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ANDRÉA DULOR FINKLER

EFEITOS COMPORTAMENTAIS DA ADOÇÃO SOBRE A RELAÇÃO MÃE-FILHOTE NO INÍCIO DO DESENVOLVIMENTO PÓS-NATAL EM RATOS

Porto Alegre
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Tese apresentada ao Programa de Pós – Graduação em Neurociências do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Doutora em Neurociências.
Linha de Pesquisa: Comportamento

Orientador: Prof. Dr. Aldo Bolten Lucion

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Aprovada em 17 de junho de 2019.

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“Assim como acontece em ratos, seres humanos carregam por toda a vida marcas permanentes do que os entes queridos lhes fazem ou deixam de fazer”.

Mc Gown

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RESUMO

As experiências da criança vividas com a sua mãe ou com seu cuidador no início da vida fornecem o modelo básico para as relações sócio afetivas na vida adulta. Dentro desta perspectiva, uma série de situações ocorridas nos primeiros anos de vida podem comprometer o desenvolvimento saudável do indivíduo. As crianças institucionalizadas, apesar de receberem cuidados adequados, apresentam importantes mudanças comportamentais na vida adulta, as quais, tem sido fortemente associada à falta de um vínculo com a figura materna no início da vida. Atualmente, ainda não se sabe como os processos de adoção humana alteram a relação mãe-bebê no início da vida. Dentro desta perspectiva, a racionalidade desta tese foi representar a complexa situação da adoção humana através de um modelo animal com o propósito de explorar o seu impacto na formação do vínculo (apego) do filho com um cuidador e, assim, contribuir com o entendimento dos processos que envolvem a adoção infantil, uma situação bastante frequente, mas pouco abordada experimentalmente. Para isso foram utilizados um total de 804 ratos Wistars (88 fêmeas prenhas, 12 machos e 704 filhotes). Inicialmente, caracterizamos o perfil comportamental dos filhotes em diferentes períodos pós-natais e o padrão de comportamento materno de ratos Wistar, no mesmo ambiente em que, posteriormente estudamos os efeitos da adoção. Posteriormente, avaliamos os efeitos comportamentais da adoção cruzada repetida de fêmeas lactantes (RCF) sobre a formação do vínculo mãe-filhote em dois períodos importantes do desenvolvimento pós-natal: no período sensível a aprendizagem do apego (dias pós-natal 1 a 3) e no período pós-sensível a aprendizagem de apego (dias pós-natal 9 a 11), tanto na perspectiva da mãe adotiva quanto na perspectiva dos filhotes adotivos. O protocolo de RCF realizado precocemente (dias pós-natal 1 a 3) reduziu a capacidade dos filhotes de preferir o odor da figura materna – filhotes machos RCF [Mdn: 0 (0-0)] vs. filhotes machos controles [Mdn: 25,67 (3,33-33,58)] e filhotes fêmeas RCF [Mdn: 0 (0-2,75)] vs. filhotes fêmeas controles [Mdn 14,17 (9,5-26,58)]. Entretanto, a adoção realizada tardiamente (dias pós-natal 9 a 11) não alterou significativamente a capacidade dos filhotes de preferirem o odor materno – filhotes machos RCF (32,53 ± 4,14) vs. filhotes controles (36,12 ± 3,84); e filhotes fêmeas RCF (37,11 ± 5,28) vs. filhotes controle (31,03 ± 5,72). Além disso, a adoção precoce reduziu o cuidado materno no dia DPN 2 – mães RCF (25,67 ± 3,50) vs. mães controles (53,75 ± 2,34); e no dia DPN 3 mães RCF (21,83 ± 2,91) vs. mães controles (37,17 ± 2,10). A adoção precoce também reduziu a motivação materna para recolher os filhotes e levá-los para o ninho – tempo para recolhimento do *primeiro filhote*: mães RCF [Mdn 39 (18-89,75)] vs. mães controles [Mdn 16 (8,25-28,50)]; e *quinto filhote*: mães RCF [Mdn 167 (79-410,5)] vs. mães controles [Mdn. 86,5 (61,75-160,3). A adoção tardia reduziu o cuidado materno no dia DPN 9 em relação as mães do grupo controle - mães RCF (23,33 ± 2,49) vs mães controle (37,17 ± 5,21). Esse estudo forneceu evidências de que o RCF, realizado durante o período sensível a aprendizagem do apego infantil (adoção precoce), criou um ambiente instável para o estabelecimento da interação mãe-filhote e, como resultado, reduziu significativamente o comportamento materno e diminuiu as respostas dos filhotes ao odor materno. Por outro lado, o procedimento de RCF realizado durante o período pós- sensível a aprendizagem do apego (adoção tardia) reduziu ligeiramente o cuidado materno, porém não alterou as respostas comportamentais dos filhotes ao odor materno.

Palavras-chave: Comportamento de apego; Aprendizado; Preferência olfatória; Comportamento materno; Mães adotivas; Comportamento de evitação; Desenvolvimento

ABSTRACT

Child experiences with their mother or caregiver early in life provide the basic model for socio-affective relationships in adult life. Within this perspective, a series of situations which have happened in the first years of life can compromise a healthy development of the individual. Institutionalized children, despite receiving adequate care, exhibit important behavioral changes in adult life, which have been strongly associated with lack of attachment to the maternal figure in early life. It is not yet known how the processes of human adoption change the mother-infant relationship early in life. In this perspective, the rationale of this thesis was to represent the complex situation of human adoption through an animal model in order to explore its impact in the formation of the offspring's attachment to a caregiver and, thus, contribute to the understanding of the processes that involve child adoption, a situation that is quite frequent but little discussed experimentally. A total of 804 Wistars rats (88 pregnant females, 12 males and 704 pups) were used. Initially, we characterized the behavioral profile of postnatal pups and the pattern of maternal behavior of Wistar rats, in the same environment where the effects of adoption were later studied. We evaluated the behavioral effects of repeated cross-fostering of lactating females (RCF) on the formation of the mother-infant bond in two important periods of postnatal development: in the sensitive period of attachment learning (postnatal days (PND) 1 to 3) and in the post-sensitive period of attachment learning (postnatal days 9 to 11), both from the perspective of the adoptive mother and from the perspective of the adopted pups. The RCF protocol performed early (PND 1 to 3) reduced the pups' ability to prefer the maternal odor – RCF male pups RCF [Mdn: 0 (0-0)] vs. control male pups [Mdn: 25,67 (3,33-33,58)]; and RCF female pups [Mdn: 0 (0-2,75)] vs. control female pups [Mdn 14,17 (9,5-26,58)]. However, late adoption (PND 9 to 11) did not affect the pups' ability to prefer maternal odor – RCF male pups (32,53 ± 4,14) vs. control male pups (36,12 ± 3,84) and RCF female pups (37,11 ± 5,28) vs. control female pups (31,03 ± 5,72). Moreover, early adoption reduced maternal care on PND 2 – RCF mothers (25,67 ± 3,50) vs. control mothers (53,75 ± 2,34); and on PND 3 – RCF mothers (21,83 ± 2,91) vs. control mothers (37,17 ± 2,10). Early adoption reduced motivation in pups retrieval test – time for f*irst pup:* RCF mothers [Mdn 39 (18-89,75)] vs. control mothers [Mdn 16 (8,25-28,50)]; and f*ifth pup*: RCF mothers [Mdn 167 (79-410,5)] vs. control mothers [Mdn. 86,5 (61,75-160,3)]. On the other hand, late adoption reduced maternal care on PND 9 – RCF mothers (23,33 ± 2,49) vs. control mothers (37,17 ± 5,21). This study provided evidence that RCF performed during the sensitive period of infant attachment learning (early adoption) developed an unstable environment for mother-infant interaction to occur in a healthy and adequate way and, as a result, significantly reduced maternal behavior and pups' response to maternal odor. On the other hand, the RCF procedure performed after the attachment learning period (late adoption) slightly reduced maternal care and it did not alter the behavioral responses of the pups to the maternal odor.

