endócrinas dos indivíduos a longo prazo.

## **ABSTRACT**

Early-life environment exerts long-term influences on rodents' brain, behavior and reproductive functions. In the rat, stimulation provided by the mother and littermates represents the most relevant source of sensory stimulation for the pups during early development and is crucial for an adequate development of the pups.

The aim of this thesis was to determine the effects of maternal behavior and social environment on rats' behavioral development, endocrine responses and reproductive functions, using two experimental approaches which intend to approximate to natural conditions.

Present results showed that maternal behavior is a plastic behavior, which could be modified by physiological or environmental factors. The different maternal and social environments caused by these variations, modify pups' early experience and provoke changes in emotional reactivity at adulthood. Besides, results showed that these variations long-term alter stress responsivity and reproductive function in a sexual dimorphic manner. These results highlight the importance of early experience as a long- term modulator of behavioral and endocrine response of individuals.

## APRESENTAÇÃO

Esta tese está organizada da seguinte forma: Introdução geral, Objetivos gerais e específicos, Abordagem metodológica, Capítulos I, II e III: Trabalhos científicos publicados ou submetidos, Discussão, Conclusões, Perspectivas e Referências bibliográficas.

Na Introdução geral se apresentam os fundamentos teóricos que nos levaram à formulação das hipóteses e da proposta de trabalho. Na seção Abordagem metodológica mostra-se uma breve explanação das abordagens experimentais utilizadas nos trabalhos. Os materiais e métodos e as referências bibliográficas específicas encontram-se no corpo de cada artigo apresentado nos Capítulos I, II e III. A seção Discussão contém a interpretação dos resultados obtidos nos diferentes artigos e aborda as conclusões gerais da tese. A seção Perspectivas discute as possibilidades de desenvolvimento de outros projetos a partir dos resultados obtidos na presente tese. Na seção Referências bibliográficas listam-se as referências citadas na Introdução geral e Discussão.

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*Table 1. Sperm quantification in single and overlapped reared adult males.*

## ABREVIATURAS

ACTH	Hormônio adenocorticotrófico
ANOVA	Análise de variância
BDNF	<i>Brain derived neurotrophic factor</i> (fator neurotrófico derivado do cérebro).
CORT	Corticosterona
CRH	Hormônio liberador de corticotrofina
GH	Hormônio de Crescimento
HL	<i>High licking mothers</i> (mães muito lambedoras)
LC	<i>Locus Coeruleus</i>
LH	Hormônio Luteinizante
LL	<i>Low licking mothers</i> (mães pouco lambedoras)
LPSL	<i>Late postpartum rats with a single litter</i>
MOL	<i>Multiparous lactating rats with overlapped litters</i>
MPOA	Área Pré-óptica média
MSL	<i>Multiparous lactating rats with a single litter</i>
NGFI-A	<i>Nerve growth factor induzible A-protein</i>
NPL	<i>Primiparous non-pregnant lactating rats</i>
PL	<i>Primiparous pregnant lactating rats</i>
PVN	Núcleo Paraventricular do Hipotálamo
RNA <sub>m</sub>	RNA mensageiro
SNC	Sistema Nervoso Central
GABA	Ácido Gama-aminobutírico



## **INTRODUÇÃO GERAL**

### **EXPERIÊNCIA PRECOCE E DESENVOLVIMENTO**

Em mamíferos, os primeiros períodos da vida são extremamente plásticos e representam estados cruciais na organização do sistema nervoso central (SNC). O período neonatal, junto com o período juvenil, são etapas críticas tanto para a formação das ligações sociais como para a relação do indivíduo com os estímulos ambientais (Anisman, Zaharia et al. 1998; Laviola e Terranova 1998; Spear 2000).

Os filhotes de rato são altriciais, durante os primeiros dias de vida estão desprovidos de pêlo, carecem de mecanismos de controle da temperatura corporal, não se locomovem por si mesmos e são incapazes de ver ou ouvir, dependendo totalmente dos cuidados maternos para sua sobrevivência. A principal fonte de estimulação sensorial (tátil, térmica, olfativa) durante as primeiras semanas de vida é fundamentalmente, provida no ninho, pela mãe e os irmãos.

As interações normais na relação mãe-filhote são cruciais para o desenvolvimento e crescimento adequados dos filhotes. A mãe é responsável pela regulação de várias respostas fisiológicas dos filhotes, como por exemplo, a frequência cardíaca, os ciclos de sono-vigília e a produção do hormônio de crescimento (GH) (Levine 2001). A privação maternal ocasiona efeitos deletérios no desenvolvimento dos filhotes, como a redução do crescimento, da diferenciação celular e da secreção do GH, provocando também um aumento da secreção de corticosterona (CORT) (Anderson e Schanberg 1972; Pauk, Kuhn et al. 1986).

Do mesmo modo, outras intervenções experimentais que afetam a interação mãe-filhote no período neonatal podem modificar drasticamente o desenvolvimento do animal, induzindo efeitos estáveis e duradouros durante a idade adulta (Ferre, Nunez et al. 1995; Laviola e Terranova 1998; Liu, Caldji et al. 2000).

Dentre os enfoques mais freqüentemente utilizados para estudar estes efeitos se acham os modelos de manipulação neonatal, privação maternal, e isolamento social. Em nosso laboratório tem sido pesquisado em profundidade o modelo da manipulação neonatal. Este procedimento induz alterações estruturais no SNC, diminuindo o número de neurônios em diferentes áreas cerebrais relacionadas à regulação das respostas ao estresse e da função reprodutiva em ratos machos e fêmeas (Lucion, Pereira et al. 2003; Winkelmann-Duarte, Todeschin et al. 2007).

Como consequência, os animais manipulados têm alterações comportamentais a longo prazo, entre as quais se encontram um aumento da atividade exploratória e atenuação do medo quando expostos a ambientes novos (Padoin, Cadore et al. 2001; Severino, Fossati et al. 2004; Madruga, Xavier et al. 2006). A manipulação neonatal induz também uma diminuição permanente da responsividade do eixo hipotálamo-hipófise-adrenal (HPA) ao estresse, determinando uma menor secreção de corticosterona CORT e prolactina ante um estímulo estressor, assim como um aumento da sensibilidade ao mecanismo de retroalimentação negativo da glândula adrenal sobre o eixo HPA promovendo um retorno mais rápido da CORT a níveis basais (Meaney e Aitken 1985; Meaney, Aitken et al. 1985; Meerlo, Horvath et al. 1999; Severino, Fossati et al. 2004). Os animais manipulados durante o período neonatal também mostram alterações na atividade do eixo hipotálamo-hipófise-gonadal (Gomes, Raineiki et al. 2005). As fêmeas manipuladas apresentam receptividade sexual

diminuída (Padoin, Cadore et al. 2001), ciclos estrais anovulatórios e alterações nas concentrações de hormônios relacionados à ovulação e ao comportamento sexual em fêmeas (Gomes, Frantz et al. 1999; Gomes, Rainecki et al. 2005). No mesmo sentido, machos manipulados apresentam diminuição do comportamento sexual (Padoin, Cadore et al. 2001; Benetti, Andrade de Araujo et al. 2007) e da espermatogênese (Mazaro e Lamano-Carvalho 2006) durante a idade adulta.

### **COMPORTAMENTO MATERNAL**

Durante a lactação ocorrem mudanças na conduta dos mamíferos orientadas a adequar a fêmea para cumprir com êxito sua função reprodutiva e garantir a sobrevivência dos filhotes. Na rata, o cuidado maternal está constituído por vários elementos comportamentais integrados, dirigidos à nutrição, estimulação e proteção da prole. Estes comportamentos incluem atividades de contato direto com os filhotes (amamentação, lambida, busca e recolhimento dos filhotes) e condutas não interativas (construção do ninho, comportamento agressivo, aumento da ingestão e diminuição do medo e da ansiedade) (Fleming e Rosenblatt 1974; Rosenblatt 1980; Hansen e Ferreira 1986). A amamentação pode ser realizada em três posturas diferentes, que se diferenciam pelo grau de arqueamento do dorso e da posição dos filhotes: postura com dorso arqueado, postura com dorso relaxado e postura supina (Stern e Lonstein 2001; Champagne, Francis et al. 2003).

O desenvolvimento do comportamento maternal está associado às mudanças hormonais que ocorrem durante a gestação e o parto (Rosenblatt, Mayer et al. 1988). A diminuição da progesterona e o aumento do estradiol são importantes para o rápido estabelecimento deste comportamento nos primeiros minutos pós-parto (Rosenblatt

1969; Rosenblatt 1975; Rosenblatt e Siegel 1975; Mayer e Rosenblatt 1980; Rosenblatt, Mayer et al. 1988). A ocitocina também é importante para a manifestação desse comportamento (van Leengoed, Kerker et al. 1987).

Uma vez estabelecido, a manutenção do comportamento maternal depende fundamentalmente da estimulação sensorial brindada pelos filhotes (Rosenblatt 1967; Rosenblatt 1975). Os principais estímulos sensoriais implicados são a estimulação da região perioral; a estimulação somatosensorial proveniente da parte ventral do tórax, realizada pelos filhotes durante a amamentação (Stern e Johnson 1990; Stern 1991; Stern e Lonstein 1996) e os estímulos olfativos (Fleming e Rosenblatt 1974; Ferreira e Hansen 1986; Fleming, Gavarth et al. 1992).

As características dos filhotes também modulam o comportamento maternal. Por exemplo, as mães lambem a zona anogenital dos machos com maior frequência que a das fêmeas (Moore e Morelli 1979; Alleva, Caprioli et al. 1989). Filhotes hiper ou hipotérmicos, ou pouco móveis são incapazes de provocar posturas de amamentação nas mães (Woodside, Pelchat et al. 1980; Stern e Lonstein 1996). As demandas fisiológicas dos filhotes também modulam a motivação das mães, promovendo alterações no comportamento maternal (Pereira, Uriarte et al. 2005; Pereira e Ferreira 2006).

Na rata, o comportamento maternal, especificamente o comportamento de lambida, naturalmente exhibe variações individuais dentro de uma população (Champagne, Francis et al. 2003). Em ratas da linhagem Long-evans, Meaney e colaboradores, desenvolveram um modelo que permite classificar as mães segundo a frequência de lamber os filhotes e de adoção da postura de amamentação com dorso arqueado (Liu,

Diorio et al. 1997; Caldji, Tannenbaum et al. 1998). Neste modelo, as mães com frequências de lambidas acima de 1 desvio padrão da frequência média da população são definidas como “muito lambedoras” (*high licking mothers*) enquanto as fêmeas com frequências abaixo da média-1 desvio padrão correspondem às “pouco lambedoras” (*low licking mothers*). Este comportamento é estável durante múltiplas lactações (Champagne, Francis et al. 2003) o que permite classificar as fêmeas durante a primeira lactação e estudar outros comportamentos associados nas sucessivas, assim como avaliar a influência dessas variações sobre o desenvolvimento dos filhotes.

As diferenças de comportamento maternal aparecem como uma variabilidade normal dentro da população. Os grupos não se diferenciam nos tamanhos e pesos das ninhadas nem na sobrevivência ou no ganho de peso dos filhotes durante a lactação mostrando que os níveis de cuidado maternal são adequados para o desenvolvimento dos filhotes (Liu, Diorio et al. 2000; Champagne, Francis et al. 2003).

Os diferentes níveis de comportamento de lambar exibidos pelas mães se relacionam diretamente à atividade do sistema ocitocinérgico (Champagne, Diorio et al. 2001). As fêmeas muito lambedoras têm níveis maiores de receptores de ocitocina na área preóptica media (MPOA), *septum* lateral, núcleo central da amígdala, núcleo paraventricular do hipotálamo (PVN), e núcleo próprio da *stria terminalis* (Francis, Champagne et al. 2000). A administração de antagonistas dos receptores para ocitocina no dia 3 pós-parto elimina completamente as diferenças comportamentais entre as fêmeas muito e pouco lambedoras (Champagne, Diorio et al. 2001).

## **EFEITOS DAS VARIAÇÕES NATURAIS DE COMPORTAMENTO MATERNAL**

O fenótipo dos filhotes é influenciado drasticamente por variações na estimulação maternal recebida durante o período neonatal (Moore 1984; Moore 1992; Liu, Diorio et al. 1997). Durante a etapa adulta, os filhotes das mães muito lambedoras têm uma liberação de corticotrofina (ACTH) e CORT reduzida em resposta a estresse por contenção, assim como uma maior sensibilidade aos efeitos inibitórios dos glicocorticóides na atividade do eixo HPA (Liu, Diorio et al. 1997).

Por sua vez, a expressão de mRNA do hormônio liberador de corticotrofina (CRH) nos neurônios parvocelulares do PVN (Liu, Diorio et al. 1997) e a densidade de receptores para CRH no *locus coeruleus* (LC) diminuiu significativamente nos filhotes de mães muito lambedoras, aumentando o efeito inibitório da regulação dos glicocorticóides sobre o eixo HPA (Caldji, Tannenbaum et al. 1998).

O comportamento maternal modula também as respostas comportamentais dos filhotes quando adultos. Os filhotes de mães muito lambedoras exibem um aumento do comportamento exploratório no campo aberto, uma latência menor e um tempo maior de alimentação em um ambiente novo, e menor tempo de imobilidade e enterramento no teste de ‘*shock-probe*’, quando comparados com filhotes de mães pouco lambedoras (Caldji, Tannenbaum et al. 1998; Menard, Champagne et al. 2004).

Tem sido demonstrado que os altos níveis de comportamento maternal observados nas mães muito lambedoras provocam efeitos a longo prazo similares aos induzidos pela manipulação neonatal (Liu, Diorio et al. 1997). Sabe-se também, que a manipulação neonatal induz um aumento das lambidas maternas (Lee e Williams 1974; Villescas,

Bell et al. 1977; Hennessy, Li et al. 1980). A partir desses achados, surgiu a hipótese da “mediação maternal” que propõe que os efeitos a longo prazo das manipulações precoces, são resultantes de mudanças do comportamento materno, especificamente do comportamento de lambar, e não pelo efeito da manipulação por si mesma (Smotherman e Bell 1980; Denenberg 1999)

#### **VARIAÇÕES NO AMBIENTE SOCIAL PRECOCE E NA COMPOSIÇÃO FAMILIAR**

Na natureza, a composição familiar dos ratos pode variar de acordo com as condições de acasalamento da mãe (Calhoun 1963; Gilbert, Burgoon et al. 1983).

As ratas fêmeas apresentam um período de receptividade que começa entre 6 e 12 horas após do parto, denominado estro pós-parto (Connor e Davis 1980; Connor e Davis 1980; Davis e Connor 1980; Gilbert, Pelchat et al. 1980).

Se o acasalamento ocorre durante o proestro no ciclo estral regular, origina-se uma ninhada simples. Entretanto, se o acasalamento ocorre no estro pós-parto, a composição familiar pode variar da unidade familiar padrão utilizadas no biotério (Gilbert, Pelchat et al. 1980). A fecundação neste período provoca a sobreposição da lactação da primeira ninhada com a gestação da segunda, e a partir do nascimento da segunda ninhada a lactação de duas ninhadas, a primeira (juvenis) e a segunda (neonatos) (Davis e Connor 1980; Bean e Estep 1981; Gilbert, Burgoon et al. 1983).

Esta situação é bastante freqüente em condições naturais, já que o acasalamento no estro pós-parto representa aproximadamente o 50% das gestações em populações selvagens de *Rattus norvegicus* (Calhoun 1963; Davis e Connor 1980). Nestes casos, as fêmeas de uma colônia podem criar sucessivas ninhadas sobrepostas, e tem sido demonstrado que irmãos mais velhos permanecem no ninho desde os 25 a 35 dias de idade, coabitando com os irmãos menores (Calhoun 1963).

Fenômenos similares têm sido descritos em outras espécies de ratos silvestres como *R. rattus* y *R. fuscipes* (Horner e Taylor 1969).

Em observações de laboratório durante os três primeiros dias após o parto da segunda ninhada, observou-se que os filhotes mais velhos permanecem no ninho, embora não estejam sendo amamentados ou a mãe não esteja no ninho (Gilbert, Burgoon et al. 1983). Além disso, o tempo que permanecem no ninho aumenta depois do nascimento da nova ninhada, podendo ser até maior que o tempo de permanência da mãe (Gilbert, Burgoon et al. 1983). Outros estudos, porém, têm reportado efeitos negativos da interação entre juvenis e neonatos (em indivíduos de ninhadas diferentes), observando-se concorrência pela amamentação e o afastamento dos filhotes recém nascidos do ninho, freqüentemente provocando a morte destes por privação de leite e/ou traumas físicos (Mennella, Blumberg et al. 1990).

A sobreposição de ninhadas poderia provocar mudanças no ambiente social onde se desenvolvem os filhotes da primeira e segunda ninhada, permitindo uma maior interação social entre filhotes de duas idades diferentes.



## **SENSIBILIZAÇÃO MATERNAL**

Nos ratos, o contacto contínuo com filhotes neonatos provoca, gradualmente, mudanças de comportamento que vão desde a indiferença, o afastamento ou inclusive canibalismo, até o aparecimento do comportamento maternal. Este fenômeno - denominado sensibilização maternal- é utilizado freqüentemente no laboratório, para estudar as bases neuroendócrinas do comportamento maternal e as mudanças comportamentais associadas à lactação. A sensibilização pode ocorrer independentemente do estado hormonal, do gênero, idade ou experiência sexual prévia (Fleming e Rosenblatt 1974; Mayer e Rosenblatt 1975; Reisbick, Rosenblatt et al. 1975; Mayer e Rosenblatt 1980).

Contudo, a idade é um fator que influi no processo de sensibilização já que a latência para o desenvolvimento de condutas maternais é menor nos animais juvenis que nos adultos (Bridges, Zarrow et al. 1974; Mayer, Freeman et al. 1979; Mayer e Rosenblatt 1979). Estes dados se relacionam com o fato de que na natureza a situação mais comum em que as ratas sem serem parturientes podem ter uma forte interação com filhotes recém nascidos, é quando os filhotes juvenis permanecem no ninho até a chegada de uma nova ninhada (Gilbert, Burgoon et al. 1983; Stern e Rogers 1988). Uma menor latência de sensibilização promoveria o rápido estabelecimento de comportamentos de cuidado dos filhotes mais novos, evitando o aparecimento de condutas prejudiciais para os neonatos (Numan e Insel 2003).

## **JUSTIFICATIVA**

Para os ratos, a mãe não somente representa a principal fonte de nutrição dos filhotes, mas, junto com os irmãos no ninho, constitui a fonte mais importante de estimulação sensorial. Esta estimulação é fundamental para a sobrevivência imediata dos filhotes,

assim como para a organização de várias respostas comportamentais e endócrinas durante as etapas prepuberal e adulta (Price & Feldon, 2003).

A influência do ambiente social no desenvolvimento comportamental dos roedores tem sido muito investigada, porém, a maior parte dos estudos tem sido realizada em modelos de privação ou isolamento social em condições artificiais. As pesquisas sobre formas mais sutis de variações no ambiente social durante o desenvolvimento comportamental podem nos proporcionar informação com maior significado etológico que aquela baseada nos experimentos em condições artificiais.

Assim, o objetivo desta tese foi mudar o foco de atenção para estudos que pretendem se aproximar às situações observadas na natureza. Baseados nos antecedentes apresentados, foram escolhidas duas abordagens experimentais para estudar os efeitos sobre o comportamento maternal e a influência exercida pela experiência precoce sobre os indivíduos. A primeira abordagem consistiu no estudo das variações naturais do comportamento maternal presentes em uma determinada população e sua influência sobre o fenótipo dos filhotes. Na segunda abordagem, estudamos o modelo de ninhadas sobrepostas, originado através do acasalamento da mãe no estro pós-parto, para avaliar os efeitos de variações na composição da unidade familiar sobre o comportamento maternal e o fenótipo dos filhotes das duas ninhadas.

## **HIPÓTESES**

1. O comportamento maternal não é uma conduta rigidamente estereotipada e sim mostra variações dentro de uma população, seja por variabilidade individual espontânea ou determinadas por diferentes condições reprodutivas.
2. Diferentes experiências, determinadas por mudanças no ambiente precoce, modulam o desenvolvimento e o fenótipo dos animais durante a idade adulta.

## **OBJETIVOS GERAIS**

1. Analisar as variações do comportamento maternal em ratas da linhagem Wistar e sua influência no fenótipo dos filhotes durante a idade adulta.
2. Analisar como variações na composição familiar no modelo de ninhadas sobrepostas afetam o comportamento maternal da rata lactante e o fenótipo dos filhotes de ambas as ninhadas.

## **OBJETIVOS ESPECÍFICOS**

- 1.a Analisar os diferentes componentes do cuidado maternal e classificar as ratas mães (*Rattus norvegicus*, linhagem Wistar) segundo a frequência do comportamento de lambar.

1.b Determinar se o desenvolvimento dos filhotes é afetado por diferentes níveis de estimulação maternal, especificamente pelo comportamento de lambar.

1.c Determinar se a inibição comportamental e a função reprodutiva dos filhotes quando adultos são afetadas por diferentes níveis de estimulação maternal, especificamente pelo comportamento de lambar.

2.a Determinar o efeito da gestação simultânea sobre o comportamento maternal de ratas lactantes.

2.b Determinar o efeito da sobreposição de duas ninhadas de diferentes idades sobre o comportamento da mãe.

2.c Determinar o efeito da convivência com irmãos neonatos no comportamento de ansiedade durante a idade adulta dos juvenis criados em ninhadas sobrepostas.

2.d Determinar o efeito de ser criado em ninhadas sobrepostas durante o período neonatal sobre a inibição comportamental, a resposta ao estresse e a função reprodutiva durante a idade adulta.

## ABORDAGEM METODOLÓGICA

Nesta seção será feita uma breve descrição da abordagem metodológica utilizada nos artigos que compõem esta tese. A descrição completa dos materiais e métodos utilizados em cada experimento encontra-se no corpo de cada trabalho nos capítulos I, II e III.

Artigo 1 (Capítulo I).

Este trabalho teve como objetivo analisar as variações do comportamento maternal em ratas da linhagem Wistar e classificá-las utilizando o modelo validado em ratas da variedade Long-Evans por Meaney e colaboradores (Liu, Diorio et al. 1997). Para isto utilizou-se uma população de 40 ratas lactantes que tiveram o comportamento maternal registrado simultaneamente, durante os 8 primeiros dias pós-parto. Uma vez concluído este registro, classificaram-se as fêmeas em dois grupos de mães: pouco lambedoras (*low licking* - LL) e muito lambedoras (*high licking* - HL) e analisaram-se os outros componentes do comportamento maternal. Os filhotes da primeira ninhada dessas mães foram deixados crescer até a idade adulta onde foram avaliadas as seguintes variáveis em machos e fêmeas:

- Inibição comportamental (avaliação do comportamento no modelo de campo aberto).
- Função reprodutiva (idade de abertura vaginal e começo da ciclicidade estral, receptividade sexual na noite do proestro e ovulação na manhã do proestro em fêmeas; comportamento sexual e avaliação espermática em machos).

Um mês após o desmame da primeira ninhada, as mães LL e HL foram acasaladas novamente. Nos filhotes da ninhada resultante foi avaliado seu desenvolvimento através do peso nos dias 1, 10 e 21 pós-parto e a idade da abertura ocular.

Artigo 2 (Capítulo II).

Este trabalho teve como objetivo analisar o comportamento maternal das ratas mães nas diferentes situações reprodutivas ocasionadas pelo acasalamento no estro pós-parto.

Para avaliar o efeito da gestação simultânea à lactação foi comparado o comportamento de ratas lactantes primíparas não gestantes (grupo NPL: fêmeas criando sua primeira ninhada, e que tiveram acasalamento no estro pós-parto, mas não emprenharam) e ratas lactantes primíparas gestantes (grupo PL: fêmeas criando sua primeira ninhada enquanto estão gestando uma segunda ninhada concebida no estro pós-parto).

Para avaliar o efeito de criar simultaneamente duas ninhadas de diferentes idades foi comparado o comportamento de ratas multíparas com ninhadas sobrepostas (grupo MOL: fêmeas criando sua segunda ninhada concebida no estro pós-parto e dos juvenis da primeira ninhada) e ratas multíparas com uma ninhada simples (grupo MSL: fêmeas criando sua segunda ninhada concebida no estro pós-parto, sem juvenis).

Para avaliar os efeitos da sobreposição de ninhadas no comportamento das mães para os juvenis, o comportamento das mães MOL foi comparado com o de ratas mães no pós-parto tardio com uma ninhada simples (grupo LPSL: fêmeas criando 2 juvenis da primeira ninhada após o dia 21 pós-parto). Além disso, foi comparado o comportamento das mães MOL para os neonatos e para os juvenis.

Para avaliar a estimulação total recebida pelos filhotes da segunda ninhada foi registrado o comportamento dos irmãos juvenis. A estimulação total recebida pelos neonatos foi calculada como a soma do comportamento da mãe e do comportamento dos dois irmãos juvenis.

O segundo experimento deste trabalho teve como objetivo determinar se a experiência com irmãos neonatos durante a idade juvenil induz efeitos a longo prazo sobre o medo e a ansiedade. Para isto, o comportamento dos juvenis criados em ninhadas sobrepostas nos testes de campo aberto e labirinto elevado em cruz foi comparado na idade adulta com o de indivíduos criados em ninhadas simples.

Artigo 3 (Capítulo III).

O objetivo deste trabalho foi determinar o efeito de ser criado em ninhadas sobrepostas durante o período neonatal sobre: a inibição comportamental, a resposta ao estresse e a função reprodutiva durante a idade adulta. Para isto obtiveram-se filhotes de mães multíparas com ninhadas sobrepostas e mães multíparas com ninhadas simples. Os animais foram deixados crescer até a idade adulta quando as seguintes variáveis foram avaliadas:

- Inibição comportamental (avaliação do comportamento no modelo de campo aberto).
- Resposta aguda ao estresse (liberação de corticosterona ante um estresse por contenção durante 20 minutos).
- Função reprodutiva (receptividade sexual na noite do proestro e ovulação na manhã do proestro em fêmeas; comportamento sexual e avaliação espermática em machos).

## **CAPÍTULO I**

### **EFFECTS OF MATERNAL CARE ON THE DEVELOPMENT, EMOTIONALITY, AND REPRODUCTIVE FUNCTIONS IN MALE AND FEMALE RATS.**

Developmental Psychobiology, 49: 451-462, 2007.



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## Effects of Maternal Care on the Development, Emotionality, and Reproductive Functions in Male and Female Rats

**ABSTRACT:** Variations in maternal behavior induce long-lasting effects on behavioral and neuroendocrine responses to stress. The aim of this study was to analyze developmental parameters, reproductive function, and anxiety-related behaviors of male and female rats raised by mothers that naturally display high and low levels of maternal licking behavior. Results showed that an increase in licking behavior received by the pups accelerated their eye opening and reduced fear behavior assessed in the open field test. Additionally, female offspring of high licking (HL) mothers showed decreased ovulation and lordosis intensity. In contrast, males from HL and low licking (LL) mothers did not differ in their reproductive function, suggesting a gender difference in maternal effects. Present results showed that individual differences in maternal behavior appear not only to be predictive of later emotionality and stress-responsivity in the offspring, but can also modulate the reproductive function of females. Maternal genetic factors, differences in the prenatal intrauterine milieu, or a combination of these cannot be excluded to explain the effects observed. © 2007 Wiley Periodicals, Inc. *Dev Psychobiol* 49: 451–462, 2007.

**Keywords:** maternal behavior; early experience; licking behavior; sexual behavior

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### INTRODUCTION

In altricial rodents, stimulation provided by the mother and littermates represents the most relevant source of sensory stimulation for the pups during early development. This “maternal environment” is crucial for an adequate development of the pups, and alterations in its characteristics could lead to complex and long-term influences on their behavioral and neuroendocrine responses (Cirulli, Berry, & Alleva, 2003; Levine, 2000; Pryce, Bettschen, Bahr, & Feldon, 2001).

Lactating females show natural individual differences in maternal behavior, specifically in pup licking. It has

been shown that mothers could be classified as either low licking (LL) or high licking (HL) based on their frequency of licking the pups (Champagne, Francis, Mar, & Meaney, 2003; Liu et al., 1997). These differences appear to be stable across multiple litters (Champagne et al., 2003).

These variations in the levels of licking behavior received by the pups profoundly affect the neural circuits that regulate their endocrine and behavioral responses to stress. Thus, the adult offspring of HL mothers showed blunted plasma ACTH and corticosterone responses to stress, increased hippocampal glucocorticoid receptor mRNA expression, enhanced glucocorticoid negative feedback sensitivity, and decreased hypothalamic CRF mRNA levels (Caldji et al., 1998; Francis, Champagne, Liu, & Meaney, 1999; Meaney, 2001).

When adults, offspring of HL mothers display lower levels of fear reactivity, evidenced by their decreased acoustic startle responses, increased exploration in a novel environment (open-field), decreased latency to begin feeding in a novel test chamber, and less shock induced

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freezing and probe burying in the shock-probe test, compared with offspring of LL mothers (Caldji et al., 1998; Liu et al., 1997; Menard, Champagne, & Meaney, 2004; Menard & Hakvoort, 2007).

Considering fear and stress response, it is interesting to note that the spontaneous high levels of licking behavior by undisturbed dams induce long-lasting effects similar to those induced by early handling. Moreover, it has been reported that, after the postnatal handling, there is an increase in maternal behavior, specifically an augment in pup licking frequency (Lee & Williams, 1974; Liu et al., 1997). From this evidence, the "maternal mediation" hypothesis was derived. It proposes that the long-term effects of early manipulations result from changes in maternal care, specifically in the licking behavior received by the pups, rather than the experimental manipulation *per se* (Caldji et al., 1998; Smotherman & Bell, 1980).

Previous results from our laboratory showed that neonatal handling reduces sexual behavior in male and female rats (Padoin, Cadore, Gomes, Barros, & Lucion, 2001), decreases ovulation (Gomes, Frantz, Sanvitto, Anselmo-Franci, & Lucion, 1999; Gomes et al., 2005), and spermatogenesis (Mazaro & Lamano-Carvalho, 2006), and reduces the number of neurons in brain areas related to stress and reproduction (Lucion, Pereira, Winkelman, Sanvitto, & Anselmo-Franci, 2003). Neonatal handling also reduces the age of eye opening of the pups (Levine, 2000).

Several studies were performed in order to investigate how maternal behavior influences stress and fear responses. However, few experiments were addressed to study the effects of natural variations in the levels of licking behavior on offspring's reproductive function. Champagne et al. (2003) showed that female offspring of LL and HL mothers did not differ in fecundity. However, Cameron et al. (2005) stated that females raised by HL mothers were less sexually receptive and had a reduced rate of pregnancy (Cameron et al., 2005), although numeric data is not shown in this work. As far as we know, there are no studies investigating the consequences of spontaneous variations in maternal licking behavior on male sexual behavior and sperm production.

Present study aimed to analyze the effects of natural variations in maternal care received by the pups on their development, reproductive function, and emotionality in Wistar rats, a strain other than the ones used by those studies. We investigated developmental parameters, ovulation, sperm production, sexual behavior, and anxiety-related behaviors of male and female Wistar rats raised by mothers that naturally displayed high (HL) and low (LL) levels of maternal licking behavior. Given that studies using the neonatal handling procedure, which increases maternal behavior after the return of the pups to the nest (Liu et al., 1997), showed a decrement in sexual

behavior and reproductive function (Padoin et al., 2001), we hypothesized that high licked pups would show reduced sexual behavior and reproductive function in adulthood.

## GENERAL METHOD

### Animals

Male and female Wistar rats were obtained from the colony maintained in the Federal University of Rio Grande do Sul Laboratory Animal Facility. Animals were kept on a 12-hr light/dark cycle (lights off at 1,800 hr) and in a stable environmental temperature (21°C) with ad lib access to rat chow (Nutrilab, Colombo, Brazil) and water. Pups were maintained with their mother until weaning (Day 21), and then housed in same-sex, same-litter groups of three to four animals per cage. All procedures used in this study followed the guidelines for the care and use of Laboratory Animals from the National Institute of Health and were approved by the Research Committee of the Federal University of Rio Grande do Sul.

### General Procedure

In experiment 1, mothers were classified in LL and HL based on the procedure used by Liu et al. (1997) and pups from their first and second litter were used. Both male and female pups from the first litter of LL and HL mothers were tested in adulthood (around 90 days of age) for open field activity (experiment 2) and reproductive function (experiment 3). Females of the first litter were also used to assess the onset of puberty by recording the day of vaginal opening and occurrence of first and second estrus. Developmental parameters (experiment 4) were recorded in the second litter of LL and HL mothers, obtained by re-mating the females 1 month after the weaning of the first litter, in order to avoid any effect of early manipulation during the classification of the mothers. These animals were not used for behavioral tests in adulthood.

The oestrus cycle of females from the first litter was monitored from the day of vaginal opening until approximately Day 50 of age, and then later in adulthood, from Day 80 of age until the testing day. Females were tested in the open field on diestrus and for sexual behavior in proestrus. For monitoring oestrus cycle, daily vaginal smears were taken, and only those females with two consecutive regular cycles were used for behavioral testing. Fifteen of 85 females were not used due to irregular estrous cycle; there were no differences between the number of female rats discarded between the two groups (LL: 4 of 37; HL: 11 of 48;  $\chi^2$  test,  $p = ns$ ).

Adult animals were used in only one behavioral test, and within each group all subjects were from different



litters. All tests were performed in a separate testing room with the same temperature and photoperiod conditions of the breeding room. Animals were individually housed, transported to the testing room, and left to acclimatize at least 1 hr before starting the tests. Behavioral tests were recorded on VHS-video tape and analyzed later using the computer software The Observer Pro<sup>®</sup> (Noldus, Wageningen, The Netherlands).

## EXPERIMENT 1: CLASSIFICATION OF THE DAMS BASED ON THEIR LICKING FREQUENCY

### Method

Breeding was accomplished by housing nulliparous females (90 days old) overnight with sexually experienced males on a proestrus night. Approximately 7 days before delivery, pregnant females were housed individually and the presence of pups was checked twice a day (at the beginning and end of the light period). On the day of birth (Day 0), the number of pups was culled into eight per dam (with usually four or at least three pups of each sex). Eleven mothers had their first litters with fewer than eight pups and were excluded from the experiment. The maternal behavior of a total of 40 primiparous lactating females was scored daily during four, 72-min observation sessions for the first 8 postpartum days. Observations occurred at regular times with three periods during the light phase (1,000, 1,300, 1,600 hr) and one during the dark phase of the light-dark cycle (1,830 hr). Within each session, the behavior of each female was scored every 3 min (25 observations per period  $\times$  4 periods per day = 100 observations/mother/day) for the following behaviors: (1) mother off pups, (2) mother licking pups (either the body surface or its anogenital region), (3) mother nursing pups in either an arched-back posture, (4) a "blanket" posture in which the mother lays over the pups, (5) or a passive posture in which the mother is lying either on her back or side while the pups nurse (Champagne et al., 2003). During this period, dams remained undisturbed and cleaning and sawdust changing began on Day 11 postpartum.

Data are reported as the percentage of observations in which pups received the target behavior (number of observations in which the target behavior was recorded divided the total number of observations  $\times$  100). HL mothers were defined as females whose scores for frequency of licking were above the highest quartile, and LL mothers were defined as females whose frequency scores were below the lowest quartile of the population observed. The comparisons between the total frequencies (the frequency of the specific behavior from a total of 800 observations per mother) of the two groups of mothers

and the frequencies of behaviors in each of the first 8 days postpartum were performed using the Mann–Whitney *U*-test. The number of second litters discarded due to insufficient number of pups or inappropriate sex ratio in the two groups was compared by the  $\chi^2$  test.

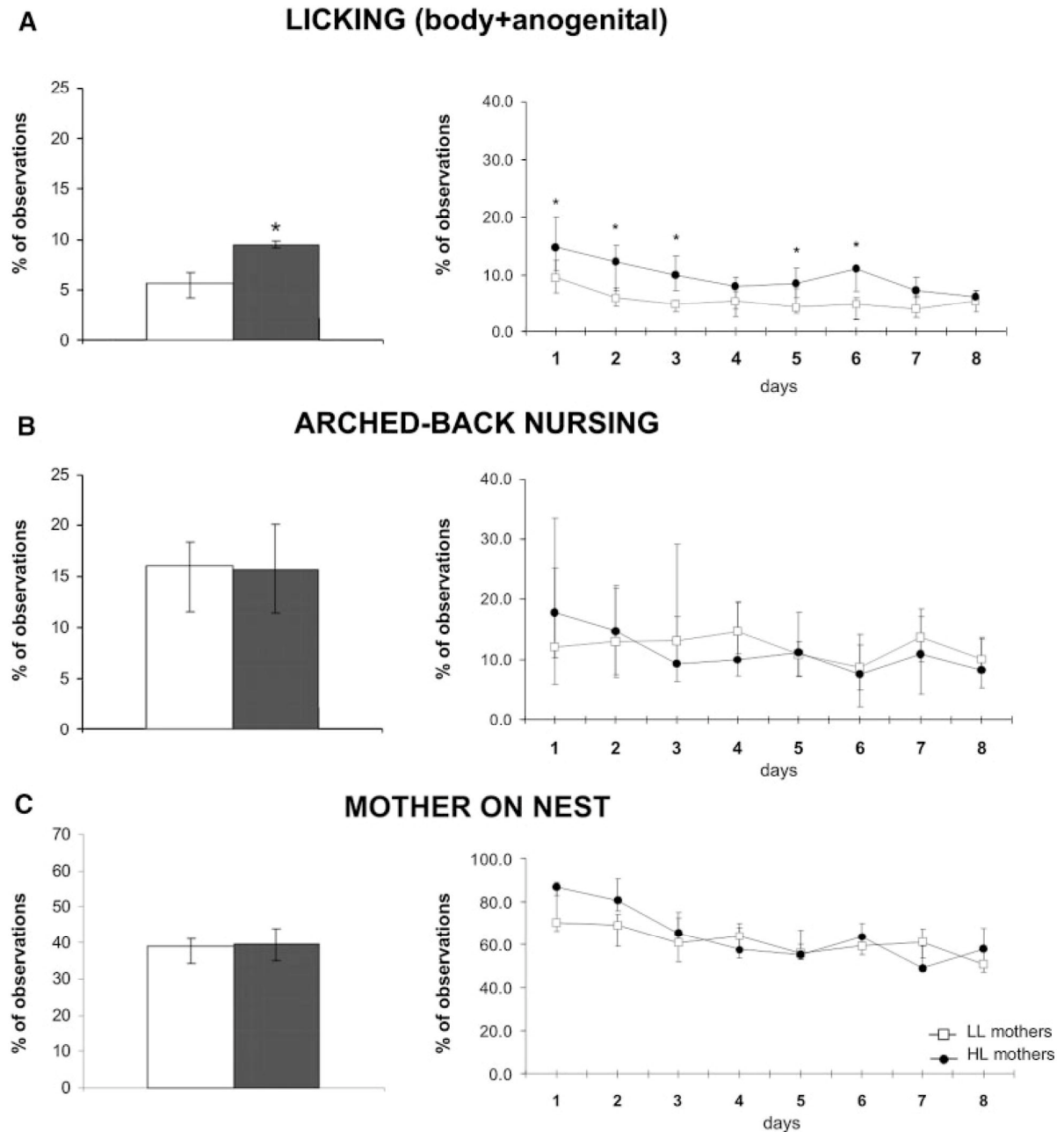
### Results

Based on the frequency distribution of licking behavior (body and anogenital licking) observed in a group of 40 Wistar lactating females with a median (IQR) of 8.1(6.9–9.2), 10 females were classified as LL mothers, with a median (IQR) of 5.6 (4.3–6.7), and 13 as HL mothers with a median (IQR) of 9.4 (9.2–9.8). LL and HL mothers did not differ in the other components of maternal behavior observed or in the time spent in the nest or in contact with pups. Figure 1 shows the median (IQR) of the percentage of total observations of licking (panel A), arched-back nursing (panel B), and time spent in nest (panel C) in LL and HL mothers during the first 8 postpartum days with detailed data of each behavior on every day. The frequency of licking of the two groups analyzed by day differs in the same way as the total latency for all days except Day 4, Day 7, and Day 8. The time in arched-back nursing posture and the time of the mother on the nest did not differ between groups in any of the first 8 days postpartum (Fig. 1, B and C). There were no differences between the number of second litters discarded between the two groups (LL: 3 of 10; HL: 5 of 13;  $\chi^2$  test,  $p = ns$ ). Litter size and mean pup weight at birth were not different between the two groups (Tab. 1).