Key-Words: Attachment behavior; Learning; Olfactory preference; Maternal behavior; Adoptive mothers; Avoidance behavior; Developmental

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LISTA DE ABREVIATURAS E SIGLAS

Em Português

RCF – Adoção Cruzada Repetida de Fêmeas Lactantes
DPN – Dia Pós-Natal
DPP – Dia Pós-Parto
CONCEA – Conselho Nacional de Controle de Experimentação Animal
CEUA – Comitê de Ética no Uso de Animais
UFRGS – Universidade Federal do Rio Grande do Sul
IQ – Dados expressados como mediana
ANOVA – Análise de Variância
CT – Grupo Controle
SEM – Erro padrão da média
CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico
CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Em Inglês

PND – *Postnatal Days*
PPD – *Postpartum Days*
GD - *Gestational Day*
NIH – *National Institutes of Health*

SUMÁRIO

CAPÍTULO I

1 INTRODUÇÃO GERAL

O presente estudo elucidou alguns efeitos comportamentais da adoção, realizada no início do desenvolvimento pós-natal, em um modelo animal que buscou mimetizar a instabilidade ambiental, a qual crianças entregues para adoção normalmente estão expostas. Neste capítulo, apresentamos um referencial teórico, a motivação para a realização deste trabalho, as perguntas a serem respondidas, as hipóteses de pesquisa, a justificativa, os objetivos gerais e específicos do estudo, e, por fim, a estrutura geral desta tese.

1.1 REFERENCIAL TEÓRICO

1.1.1 Adoção

Crianças entregues para adoção geralmente vivenciam situações avassaladoras e, se essas experiências iniciais forem experimentadas através de maus-tratos, negligência da família biológica ou das instituições de abrigo, elas poderão apresentar muitas dificuldades em formar vínculos afetivos com seus novos pais adotivos. Da mesma forma, isso também pode acontecer em crianças que passaram muito tempo em instituições de acolhimento, apesar de terem recebido cuidados físicos adequados.

Um aspecto importante a ser levado em consideração, é que as experiências iniciais da criança com a sua mãe ou cuidador fornecem o modelo básico para as relações sócio afetivas na vida adulta (Moriceau et al., 2010; Sullivan et al., 2015). Dentro desta perspectiva, uma série de situações ocorridas nos primeiros anos de vida podem comprometer o desenvolvimento saudável do indivíduo. Estudos mostraram que um ambiente precoce afetuoso, bem estruturado e com cuidados maternos estáveis, refletiram em padrões comportamentais estáveis na criança e produziram sentimentos de segurança e confiança. Entretanto, ambientes caracterizados por mudanças significativas, instabilidade, falta de afeto e de segurança desencadearam comportamentos menos estáveis, sentimento de insegurança e falta de confiança (Lebovici, 1987).

Nas instituições de acolhimento, nem sempre os cuidadores conseguem suprir a necessidade afetiva e psicológica das crianças em função do elevado número de residentes. Assim, essa é uma variável importante que pode influenciar na formação de laços afetivos entre a criança e seus pais adotivos. Diversos estudos envolvendo crianças adotadas ou em acolhimento adotivo relataram uma melhora rápida após a colocação da família substituta, nos parâmetros físicos da criança - peso, altura e circunferência craniana - juntamente com uma recuperação social-emocional impressionante, embora incompleta (Juffer & Van IJzendoorn, 2009; Palacios & Brodzinsky, 2010).

Tem sido demonstrado diferenças no desenvolvimento e na saúde mental entre crianças adotadas e não adotadas (Askeland et al., 2015; Askeland et al., 2017; Westermeyer et al., 2015; Westermeyer et al., 2014). Além disso, o período em que a adoção ocorreu, se foi na fase inicial da vida ou na infância tardia, foi associado a um menor ou maior comprometimento respectivamente do desenvolvimento cognitivo e afetivo (Grotevant & McDermott, 2014). Também foi evidenciado que crianças adotadas podem ter maior tendência a apresentar comportamentos de risco ou comportamentos tipo suicida (Keyes et al., 2013), abuso de substâncias como álcool e drogas (Yoon et al., 2012) assim como respostas cognitivas inferiores quando comparadas a crianças não adotadas (Delcenserie & Genesee, 2014).

A institucionalização, embora possa ser melhor que as alternativas, como abandono ou maus-tratos em casa, proporciona um ambiente de cuidado inconsistente devido à rotatividade de cuidadores profissionais e a vários fatores desafiadores para o desenvolvimento de uma criança saudável que possivelmente prejudicam sua relação sócio emocional (Dozier & Rutter, 2016).

A adoção tem sido pouco estuda experimentalmente em modelos biológicos. Um modelo de adoção em animais, conhecido como adoção cruzada repetida (RCF) é um procedimento de rotina utilizado em muitos laboratórios com o objetivo de modelar a instabilidade ambiental precoce em humanos encontrada em muitas instituições de acolhimento (Cittaro et al., 2016; Luchetti et al., 2015). Este procedimento, consiste em entregar filhotes a uma fêmea lactante, diferente da mãe biológica, geralmente dentro das primeiras 24 horas após o nascimento e repetir o mesmo procedimento todos os dias durante os primeiros 4 dias de vida do filhote, sendo que em cada dia os filhotes são entregues a uma fêmea lactante diferente (Luchetti et al., 2015). Essa imprevisibilidade no ambiente pós-natal inicial, provocada pelo protocolo do RCF, pode representar uma condição estressante para os filhotes que tem efeitos a longo prazo (Luchetti et al., 2015). É importante ressaltar que a estabilidade do ambiente neonatal, durante o período sensível ao aprendizado do apego pela figura materna (cuidador) é crucial para o desenvolvimento comportamental do filhote e para o estabelecimento do apego mãe-filhote.

1.1.2 Apego mãe – filho

A Teoria do Apego evidencia a importância da ligação emocional que se desenvolve entre o bebê e seu “cuidador”, para orientar o desenvolvimento afetivo, cognitivo e social da criança. A partir de uma perspectiva etológica, (Bowlby, 1958, 1965, 1982) constatou que os bebês, assim como os pássaros, também apresentaram um “período sensível”, em que se encontram mais dispostos a formar vínculos com suas mães. Dessa maneira, eles revelaram esse vínculo (chamado apego) através do comportamentos como (sorrir, chorar, sugar o polegar, olhar em direção à mãe, buscar proximidade, contato físico) destinado a atrair a mãe para perto deles (recém-nascidos), ou levá-los (quando maiores) em direção a sua mãe.