## EXPERIMENT 2: EFFECTS OF VARIATIONS IN RECEIVED LICKING BEHAVIOR ON EMOTIONALITY

### Method

Behavior of male and female adult rats was measured in the open field test during the light period of the cycle (diestrus morning in females). The apparatus was a 100-cm square open field that was evenly illuminated by overhead fluorescent lights. The arena was divided into 20-cm squares painted on the floor. Each animal was placed in one corner of the open field and its behavior was videotaped over a 5-min session. The following behaviors were analyzed: duration(s) of locomotion, number of entries into, time(s) spent in and latency(s) to enter into the central quadrants. The open field was cleaned thoroughly between test sessions. The duration of locomotion, time in central quadrants, and latencies to enter in central quadrants (means  $\pm$  SEM) were analyzed by a two-way ANOVA (gender  $\times$  group).



**FIGURE 1** Maternal behavior of LL and HL mothers. Data are plotted as a median (IQR) percentage of observations from a total of 800 observations per mother (100 observations/mother/day during the first 8 days postpartum) (left panels) and separated by measures for each of the 8 days postpartum (right panels); (A) licking (includes body and anogenital licking); (B) arched back nursing posture; (C) time that the mother spent on the nest, Mann–Whitney  $U$ -test,  $*p < .05$ .

## Results

Offspring of HL mothers (females,  $n = 9$ ; males,  $n = 10$ ) showed increased locomotion in the open field compared with offspring of LL mothers (females,  $n = 8$ ; males,

$n = 9$ ) as revealed by ANOVA analyses (group effect  $F(1,33) = 9.28$ ,  $p < .01$ ). There were no main effects of gender ( $F(1,33) = .07$ ,  $p = ns$ ) or interaction effects between the two factors ( $F(1,33) = .16$ ,  $p = ns$ ). The time spent in central quadrants did not differ between groups

**Table 1.** Litter Size, Mean Pup Weight, and Mean Age (Day After Birth) of Eye Opening of Pups from the Second Litter of LL and HL Mothers (Means  $\pm$  SEM)

	LL Pups	HL Pups
Litter size, <i>n</i>	10.0 $\pm$ .5	9.5 $\pm$ .5
Pup weight on Day 1 (g)	6.5 $\pm$ .3	7.0 $\pm$ .2
Pup weight on Day 10 (g)	17.2 $\pm$ .1	18.8 $\pm$ .7
Pup weight on Day 21 (g)	39.1 $\pm$ 1.3	37.9 $\pm$ 1.1
Age of eye opening (days)	14.3 $\pm$ .2	13.4 $\pm$ .3*

\* $p < .05$ , Student's *t*-test.

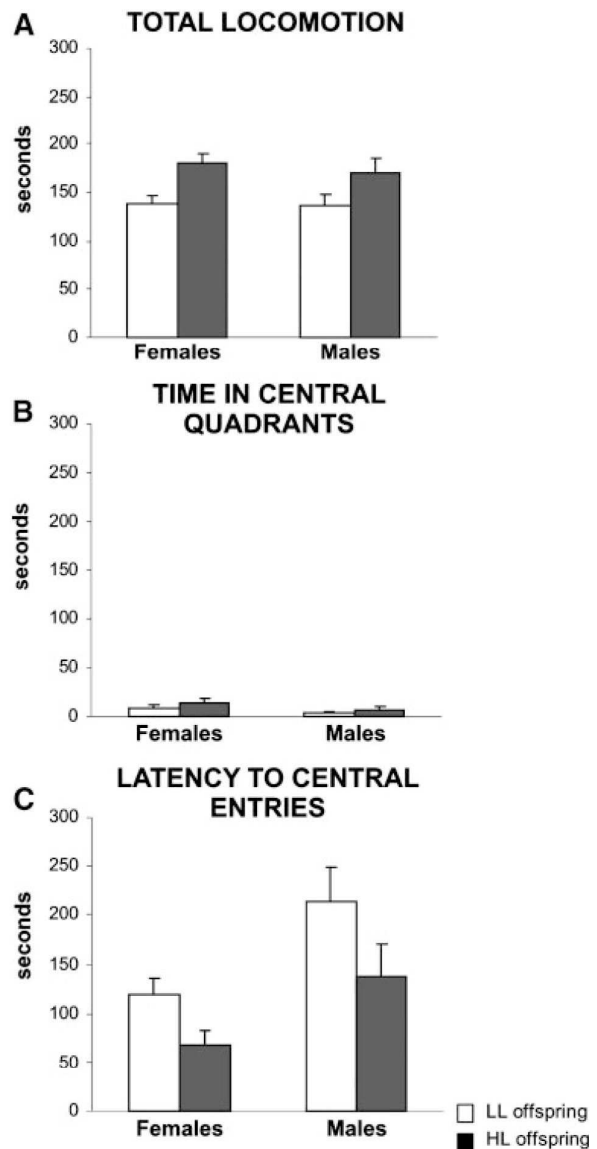
( $F(1,33) = 3.83$ ,  $p = \text{ns}$ ) or genders ( $F(1,33) = 1.41$ ,  $p = \text{ns}$ ), and there was no interaction between factors ( $F(1,33) = .05$ ,  $p = \text{ns}$ ). HL offspring also showed a shorter latency to enter into the central quadrants of the open field compared with LL offspring (group effect  $F(1,33) = 5.56$ ,  $p < .05$ ), and a main effect of gender was detected ( $F(1,33) = 8.97$ ,  $p < .01$ ), showing that females had shorter latencies than males, but there was no interaction between the two factors ( $F(1,33) = .21$ ,  $p = \text{ns}$ ), see Figure 2.

### EXPERIMENT 3: EFFECTS OF VARIATIONS IN RECEIVED LICKING BEHAVIOR ON REPRODUCTIVE FUNCTION

#### Method

**Assessment of Female Puberty Onset.** Starting at 30 days of age, female rats were checked daily for vaginal opening. After that, daily vaginal smears were taken to establish the age of occurrence of first and second estrus.

**Female Sexual Behavior.** On the proestrus day, during the first 2 hr after the beginning of the dark cycle, virgin females were tested with a proven breeder male. Rats were videotaped for 15 min in steel cages (70 cm  $\times$  70 cm  $\times$  35 cm) with a front wall of glass, which allowed complete viewing of the rats. The indexes of sexual receptiveness used were the lordosis quotient (the proportion of mounts that are accompanied by lordosis) and the mean lordosis intensity (the "quality" of lordosis) (Hardy & DeBold, 1972; Sodersten & Hansen, 1977). The lordosis quotient was calculated by dividing the total number of lordosis by the number of mounts. The intensity of lordosis was assessed using a three-point scale: 1 = slight flex of spin, slightly raised head and hips with tail base elevated from floor; 2 = spinal flex, head at an approximate angle of 30° with the horizontal, front paws placed slightly forward and hind legs straightened up stiffly; and 3 = pronounced spinal flex, head at an angle of 45° or more with the horizontal. The lordosis intensity index was calculated by



**FIGURE 2** Effects of variations in received licking behavior on open-field test activity of female and male adult rats from the first litter of LL and HL mothers; (A) total locomotion, (B) time spent in central quadrants, (C) latencies to enter into the central quadrants. Data are presented as means  $\pm$  SEM and were analyzed by a two-way ANOVA (group  $\times$  gender).

dividing the total points of each rat by the total number of lordosis displayed. Data are expressed as median (IQR) and were compared using the Mann–Whitney *U*-test.

**Ovulation.** On the morning of the following estrus day, females were decapitated, the ovaries were removed and the oviducts were dissected and compressed between two glass slides. The number of oocytes of both



oviduct ampullae was counted under microscope (Zeiss, Goettingen, Germany) with a  $2.5\times$  lens. The number of oocytes from both oviducts was summed and data were compared using the Mann–Whitney  $U$ -test.

**Male Sexual Behavior.** Sexually inexperienced adult males were tested for sexual behavior 1–2 hr after the beginning of the dark phase. The male was habituated to the testing environment (70 cm  $\times$  70 cm  $\times$  35 cm) for 10 min before behavioral testing. After this period, a receptive female was introduced into the observation cage, and the behavior was videotaped. Each test lasted until one copulatory series was completed. Each series included the events from the first mount to the end of the postejaculatory interval (the first intromission of the second ejaculatory series). If a male failed to ejaculate in a period of 30 min, the test was finished. The number of animals showing mounts intromissions and ejaculation was registered. In those males that ejaculated, the following parameters were analyzed: latency of the first mount, number of mounts, latency of the first intromission, number of intromissions, and the latency of ejaculation (Fernandez-Guasti, Larsson, & Beyer, 1986; Rhees, Lephart, & Eliason, 2001). Females used to test the sexual behavior of the males were previously ovariectomized and brought to sexual receptiveness by subcutaneous injections of estradiol benzoate (20  $\mu$ g/rat) and progesterone (500  $\mu$ g/rat) plus estradiol benzoate (20  $\mu$ g/rat) 54 h and six before the test, respectively. All females were first tested with a sexually active male and only those proven to be lordotic were used.

Male reproductive parameters are expressed as median (IQR) and were compared using the Mann–Whitney  $U$ -test. The number of ejaculating males in the two groups was compared by the  $\chi^2$  test.

**Spermatozoa Quantification.** Homogenization-resistant testicular spermatids in the testes and sperm in the caput/corpus epididymidis and cauda epididymidis were enumerated in Neubauer chambers. Daily sperm production per testis was determined by dividing the total number of homogenization-resistant spermatids per testis by 6.1 days, the number of days of a seminiferous cycle in which these spermatids are present. Transit times through the caput/corpus epididymidis and cauda epididymidis were calculated by dividing the number of sperm within each of these regions by the daily sperm production. The data (means  $\pm$  SEM) were compared by the Student's  $t$ -test.

## Results

**Female Puberty Onset.** No significant differences were found in the time of vaginal opening or in the age of first

**Table 2.** Puberty Parameters (Means  $\pm$  SEM) and Ovulation During Adulthood (Median (IQR)) in Female Pups from the First Litter of LL and HL Mothers

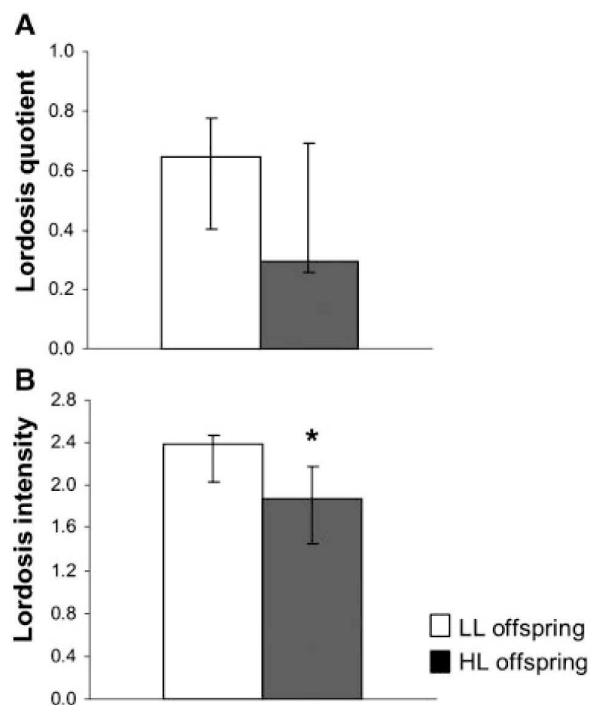
	LL Offspring	HL Offspring
Age of vaginal opening (days)	36.1 $\pm$ 1.0	37.3 $\pm$ .8
Age of first estrus (days)	40.7 $\pm$ 1.4	40.5 $\pm$ 1.1
Age of second estrus (days)	48.2 $\pm$ .8	48.8 $\pm$ 1.8
Number of oocytes	10 (9–11)	7 (7–8)*

\* $p < .001$ , Mann–Whitney  $U$ -test.

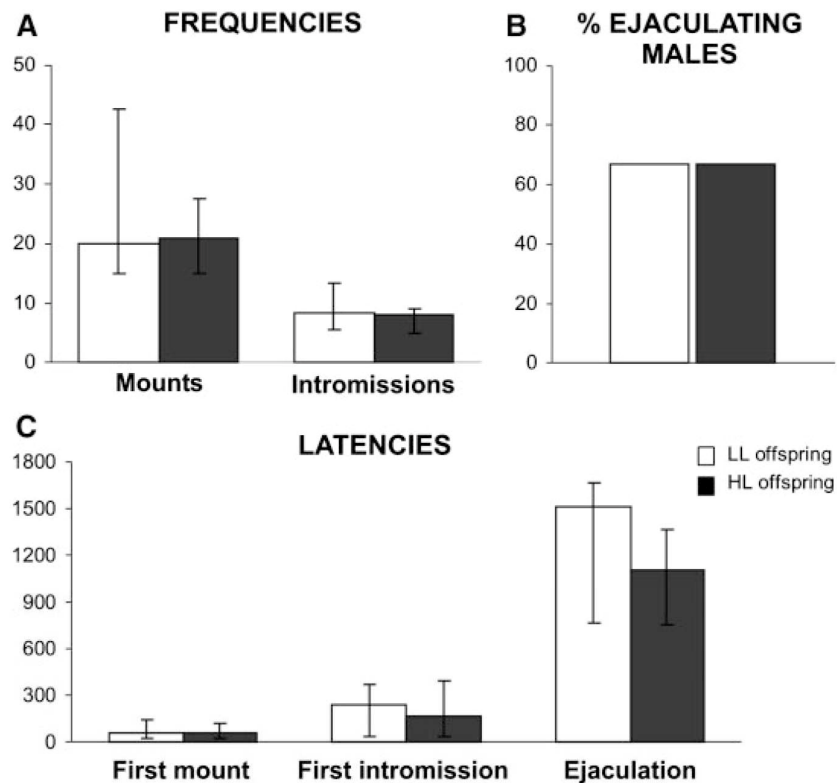
and second estrus appearance between LL and HL female offspring (see Tab. 2).

**Female Sexual Behavior.** Although no statistical differences were obtained in the lordosis quotient between offspring of LL ( $n = 10$ ) and HL ( $n = 13$ ) mothers (Mann–Whitney  $U = 45.50$ ), the lordosis intensity was significantly lower in HL female offspring (Mann–Whitney  $U = 32.00$ ,  $p < .05$ ) (see Fig. 3A and B).

**Ovulation.** Adult female offspring of HL mothers exhibited a significant reduction in ovulation. The number of oocytes in HL females ( $n = 10$ ) was significantly



**FIGURE 3** Effects of variations in received licking behavior on lordosis quotient (A) and lordosis intensity (B) of female pups from the first litter of LL and HL mothers. Data are plotted as median (IQR) and analyzed by Mann–Whitney  $U$ -test, \* $p < .05$ .



**FIGURE 4** Effects of variation in received licking behavior on the male sexual behavior of male pups from the first litter of LL and HL mothers; (A) frequency of mounts and intromissions, (B) percentage of ejaculating males, and (C) latencies to the first mounts, first intromission and ejaculation. Data are plotted as median (IQR) and analyzed by Mann–Whitney  $U$ -test,  $p < .05$ .

lower than that observed in LL females ( $n = 13$ ) (Mann–Whitney  $U = 20.0$ ,  $p < .001$ ) (see Tab. 2).

**Male Sexual Behavior.** The number of ejaculating males in the 30-min session was not different between LL (6/9, 66.7%) and HL males (8/12, 66.7%) ( $\chi^2$  test). As shown in Figure 4, the number of mounts and intromission, as well as the latency to perform those behaviors did not

significantly differ between HL and LL adult male offspring (that ejaculated in the 30-min test).

**Sperm Quantification.** The number of spermatids per testis, daily sperm production, number of spermatozoa, and sperm transit time through per caput + corpus and cauda of the epididymis did not differ between the two groups of males (LL:  $n = 9$ , HL:  $n = 10$ ) (see Tab. 3).

**Table 3. Sperm Quantification in Adult Male Offspring from the First Litter of LL and HL Mothers (Means  $\pm$  SEM)**

	LL Males	HL Males
Number of spermatids ( $10^6$ /testis)	231.1 $\pm$ 9.6	225.1 $\pm$ 7.5
Number of spermatids ( $10^6$ /g/testis)	161.4 $\pm$ 6.6	156.6 $\pm$ 5.4
Daily sperm production (number of spermatids $\times 10^6$ /testis/day)	37.8 $\pm$ 1.6	36.9 $\pm$ 1.2
Number of spermatozoa $\times 10^6$ /caput + corpus of epididymis	132.3 $\pm$ 7.4	127.9 $\pm$ 9.3
Number of spermatozoa $\times 10^6$ /g/caput + corpus of epididymis	523.5 $\pm$ 29.4	501.7 $\pm$ 29.7
Number of spermatozoa $\times 10^6$ /cauda of epididymis	131.4 $\pm$ 12.4	142.7 $\pm$ 12.4
Number of spermatozoa $\times 10^6$ /g/cauda of epididymis	707.9 $\pm$ 57.4	741.0 $\pm$ 55.8
Sperm transit time (days) through caput/corpus of epididymis	3.5 $\pm$ .2	3.5 $\pm$ .3
Sperm transit time (days) through Cauda of epididymis	3.5 $\pm$ .3	3.8 $\pm$ .3

## EXPERIMENT 4: EFFECTS OF RECEIVED LICKING BEHAVIOR ON DEVELOPMENTAL PARAMETERS

### Method

**Body Weight.** Weights of the second litters of LL and HL mothers were registered on Day 0 (day of birth), Day 10, and Day 21 (day of weaning). The weight (g) of the whole litter (male + female pups) was divided by the total number of pups. Thus, we obtained an average value of the body weight for each pup, which was used for statistical analysis.

**Assessment of Eye Opening.** Age of eye opening was assessed on the second litter of LL and HL mothers. Beginning on postnatal Day 10, the litter was removed from the maternal cage; each pup was gently inspected for eye opening and then returned to the nest. This procedure took no more than 1 min, and it was repeated daily until all the pups opened their eyes. Eye opening was defined as the slightest break in the membrane sealing the lids of both eyes.

Pups' developmental parameters are expressed as means  $\pm$  SEM (LL and HL) and were analyzed by the Student's *t*-test.

### Results

Pups from HL mothers ( $n = 8$ ) opened their eyes earlier than pups of LL mothers ( $n = 7$ ); however, the two groups did not differ in the size (number of pups) or body weight of litters on Day 1 postnatal or in the mean weight of the culled litter on Day 10 postnatal and at weaning (21 days of age) (Tab. 1).

## DISCUSSION

### Maternal Behavior of LL and HL Mothers

Present results showed that Wistar lactating rats could also be classified into high (HL) and low (LL) licking dams, as observed by Meaney and coworkers in the Long-Evans strain (Caldji et al., 1998; Champagne et al., 2003). However, we found some differences with the previously reported data, since our LL and HL mothers did not show differences in the frequency of arched-back posture, while in Long-Evans rats the frequency of licking behavior was significantly correlated with the frequency of adopting an arched-back nursing posture (Caldji et al., 1998; Champagne et al., 2003). As in the present study, others (Moore, Wong, Daum, & Leclair, 1997) have found that

licking occurs when the dam is in the nest but not during crouching. This discrepancy could be attributed to strain differences in maternal behavior. Indeed, using the neonatal handling procedure, a previous study (Pryce, Bettschen, & Feldon, 2001) showed differences in the behavior of Wistar mothers after the return of the pups to the nest (unchanged licking behavior and less augment in arched back posture), as compared to the Long-Evans strain (Liu et al., 1997).