Dizer que uma criança tem apego por alguém significa que ela está fortemente disposta a buscar proximidade e contato com uma figura específica, principalmente quando está cansada, assustada ou doente. Para isso, considerou-se o apego uma ligação contínua e íntima, apresentada pela criança em relação à mãe ou cuidador; enquanto o comportamento de apego foi considerado qualquer forma de comportamento que uma criança comumente adota para conseguir e/ou manter uma proximidade com algum outro indivíduo claramente identificado, por exemplo, a mãe. Assim, a característica essencial comum entre apego e comportamento de apego é que dois parceiros tendem a manter-se próximos um do outro (Klaus et al., 2000).

Um fator importante no comportamento de apego é a intensidade da emoção que o acompanha. Essa emoção surge conforme se desenvolve a relação entre o indivíduo apegado e a figura de apego. A partir dessas considerações Lebovici (1987), reforçou que se tudo está bem na relação entre os indivíduos apegados, há satisfação e um senso de segurança; porém, se esta relação está ameaçada, existem ansiedade e angústia. Caso ocorra uma ruptura, há dor e depressão. No caso de privação materna, seja este afastamento físico ou emocional, muitas são as consequências, tanto de ordem física quanto intelectual e social, podendo, inclusive, desencadear o aparecimento de doenças físicas e mentais (Oppenheim et al., 2001).

Uma criança aprende sobre o mundo através do seu cuidador e, isso inclui a capacidade de experimentar o mundo por meio dos sinais de segurança e ameaça que o cuidador transmite. O apego no início da vida é um dos comportamentos sociais mais conservados nas espécies altriciais, incluindo os humanos, o qual se caracteriza por um repertório de comportamentos de procura-proximidade que o infante exibe com a mãe ou cuidador, principalmente durante o período de imaturidade sensorial e motora, os quais garantem que os filhotes permaneçam apegados a sua mãe ou ao seu cuidador, independente da qualidade de cuidado que possam receber (Moriceau, 2005; Sullivan, 2017).

1.1.3 Apego mãe-filhote em roedores

Estudos em roedores mostraram que os filhotes de ratos possuem um repertório de respostas comportamentais específicas que os diferem das respostas observadas em animais adultos. É possível que tais especificidades tenham sido selecionadas ao longo da evolução por serem importantes para a manutenção do vínculo do filhote com a sua mãe (Opendak et al., 2017; Sullivan, 2017).

Ao estudar o desenvolvimento da aprendizagem infantil é importante entender que o cérebro do filhote de rato não é uma versão imatura do cérebro do adulto. Em vez disso é constituído para garantir que a aprendizagem do filhote mude para se adequar a cada época do desenvolvimento pós-natal, tipicamente usando “períodos sensíveis” temporários que filtram a aprendizagem, aprimorando alguns aspectos e inibindo outros (Sullivan, 2017).

Vários estudos mostraram, de maneira consistente, que o apego filhote-mãe se dá por um processo de aprendizado e formação de memória desta relação filial inicial (Sullivan & Wilson, 1991; Landers & Sullivan, 2012; Opendak & Sullivan, 2016). Tanto em humanos como em animais, o apego requer uma breve experiência de aprendizado das caraterísticas sensoriais da figura materna (odor, textura, cor e som), que se produz durante as primeiras interações do neonato com seu cuidador (Debiec & Sullivan, 2017; Raineki et al., 2010; Sullivan & Holman, 2010).

Os filhotes altriciais recém-nascidos devem aprender o odor da sua mãe e usá-lo para orientar seus deslocamentos. Como a mãe é a única fonte de alimento, calor e proteção, aprender esse odor é fundamental para a sobrevivência do recém-nascido (Sullivan, 2003; Landers & Sullivan, 2012). Especificamente, o odor materno controla as interações dos filhotes de ratos com a mãe, incluindo respostas de aproximação, comportamento social e apego ao mamilo (Raineki et al., 2010). O aprendizado olfatório é expresso e pode ser medido pela resposta de preferência do filhote pelo odor da mãe, através de comportamentos de orientação e aproximação da fonte deste odor. Ele ocorre numa janela temporal para sua formação, chamada nos roedores de período sensível ao apego. Em ratos, esse período corresponde aos primeiros 10 dias de vida. O aprendizado é fortemente dependente dos repertórios comportamentais que a mãe direciona para o filhote, e da responsividade do sistema nervoso do filhote para com os estímulos táteis maternos (Sullivan & Wilson, 2003).

Estudos mostraram que, o condicionamento realizado fora do ninho, ainda no período sensível ao apego, utilizando um odor artificial pareado com estímulos que mimetizam o cuidado materno, como por exemplo o comportamento de lamber o filhote, induz rapidamente uma resposta comportamental de preferência olfatória do filhote a esse odor que foi condicionado (Raineki et al., 2010; Sullivan et al, 1990).

A partir desses trabalhos tem sido proposto que o odor da mãe é aprendido mediante os mesmos mecanismos associativos dentro do ninho (Debiec & Sullivan, 2017; Opendak & Sullivan, 2016), pois os filhotes estão expostos simultaneamente ao odor da mãe e às contingências dos estímulos maternos termo táteis. Dessa forma, quando os filhotes foram avaliados fora do ninho, em um labirinto de duas escolhas para o odor do próprio ninho ou odor da mãe *versus* odor de maravalha limpa, os filhotes apresentaram respostas de preferência olfatória para o odor materno ou para o odor do próprio ninho (Meyer & Alberts, 2016). Por outro lado, tem sido sugerido também que as experiências com a mãe dentro do ninho, tanto antes como depois do dia pós-natal 10 são capazes de induzir respostas comportamentais de preferências para os congêneres.

O reportório de comportamento materno da rata lactante é intenso durante os primeiros dias pós-natal. À medida que o período pós-natal avança os comportamentos maternos diminuem e gradualmente outros comportamentos aumentam. O aprendizado do odor da mãe e do ninho é coincidente com o período em que o cuidado materno é intenso e, no qual, o filhote está exposto dentro do ninho. Entretanto, o aprendizado de preferência por congêneres ocorre em uma janela temporal maior. Possivelmente, certos padrões de comportamento materno após o dia pós-natal 10 sejam importantes para induzir essas preferências. Porém, ainda não está claro como se desenvolvem naturalmente as respostas de preferência olfatória para odores da mãe, do próprio ninho, dos congêneres e outros odores biologicamente relevantes. A maior parte da literatura sobre as preferências olfatórias baseadas na experiência inicial deriva de estudos em filhotes de ratos e, entre estes, as medidas comportamentais envolveram principalmente a seleção de dieta (Galef & Henderson, 1972), preferências para o amontoamento (*huddling*) (Alberts & Brunjes, 1978; Alberts & May, 1984) e outras formas mais complexas de afiliação social (Mainardi et al, 1965).

1.2 MOTIVAÇÃO DO ESTUDO

A motivação para a realização do presente estudo surgiu da necessidade de compreender melhor as bases comportamentais do apego e, principalmente, os efeitos do rompimento do vínculo mãe-filhote (apego) no início do desenvolvimento pós-natal, como ocorre nos casos de adoção, sobre os comportamentos filiais, os quais são determinantes para uma vida saudável no futuro. Além disso, este estudo pode lançar mais luz sobre os desvios do desenvolvimento infantil normal e sobre a capacidade das crianças de recuperar o atraso após sofrerem privações significativas, as quais normalmente ocorrem nos casos de crianças entregues para adoção.