Accordingly with the results obtained by Champagne et al. (2003), LL and HL mothers did not differ in the time spent in contact with the pups or in the total time spent crouching. Also, here we show that the body weight of the pups on postnatal Day 10 and Day 21 was similar in the two groups of mothers. Thus, despite the differences, the natural variations in the maternal behavior provided by both groups of Wistar rats (HL and LL) lie within a normal range of adequate level of maternal care.

### Emotionality of LL and HL Offspring

Results show that Wistar rats born and raised by HL mothers were less inhibited in the open field test, in accordance with results obtained by Caldji et al. (1998) using Long-Evans rats. Moreover, Menard et al. (2004) showed that Long-Evans male offspring of HL mothers were less fearful in the shock-probe burying test. These authors also demonstrate that HL offspring (but not LL offspring) displayed shock-induced increases in c-Fos-IR in limbic structures (dentate gyrus, ventral subiculum, lateral septum, nucleus accumbens shell, and periaqueductal gray) implicated in the reduction of fear. It has been proposed that maternal licking behavior modifies neural circuits associated with fear reduction, leading to less fearful animals in adulthood (Menard et al., 2004). Moreover, natural differences in maternal behavior can induce alterations in neuroendocrine systems implicated in fear regulation. Indeed, HL offspring have decreased CRF receptor levels in the locus coeruleus (LC) as well as increased GABA<sub>A</sub> and benzodiazepine receptor levels in the basolateral amygdala (Caldji et al., 1998).

On the other hand, a main gender effect was observed in the latency to enter into the center of the arena, with females having shorter latencies than males. Interestingly, similar sex differences were found in previous studies in our laboratory in handling experiments (Padoin et al., 2001), where handled females showed higher levels of exploration than did males. These results are in accordance with several studies showing gender differences in emotional behavior, where female rats exhibited less fear or anxietylike behaviors than did males (Fernandes, Gonzalez, Wilson, & File, 1999; Johnston & File, 1991; Steenbergen, Heinsbroek, Van Haaren, & Van de Poll, 1989).



## Reproductive Function of LL and HL Offspring

The age of puberty onset, evaluated by the time of vaginal opening and the occurrence of first and second estrus, did not differ between females born and raised by LL and HL mothers. Therefore, the difference in the amount of licking behavior received by the pups appears to have no influence on the onset of puberty of females, at least with the variables analyzed in the present study. In contrast, Cameron et al. (2005) found that females from LL mothers showed vaginal opening earlier than did the offspring of HL mothers. This discrepancy could also be due to strain differences.

Interestingly, here we show that natural variations in maternal care altered reproductive function of females in adulthood. Females born and raised by HL mothers had a significant reduction in ovulation and lordosis intensity compared with females of LL mothers. Thus, the effect of licking seems to be specific for the intensity of the lordosis with no influence on the frequency of the behavior. Differences in lordosis reflex intensity have been related with different levels of estrogenization and LH levels and in induction of pregnancy (Gans & McClintock, 1993; Lehmann & Erskine, 2004). In the same line, Cameron et al. (2005) showed that the sexual receptivity and the rate of pregnancy were reduced in females of HL mothers compared with the LL ones. Conversely, Champagne et al. (2003) found no difference in the fertility of the offspring of LL and HL mothers. However, it is important to notice that the impairment in reproductive function presented by HL female offspring was partial as they still maintain their reproductive capability. All females still ovulate and were sexually receptive.

The effects observed were much less pronounced than those reported by studies using the neonatal handling procedure (Gomes et al., 1999). Handled females showed a drastic reduction in ovulation, most of them presented anovulatory cycles and had a drastic reduction in sexual receptiveness. These results could be explained by taking into account that the variations in licking behavior observed here fall into a normal range of maternal behavior, while the augment of licking behavior induced by the handling procedure could be more intense. This latter possibility remains speculative, as we did not perform handling experiments.

In contrast, males born and raised by HL and LL mothers did not differ in sexual behavior. Moreover, no differences were detected in daily sperm production and maturation or in epididymal sperm storage capacity and epididymal transit.

Thus, reproductive parameters of male rats appear to be less susceptible to the mother's behavior than those of females. One putative explanation for this gender difference could rely on the sexually biased nature of

the anogenital licking (Moore & Morelli, 1979). Maternal licking may be divided in anogenital licking that is directed to the perineum of pups, and body licking, which includes snout and oral contact with the general body surface. Male pups receive more anogenital licking than do their female siblings (Moore, 1992; Moore & Morelli, 1979). It is possible that male pups are less sensitive to the higher frequency of licking displayed by HL mothers because they already receive high levels of anogenital licking. Therefore, a slight augment of licking may be without significant effect. It is important to note that we are showing variations in total licking of the pups (anogenital licking and body licking), and possibly for that reason our data diverges from the classical effect of maternal anogenital licking on offspring male reproduction (Moore & Morelli, 1979).

An alternative explanation for that gender difference could be a gender difference in the licking behavior. We may speculate that the HL mothers would have a basal level of licking directed to both male and female pups that would not differ from the LL ones. The higher level of licking of the HL mothers could mainly be due to an enhancement in the licking directed to female pups only. However, Champagne et al. (2003) did not find significant differences in total licking received by male as compared to female pups, although a different strain of rat than the present study was used.

## Relation between the Effects of the Maternal Behavior on Emotionality and Reproductive Function

Present results showed that individual differences in maternal behavior appear not only to be predictive of later emotionality and stress-responsivity in the offspring, but can also modulate the reproductive function in females. Besides being activated by stressful stimuli (Pacak & Palkovits, 2001; Van Bockstaele, Bajic, Proudfit, & Valentino, 2001), the LC participates in the control of luteinizing hormone (LH) secretion, and its activation is important for the occurrence of gonadotropin surges (Anselmo-Franci, Franci, Krulich, Antunes-Rodrigues, & McCann, 1997; Helena, Franci, & Anselmo-Franci, 2002; Liu, Diorio, Day, Francis, & Meaney, 2000; Lucion et al., 2003). LC is an integrative noradrenergic nucleus being an important modulator of both the hypothalamic–pituitary–adrenal and the hypothalamic–pituitary–gonadal axis. High maternal licking is associated with increased binding of  $\alpha 2$  inhibitory autoreceptor in the LC, and, as a consequence, an attenuated noradrenergic response to stress (Caldji et al., 1998; Liu et al., 2000). Thus, the decreased LC activity induced by the augment in maternal licking behavior could result in a diminished release of noradrenaline in the medial preoptic area (MPOA) and,

consequently, a low surge of LH and, as a consequence, a reduced ovulation.

### Developmental Parameters of LL and HL Pups

Present results showed that pups born and raised by HL mothers opened their eyes earlier than pups of LL mothers. This result is in line with neonatal handling experiments, where handled pups showed precocious eye opening (Levine, 2000). Accordingly, in a previous study (Sale et al., 2004), it was found that mice pups raised in an enriched environment received increased licking behavior and presented a significant acceleration in the age of eye opening when compared with pups raised in standard conditions. It was suggested that the acceleration of the development could be maternally mediated (Sale et al., 2004), however, another study (Welberg, Thiruvikraman, & Plotsky, 2006) showed that the environmental enrichment during the neonatal period does not affect maternal licking in rats, but it decreases the time in nest. Present results confirm the hypothesis that the stimulation provided by the mother, in the form of licking, affects pups' development. It has been shown that variations in maternal care can affect neurotrophin levels such as brain derived neurotrophic factor (BDNF) (Liu et al., 2000). Moreover, the acceleration in pups' eye opening was related to increased BDNF levels in the visual cortex (Sale et al., 2004). Taken together, these results support the hypothesis concerning maternal involvement in mediating this phenomenon. It could be argued that differences in the time of eye opening found in the present work could be related to variations in the normal growth of the pups. However, other developmental parameters, such as pup growth (measured as body weight gain), were not affected by different maternal licking levels. Thus, it seems that the developmental effects exerted by variations in maternal stimulation received by the pups were rather specific for precocious eye opening.

### GENERAL DISCUSSION

Present results showed that individual differences in maternal behavior appear not only to be predictive of later emotionality and stress-responsivity in the offspring but can also modulate pups' development and the reproductive function of female offspring. It is important to note that in the present study litters were not cross-fostered, therefore, it is difficult to discern whether the observed effects are a result of maternal behavior, maternal genetic factors, differences in the prenatal intrauterine milieu, or a combination of these.

In this line, experiments with two different strains of mice, showed that an interaction of both genetic and

environmental factors determine stress and anxiety responses (Priebe et al., 2005).

As we hypothesized, high licked female pups showed a reduction in reproductive function in adulthood. This phenomenon seems to contradict the expected outcome of a greater maternal care, and could question the beneficial effects of licking, as the impairment of female reproductive function suggests a maladaptive effect. However, it is important to note that although reduced, all female pups from HL mothers preserve their reproductive capability.

In the present case, as in many other physiological functions, a behavioral homeostasis is probably required for an appropriate development. Female pups raised by LL mothers seem to follow a normal pattern in terms of number of oocytes and lordosis behavior. It is interesting to note that maternal separation induced no long-lasting change on sexual behavior and reproductive physiology in females, whereas it decreased sexual behavior in males (Rhees et al., 2001). Thus, while sexual behavior and reproductive function in males are more affected by reduced maternal behavior, females appear to be more susceptible to a higher level of maternal behavior. Nevertheless, it is important to note, that the effects of maternal separation on offspring stress responses appear not to be mediated solely by alterations in maternal behavior (Macri, Mason, & Wurbel, 2004).

As mentioned above, the differences in licking behavior apparently lie within the normal range of parental behavior (Champagne et al., 2003). Why then do these spontaneous variations exist and why they induce effects on female's reproduction and affective behavior? It is possible that maternal behavior serve as an interface between the offspring and environmental conditions and that the differences in maternal licking behavior reflect individual strategies for programming offspring's rudimentary responses to future environmental demands (Caldji et al., 1998; Fish et al., 2004). Meaney and coworkers have shown that gene expression is significantly altered in the hippocampus of adult rats as a repercussion of maternal care received during lactation. This maternal programming involves modifications of epigenetic mechanisms, including DNA methylation and histone modification of a nerve growth factor inducible protein A transcription factor binding site on a brain-specific glucocorticoid receptor promoter (Weaver et al., 2004). These differences in gene expression may, at least in part, form the molecular basis for the effect of early-life experience on the development HPA responses to stress in the offspring, which are endured throughout life (Weaver, Meaney, & Szyf, 2006).

Similar mechanisms could be responsible for the effects on female reproductive function through acting in brain areas related to reproduction. It would be interesting to explore this possibility.



## NOTES

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## **CAPÍTULO II**

### **OVERLAPPING LITTERS IN RATS: EFFECTS ON MATERNAL BEHAVIOR AND OFFSPRING EMOTIONALITY**

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## Overlapping litters in rats: Effects on maternal behavior and offspring emotionality

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### Abstract

Female rats have a fertile postpartum estrus, which can result in a simultaneous gestation and lactation and later in the overlapping of two different-age litters. These different physiological and contextual situations may affect the maternal behavior of lactating rats and provoke long-lasting changes in the affective behavior of the litter. Therefore, we aimed to assess the effect of pregnancy and of litter overlapping on the maternal behavior of lactating rats and to describe the maternal- and anxiety-like behaviors of the juveniles that remained in contact with their younger siblings. Results showed that pregnant lactating rats spent more time outside the nest and less time nursing than non-pregnant mothers. On the other side, mothers with overlapping litters licked less the newborn pups than mothers with single litters. These deficits in maternal licking received by neonates were overridden by the juveniles' licking behavior to their younger siblings. Adult male and diestrous female rats reared with younger siblings showed a reduced anxiety-like behavior as compared to age-weaning matched animals without this experience. Thus, natural changes in the reproductive conditions and in the early experience, affect the maternal behavior and long-term modulate affective behavior of the individuals.

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**Keywords:** Postpartum estrus; Early experience; Maternal behavior; Anxiety

### 1. Introduction

Female rats can conceive in the postpartum estrus, which normally occurs during the first 6 to 15 h following parturition [1,2]. This enables them to gestate one litter while simultaneously nurturing another. Even though the maintenance phase of maternal behavior is primarily based on pups' sensory stimulation and not on hormonal factors [3,4], no study to our knowledge has investigated how a concurrent gestation affects the maternal behavior of lactating rats.

Following the birth of a new litter, older offspring may remain in the maternal burrow and share the nest with their younger siblings [5,6]. As the maternal behavior primarily

depends on the stimulation of the pups, it could be hypothesized that during litter overlapping, the mothers adapt their behavior according to the characteristics and demands of the two different-age litters.

The behavior of juvenile rats toward neonates has been well studied in the context of maternal sensitization experiments (induction of maternal behavior through continuous exposure to neonatal pups) [7–10]. However, few studies [5,11] have explored this behavior in a more ethological context, as would be the case with the overlapping of successive litters. While some studies reported that older pups may show care-giving behaviors toward neonates and therefore complement the maternal care of the lactating female [5,11], others suggested that juveniles compete with their younger siblings for maternal attention [12].

Early-life environmental factors exert long-term influences on rodents' affective behavior [13–17]. In the present model of

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overlapped litters, the pre-weaning experiences of the juveniles include not only those acquired during interaction with the mother and age-matched littermates, but also with the newborn siblings. Based on previous findings showing that a previous maternal experience reduces anxiety-like behavior later in life [18], we hypothesized that the early experience of juveniles with younger siblings, which provokes maternal-like behaviors [11], would produce a long-term decrease in the emotionality of overlapped reared juveniles.

In this study, we aimed to assess if: 1) postpartum pregnancy modifies maternal behavior, 2) the maternal behavior is affected by the presence of older siblings and 3) the experience with younger siblings affects the emotionality of adult offspring.

## 2. Methods

### 2.1. Animals

Male and female Wistar rats were obtained from the colony maintained in the Federal University of Rio Grande do Sul Laboratory Animal Facility. Animals were kept under 12-h light/dark cycle conditions (lights off at 18:00 h) and in a stable environmental temperature (21 °C) with ad-lib access to rat chow (Nutrilab, Colombo, Brazil) and water. All procedures used in this study followed the guidelines for the care and use of Laboratory Animals from the National Institute of Health and were approved by the Research Committee of the Federal University of Rio Grande do Sul.

### 2.2. Experiment I: maternal behavior and total stimulation received by pups following postpartum mating

#### 2.2.1. Experimental groups and comparisons

Experimental groups are illustrated in Fig. 1.

To assess the effect of pregnancy on the maternal behavior of lactating rats, the following groups of mothers were compared:

- 1) Primiparous non-pregnant lactating rats (group NPL,  $n=10$ ): females nursing the first litter, mated in postpartum estrus but that did not become pregnant.
- 2) Primiparous pregnant lactating rats (group PL,  $n=14$ ): females nursing the first litter while pregnant with a second litter conceived at the postpartum estrus.

To assess the effect of raising two different-aged litters on the maternal behavior of postpartum females to the newborn pups, the following groups were compared:

- 3) Multiparous lactating rats with overlapped litters (group MOL,  $n=14$ ): females nursing the second litter conceived at postpartum estrus and two juveniles from the first litter.
- 4) Multiparous lactating rats with a single litter (group MSL,  $n=10$ ): females nursing the second litter conceived at postpartum estrus; the first litter was removed before parturition of the second litter.

To determine the effects of litter overlapping on maternal behavior toward juveniles we compared the behavior of MOL mothers with that of:

- 5) Late postpartum rats with a single litter (group LPSL,  $n=10$ ): females raising two juveniles from the first litter, after PPD 21.

In addition, the MOL dams' behavior toward their juveniles vs. newborn pups was compared.

To compare the total stimulation received by the newborn pups reared in single vs. overlapped litters, the behavior of the two juveniles (older litter of MOL mothers) toward their younger

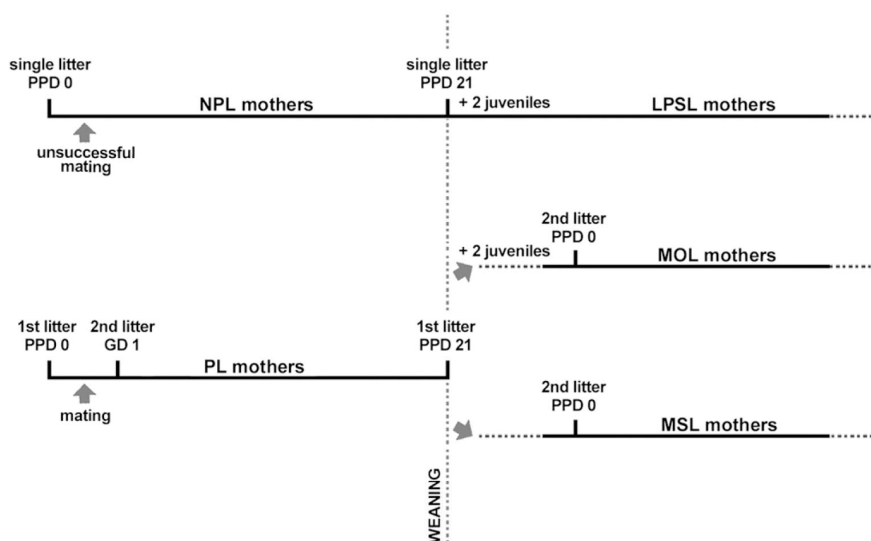


Fig. 1. Schematic representation of experimental groups of mothers studied. NPL: primiparous non-pregnant lactating rats, PL: primiparous pregnant lactating rats, MOL: multiparous lactating rats with overlapped litters, MSL: multiparous lactating rats with a single litter, LPSL: late postpartum rats with a single litter.

siblings was registered. The total stimulation received by newborn pups in the overlapped litter was calculated by adding the behavior of the mother and the behavior of the two juveniles.

### 2.2.2. Breeding procedure

Breeding was synchronized by monitoring the estrous cyclicity of females. Vaginal smears were taken daily and nulliparous females (90 days old) on their second regular proestrus were placed overnight with a sexually experienced male. Successful mating was confirmed the next morning by the presence of spermatozooids in the vaginal smear and that day was designated as day 1 of gestation (GD1). Animals were marked with a permanent-ink pen in the tail for individual identification and housed in white polycarbonate cages (16 cm×28 cm×42 cm) in same-sex groups of 3 to 4 females and of two males. Approximately 7 days before delivery, pregnant females were separated and housed in transparent polycarbonate cages (16 cm×28 cm×42 cm) with the same males as before. Starting on GD20, the presence of pups was checked daily at the beginning and end of the light period, and on the day of birth (PPD0) the behavior of parturient females and males was observed to avoid agonistic behaviors or cannibalism of the pups. On the following day (second litter GD1), the male was removed, the presence of spermatozooids was checked, the litter was weighed and the number of pups was culled to 8 per dam (with usually 4 or at least 3 pups of each sex). At weaning (PPD21), juveniles of the first litter were removed, weighed and housed in same-sex same-litter cages.

In the case of mothers that remained with juveniles (MOL and LPSL), one male and one female were returned to the home cage. Female juveniles were marked in the ears and tail with a permanent-ink pen for individual identification in behavioral observations. Beginning on GD21 of the second litter, the presence of newborn pups was checked twice a day, and on PPD1 the second litter was weighed and culled in the same way as the first one.

### 2.2.3. Behavioral observations

The behaviors of lactating females and of juveniles (if present) were scored daily during four, 72-min observation sessions from PPD1 to PPD10. Observations occurred at regular times with three periods during the light phase (1000, 1300, 1600 h) and one during the dark phase of the light–dark cycle (1830 h). Within each session, the behavior of each subject was scored every 3 min (25 observations per period×4 periods per day=100 observations/subject/day). Data are shown as means±SEM of the percentage frequencies from a total of 1000 observations for each rat (100 observations per day during the first ten day postpartum) [19].

The following behaviors of the mothers were registered: *Mother off pups*, the mother is away from the nest and without physical contact with the pups; *Licking the pups* (either the body surface or its anogenital region); *Nursing*, the mother nursing the pups in either an *arched-back posture*, a *blanket posture* in which the mother lays over the pups, or a *supine posture* in which the mother is lying either on her back or side while the pups nurse; *Retrieving and mouthing*, retrieval of the displaced pups or oral repositioning of the pups into the nest; *Nest building*, the female pushes or picks up nest material in

her mouth; *Aggressive behavior*, any agonistic behavior toward juveniles, that usually consists in dominating postures and mild attacks.

Additionally, the following behaviors of the juveniles were registered: *Lying in contact* or *hovering over* the pups, *Licking*, *Retrieving* and *Off neonatal pups*, defined as in the mother's behavior and *Play behavior*, any playful behavior directed to the juvenile sibling or the mother. The occurrence of cannibalism or nipple displacement was also registered. The frequency with which neonatal pups were observed away from the nest and the time that the second litter was effectively left alone (absence of the mother and the two juvenile siblings) in the nest was also recorded.

### 2.2.4. Statistics

Behavioral data fit parametric testing assumptions (Kolmogorov–Smirnov and Cochran *C* test) and therefore were analyzed by parametric tests. Maternal behavior was analyzed by the Student's unpaired *t* test except for the comparison of MOL maternal behavior toward juvenile vs. newborn pups, and of male and female juveniles' behavior, which were performed by the Student's paired *t* test. Correlation analysis between the length of gestation and the number of pups was performed using the Pearson correlation analysis. The number of females that conceived/not conceived during the first mating or the postpartum estrus was compared by the Fisher exact probability test.

## 2.3. Experiment II: effect of litter overlapping on the emotionality of overlapped reared juveniles

### 2.3.1. Experimental groups

To assess the long-term effects induced by the early experience with newborn siblings on emotionality, we analyzed the anxiety-like behavior of overlapped reared juveniles (senior offspring of MOL mothers: males, *n*=15; females, *n*=11) and single reared late-weaned juveniles (offspring of LPSL mothers: males, *n*=11; females, *n*=8) in the open field and the elevated plus maze tests.

### 2.3.2. Procedure

Adult male and female offspring from MOL and LPSL mothers were used in this study. Pups were maintained with their mothers until weaning (PPD35–39), and then housed in same-sex, same-litter groups of three to four animals per cage and left undisturbed until the time of testing (approximately 90 days of age). The behavior of adult male and female rats was evaluated in the open field test and four days later the same animals were tested in the plus maze test. Females were tested in diestrus. For monitoring oestrus cycle, daily vaginal smears were taken, and only those females with two consecutive regular cycles were used for behavioral testing.

### 2.3.3. Behavioral tests

Behavioral tests were performed in a separate testing room with the same temperature and photoperiod conditions of the breeding room, during the light period of the cycle. Animals were individually housed, transported to the testing room and



Table 1  
Characteristics of single vs. overlapping litters

	Single litter	Overlapped litter
Litter size ( <i>n</i> )	8.5±0.5	9.6±0.7
Sex ratio ( <i>n</i> males/total)	0.52±0.04	0.48±0.05
Litter weight on day 1 pp (g)	68.2±3.7	76.9±8.5
Mean pup weight on day 10 pp (g)		
Males	20.3±0.9	21.4±0.7
Females	19.8±0.9	21.0±0.6
Mean pup weight on day 21 pp (g)		
Males	41.3±1.6	41.5±1.5
Females	40.1±1.2	40.1±1.4

\* $p < 0.05$  Student's *t* test.

left to acclimatize at least 1 h before testing. After each test, the apparatus used was cleaned thoroughly with 70% ethanol and allowed to dry completely between test sessions. Behavioral tests were recorded on VHS-video tape and analyzed later using the computer software: The Observer Pro® (Noldus, Wageningen, The Netherlands) by an experienced observer blind to the subjects' experimental condition.

#### 2.3.4. Open field test

The apparatus was a 100 cm square open field that was evenly illuminated by overhead fluorescent lights. The arena was divided into twenty-five 20-cm squares painted on the floor.

Central area was defined as the nine inner quadrants of the arena. Each animal was placed in one corner of the open field and its behavior was videotaped over a 5-min session. The following behavioral parameters were recorded: frequency of entries and time spent in central quadrants of the arena, duration (s) of locomotion and frequency of rearing [20].

#### 2.3.5. Elevated plus maze test

This model consists of an elevated (40 cm above the floor) plus-shaped maze with two opposite enclosed (50 cm-high walls) and two open arms, each measuring 50 cm long × 10 cm wide. Each animal was placed in the center of the maze facing a closed arm and allowed to freely explore the maze for 5 min. The behavioral parameters assessed were: frequency of total entries (arm entry, all four paws into an arm), percentage of open arms entries ( $100 \times \text{open}/\text{total}$ ), percentage of time spent in the open arms ( $100 \times \text{open}/\text{total}$ ) and frequency of head dipping, stretched-attend posture and rearing [21].

#### 2.3.6. Statistics

Data obtained in the open field and elevated plus maze tests were compared by a two-way analyses of variance (ANOVA) using group (rearing condition) and sex as factors. Post hoc comparisons were carried out using Newman–Keuls test. In all cases, a  $p < 0.05$  was considered significant.

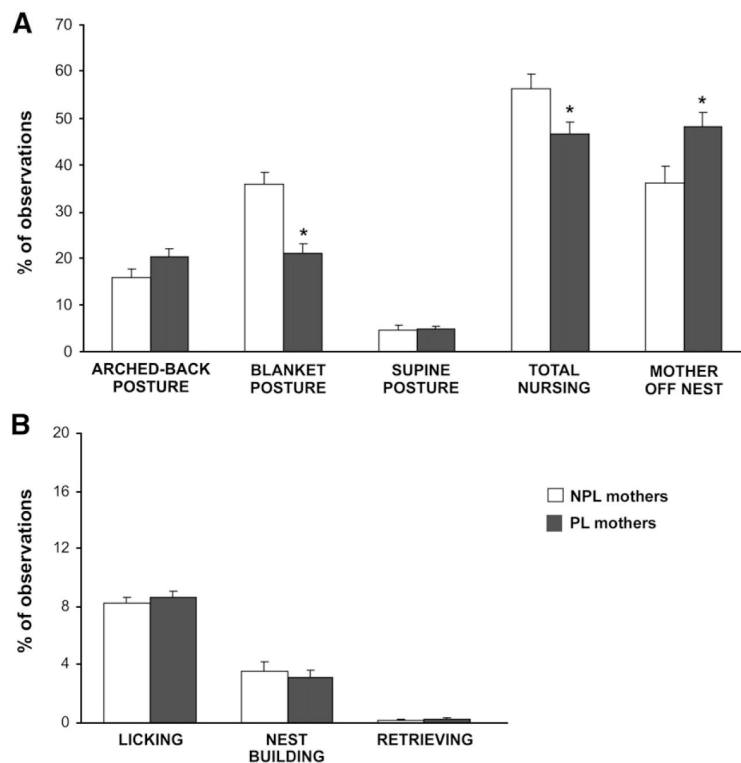


Fig. 2. Maternal behavior toward neonates displayed by: primiparous non-pregnant lactating rats (NPL mothers), and primiparous pregnant lactating rats (PL mothers). A. Nursing postures, total nursing behavior and frequency of the mother outside the nest. B. Active components of maternal behavior: licking, nest building, and retrieving of the pups. Data are shown as means±SEM of the percentage frequencies from a total of 1000 observations for each rat (100 observations per day). \*Represents significant differences, Student's unpaired *t* test,  $p < 0.05$ .

### 3. Results

#### 3.1. Experiment I

##### 3.1.1. Characteristics of gestations and pups

From a total of 28 females that were mated on the postpartum estrus, 15 (53.6%) became pregnant and successfully delivered a second litter. This percentage was not different from that of successful pregnancies obtained in the first mating (46.9%) (Fisher exact probability test,  $p=0.64$ ).

The pregnancy duration (days) of the lactating mothers ( $27.6\pm 0.8$ ) was significantly longer than that of the non-lactating mothers ( $22.9\pm 0.2$ ),  $p<0.05$ , Student's  $t$  test.

The number of pups per litter and the sex ratio did not differ between single and overlapped litters. No significant correlation was detected between the length of the second gestation and the number of pups per litter (Pearson  $r=-0.17$ ,  $p=ns$ ). The weights of single and overlapped litters on PPD 1, 10 or 21 did not differ (see Table 1).

##### 3.1.2. Effect of a concurrent pregnancy on dams' maternal behavior

As shown in Fig. 2, some components of maternal behavior were affected by the concurrent gestation and lactation. Thus, the total frequency that mothers were registered in a nursing position

was lower in pregnant (PL) as compared to non-pregnant (NPL) lactating females ( $p<0.05$ , Student's  $t$  test). PL mothers showed a significant decrease ( $p<0.05$ , Student's  $t$  test) in the blanket posture frequency compared to NPL females, while the other nursing postures (arched-back and supine) did not differ between the two groups ( $p=ns$ , Student's  $t$  test). Fig. 2 (panel B) also shows that the frequency that PL mothers were observed outside the nest leaving the pups alone was significantly higher than that observed in the NPL group ( $p<0.05$ , Student's  $t$  test).

##### 3.1.3. Effect of the litter overlapping on the dams' maternal behavior to newborn pups

The frequency of nursing the newborn pups did not differ between mothers with single (MSL) or overlapped litters (MOL), however, as shown in Fig. 3, the frequency in which the mother was outside the nest showed a tendency to be higher when litters were overlapped ( $p=0.06$ , Student's  $t$  test). While retrieving of neonates was not different between MSL and MOL mothers, licking behavior was significantly lower in MOL mothers ( $p<0.05$ , Student's  $t$  test), see Fig. 3.

##### 3.1.4. Effect of the litter overlapping on dams' maternal behavior to juveniles

The mothers were observed with significantly less frequency nursing the juveniles than the neonates (total frequency of

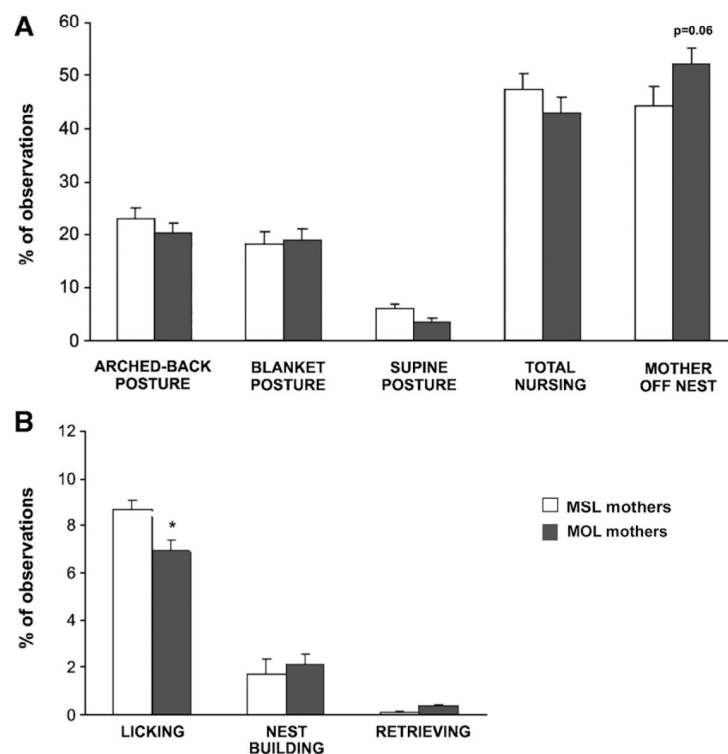


Fig. 3. Maternal behavior toward neonates displayed by multiparous lactating rats with overlapped litters (MOL mothers) and multiparous lactating rats with a single litter (MSL mothers) A. Nursing postures, total nursing behavior and frequency of the mother outside the nest. B. Active components of maternal behavior: licking, nest building and pup retrieving. Data are shown as means  $\pm$  SEM of the percentage frequencies from a total of 1000 observations for each rat (100 observations per day). \*Represents significant differences, Student's unpaired  $t$  test,  $p<0.05$ ).

nursing, neonates:  $41.45 \pm 1.75$  and juveniles:  $4.76 \pm 2.19$ ;  $p < 0.01$ , Student's paired *t* test). In addition the frequency of mothers' licking behavior ( $0.13 \pm 0.04$ ) and retrieving ( $0.12 \pm 0.10$ ) to juveniles was almost absent. MOL females' maternal and aggressive behaviors directed to juveniles differed from that of LPSL females. Nursing received by juveniles was very low during pre-pubertal period in the absence of a second litter, however, when younger siblings were present, the mothers accepted, in a more extent, juveniles' attempts to suckle (total frequency spent nursing, MOL:  $5.13 \pm 2.33$ ; LPSL:  $0.04 \pm 0.04$ ,  $p < 0.05$ , Student's *t* test). The juveniles in overlapping litters received less maternal licking (MOL:  $0.16 \pm 0.05$ ; LPSL:  $0.64 \pm 0.16$ ,  $p < 0.001$ , Student's *t* test) and some maternal aggressive behavior (MOL:  $0.43 \pm 0.13$ ; LPSL:  $0.03 \pm 0.02$ ,  $p < 0.05$ , Student's *t* test) when compared with single juveniles. However, despite some episodes of maternal aggression, juveniles in overlapped litters spent significantly more time in contact with the mother than juveniles from LPSL mothers (MOL:  $67.14 \pm 3.93$ ; LPSL:  $34.25 \pm 2.54$ ,  $p < 0.05$ , Student's *t* test).

### 3.1.5. Effect of litter overlapping on the behavior of the juveniles

Neither cannibalism, aggressive behavior toward neonatal or attempts to remove them from the nipples of the mother or from the nest were observed. Moreover, juveniles spent considerable time in the nest in close contact with their younger siblings, in the presence and even in the absence of the mother and licked younger pups (Fig. 4). No differences were found in the behavior of male and female juveniles (frequency of lickings: female:  $0.26 \pm 0.07$  and male  $0.32 \pm 0.08$ ; time (s) in contact: female:  $37.09 \pm 5.75$  and male  $36.41 \pm 5.78$ ; play behavior: female:  $2.45 \pm 0.29$  and male:  $2.52 \pm 0.29$ ).

### 3.1.6. Stimulation received by newborns in the overlapped litters

The social environment in which the newborn pups developed in the overlapping litters was quite different from

the standard familiar composition of single lactations. Thus, both the mother and the two older siblings shaped the stimulation that they received. MOL mothers licked less the neonates than MSL ones ( $p < 0.05$ , Student's *t* test), noteworthy, the total licking stimulation, received by the newborn pups raised by both groups, did not differ ( $p = \text{ns}$ , Student's *t* test), due to the licking behavior displayed by juveniles. Moreover, even though MOL mothers spent less time in the nest (Fig. 3), the frequency in which the newborns were left unattended did not differ between single or overlapped litters, due again to the presence of the juvenile siblings in the nest ( $p < 0.05$ , Student's *t* test) (see Fig. 4).

### 3.2. Experiment II. Effect of litter overlapping on the emotionality of juveniles

As shown in Fig. 5A, the behavior of adult rats in the open field did not significantly differ as a function of rearing condition or sex. The duration (s) of locomotion (group:  $F(1,41) = 0.13$ ; sex:  $F(1,41) = 0.01$ ), time spent (group:  $F(1,41) = 1.57$ ; sex:  $F(1,41) = 0.74$ ) and frequency of entries in central quadrants of the arena (group:  $F(1,41) = 1.77$ ; sex:  $F(1,41) = 0.03$ ) were not different between male and female overlapped reared juveniles and single reared late-weaned juveniles.

Interestingly, in the plus maze test, the experience with neonates during juvenile period reduced anxiety-like behavior (see Fig. 5B). Thus, overlapped reared juveniles significantly increased the percentage of entries ( $F(1,36) = 6.72$ ,  $p < 0.05$ ) and time spent in open arms ( $F(1,36) = 4.69$ ,  $p < 0.05$ ), and decreased the frequency of stretched-attend postures (males:  $11.29 \pm 1.54$  vs.  $8.00 \pm 4.30$ ; females:  $8.33 \pm 0.88$  vs.  $6.54 \pm 0.62$ ;  $F(1,36) = 5.97$ ,  $p < 0.05$ ) as compared to non-overlapped offspring during adulthood.

A significant interaction between rearing condition and sex was found for rearing behavior ( $F(1,36) = 6.32$ ,  $p < 0.05$ ), with overlapped reared females performing more rearing than non-

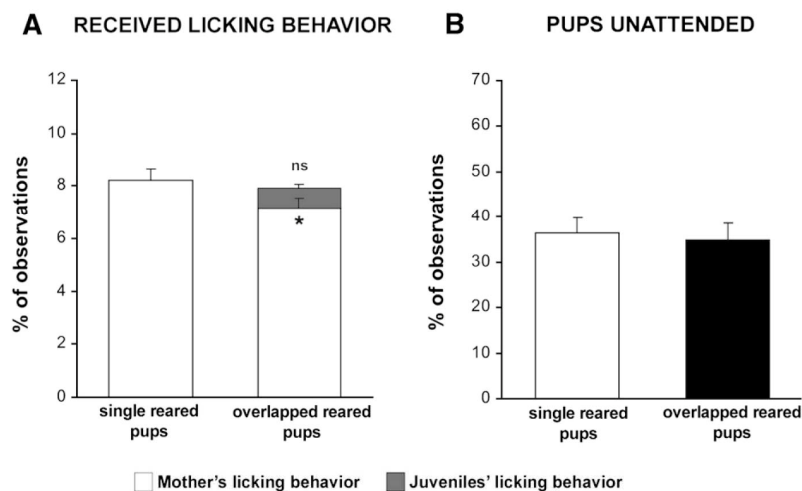


Fig. 4. A. Licking behavior received by neonatal pups reared in single or overlapped litters. B. Frequency of the mother spent off the nest and that pups remained alone in the nest, note that in overlapped litters either the mother or the juveniles could be attending to the pups on the nest. Data are shown as means  $\pm$  SEM of the percentage frequencies from a total of 1000 observations for each rat (100 observations per day). \*Represents significant differences, Student's unpaired *t* test,  $p < 0.05$ ).



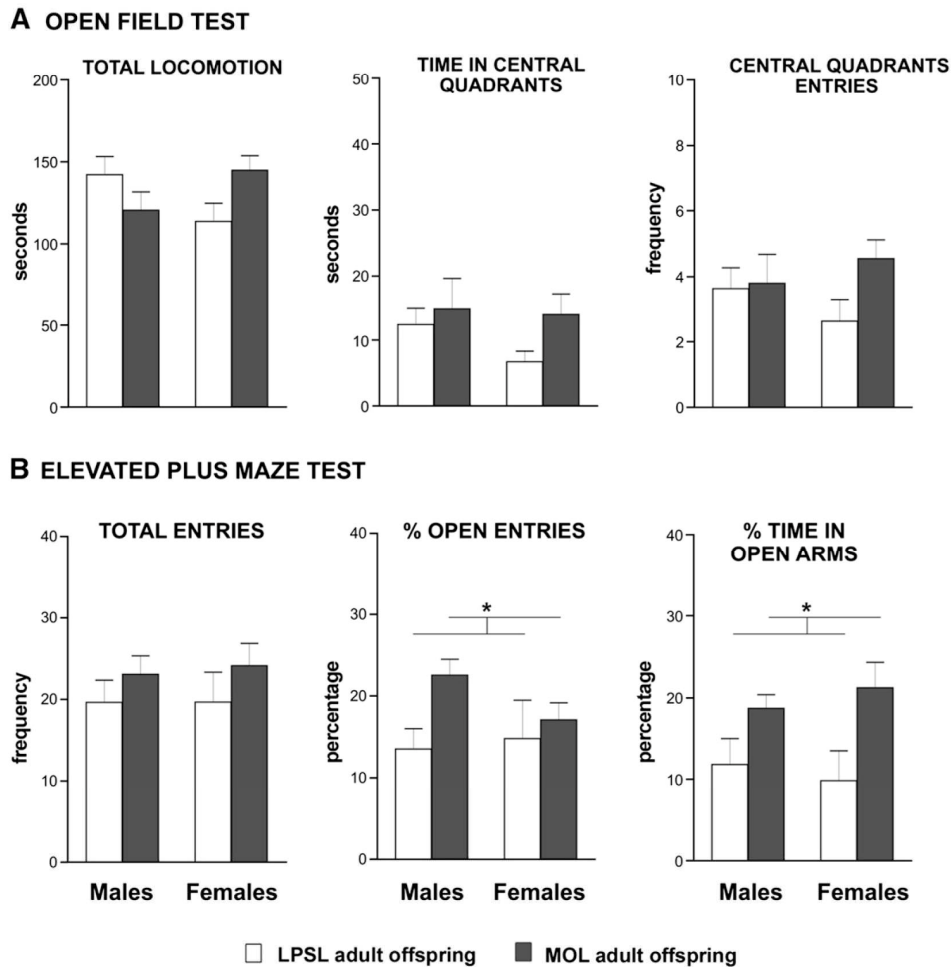


Fig. 5. Affective behavior of adult male and female single litter late-weaned pups (LPSL offspring) and overlapped reared juveniles (MOL offspring): A. Open field test, B. Elevated plus maze test. Data are presented as means  $\pm$  SEM and were analyzed by a two-way ANOVA (group  $\times$  sex). \*Represents significant differences,  $p < 0.05$ .

overlapped ones ( $17.7 \pm 2.2$  vs.  $10.3 \pm 0.9$ ,  $p < 0.05$ ) and non-overlapped reared females showing significantly less frequency than non-overlapped males ( $10.3 \pm 0.9$ ,  $p < 0.05$  vs.  $16.7 \pm 1.6$ ,  $p < 0.05$ ). As shown in Fig. 5B, the experience with neonates during juvenile period did not affect total activity in the plus maze test, since the number of total entries did not differ between groups ( $F(1,36) = 1.89$ ).