1.3 PRINCIPAIS PERGUNTAS DE PESQUISA

O presente estudo focou na abordagem das seguintes perguntas:

Pergunta 1: Qual o perfil do desenvolvimento natural das respostas comportamentais de filhotes de ratos a odores biologicamente relevantes e, como essas respostas se alteram durante as três primeiras semanas de vida pós-natal?

Pergunta 2: Quais são as características naturais do comportamento maternal que ocorrem antes, durante e depois do aparecimento das respostas comportamentais de filhotes de ratos a odores biologicamente relevantes?

Pergunta 3: Quais são os efeitos comportamentais da adoção realizada durante dois períodos importantes do desenvolvimento pós-natal (período sensível e período de transição para o pós-sensível a aprendizagem do apego) sobre os comportamentos de filhotes de ratos?

Pergunta 4: Quais são os efeitos comportamentais da adoção realizada durante dois períodos importantes do desenvolvimento pós-natal (período sensível e período de transição para o pós-sensível a aprendizagem do apego) sobre o comportamento materno das fêmeas lactantes cuidadoras?

1.4 HIPÓTESES ESPECÍFICAS

As hipóteses desta pesquisa foram fundamentadas nas experiências iniciais do filhote com o odor materno.

Hipótese 1: O procedimento de adoção cruzada repetidas de fêmeas lactantes durante o período sensível ao aprendizado do apego (RCF precoce) altera a formação do apego mãe-filhote. No entanto, quando este procedimento é realizado durante o período pós-sensível ao aprendizado do apego (RCF tardio) não altera o vínculo mãe-filhote.

Hipótese 2: A troca repetida de filhotes, embora de mesma idade, afeta negativamente o comportamento de cuidado materno para os filhotes que não fossem os seus biologicamente.

1.5 ESTRUTURA DA TESE

A presente tese está organizada em 5 capítulos e 1 anexo. A seguir descrevemos brevemente cada capítulo.

O Capítulo 1 faz um breve embasamento teórico que permitiu elaborar as perguntas de pesquisa, as hipóteses do trabalho e os objetivos da tese, apresentando a principal razão que motivou a realização deste trabalho.

No Capítulo 2 descrevemos o primeiro estudo experimental (artigo 1), que teve por objetivo principal traçar o perfil do desenvolvimento comportamental de filhotes neonatos e o padrão do comportamento materno de ratos *Wistar* no mesmo ambiente onde posteriormente foi realizado o estudo dos efeitos da adoção. Neste capítulo expomos a introdução, a metodologia e os resultados, bem como apresentamos uma discussão a cerca da possível relação funcional entre a variação dos repertórios do comportamento materno ao longo do desenvolvimento pós-natal inicial e o aparecimento das respostas comportamentais dos infantes para odores biologicamente relevantes.

O capítulo 3 contém o segundo estudo experimental (artigo 2), realizado para investigar os efeitos comportamentais da adoção cruzada repetida de fêmeas lactantes (RCF) sobre a formação do vínculo mãe-filhote em dois períodos importantes do desenvolvimento pós-natal: no período sensível a aprendizagem do apego (dias pós-natal 1 a 3) e no período pós-sensível a aprendizagem de apego (dias pós-natal 9 a 11). Da mesma forma, este capítulo contém introdução, metodologia, resultados e uma discussão específica sobre os achados tanto na perspectiva da mãe adotiva como na perspectiva dos filhotes adotados.

O Capítulo 4 apresenta uma discussão geral de ambos estudos e, por fim, o Capítulo 5 encerra essa tese com as conclusões gerais sobre a pesquisa, apontando as principais contribuições deste trabalho e além disso apresenta perspectivas de trabalho decorrentes da própria pesquisa. No Anexo, encontra-se os comprovantes de submissão dos dois artigos para as respectivas revistas.

1.6 JUSTIFICATIVA

A Neurociência tem feito progressos no estudo sobre as bases neurais do apego através de estudos em humanos (Feldman, 2016; Lenzi et al., 2015) e em outras espécies, particularmente roedores (Moriceau et al., 2009; Sullivan et al., 2015), e também os eventos ambientais que podem afetar essa relação mãe-filhote (Lucion & Bortolini, 2014). Entretanto existe muitas questões que ainda precisam ser elucidadas, como os efeitos da adoção sobre o comportamento de apego do indivíduo adotado, por exemplo.

Baseado no fato de que as experiências iniciais da criança com a sua mãe (cuidador) fornecem o modelo básico para as relações sócio afetivas na vida adulta e que uma série de situações ocorridas nos primeiros anos de vida, como por exemplo a adoção, podem comprometer o desenvolvimento saudável do indivíduo, é de fundamental importância aprofundar o estudo sobre este tema.

Crianças institucionalizadas, apesar de receberem cuidados adequados, apresentam alterações comportamentais importantes na vida adulta. Essas alterações têm sido fortemente associadas com a falta de uma relação de apego seletivo e consistente com a figura materna no início da vida Bruce et al (2009), porém, ainda não está claro como os processos de adoção alteram a relação mãe-infante no início da vida.

Considerando que crianças adotadas possam estar expostas a experiências de maus-tratos, negligência da família biológica ou das instituições de abrigo, e que elas possam apresentar muitas dificuldades em formar ligações afetivas com seus novos pais adotivos, como por exemplo, uma dependência exagerada da figura de apego, distanciamento emocional, comportamentos de insegurança (Oppenheim et al, 2001), este estudo pode lançar mais luz sobre os desvios do desenvolvimento infantil normal e sobre a capacidade das crianças de recuperar o atraso após sofrerem privações significativas, as quais normalmente ocorrem nos casos de crianças entregues para adoção.

2 OBJETIVO GERAL

Avaliar os efeitos comportamentais da adoção cruzada repetida de fêmeas lactantes (RCF) sobre a formação do vínculo (apego) mãe-filhote em dois períodos importantes do desenvolvimento pós-natal: no período sensível e no período de transição para o pós-sensível a aprendizagem de apego.

2.1 OBJETIVOS ESPECÍFICOS

1. Caracterizar as preferências iniciais para odores biologicamente relevantes de filhotes de ratos machos e fêmeas nos dias pós-natal 3, 7, 11 e 15 *(Artigo 1);*

2. Conhecer o papel das variações naturais do comportamento materno nos dias pós-parto 2, 6, 10 e 14 sobre o processo de aprendizado de preferências olfatórias dos filhotes em um ambiente natural *(Artigo 1);*

3. Analisar os efeitos da adoção cruzada repetida de fêmeas lactantes sobre as respostas de preferência olfatória para o odor da mãe biológica e da mãe adotiva em filhotes de ratos, machos e fêmeas, nos dias pós-natal 7 e 15 *(Artigo 2);*

4. Elucidar os efeitos da adoção cruzada repetida de fêmeas lactantes sobre o sistema de inibição do comportamento de aproximação e ou evitação para o odor de um predador natural, em filhotes de ratos, machos e fêmeas, nos dias pós-natal 7 e 15 *(Artigo 2*);

5. Investigar os efeitos da adoção cruzada repetida de fêmeas lactantes cuidadoras sobre o comportamento maternal nos dias pós-parto 1 a 6 e 9 a 14 *(Artigo 2)*;

CAPÍTULO II

ARTIGO I – Este artigo foi submetido para a revista *"Journal of Comparative Psychology"*, conforme comprovante no Anexo A.