#### 4. Discussion

The main findings of the present study are:

1. Pregnancy decreased total nursing behavior and nest attendance of lactating dams, without affecting the growth of pups or the active components of maternal behavior.
2. Neonates reared with older siblings received less licking behavior from their mothers than those singly reared, but this deficit was compensated by licking performed by older siblings.

3. Being reared in close contact with younger siblings reduced anxiety-like behavior at adulthood in males and diestrous females as compared to control animals.

##### 4.1. Characteristics of gestations and pups

The duration of gestation that occurs concurrently with lactation was longer than a regular pregnancy [5,11,22], probably due to a suckling-induced delay in embryo implantation [23]. As reported in previous studies [5,24] a similar percentage of successful pregnancies occurred following mating in regular or postpartum estrus and the litter size and sex of the pups born after single gestations did not differ from those born after a gestation concomitant with lactation. According to previous reports [5,22,25,26], no differences were found in the growth of the pups reared in single or overlapped litters, suggesting that females that conceived during postpartum estrus may allocate the same resources to neonates in both reproductive conditions.

#### 4.2. *Pregnancy decreased nest attendance and total nursing behavior*

The behavior of mothers that were concomitantly gestating and lactating (PL) did not show major differences from that of non-pregnant mothers (NPL), consistently with findings in mice [27]. However, the frequency of being outside the nest was significantly higher in PL mothers, possibly due to an increase in body temperature forcing the termination of nest bouts to dissipate the heat load [28,29].

Interestingly, PL mothers showed decreased frequency of nursing relative to NPL mothers, due, at least in part, to the lower duration of the blanket posture. However, since the growth of the pups did not differ according to the mother's reproductive condition, it can be inferred that total milk transference was similar in both groups. In this sense, the blanket posture has been considered the least effective nursing position for milk letdown [30]. In addition, the arched-back nursing, considered the most effective posture for pups' suckling [30] was displayed similarly by both groups.

#### 4.3. *Raising overlapped litters affected dams' maternal behavior toward younger pups*

MOL mothers licked pups less than MSL mothers, showing that the presence of juveniles affected this maternal behavior component, accordingly with results found in prairie voles [31]. However, the total licking received by neonates raised together with juveniles was similar to that gained by pups in single litters due to licking received from the juveniles (present results, [31]). The mechanisms by which these changes in maternal licking behavior occur are unknown, although it could be speculated that MOL mothers experienced specific physiological changes that induced a decrease in licking behavior. Hormonal status appears not to be the responsible factor for these differences, since both MOL and MSL mothers were mated at postpartum estrus and were pregnant concurrently with the lactation, thus having similar endocrine profiles. We may also discard possible differences in pup-raising experience, since the two groups of dams had previous maternal experience.

A putative explanation for the reduced maternal licking behavior can be based on the pups' role in promoting their own care as a function of their physiological needs [32–34]. Less demanding pups, as could be those reared with juveniles, will elicit less maternal behavior.

Since licking behavior is an important modulator of several functions [15,35–37] the maintenance of this behavior in a constant amount during the overlapping of litters could be important. In this line, we have found that spontaneous high frequency of licking behavior during the neonatal period induces deleterious long-lasting effects in female's reproductive function [15].

#### 4.4. *The presence of a new litter modified the maternal behavior toward older pups*

As previously reported [5,11], we found that the birth of a second litter as a consequence of postpartum mating did

not automatically promote the weaning of the first litter. Furthermore, it appears that mothers continue to nurse juveniles only if a second litter is present, as juveniles that remained with mothers beyond PPD 21 without a younger litter (LPSL mothers), were rarely seen suckling. However, even though the presence of younger siblings increased the frequency of nursing, most commonly the mother nursed the neonates while the juveniles were in close contact with her without being nursed. This result is in accordance with previous findings showing extended suckling in rats (up to 60 days old) if they have access to foster dams that are actively nursing [38].

In addition, MOL, but not LPSL mothers displayed some aggressive behaviors toward juveniles (never to neonates). This behavior was not associated with juveniles' suckling attempts, but with approaches to the nest site when playing (present results, [5]), suggesting that aggression directed towards the older pups does not constitute a weaning mechanism but is probably related to the protection of the neonates.

The present results show that when pups of two different-age litters share the same nest environment, the mothers can adapt their behavior and direct maternal care preferentially toward the newborns. Maternal behavior and maternal motivation decline as lactation progresses [39,40], probably because older pups elicit less maternal behavior than newborns.

#### 4.5. *Juveniles showed parental behavior toward younger siblings*

In agreement with previous results [11], all juveniles licked their newborn siblings and spent significant amounts of time in contact with them, even when the mother was not present in the nest.

Juveniles may obtain direct benefits from the increased social and parental experience gained by interacting with the younger siblings [31,41]. Indeed, prior cohabitation with newborn pups reduces the latency to become maternal in adulthood [10,11,42,43]. Also, as discussed previously, the heightened parental responsiveness of juvenile rats could be a mechanism that evolved via kin selection to ensure that an older litter would not attack its younger siblings [44].

#### 4.6. *Experience with younger siblings affects emotionality of overlapped reared juveniles during adulthood*

Being reared in close contact with younger siblings induced licking behavior in juveniles and reduced anxiety-like behaviors in the plus maze test in males and diestrous females compared with age-weaning matched animals without newborn experience. In this line, primiparous rats are less anxious in the elevated plus maze than nulliparous during proestrus [18]. Also, prior maternal experience, following pregnancy and parturition or through a sensitization procedure, establishes a "maternal memory" that persists for up to 80 days, inducing similar diminished levels of anxiety [45]. Taken together, these results and present study support the idea that the prior maternal experience can differentially affect animals' responses to



environmental stimuli. It is possible that under more stressful or challenging circumstances than the typical laboratory conditions, females with prior experience with younger siblings may be more successful mothers with their first litter [11].

Alternatively, the overlapping litter model could be viewed as an early social-enriched environment. In this line, rabbits that have higher degrees of social interaction during the juvenile period, exhibit less fear behavior when confronted to experimental aversive stimuli in adulthood [46].

As discussed in a recent study [14], when reared in a complex social condition, factors other than maternal behavior occurring in the early postnatal life could shape the adult emotional response. Interestingly, opposite results were found in the communal rearing model, as a highly social stimulating environment (three mothers keep their pups together and share care-giving behavior) increases levels of anxiety in adult mice and brain-derived neurotrophic factor (BDNF) levels [47]. We can hypothesize that the interaction with younger siblings could promote a decrease of BDNF levels in hippocampus or other brain areas related to anxiety regulation.

It is worth mentioning that juveniles in overlapped litters received less total nursing during the neonatal period from their pregnant mothers than their controls. As differences in maternal behavior importantly affect offspring's responses [15,48], this factor must be taken into account. However, the behavioral component that is critically involved in these effects is the licking behavior [15], that was not different between pregnant and non-pregnant mothers. Also, during the juvenile age, overlapped offspring received more nursing behavior and less licking behavior than their weaning-matched controls. We may also discard this factor in the emotionality effects as it has been shown that the influence of maternal care occurs during the early postpartum period [15,48] but not at later stages of lactation [49,50]. Taken together, these data suggest that there is no influence of maternal behavior on the effects observed in overlapped reared juveniles' emotionality during adulthood.

The decreased anxiety of parentally experienced animals was observed only in the plus maze test while behavioral responses in the open field test were not different, suggesting a context specific response [46]. The difference between the behavior of animals in the two models could be attributed to a more aversive situation induced in the open field. Indeed, mother rats are less anxious in the plus maze test [51] but not when confronted with a bright white light illuminated open field [52,53], which is considered a highly anxiogenic situation [54].

In summary, postpartum mating give rise to a more complex family unit consisting of the mother and two overlapped litters in which she can adapt her behavior to the different characteristics of the pups. The overlapping of litters provides a highly stimulating social environment for juveniles, as it allows an increased interaction with their mother and the development of parental behavior toward younger. These changes in early experience long-term modified the affective behavior of both adult male and female rats.

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## **CAPÍTULO III**

### **EFFECTS OF LITTER-OVERLAPPING ON EMOTIONALITY, STRESS RESPONSE AND REPRODUCTIVE FUNCTIONS IN MALE AND FEMALE RATS.**

A ser submetido para Developmental Psychobiology



## **Effects of litter-overlapping on emotionality, stress response and reproductive functions in male and female rats**

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## **Abstract**

In rats, mating at postpartum estrus and delayed dispersal of the young would result in the overlapping of two different-age litters. As a consequence, newborn pups' early experience will include not only that acquired during the interaction with the mother and age-matched littermates, but also with older siblings. As early-life experience induces modulates rodents' brain function, behavior and reproduction, we aimed to assess how changes in the early environment provoked by the overlapping of litters would affect emotionality, stress response and reproductive functions of male and female pups during adulthood. Results showed that overlapped reared pups, both males and females, exhibited a reduced behavioral inhibition in the open field test during adulthood. In addition, overlapped reared adult females, but not males, showed a blunted corticosterone (CORT) response to an acute stressor during diestrus and a reduction in sexual behavior. In summary, natural changes in early experience provoked by the overlapping of litters, long-term modulate affective behavior, and the stress and reproductive function in a sex dimorphic manner.

Key words: overlapping litters, early experience, anxiety, stress, reproduction

## **Introduction**

Mating at postpartum estrus is a common situation for rodents in nature (Calhoun, 1963; Hayes, 2000). If older offspring remain in the maternal burrow following the birth of a new litter conceived at postpartum estrus, temporal overlapping of successive litters will occur (Calhoun, 1963; Gilbert, Burgoon, Sullivan, & Adler, 1983). In a previous study, we showed that the maternal behavior of female rats toward newborn pups is affected by the presence of older siblings. When raising overlapping litters (two juveniles from the former litter and the newborn from the second one), mothers licked the newborn pups less than mothers with single litters (Uriarte, Ferreira, Rosa, Sebben, & Lucion, 2008). Interestingly, juveniles, both males and females, developed maternal-like behaviors toward their newborn siblings and overrode the deficit in maternal licking behavior received by newborn of the second litter (Uriarte et al., 2008).

As early-life environment exerts long-term influences on rodents' brain, behavior and reproductive functions (Benetti, Andrade de Araujo, Sanvitto, & Lucion, 2007; Branchi & Alleva, 2006; Caldji, Tannenbaum, Sharma, Francis, Plotsky, & Meaney, 1998; Laviola & Terranova, 1998; Uriarte, Breigeiron, Benetti, Rosa, & Lucion, 2007), we hypothesized that the altered pattern of sensory stimulation received by pups reared in overlapped litters can modify their emotionality, stress response and reproductive functions in adulthood. Therefore, the present study aimed to characterize the anxiety-like behavior in the open field test, the endocrine stress response to an acute stressor, and

the reproductive function and sexual behavior of adult male and female rats reared in overlapped litters.

## **Material and Methods**

### ***Animals***

Male and female Wistar rats were obtained from the colony maintained in the Federal University of Rio Grande do Sul Laboratory Animal Facility. Animals were kept on a 12-h light/dark cycle (lights off at 18:00 h) and in a stable environmental temperature (21°C) with ad-lib access to rat chow (Nutrilab, Colombo, Brazil) and water. All procedures used in this study followed the guidelines for the care and use of Laboratory Animals from the National Institute of Health and were approved by the Research Committee of the Federal University of Rio Grande do Sul.

### ***Experimental groups and general procedure***

Nulliparous females (90 days old) were placed overnight with a sexually experienced male. Successful mating was confirmed next morning by the presence of spermatozooids in the vaginal smear and that day designated as day 1 of gestation (GD1). Approximately 7 days before delivery, pregnant females were re-housed with the same males to impregnate in the postpartum estrus. Starting on GD20, the presence of pups was checked daily at the beginning and end of the light period, and on the day of birth (PPD0) the behavior of parturient females and males was observed to avoid agonistic behaviors or cannibalism of the pups. On the following day (second litter GD1), the male was removed, the presence of spermatozooids was checked,

and the newborn litter was culled to 8 pups per dam (with usually 4 or at least 3 pups of each sex). Before parturition of the second litter, 21 day-old juveniles of the first litter were weaned. In the single reared condition (SR), the mothers remained with the second litter conceived at postpartum estrus while in the overlapped rearing condition (OR), two juveniles (one male and one female) were returned to the home cage and remained for 10 days after the birth of the second litter. The presence of newborn pups was checked twice a day from GD20, and on PPD1 the second litter was weighed and culled in the same way as the first one. In both groups, the second litter was maintained with their mother until weaning (PPD21), and then housed in same-sex, same-litter groups of three to four animals per cage. Experimental tests were performed when animals were three months old. Adult animals were used in only one behavioral test and within each group all subjects were from different litters.

### ***Estrous cycle***

Estrous phase was determined by microscope examination of daily vaginal smears. Only females showing two consecutive regular cycles were used for behavioral testing and ovulation assessment.

### ***Anxiety-like responses***

The behavior of adult male and female rats was evaluated in the open field test during the light period of the cycle. Females were tested in diestrus. Behavioral tests were performed in a separate testing room with the same temperature and photoperiod conditions of the breeding room. Animals were individually housed, transported to the testing room and left to acclimatize at least one hour before testing. After each test, the apparatus was cleaned thoroughly with 70% ethanol and allowed to dry completely between test sessions. Behavioral tests were recorded on VHS-video tape and analyzed later using the computer software: The Observer Pro® (Noldus, Wageningen, The Netherlands) by an experienced observer blind to subjects' experimental condition.

### ***Open field test***

The apparatus was a 100 cm square open field that was evenly illuminated by overhead fluorescent lights. The arena was divided into twenty-five 20-cm squares painted on the floor. Central area was defined as the nine inner quadrants of the arena. Each animal was placed in one corner of the open field and its behavior was videotaped over a 5-min session. The following behavioral parameters were recorded: frequency of entries and time spent in central quadrants of the arena, duration (s) of locomotion and frequency of rearing (Padoin, Cadore, Gomes, Barros, & Lucion, 2001).

## ***Stress Response***

### *Jugular cannulation*

The jugular catheter implantation was performed between 1500 and 1700 h on the day before (metaestrus in the case of the females) the animals were subjected to an acute stressor. Animals were anesthetized with 2.5% tribromoethanol (1 ml/100 g body weight ip) and a silastic cannula was inserted through the external jugular vein into the right atrium (Harms & Ojeda, 1974). Following surgery, animals were individually housed until testing on the following day.

### *Stress procedure and blood sampling*

On the day of testing (diestrus in the case of the females), rats were transported and allowed to acclimatize to the experimental room for 120 minutes. Immediately after, animals were submitted to restraint for 20 minutes in individual flat bottom restrainers (Plas-Labs) (6,35 cm diameter × 15,24 cm long for females; 8,26 cm diameter × 20,32 cm long for males) with four holes in the front and an opening at the back for the tail) for 20 min. Blood samples (0.4 ml) were collected in heparinized syringes with an equivalent volume of sterile 0.9% NaCl solution replaced after each bleeding at 0, 10, 20, 40, 60 and 80 min after the beginning of the stress. Samples were centrifuged at 4 °C at 3000 rpm, and the obtained plasma was stored at –80 °C until assay.

### *Corticosterone (CORT) determination*

Determination of CORT in blood plasma was performed using a commercial ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA). Plasma samples were extracted with ethyl acetate, evaporated to dryness and dissolved into assay buffer. The sensitivity of the assay was 24 pg/ml and the intra assay coefficient of variation was 15%. Results are expressed in ng/ml.

### ***Reproductive Function***

#### *Female sexual behavior*

Virgin females in proestrus were tested for sexual receptivity during the first two hours of the dark cycle. Females were placed with a proven breeder male in a steel cage (70 cm long x 35 cm wide x 70 cm high) with a front wall of glass, which allowed complete viewing of the couple, and its behavior was videotaped over a 15-min session. The indexes of sexual receptiveness used were the lordosis quotient (the proportion of mounts that are accompanied by lordosis) and the mean lordosis intensity. The lordosis quotient was calculated by dividing the total number of lordosis by the number of mounts. The intensity of lordosis was assessed using a three-point scale: 1=slight flex of spin, slightly raised head and hips with tail base elevated from floor, 2=spinal flex, head at an approximate angle of 30° with the horizontal, front paws placed slightly forward and hind legs straightened up stiffly, and 3=pronounced spinal flex, head at an angle of 45° or more with the horizontal. The lordosis intensity index was calculated by dividing the total points of each rat by the total number of lordosis displayed (Hardy & DeBold, 1972; Sodersten & Hansen, 1977).



### *Ovulation*

The following morning, females were decapitated, the ovaries were removed and the oviducts were dissected and compressed between 2 glass slides. The number of oocytes of both oviduct ampullae was counted under microscope (Zeiss, Goettingen, Germany) with a 2.5 x lens (Gomes, Raineiki, Ramos de Paula, Severino, Helena, Anselmo-Franci, Franci, Sanvitto, & Lucion, 2005).

### *Male sexual behavior*

Sexually inexperienced adult males were tested for sexual behavior 1-2 hours after the beginning of the dark phase. The male was habituated to the testing environment (70 cm long x 70 cm high x 35 cm wide) for 10 min before behavioral testing. After this period, a receptive female was introduced into the observation cage, and the behavior was videotaped. Each test lasted until one copulatory series was completed. Each series included the events from the first mount to the end of the postejaculatory interval (the first intromission of the second ejaculatory series). If a male failed to ejaculate in a period of 30 min, the test was finished. The number of animals showing mounts, intromissions and ejaculation was registered. In those males that did ejaculate, the following parameters were analyzed: latency to the first mount, number of mounts, latency to the first intromission, number of intromissions, and the latency to ejaculation (Fernandez-Guasti, Larsson, & Beyer, 1986; Rhees, Lephart, & Eliason, 2001). Stimulus females were ovariectomized females brought into sexual receptiveness by subcutaneous injections of estradiol benzoate (20 µg/rat) and progesterone (500 µg/rat) plus estradiol

benzoate (20 µg/rat) 54 h and 6 h before the test, respectively. All females were first tested with non-experimental sexually active males and only those proven to be lordotic were used.

### *Spermatozoa quantification*

Male rats were decapitated, and their testes collected for measurement of the spermatids quotient (number of the spermatids $\times 10^6$ ) and epididymides time (transit time/days for the maturation of the sperm in epididymides). The testes were weighed, homogenized and sonicated in 8 mL of a T-X solution (NaCl 0.9% and Triton X-100 0.05%). Portions of 100 µl of the homogenate were diluted in 900 µL of the T-X solution. Homogenization-resistant testicular spermatids in the testes and sperm in the caput/corpus epididymidis and cauda epididymidis were enumerated in Neubauer chambers. Daily sperm production per testis was determined by dividing the total number of homogenization-resistant spermatids per testis by 6.1 days (the number of days of a seminiferous cycle in which these spermatids are present). Transit times through the caput/corpus epididymidis and cauda epididymidis were calculated by dividing the number of sperm within each of these regions by the daily sperm production (Breigeiron, Lucion, & Sanvitto, 2007).

## **Statistics**

Data are expressed as means  $\pm$  SEM. Open field behavior was analyzed using a two-way ANOVA (group x sex). CORT plasma levels between groups were analyzed using a three-way ANOVA (group x sex x time) with repeated measures on time course followed by the Newman-Keuls test. The differences in the areas under the curve (AUCs) between the groups were determined by two-way ANOVA (group x sex). Sexual behavior, number of oocytes and spermatozoa count comparisons between SR vs OR animals were performed by the Student's t test. The number of ejaculating males between groups was compared by the Chi square test. In all cases the level of significance was set at  $p < 0.05$ .

## **Results**

### ***Anxiety like responses***

The two-way ANOVA analyses did not detect any group ( $F(1,50)=0.321$ ,  $p=ns$ ) or sex ( $F(1,50)=0.201$ ,  $p=ns$ ) effects in the total locomotion (s) assessed in the open field. Thus, single (SR, males:  $n=14$ , females:  $n=13$ ) and overlapped (OR, males:  $n=14$ , females:  $n=13$ ) reared rats did not differ in general locomotor activity (see Fig.1A).

As shown in Fig. 1B-C, OR animals, both males and females, showed increased frequency of entries (group:  $F(1,50)=8.069$ ,  $p < 0.01$ ; sex:  $F(1,50)=0.002$ ,  $p=ns$ ) and time spent (group:  $F(1,50)=8.069$ ,  $p < 0.01$ ; sex:  $F(1,50)=0.002$ ,  $p=ns$ ) in the central quadrants of the open field compared with SR ones.

### ***Stress response***

Fig. 2 shows the CORT plasma levels (ng/ml) of male and female rats from SR and OR litters (each group n=6).

Significant effects of sex [ $F(1,20)=34.183$ ,  $p<0.0001$ ] and time [ $F(5,100)=15.256$ ,  $p<0.01$ ] were observed, as well as significant interactions between sex and group [ $F(1,20)=9.104$ ,  $p<0.01$ ] and sex and time [ $F(1,20)=2.249$ ,  $p<0.05$ ].

A significant interaction between the three factors (sex x group x time) was found [three-way ANOVA,  $F(5,100)=3.810$ ,  $p<0.005$ ].

As shown in Fig. 2A, SR and OR males showed a significant increase of CORT levels in response to the stressor which did not differ between the groups at the times sampled. By contrast, OR and SR females showed different responses profiles. While SR females showed enhanced corticosterone levels following (similar to SR males), OR females displayed a blunted CORT response.

The two way analyses revealed significant effects of sex [ $F(1,20)=40.766$ ,  $p<0.0001$ ] without main effects of group [ $F(1,20)=0.537$ ,  $p=ns$ ] in AUC. A significant interaction was detected between sex and group [ $F(1,20)=9.900$ ,  $p<0.01$ ]. Post-hoc Newman Keuls test showed that OR and SR males did not statistically differ in AUC, whereas SR females had significantly higher AUC than OR females.

## ***Reproductive function***

### *Male Sexual Behavior*

The number of ejaculating males in the 30-min session did not differ between SR (9/14, 64.3%) and OR males (8/12, 66.7%). As shown in Fig. 3, the number of mounts and intromissions, as well as the latency to perform those behaviors did not significantly differ between SR and OR adult males (that ejaculated in the 30-min test).

*Sperm Quantification.* The number of spermatids per testis, daily sperm production, number of spermatozoa, and sperm transit time through per caput/corpus and cauda of the epididymis did not differ between the two groups of males (SR: n=8, OR: n=8) (see Table 1).

### *Female Sexual Behavior.*

Lordosis quotient was significantly lower in OR females (Student's t test=2.2,  $p<0.05$ ) although no statistical differences were observed in the lordosis intensity between SR (n=14) and OR (n=13) female rats (see Fig. 4A and B).

### *Ovulation.*

OR females did not show significant differences in ovulation as compared to SR reared ones (number of oocytes, SR:  $10.50 \pm 0.45$ , n=12; OR:  $10.00 \pm 0.54$ , n=12; Student's t test=0.71,  $p=ns$ ).

## Discussion

The main findings of the present study are:

- 1- Being reared in an overlapped litter, with the mother and two older siblings during the first postnatal days, induced a decrease in behavioral inhibition in the open field test in both male and female adult rats.
- 2- In adulthood, overlapped reared females, but not males, showed a blunted CORT secretion in response to restraint stress, and a reduction in sexual receptiveness during proestrus.

The decreased fear-like behavior exhibited by adult OR male and female rats compared to SR ones supports previous findings showing that early rearing environment can modulate neural systems controlling emotionality (Fernandez-Teruel, Escorihuela, Castellano, Gonzalez, & Tobena, 1997; Uriarte et al., 2007). We have previously shown that the licking behavior received by the pups from the mother decreased in the overlapped rearing condition and that this deficit was overridden by the licking provided by juvenile siblings in terms of quantity (Uriarte et al., 2008). However, it is possible that the quality of the sensory stimulation provided by juveniles differed from that of the mother, resulting in a different total stimulation received by OR newborns. This altered pattern of sensory stimulation may be responsible for their modified emotionality during adulthood. Indeed, it has been shown that natural differences in maternal stimulation affect the development of the young and induce long-term changes in their affective behavior (Caldji et al., 1998; Liu, Diorio, Tannenbaum, Caldji, Francis,

Freedman, Sharma, Pearson, Plotsky, & Meaney, 1997; Menard, Champagne, & Meaney, 2004; Uriarte et al., 2007).

In addition, changes in licking received by the pups could modify neural circuits associated with fear leading to less fearful animals in adulthood (Menard et al., 2004). In this line, it has been shown that natural differences in licking behavior received by the pups can induce alterations in some of their neuroendocrine systems involved in fear regulation, such as changes in the receptor levels of CRF in the locus coeruleus and in GABA<sub>A</sub> in the basolateral amygdala (Caldji et al., 1998).

On the other hand, the overlapping-litters model could be viewed as a precocious social- enriched environment. Rearing animals in socially and physically enriched environments has long-lasting effects, leading to a decreased behavioral inhibition of adult animals in novel environments (Elliott & Grunberg, 2005). Thus the effects observed in present model of overlapping litters could be due to the increased social interaction during the neonatal period independently of the changes in maternal behavior (Branchi & Alleva, 2006).

The lack of differences in basal CORT plasma levels between male and female SR animals is in accordance with studies showing that, at the time of the circadian rhythm and the stage of estrous cycle here considered, males and females do not show differences in CORT plasma levels (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomp, 1963; Diehl, Silveira, Leite, Crema, Portella, Billodre, Nunes, Henriques, Fidelix-da-Silva, Heis,

Goncalves, Quillfeldt, & Dalmaz, 2007). However, in present study, while adult males showed a rise in plasma CORT to the acute stressor independently of their rearing condition, OR females showed a blunted CORT release compared to SR ones. The finding that early environment effects on stress responses are influenced by the gender of the animal is in agreement with several studies (Pryce, Bettschen, Bahr, & Feldon, 2001; Smythe, McCormick, Rochford, & Meaney, 1994; Weinberg & Levine, 1977).

However, some contradictory data appears related to these issues. Whilst in some studies females appear to be less susceptible to the effects of early manipulations (Pryce et al., 2001) or natural variations in maternal licking received (Barha, Pawluski, & Galea, 2007), in others, accordingly with our results, males appear less susceptible or unaffected by them (Smythe et al., 1994; Weinberg & Levine, 1977; Welberg, Thirivikraman, & Plotsky, 2006). Interestingly, the reduced fear response of OR males was not accompanied by an attenuation of the endocrine stress response as in OR females, indicating a dissociation in the mechanisms that regulate both behavioral and endocrine responses to aversive stimuli. In other studies, periodic long maternal deprivation during the neonatal period increases anxiety-like behaviors in both males and females, but enhances the HPA axis responsiveness only in males (Wigger & Neumann, 1999). Taken together, these results point toward gender-dependent variations in strategies for coping with stress, which may be influenced by differences in the functional development of male and female stress circuitry and in the modulatory effect of early life experience (Welberg et al., 2006; Wigger & Neumann, 1999).



Adult OR females showed a reduction in sexual receptiveness during proestrus. In contrast, OR males did not differ in sexual behavior from SR ones. Moreover, no differences were detected in daily sperm production and maturation or in epididymal sperm storage capacity and epididymal transit. In the same line, we have previously shown that natural variations in maternal care long-term affect the reproductive function of female offspring without affecting male sexual behavior or sperm production and maturation (Uriarte et al., 2007). Thus, reproductive parameters, as CORT response to stress, appear to be less susceptible to changes in early social environment in males than in females.

As aforementioned, in the model of overlapped litters, stimulation received by OR newborns could be different than that received by control pups, and this could be the reason for the reduced reproductive performance in females. From an eco-ethological point of view, one putative hypothesis is that, in nature, the presence of juveniles in the nest and the occurrence of litter overlapping is related to a delayed dispersal of the young (Gilbert et al., 1983) as a consequence of ecological constraints (i.e. high population density, insufficient resources) (Emlen, 1994). As discussed by Branchi and Alleva (2006), if a species has a short life span, as it is the case of the rat, an individual usually will spend its adult life in an environment similar to the one in which it was born. Thus, shaping behavior in early life may be of adaptive value to the adult. In a hostile environment, the occurrence of overlapped litters will probably increase. In this situation, it would be more adaptive to be less reactive to stressors and less reproductive. We may speculate that the

diminished reproductive parameters observed only in females could be due to the greater energetic expenditure that they will afford in a reproductive event compared with males.

Thus, an unsuccessful reproductive attempt would have a greater energetic prejudice to females than to males (Bronson, 1985).

Summarizing, present study shows that the complex precocious rearing environment induced by the overlapping litters appears to modulate the affective behavior of adult males and females, the stress response, and the reproductive function in females. We can speculate that these changes in the offspring responses could be due to an altered quality/quantity of environmental stimulation received from mothers and/or littermates.

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**Table 1.** Sperm quantification in single (SR) and overlapped (OR) reared adult males (means  $\pm$  SEM).

	SR males	OR males
Number of spermatids ( $10^6$ /testis)	331.8 $\pm$ 28.3	331.2 $\pm$ 13.3
Number of spermatids ( $10^6$ /g/testis)	242.3 $\pm$ 17.0	228.9 $\pm$ 18.0
Daily sperm production (number of spermatids $\times$ $10^6$ /testis/day)	54.4 $\pm$ 4.6	54.3 $\pm$ 2.2
Number of spermatozoa $\times$ $10^6$ /caput+corpus of epididymis	124.2 $\pm$ 11.0	119.6 $\pm$ 7.9
Number of spermatozoa $\times$ $10^6$ /g/caput+corpus of epididymis	514.3 $\pm$ 33.0	471.7 $\pm$ 24.3
Number of spermatozoa $\times$ $10^6$ /cauda of epididymis	107.6 $\pm$ 9.2	89.8 $\pm$ 12.4
Number of spermatozoa $\times$ $10^6$ /g/cauda of epididymis	747.8 $\pm$ 42.0	629.1 $\pm$ 112.6
Sperm transit time (days) through caput/corpus of epididymis	2.4 $\pm$ 0.4	2.3 $\pm$ 0.2
Sperm transit time (days) through Cauda of epididymis	2.1 $\pm$ 0.2	1.4 $\pm$ 0.2



Figure 1

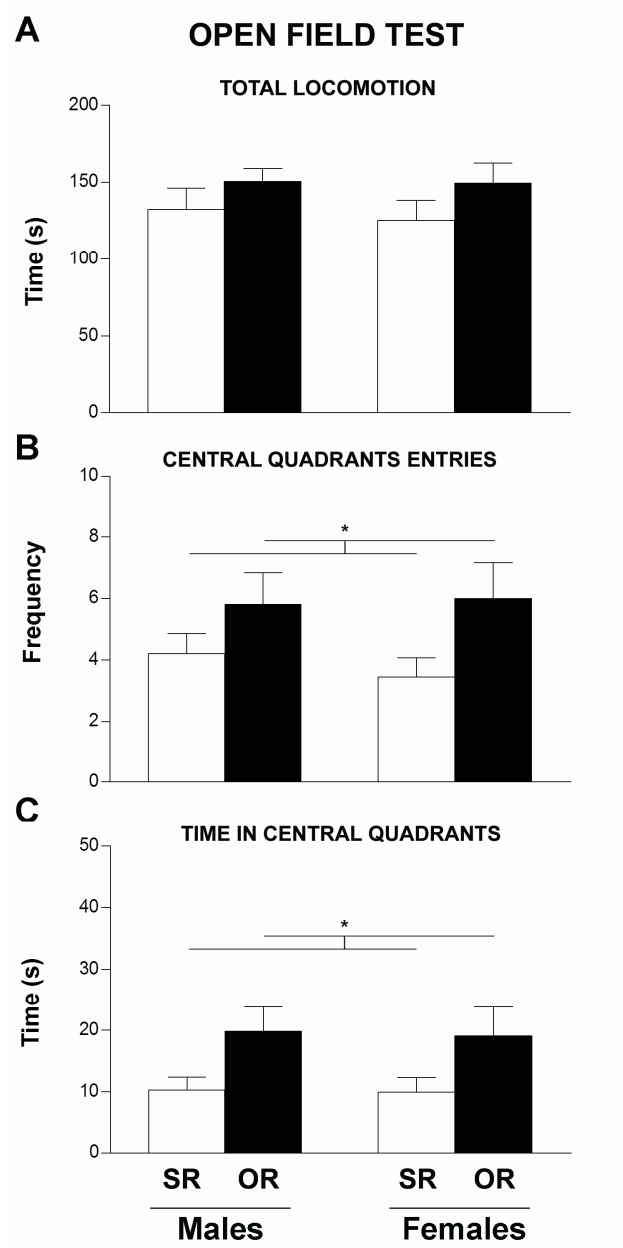


Figure 1. Open field behavior during a 5 min session of SR and OR adult males and females: (A) total locomotion, (B) frequency to enter and (C) time spent in central quadrants. Data are presented as means  $\pm$  SEM.

**Figure 2**

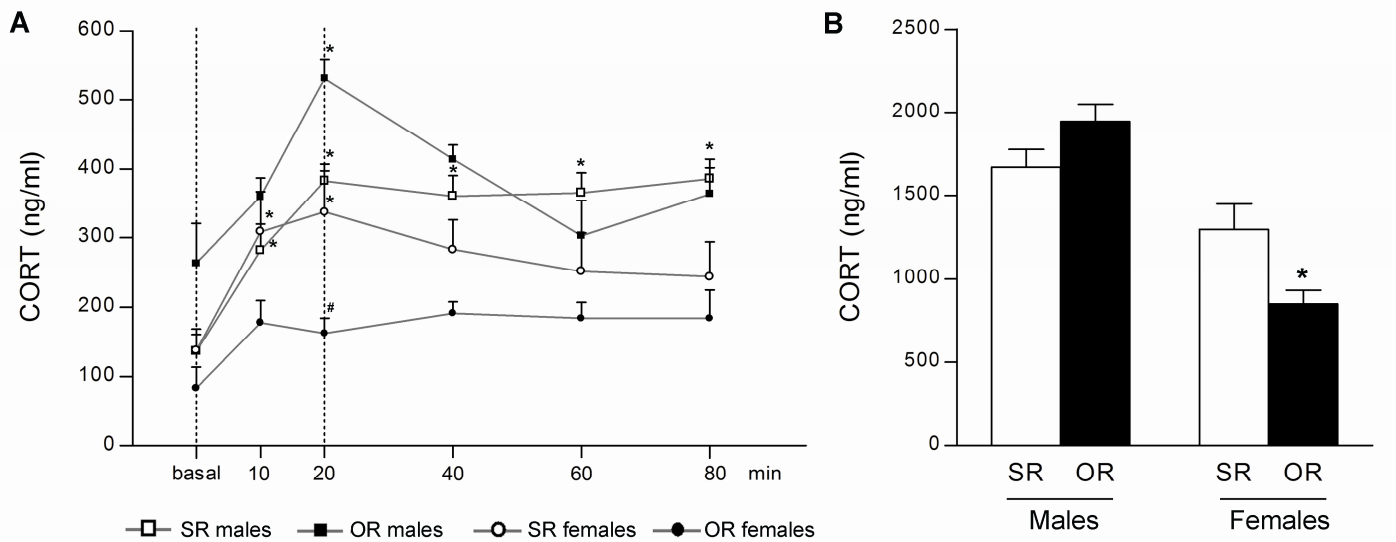


Figure 2. Mean ( $\pm$  SEM) CORT plasma levels (ng/ml) at baseline, 10, 20, 40, 60 and 80 min after the onset of acute restraint stress (A) and area under the curve (AUC) (B) of male and female SR and OR adult rats. \* significant differences from basal levels in the same group, # significant differences between SR vs. OR females.

**Figure 3**

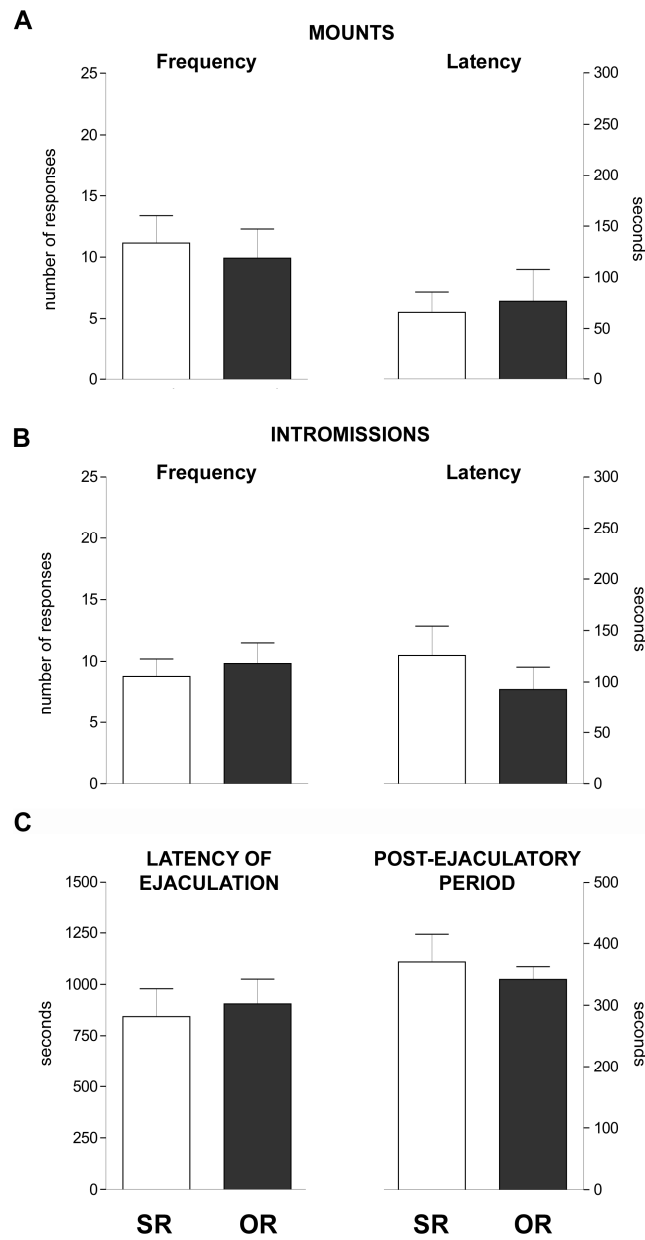


Figure 3. Male sexual behavior of SR and OR adult rats: A) frequency and latency of mounts, B) frequency and latency of intromissions, C) percentage of ejaculating males and duration of the post-ejaculatory period. Data are presented as means  $\pm$  SEM. \* $p < 0.05$ , Student's t test.