DEVELOPMENT OF BEHAVIORAL RESPONSES TO BIOLOGICALLY RELEVANT ODORS IN INFANT RATS

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ABSTRACT

Rat pups born immature but with olfactory system sufficiently functional that allows them to identify the odor of the mother, the nest, conspecifics, and predators. In response to these odors, infants exhibit approach or avoidance behaviors. While the behavioral response to predator odor is innate, it is presumed that the behavioral response to the odor of the mother, nest, and conspecifics is learned during the mother-infant interaction inside the nest. Here, we investigated how the behavioral responses to different biologically relevant odors in rat pups develops during postnatal days (PND) 3, 7, 11, and 15 and how the maternal behavior changed naturally during those periods (postpartum days 2, 6, 10 and 14).

We tested rat pups in a two choice Y-maze using the own nest odor, juvenile rats nest odor, adult male rats nest odor, and sand with cat urine (predator odor) versus the clean shavings as an odor stimuli. We found that preference responses to the own nest odor, juvenile rats nest odor, and even avoidance responses to adult male odor were evident from the PND 7 and not earlier. Although the responses remain unchanged between PND 7 and 11, pups at PND 15 showed an increase in olfactory preference for own nest odor and male rat nest odor. For the odor of congeners, the preference remained stable between PND 7 and PND 15. And for a predator odor rat pups showed avoidance responses from PND 11, not before. We did not find differences in the responses of male and female pups to any of the odors tested. Parallel to the postnatal development of pups, we also observed a significant decrease in the high crouch and licking posture and a significant increase in the number of offspring events between PND 2 and PND 6.The possible functional relationship between the variation of maternal behavior repertories throughout early postnatal development and the appearance of the infants' behavioral responses to biologically relevant odors are discussed.

Keywords: development, olfactory preference, infant attachment, maternal behavior, avoidance.

1 INTRODUCTION

At birth, rats exhibit most of their motor and sensory systems as immature. However, the olfactory system of these animals is already quite functional, which allows them to identify and discriminate biologically relevant odors, triggering different behaviors essential for the survival of the pup. Guided by odors, the pup is able to find the mother's nipple (Al Aïn et al., 2013; Raineki et al., 2010; Teicher & Blass, 1977) orienting and approaching close to the mother (Al Aïn et al., 2016; Gregory & Pfaff, 1971; Sullivan & Perry, 2015), and the congeners (Alberts & Brunjes, 1978; Gregory & Pfaff, 1971). While behavioral responses to predator odor are innate (Pinel et al., 1989; Van Der Poel, 1979), behavioral responses to the mother, the nest, and congener odors need to be learned through associative processes during mother-infant interaction in the first weeks of postnatal life.

Studies show that the conditioning performed outside the nest, even in the attachment-sensitive period, using an artificial odor paired with stimuli that mimic maternal care, such as licking, induces rapidly a behavioral response of olfactory preference to the conditioned odor (Raineki et al., 2010; Sullivan et al., 1990). From these studies, it has been proposed that the mother's odor is learned through the same associative mechanisms within the nest, since the pups are exposed simultaneously to the odor of the mother and to the contingencies of the maternal thermo-tactile stimuli. Thus, when offspring are evaluated out of the nest, in two-choice maze for the nest odor or mother odor versus clean odor, the pups present olfactory responses to the mother's odor or nest odor (Meyer & Alberts, 2016). On the other hand, it has also been suggested that the experiences with the mother inside the nest, both before and after PND 10 are able to induce behavioral responses of preferences for the congeners (Kojima & Alberts, 2009).

The repertoire of maternal behavior in the rat is intense during the first postnatal days, where thermo-tactile contact (nursing postures and licking) is quite intense. As the postnatal period progresses, maternal behaviors decrease and other behaviors gradually increase, such as supine posture and the number of times the mother leaves the nest (dam off the nest). Learning the odor of the mother and the nest coincides with the period in which maternal care is intense, to which the pup is exposed inside the nest. Since huddling preference learning takes place over a longer time window, it is possible that certain patterns of maternal behavior after PND 10 are important to induce such preferences. However, it is still unclear how the olfactory preference responses to the mother odors, the own nest, congeners and other biologically relevant odors naturally develop. Most of the literature on olfactory preferences based on the initial experience is derived from studies with rat pups, and among these, behavioral measures mainly involve diet selection (Galef & Henderson, 1972), huddling preferences (Alberts & Brunjes, 1978; Alberts & May, 1984) and other more complex forms of social affiliation (Fillion & Blass, 1986; Mainardi et al., 1965). Consequently, it becomes important to know at what point in the postnatal development, rat pups begin to respond to the mother, the nest, juvenile, and predatory odors and how these responses change in the first three weeks of postnatal development. In addition, we analyze the characteristics of maternal behavior that occur before, during, and after the onset of these offspring behaviors. Considering the evidence from studies that correlate disorders in primary mother-child relationships with the development of psychopathologies, we believe that the characterization of these behavioral responses can improve our understanding of how these behaviors, that are essential for the formation and maintenance of the mother-puppy attachment, are developed.

Thus, in this work we explore the development of behavioral responses to biologically relevant odors during the first three weeks of the postnatal period of rat pups and characterize the profile of maternal behavior during this period. First, we evaluated the responses to the own nest odor and the of juvenile rat nest odor in male and female offspring on PND 3, 7, 11, and 15 and sought to determine when pups show preference for own nest odor and juvenile rat nest odor in relation to a neutral odor. To our knowledge, response to the juvenile rat nest odor has not been studied in rat pups. Second, we evaluated responses to the adult male rat nest odor and sand with cat urine at PND 3, 7, 11, and 15 to establish when pups show odor aversion in adult male rat nest odor and predator odor relative to a neutral odor. Third, we evaluated maternal behavior on PPD 2, 6, 10, and 14 to identify characteristics in maternal behavior that contribute to the normal development of the offspring.

2. MATERIAL AND METHODS

2.1 ANIMALS AND ETHICS STATEMENT

Animal care and all behavioral experiments were performed in accordance with the *Guidelines for Animal Care and Use of Laboratory Animals* of the National Institutes of Health (NIH) and the home-research institution Animal National Council for Control of Animal Experimentation (CONCEA), and approved by the Ethics Committee in Use of Animals (CEUA) from the Federal University of Rio Grande do Sul, UFRGS (Process no. 28367/2015) (Appendix B).

2.1.1 SUBJECTS AND HOUSING CONDITIONS

A total of 320 Wistar rat pups (equal number of males and females) belonging to 40 litters were used in this study. Animals were bred, and pups were born in the Vivarium of the Department of Physiology at the Federal University of Rio Grande do Sul (UFRGS), descendants from the Center for Reproduction and Experimentation of Laboratory Animals of UFRGS were used.

Dams were group-housed in transparent acrylic boxes with dimensions (45x31x21cm) at temperature (22 ± 1˚C), and humidity-controlled (38%) animal facility. Dams were maintained under a 12h light-dark cycle (lights on at 06:00) with *at libitum* access to food (Nutrilab, Colombo, Brazil) and water. On gestational day (GD) 19, the pregnant rats were individually housed in acrylic cages covered with ample clean shavings for nest building. On postnatal day 1 (PND 1; birth is designated PND 0), litters were culled to 8 pups, with equal number of pups of each sex when possible, and the animals were randomly assigned to PND 3, PND 7, PND 11, and PND 15 group (n = 80, with equal number of male and female pups), for PND 7, 11, and 15 group, the exchange for clean shavings occurred at PND 3, PND 7, and PND 11, respectively (for PND 3 group, shavings were not exchanged and for PND 15, the exchange occurred twice, at PND 7 and 11).

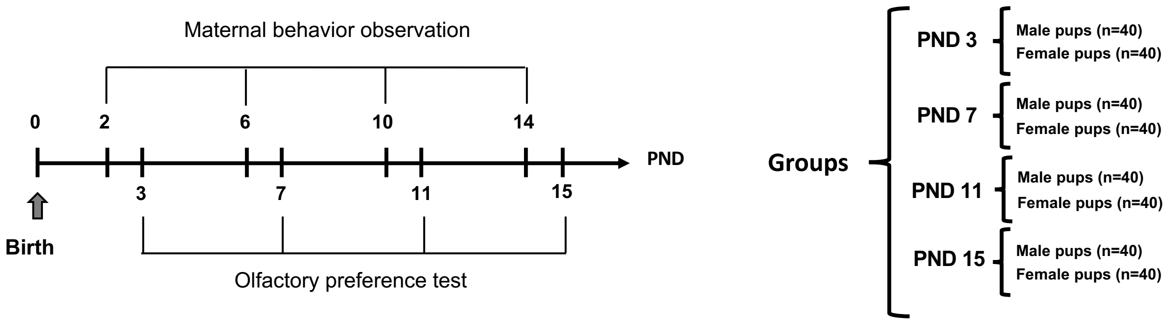

Figure 1: Representation of the experimental design

2.2 BEHAVIORAL STUDIES

2.2.1 Olfactory Preference Test

On PND 3, 7, 11, and 15, one male and female pup (Fig. 1) from each litter were randomly removed from the nest and transported into a small box lined with paper towels to the behavior room, which was at temperature 29 ±˚C . For the test, the pup was placed in the center of the Y-maze and given five trials to choose between two arms (L8,5xW8xH20cm) for pups with PND 3 and 7 and (L12xW10xH20cm) for pups with PND 11 and PND 15. The odors tested and the neutral odor (clean shavings) were placed at the end of one arm into acrylic boxes (L12xW12xH20cm) with several holes in the wall. For experimental odors, we used 300 ml of shavings from own nest, juvenile rat nest, adult male nest, or 60 g of sand with cat urine in a petri dish and 300 ml of clean shavings. Immediately before the test, the pups were removed from their housing boxes (the first always randomly removed), placed in a small box lined with paper towels and transported to the behavior room, which was between 28 ° C to 30 ° C. From each litter a female and a male were chosen to be tested. Different pups of the same litter were used for each tested odor. For each trial, the pup was given one min to make a choice, with 30 seconds of intervals between tests for cleaning the Y-maze and switching the boxes containing experimental and control odors.

Using EthoWatcher® software (Crispim Junior et al., 2012), we recorded the time spent in each arm of the Y-maze (for own nest and juvenile odors). The average time spent in each arm of the Y-maze was calculated for every pup and the result is expressed as a percentage of time the pup remained in each arm (sum of time spent in 5 trials divided by total duration of the test 300 s and multiplied by 100). For odors considered biologically aversive (adult male rat nest odor and predator odor), we recorded the number of choices made by the pups in each trial, with each pup having one minute to make a single choice. We considered as a choice when the pup entered with its whole body in the Y maze arm (Moriceau et al., 2009; Raineki et al., 2010; Sullivan & Wilson, 1991; Perry et al., 2016). Pups that did not make any choice during the five trials were considered non-responsive pups and were excluded from the data analysis. The result was expressed by adding the number of choices made in the five test runs. Pups that did not enter either arm during the five test trials were considered non-responsive pups and were not included in the data analysis.

2.2.2 Maternal Behavior

On postpartum days (PPD) 2, 6, 10, and 14 the maternal behavior of the lactating females was recorded (Fig. 1) through video cameras positioned laterally to the housing cages. For this, we used the protocol developed by (Champagne et al., 2003) and adapted in previous studies (Myers et al., 1989; Pardo et al., 2016; Reis et al., 2014), maternal behavior was recorded four times per day, including 3 periods during the light cycle phase (09:00 h, 12:00 h, 15:00 h) and once at the beginning of the dark cycle (18:00 h). At the observation session, the behavior of each lactating female at that specific time was scored every three minutes (25 observations in four periods per day = 100 total observations / mother / day). For each observation, the location of the mother (inside or outside the nest) was noted. If the mother was in the nest, the following nursing postures were observed: *high crouch posture* (mother nursing pups in an arched-back posture); *low crouch posture* (mother nursing pups in a "blanket" low arched back posture); *supine posture* (a passive posture in which the mother is lying on her back or side while the pups nurse) along with *licking of the pups* (licking the surface of their bodies and their anogenital regions); and *mother off the nest* (the lactating female is out of the nest).

Analysis of maternal behavior was done in two ways: 1) accumulated maternal behavior was calculated through the sum of nursing postures and pup lickings during the recording periods (09:00 h, 12:00 h, 15:00 h, and 18:00 h). To evaluate the variation in nursing behavior we separately compared the cumulative mean scores of the four postpartum period (PPD 2, 6, 10, and 11, respectively). 2) Maternal behavior of the four daily periods of observation (09:00 h, 12:00 h, 15:00 h, and 18:00 h) in PPD 2, 6, 10, and 14. The increase or decrease of maternal behavior was correlated with the behavior of the pups during the same observation period

2.3 STATISTICAL ANALYSIS

All statistics were run in Software GraphPad Prism 8 (San Diego, CA). All data is expressed as median (IQ) or mean ± SEM. To access the differences in the olfactory preference test and maternal behavior variation between groups, we used One-Way ANOVA (for parametric data) or Friedman test (for non-parametric data). The number of animals per group is expressed as “n”. Differences were considered significant when $p<0.05$ and significant results are indicated by asterisk (*) in associated figures and tables.

3. RESULTS

3.1 DEVELOPMENT OF OLFACTORY PREFERENCE BEHAVIORAL RESPONSES TO OWN NEST ODOR

In order to understand how the olfactory preference behaviors of the newborns, especially those directed to the mother, are developed, we subjected rat pups to the olfactory preference test in the Y-maze. On postnatal days 3, 7, 11, and 15, we tested the time that the pups spent in the area with the own nest odor *vs*. neutral odor (clean shavings) *vs*. escape area (odorless). About 60% of male pups (6 of 10 tested) and 40% of females (4 of 10 tested) with PND 3 were responsive to the test and entered at least one of the Y-maze arms during the five trials.

Male and female pups at PND 3 spent more time in the area with the own nest odor compared to neutral odor or escape area, but without statistical significance ($p> 0.05$). However, male and female pups at PND 7, 11, and 15 (10 of 10 tested, respectively) were 100% responsive to the test and spent significantly more time in the maze arm with the own nest odor when compared with neutral odor or in the escape area ($p <0.05$), details in (Table 1, Box 1).

Table 1: Olfactory behavioral responses to own nest odor or juvenile rat nest odor versus clean shavings in male and female infant rats on PND 3, 7, 11, and 15.