**Figure 4**

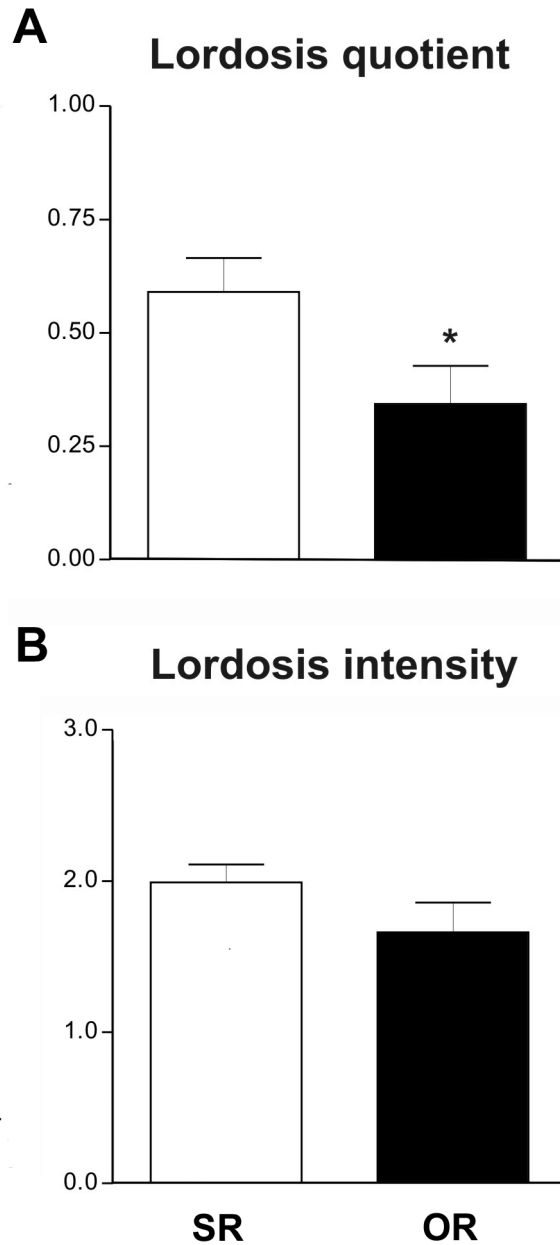


Figure 4. Female sexual receptiveness during proestrus of SR and OR adult pups: A) lordosis quotient, B) lordosis intensity. Data are presented as means  $\pm$  SEM, \* $p < 0.05$ , Student's t test.

## **Notes**

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## DISCUSSÃO

Considerando a análise do comportamento das ratas lactantes durante os primeiros dias pós-parto realizada nos capítulos I (diferenças espontâneas na população) e II (alterações de acordo ao estado reprodutivo) podemos concluir que o comportamento maternal não é uma conduta rigidamente estereotipada e sim apresenta variações individuais. Este comportamento aparece como uma conduta modificável, por fatores internos ou ambientais.

O comportamento maternal, especificamente o comportamento de lambida mostrou variações espontâneas dentro de uma população. As diferentes condições reprodutivas (gestação simultânea à lactação, lactação de ninhadas sobrepostas) também modularam o comportamento da mãe. Possivelmente, estas mudanças aparecem para adaptar o comportamento às características específicas da prole.

As diferenças espontâneas no comportamento de lambar reportadas no primeiro trabalho se encontram dentro de valores normais de comportamento parental para o crescimento adequado dos filhotes, de acordo como foi demonstrado também em ratas Long-Evans (Champagne, Francis et al. 2003). Aparece então uma questão relevante: o porquê da existência dessas variações e por que afetam características dos filhotes (por exemplo, a inibição comportamental e a função reprodutiva das fêmeas).

Possivelmente o comportamento maternal opere como uma interface entre os filhotes e as condições ambientais, sendo estas diferenças no comportamento maternal estratégias individuais para programar respostas rudimentares às demandas ambientais futuras da prole (Fish, Shahrokh et al. 2004). Outros estudos apóiam esta hipótese,

recentemente demonstrou-se que a exposição a odor de predador logo depois do parto altera o comportamento maternal das ratas lactantes (aumento do comportamento de lamber), bem como o das filhas quando adultas, embora elas não tiverem sido expostas ao odor de predador (McLeod, Sinal et al. 2007). Também tem sido demonstrado que em fêmeas de camundongos a exposição a odor de predador (rato) durante a lactação induz um aumento do comportamento de lamber e diminuição da reatividade emocional dos filhotes quando adultos (Coutellier, Friedrich et al.).

As variações na frequência do comportamento de lamber têm sido associadas a um aumento da expressão dos receptores de estrógeno  $\alpha$  e uma maior sensibilidade ao estrógeno na MPOA (Champagne, Weaver et al. 2003), onde este hormônio age aumentando o *binding* dos receptores de ocitocina (Caldwell, Walker et al. 1994). Assim, mães muito lambedoras têm um *binding* de ocitocina aumentado em áreas que participam na regulação do comportamento maternal (Francis, Champagne et al. 2000; Champagne, Diorio et al. 2001). Também, as altas frequências de lambida se associam com um aumento da atividade dopaminérgica no sistema mesolímbico (Champagne, Chretien et al. 2004), fortemente envolvido na regulação do componente motivacional do comportamento maternal (Pereira, Uriarte et al. 2005).

Os mecanismos pelos quais ocorrem as variações no comportamento de lamber nas mães de ninhadas sobrepostas poderiam incluir mudanças fisiológicas específicas. Os hormônios parecem não ser os responsáveis devido a que as mães de ambos os grupos (MOL e MSL) foram acasaladas no estro pós-parto e estiveram prenhas concomitantemente à lactação, tendo então perfis endócrinos similares. Podemos descartar também possíveis diferenças na experiência prévia de cuidado dos filhotes,

pois as mães dos dois grupos eram múltíparas, tendo passado por uma lactação e experiência maternal.

Uma explicação possível para a redução do comportamento de lambar poderia ser baseada no papel dos filhotes em promover seus próprios cuidados em função de suas demandas fisiológicas (Brouette-Lahlou, Vernet-Maury et al. 1992; Stern e Keer 2002; Pereira e Ferreira 2006). Os filhotes menos demandantes, como poderiam ser os criados junto com os juvenis (que suplementam parcialmente alguns comportamentos) induziriam menos comportamento maternal.

A partir dos resultados obtidos nos trabalhos apresentados nos capítulos I, II e III concluímos que variações do ambiente neonatal precoce, por variações do comportamento maternal recebido ou por alterações na composição da unidade familiar e o ambiente social, induzem alterações a longo prazo de diversas funções fisiológicas, entre elas a inibição comportamental frente a estímulos aversivos, resposta ao estresse agudo e funções reprodutivas.

Quais são os mecanismos envolvidos na modulação destes efeitos em longo prazo provocados pelas mudanças no ambiente precoce?

Tem sido demonstrado que o aumento do comportamento de lambar recebido durante os primeiros dias de idade provoca um aumento da ativação (avaliada através da marcação c-Fos-IR) em áreas do sistema límbico envolvidas na redução do medo, quando os animais são submetidos a um estímulo altamente aversivo na idade adulta (Menard, Champagne et al. 2004). Também tem sido demonstrado que os filhotes de mães muito lambedoras têm níveis menores de receptores para CRF no *locus coeruleus* e níveis maiores de receptores para GABA e benzodiazepinas na amígdala



basolateral (Caldji, Tannenbaum et al. 1998). Assim, foi sugerido que o comportamento de lambar modificaria circuitos neurais associados à redução do medo, levando a diminuição da inibição comportamental em ambientes aversivos durante a idade adulta (Menard, Champagne et al. 2004).

No caso dos neonatos criados em ninhadas sobrepostas (capítulo III), variações na estimulação (comportamento de lambar) também poderiam ser as responsáveis pela modificação das respostas de medo durante a idade adulta. No capítulo II mostramos que as mães com ninhadas sobrepostas lambem menos os filhotes neonatos, mas que a estimulação total, em termos de quantidade, recebida pelos filhotes recém nascidos não é diferente, devido a que comportamento de lambar provido pelos juvenis compensa esse déficit. Contudo, é possível que a qualidade da estimulação sensorial brindada pelos neonatos seja diferente da provida pela mãe. Considerando esses resultados em conjunto, podemos hipotetizar que os padrões alterados de estimulação sensorial recebida pelos filhotes criados em ninhadas sobrepostas são os responsáveis pelas alterações das respostas emocionais dos animais adultos.

No caso dos filhotes da primeira ninhada (capítulo II), a modificação na experiência precoce ocorre durante o período juvenil, onde o comportamento da mãe não teria já influências tão importantes sobre o desenvolvimento (Weaver, Cervoni et al. 2004). Provavelmente, estes efeitos estejam determinados pela experiência maternal que tiveram ao cuidar de seus irmãos mais novos. Neste sentido, sabe-se que a experiência reprodutiva modifica a ansiedade de fêmeas adultas: as ratas primíparas são menos ansiosas no labirinto em cruz elevado quando comparadas com fêmeas nulíparas durante o proestro (Byrnes & Bridges, 2006). Também a experiência maternal prévia,

obtida depois da gestação e do parto ou através do procedimento de sensibilização, estabelece uma “memória maternal” em ratas nulíparas e primíparas que persiste por vários meses, e que induz níveis reduzidos de ansiedade (Scanlan, Byrnes, & Bridges, 2006). Analisados em conjunto, esses dados e os presentes resultados apóiam a hipótese de que a experiência maternal prévia afeta a resposta dos animais aos estímulos ambientais. É possível que, sob condições mais estressantes ou desafiantes que as condições padrão do laboratório, as fêmeas com experiência previa com filhotes sejam mães com mais sucesso na sua primeira ninhada (Stern e Rogers 1988).

Alternativamente, o modelo de sobreposição de ninhadas pode ser considerado um modelo de enriquecimento social precoce. Em este sentido, tem sido demonstrado que coelhos que tiveram altos níveis de interação social durante o período juvenil mostram menos medo quando confrontados a estímulos aversivos durante a idade adulta (Rodel, Monclus et al. 2006). Como discutido num estudo recente (Branchi e Alleva 2006), quando criados em uma condição socialmente complexa, outros fatores além do comportamento maternal, que ocorrem durante o período pós-natal, poderiam modificar as respostas emocionais do animal adulto.

Entretanto, resultados opostos foram observados no modelo de cria comunal em camundongos (três mães cuidando de suas ninhadas juntas e compartilhando o comportamento maternal), observando-se um aumento dos níveis de ansiedade e do fator de crescimento nervoso BDNF no hipocampo de camundongos adultos (Branchi, D'Andrea et al. 2006). No presente trabalho podemos hipotetizar que a interação com os juvenis da primeira ninhada no caso dos neonatos, ou com os irmãos mais novos no

caso dos juvenis, poderia promover uma diminuição dos níveis de BDNF no hipocampo e em outras áreas relacionadas à regulação da ansiedade.

Os presentes resultados mostraram que diferenças individuais no comportamento maternal não só podem prever a reatividade emocional e a responsividade ao estresse, senão também podem modular a função reprodutiva em fêmeas. Estes efeitos poderiam ser explicados analisando a influência do comportamento de lambe sobre o *locus coeruleus* (LC). Além de ser ativado por estímulos estressores (Pacak e Palkovits 2001; Van Bockstaele, Bajic et al. 2001), este núcleo participa no controle da secreção do hormônio luteinizante (LH), e sua ativação é importante para a ocorrência do pico de gonadotrofinas (Anselmo-Franci, Franci et al. 1997; Helena, Franci et al. 2002; Lucion, Pereira et al. 2003). O LC é um núcleo noradrenérgico integrador e é um importante modulador dos eixos hipotálamo-hipófise-adrenal e hipotálamo-hipófise-gonadal.

Altos níveis de comportamento maternal se associam com um aumento do binding dos auto-receptores noradrenérgicos  $\alpha_2$  inibitórios no LC, e, como consequência uma diminuição na resposta noradrenérgica ao estresse (Caldji, Tannenbaum et al. 1998; Liu, Diorio et al. 2000). A diminuição da atividade do LC induzida pelo aumento do comportamento de lambe poderia resultar em uma liberação de noradrenalina diminuída na MPOA e conseqüentemente um pico de LH menor levando à redução da ovulação.

Em contraste às fêmeas, os parâmetros reprodutivos dos ratos machos aparecem como menos suscetíveis às variações da experiência neonatal e do comportamento maternal

recebido. Uma explicação para esta diferença entre os sexos poderia ser baseada no fato que o comportamento maternal das ratas lactantes tem um viés para os filhotes machos (Moore e Morelli 1979). O comportamento de lambar se classifica em lambida anogenital e lambida corporal. Enquanto os filhotes machos recebem mais comportamento de lambida anogenital que as irmãs (Moore e Morelli 1979; Moore 1992), não se acharam diferenças no comportamento de lambida corporal dirigido a filhotes fêmeas ou machos (Champagne, Francis et al. 2003). É possível que os filhotes machos sejam menos sensíveis às variações das frequências do comportamento de lambar, devido a que naturalmente recebem altos níveis desse comportamento. Assim, pequenas variações no comportamento de lambar, poderiam não ter um efeito significativo. É importante notar que as variações que mostramos neste trabalho são variações totais no comportamento de lambar (anogenital+corporal) e possivelmente por essa razão nossos dados divergem dos reportados por Moore e colaboradores e dos efeitos clássicos do comportamento de lambar anogenital e comportamento sexual em machos (Moore e Morelli 1979; Moore 1984).

Uma explicação alternativa seria que as mudanças de comportamento de lambar observadas nos dois experimentos seriam dirigidas exclusivamente aos filhotes fêmeas. Contrariamente a esta hipótese, Champagne et al (2003) não observaram diferenças significativas na frequência total de lambidas recebidas entre machos e fêmeas.

A redução da função reprodutiva nas filhas de mães muito lambedoras aparece como contraditória com a resposta esperada de um comportamento maternal “melhor” ou

“aumentado”, e questiona os efeitos benéficos do comportamento de lambe, pois o prejuízo da função reprodutiva das fêmeas sugere um efeito contra-adaptativo.

No entanto, é importante notar que ainda reduzida, todas as fêmeas filhas de mães lambedoras mantiveram sua capacidade reprodutiva.

Desde uma perspectiva eco-etológica, uma hipótese possível do dimorfismo sexual nos efeitos do ambiente precoce sobre a função reprodutiva, é que na natureza, a presença dos juvenis no ninho e a ocorrência de ninhadas sobrepostas estariam relacionadas a uma dispersão demorada dos juvenis (Gilbert, Burgoon et al. 1983) como consequência de limitantes ecológicas (por exemplo, uma alta densidade populacional, recursos insuficientes) (Emlen 1994).

Como discutido por Branchi e Alleva (2006), se uma espécie tem um período de vida curto, como no caso dos ratos, um indivíduo geralmente passará sua vida adulta em um ambiente similar a aquele onde nasceu. Desse modo, moldar o comportamento nos estádios iniciais da vida teria um valor adaptativo para o adulto.

Em um ambiente hostil, a probabilidade de ocorrência de ninhadas sobrepostas aumentaria e seria mais adaptativo ter uma menor reatividade ao estresse e uma atenuação da reprodução. Podemos especular que a razão para a diminuição da reprodução só nas fêmeas é a grande inversão energética que elas têm que afrontar em cada evento reprodutivo comparadas com os machos, e que uma tentativa reprodutiva frustrada teria um maior prejuízo energético para elas (Bronson 1985).

Meaney e colaboradores demonstraram que a expressão gênica está significativamente alterada no hipocampo de ratas adultas como resultado do comportamento maternal recebido durante a lactação. Esta ‘programação maternal’ envolve modificações

através de mecanismos epigenéticos de modificação da cromatina, incluindo metilação do DNA, acetilação de histonas e alteração da união do fator de transcrição NGFI-A ao promotor de receptores de glicocorticóides (Weaver, Cervoni et al. 2004). Essas diferenças na expressão gênica poderiam, em parte, constituir a base molecular para os efeitos da experiência precoce no desenvolvimento das respostas do eixo HPA ao estresse nos filhotes, as quais perduram ao longo da vida (Weaver, Meaney et al. 2006). Mecanismos similares poderiam ser os responsáveis pelos efeitos na função reprodutiva da fêmea por meio de ação em áreas relacionadas à reprodução.

Em resumo, na presente tese mostramos que o comportamento maternal das ratas é uma conduta flexível e modificável por variáveis internas e ambientais. Os diferentes ambientes maternos ou sociais decorrentes dessas variações modificam a experiência precoce que recebem os filhotes e provocam mudanças na sua reatividade emocional durante a idade adulta. Também mostramos que essas variações induzem efeitos em longo prazo sobre a responsividade ao estresse e a função reprodutiva, em forma sexualmente dimórfica.

Assim, os resultados obtidos nesta tese evidenciam a importância da experiência precoce como moduladora das respostas comportamentais e endócrinas dos indivíduos adultos. Também mostramos que variações espontâneas no comportamento de lambar e variações na composição da unidade familiar, os modelos de experiência precoce utilizados neste trabalho são suficientes para alterar o fenótipo dos indivíduos e podem constituir modelos experimentais úteis nesta disciplina, brindando informações com importante significado etológico.

## CONCLUSÕES

1. As ratas mães da linhagem Wistar mostram variações espontâneas da frequência do comportamento de lambe os filhotes durante os 8 primeiros dias pós-parto, permitindo sua classificação em mães “muito lambedoras” e “pouco lambedoras”.
2. O aumento da estimulação maternal (frequência de lambidas) recebida durante os primeiros dias de vida provoca a aceleração do desenvolvimento dos filhotes, avaliado pela idade de abertura ocular.
3. O aumento da estimulação maternal durante o período neonatal induz uma redução permanente na inibição comportamental dos filhotes machos e fêmeas.
4. O aumento da estimulação maternal diminui a função reprodutiva das fêmeas (avaliada pela intensidade de lordose e número de óvulos) sem afetar a função reprodutiva dos filhotes machos.
5. A gestação simultânea afeta o comportamento maternal de ratas lactantes, provocando uma redução do tempo total de amamentação e o tempo de permanência no ninho.
6. A sobreposição de duas ninhadas de diferentes idades afeta o comportamento da mãe, provocando mudanças no comportamento dirigido a juvenis (aumento da agressão) e neonatos (diminuição do comportamento de lambe).
7. A convivência com irmãos neonatos durante a sobreposição de ninhadas induz comportamento parental nos juvenis, que durante a idade adulta mostram níveis de ansiedade reduzidos.
8. A sobreposição de ninhadas provoca, tanto em machos quanto em fêmeas, uma redução da inibição comportamental durante a idade adulta. Entretanto a resposta ao

estresse e a função reprodutiva são afetadas de maneira sexualmente dimórfica, sendo unicamente as fêmeas afetadas por este tipo de experiência precoce.



## PERSPECTIVAS

Um possível caminho para dar continuidade a este trabalho seria verificar a participação dos hormônios gonadais nos efeitos induzidos pelas variações na experiência neonatal. Uma vez que os resultados tanto de variações espontâneas no comportamento maternal (capítulo I) quanto de variações no ambiente social precoce (capítulo III) foram diferentes entre os animais machos e fêmeas, seria interessante identificar o papel dos hormônios gonadais durante o período neonatal, a puberdade e a idade adulta nos efeitos exercidos por mudanças ambientais.

A partir dos resultados obtidos no capítulo II; onde os juvenis criados em ninhadas sobrepostas desenvolveram atividades de cuidado dos irmãos neonatos e mostraram menos ansiedade durante a idade adulta, seria interessante estudar mais detalhadamente os mecanismos pelos quais surgem estes efeitos. Para isso, seria importante determinar se as áreas cerebrais envolvidas na diminuição da ansiedade provocada por variações na experiência precoce são as mesmas envolvidas na regulação da ansiedade em adultos e através de que processos a experiência maternal está agindo.

Seria interessante avaliar se as variações na experiência precoce devidas ao aumento na complexidade do ambiente familiar no modelo de ninhadas sobrepostas (capítulos II e III) ‘protege’ os filhotes contra os efeitos deletérios provocados por eventos estressantes durante os primeiros períodos da vida. Se fosse assim, poderíamos dizer que o aumento da ansiedade e da resposta endócrina ao estresse produzido pela separação maternal repetida durante o período neonatal ou a exposição a estresse

repetido durante a adolescência poderiam ser revertidos pela experiência no modelo de ninhadas sobrepostas.

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