Test	PND	Sex	*n*	Time over area			*F (2.18) or* X^2	*P value*
				Clean shavings	**Tested odor**	**Escape**		
Own nest odor	3	M	6	0 (0-0)	0(0-15.67)	0 (0-6.91)	3.600	0.1852
		F	4	0 (0-4.50)	0(0-13)	0 (0-0)	3.875	0.1728
	7	M	10	0 (0-0.58)	**21 (0-71.75)***	0 (0-3.75)	7.000	**0.0261**
		F	10	0 (0-1.66)	**24.67 (0-46.08)***	0 (0-0)	8.615	**0.0075**
	11	M	10	13.32 ± 4.50	**32.82 ± 5.70**$^{\#}$	12.72 ± 2.41	5.113	**0.0263**
		F	10	12.53 ± 2.41	**30.93 ± 8.82***	5.30 ± 2.30	4.891	**0.046**
	15	M	10	20 ± 3.44	**50.73 ± 5.31***$^{\#}$	12.23 ± 2.92	17.42	**0.0005**
		F	10	23.80 ± 4.29	**45.03 ± 4.70**$^{\#}$	13.27 ± 1.81	12.23	**0.0027**
Juvenile rat nest odor	3	M	3	0 (0-0)	0 (0-1.83)	0 (0-0)	2.00	0.7778
		F	3	0 (0-0)	0 (0-5.66)	0 (0-0)	6.00	0.1111
	7	M	10	8.66 (0-21.42)	**39.67 (2.5-49.83)**$^{\#}$	0 (0-10.08)	7.08	**0.0008**
		F	10	0 (0-0.34)	**29.67 (9.75-62)***$^{\#}$	0 (0-0.83)	14.30	**0.0005**
	11	M	10	17.70 ± 4.69	**30.10 ± 6.822**$^{\#}$	7.60 ± 2.74	4.171	**0.0425**
		F	10	18.73 ± 4.56	**34.37 ± 6.79***	11.87 ± 3.93	4.646	**0.0357**
	15	M	10	18.47 ± 3.90	**36.97 ± 5.88***	15.83 ± 4.84	4.469	**0.0431**
		F	10	21.33 ± 7.18	36.60 ± 6.98	16.80 ± 5.02	1.934	0.1798

Data are median values (IQ) or mean ± SEM of time spent over the areas of the Y-maze. Data correspond only to responsive pups in the olfactory preference test. Percentage of time spent in Y-maze areas (time spent in the stimulus area / total approximations * 100) of male and female pups on days 3, 7, 11, and 15 PN. Friedman Test or One-Way ANOVA test significant differences ($p<0.05$) are indicated in bold. * Different from clean shavings and $^{\#}$ different from escape area (Dunn's Test or Tukey's *post hoc* tests).

The comparison of time spent in the area with own nest odor, between males and females at the same postnatal day did not show a statistically significant difference ($p> 0.05$), data in (Table 3), we combined the means of pups of both sexes at the same postnatal day in the same group and analyzed through One-Way ANOVA if the time spent near the own nest odor decreased as the pups' age increased, especially during the period of transition from the sensitive period to the post sensitive for attachment learning (PND 10-14).

We observed that the search behavior and approach to own nest odor tended to increase as the age of the offspring advanced [$F(3,76) = 12.45$, $p\leq0.0001$]. This preference was significantly higher on PND days 7 and 11 when compared to PND 3 ($p <0.05$), but when we compared PND days 7 and 11 with each other, we did not find a significant difference ($p> 0.05$). In PND 15 the pups preferred significantly more own nest odor than in other developmental periods (p <0.05) (Figure 2 A). Thus, we found no decrease in olfactory preference for own nest odor in any of the postnatal days studied. In other words, as the age of the pups advanced, they preferred the own nest odor.

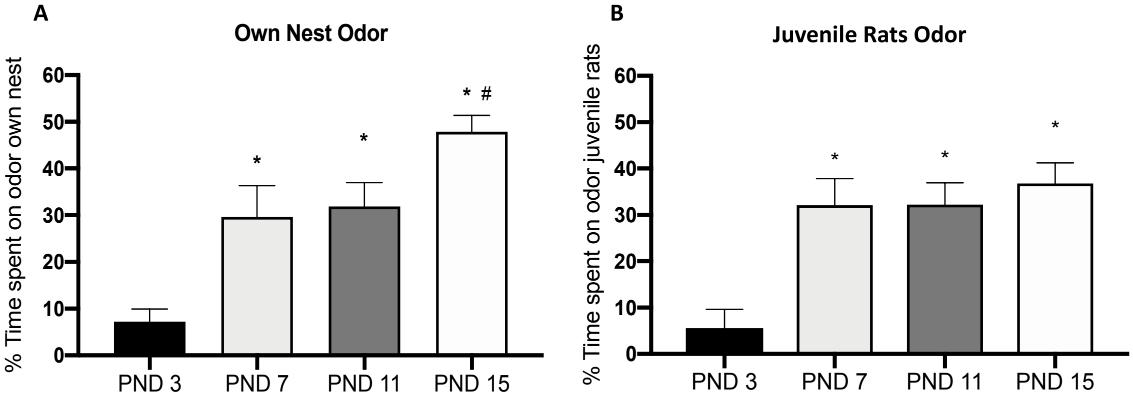

Figure 2: Olfactory preference profile to own nest odor and juvenile rats odor during early development. A) Olfactory preference of rat pups (male and female) at PND 3 (n=10), PND 7 (n=20), PND 11(n=20), and PND 15 (n=20) for the own nest odor. B) Olfactory preference of rat pups (male and female) at PND 3 (n=6), PND 7 (n=17), PND 11(n=20), and PND 15 (n=20) to the juvenile rats odor. Data correspond only to responsive pups in the olfactory preference test and data are expressed as mean ± SEM. One-way ANOVA was performed, followed by Tukey's *post hoc* test when appropriate. p< 0.05, significantly different from the other group. * Different from PND 3; $^{\#}$Different from PND 7 and PND11.

3.2 DEVELOPMENT OF OLFACTORY PREFERENCE BEHAVIORAL RESPONSES TO JUVENILE RAT NEST ODOR

To verify the presence of olfactory behaviors by other congeners, we subjected rat pups to the olfactory preference test in the Y-maze on postnatal days 3, 7, 11, and 15. For this, we evaluated the time the pups spent in the area of the maze with juvenile rat nest odor versus clean shavings (neutral odor) or escape area (odorless).

Only 30% (3 out of 10 of the male offspring tested) and 30% (3 out of 10 of the females tested) at PND 3 were responsive to the odor preference test for juvenile rat nest odor. When comparing the time spent in the maze area with juvenile rat nest odor, neutral odor or escape area no significant difference (p> 0.05) was observed for this age. On the other hand, 100% of the pups at PND 7, 11, and 15 were responsive, totalizing (10 males and 10 females tested) for each postnatal age. Male and female pups at PND 7 and 11 spent significantly more time in the juvenile rat nest odor area when compared to the neutral odor area or escape area (p <0.05). Similarly, male offspring at PND 15 also came closer to the juvenile rat nest odor; however, olfactory preference responses for females at PND15 did not show any significant difference between labyrinth areas, for more details see (Table 1, Box 1).

To test differences in olfactory preference between the sexes, we compared the time spent in the area with juvenile rat nest odor, between male and female pups at the same postnatal age and did not find a statistically significant difference (p <0.05), data (Table 3). In this way, we combined the means of the pups of both sexes with the same postnatal age in the same group and evaluated whether the time spent in the juvenile rat nest odor decreased or increased, as the age of the offspring advanced, especially the transition period changed from sensitive to attachment then to post-sensitive (PND 10-14).

One-Way ANOVA showed that in PND 7 the pups presented a significant olfactory preference for the juvenile rat nest odor [F (3,76) = 8,875, p <0.0001], but this preference did not increase as the age advanced, that is, it remained stable after PND 7, with a slight increase in the mean time spent on the juvenile rat nest odor only in PND 15 (36.78 ± 4.44) when compared to the mean on days PND 7 (32.07 ± 5.75) and 11 (32.23 ± 4.71), but without statistical significance (Figure 2 B). Thus, our data showed that from PND 7 the pups already presented olfactory preference responses by other olfactory stimuli besides the own nest odor. However, the profile of the responses to the juvenile rat nest odor was different from the responses to maternal odor on PND 7, 11, and 15, that is, for the juvenile rat nest odor the preference remained stable from PND 7, and for own nest odor the preference increased significantly as the postnatal age of the pups progressed.

3.3 DEVELOPMENT OF OLFACTORY AVERSIVE BEHAVIORAL RESPONSES TO PREDATOR ODORS

To understand how the first rat pups' avoidance responses to stimuli considered aversive develop, that is, the adult male rat nest odor and predator odor, we subjected rat pups to the olfactory / avoidance preference test in the Y-maze. On postnatal days 3, 7, 11, and 15, we tested the number of entries in the maze area with neutral odor (clean shavings) versus the escape area (no odor) or the area of aversive odor (adult male rat nest odor or predator odor).

3.3.1 Development of olfactory aversive behavioral responses to adult rat nest odor

Male and female offspring at PND 3 were not responsive to the test and were excluded from the analysis. Of the offspring at PND 7, 70% of males (7 of 10 tested) and 50% of females (5 out of 10 tested) were responsive to the test and animals at PND 11, 80% of males (8 of 10 tested), and 100 % of females (10 out of 10 tested) were also responsive. Finally, 100% of males and females (10 of 10 tested for each sex) at PND 15 were also responsive to the odor avoidance test of adult male rat nest odor.

Male offspring at PND 7 had a median of 3 (0-3) for adult male odor ($p <0.05$), however, when comparing the areas of the Y-maze with each other, the *post hoc* did not show a significant difference ($p> 0.05$). Females at PND 7 and 15, and males at PND 15 did not present preference or avoidance responses to adult male rat nest odor ($p> 0.05$), however females at PND 11 had a higher median adult male rat nest odor 2 (0-4.25) with *post hoc* indicating that they got closer to adult male rat nest odor than to other areas of the maze ($p = 0.0138$), more details in (Table 2, Box 1).

Table 2: Olfactory behavioral responses to adult male rat nest odor or sand with cat urine (predator odor) versus clean shavings in male and female infant rats on PND 3, 7, 11, and 15.

	PND	**Sex**	***n***	**Number of choices for the areas**			***X2 or F (2,18)***	***P value***
				Clean shavings	**Tested odor**	**Escape**		
Adult male nest odor	7	M	7	0 (0-0.25)	3 (0-3)	0 (0-1)	7.440	**0.0204**
		F	5	0 (0-1)	0 (0-1.25)	0 (0-0)	3.500	0.2593
	11	M	8	2 (0-4.25)	2 (0-4.25)	0.50 (0-1)	5.000	0.0905
		F	10	2 (1-4)	**2 (2-3.5)$^{\#}$**	1 (0.75-1.25)	8.061	**0.0138**
	15	M	10	3 (1.75-4)	4 (2.75-4)	3 (0.75-3)	8.240	**0.0105**
		F	10	3 (2-4)	4 (2.75-5)	3 (2-4)	9.083	**0.0099**
Sand with cat urine odor	7	M	5	0.5 (0-3)	0 (0-0)	0 (0-0.25)	7.412	**0.0185**
		F	7	0 (0-0.25)	0 (0-0)	0.5 (0-1)	3.714	0.2455
	11	M	9	2.30 ± 0.47	**0.60 ± 0.26***	1.20 ± 0.44	4.208	**0.0430**
		F	10	1.50 ± 0.47	**0.60 ± 0.26$^{\#}$**	2.70 ± 0.44	7.364	**0.0117**
	15	M	10	3.50 ± 0.37	2.00 ± 0.53	3.00 ± 0.57	3.000	0.0777
		F	10	3.00 ± 0.47	**1.60 ± 0.33***	1.80 ± 0.44	5.568	**0.0153**

Data are median values (IQ) of number of choices for the areas of the Y-maze. Data correspond only to responsive pups in the avoidance test. Number of choices in Y-maze areas of male and female pups on days 7, 11, and 15 PN. Parametric and non-parametric data were analyzed using One-Way ANOVA or *Friedman Test*, followed by Dunn's Test or Tukey's Test. * Different from clean shavings and $^{\#}$ different from escape area. P<0.05.

We compared the responses obtained in the adult male rat nest odor test between male and female pups at the same postnatal age and did not find differences of preference or avoidance in the studied ages (Table 3), so we combined the data of male and female pups on PND 7, 11, and 15 in the same group and analyzed through One-Way ANOVA the behavioral responses of preference or avoidance in the studied postnatal periods.

We found a significant difference between the adult male rat nest odor area vs. clean odor and escape area [F (2.38) = 8.087; p = 0.0031] for pups at PND 7 and, *post hoc* showed that the pups chose on average (1.30 ± 0.35) times more the area with adult male rat nest odor than the area with clean odor (0.40 ± 0.18) or escape area (0.20 ± 0.09), (p = 0.0097). When we tested pups at PND 11 and 15 One-Way ANOVA also showed a significant difference for maze areas [F (2, 38) = 6.801; p = 0.0066] and [F (2.38) = 9.983; p = 0.0003], with *post hoc* indicating that the pups on PND 11 more often chose the area with adult male rat nest odor (2.45 ± 0.37), when compared to the area with clean odor (2.25 ± 0.39) and the escape area (0.85 ± 0.20), (p = 0.024). This pattern was also observed in the *post hoc* for the PND 15 pups, which also chose the adult male rat nest odor area (3.65 ± 0.26) as compared to the area with the clean odor (2.95 ± 0.24), (p = 0.024) and with the escape area (2.5 ± 0.26), (p = 0.0009).

Table 3: Comparison of male and female pup olfactory behavioral responses on PND 3, 7, 11, and 15 to biological relevant odors.

Test	**PND**	**n**	**Median (IQ) or Mean ± SEM**		**U or *t (18)***	***P value***
			Female	**Male**		
Own nest odor	3	10	0 (0-13)	0 (0-15.67)	49	0.9600
	7	20	24.67 (0-46.08)	21 (0-71.75)	47	0.8497
	11	20	30.93 ± 8.82	32.82 ± 5.70	0.1796	0.8595
	15	20	45.03 ± 4.70	50.73 ± 5.31	0.8029	0.4325
Juvenile rat odor	3	6	0 (0-5.66)	0 (0-1.83)	46	0.8607
	7	20	32.77 ± 8.24	31.37 ± 8.46	0.1185	0.9070
	11	20	34.37 ± 6.79	30.10 ± 6.82	0.4431	0.6630
	15	20	36.60 ± 6.98	36.97 ± 5.88	0.04016	0.9684
Adult male rat odor	3	0	-	-	-	-
	7	12	0 (0-1.25)	3 (0-3)	31.50	0.1287
	11	18	2.70 ± 0.42	2.20 ± 0.62	0.6597	0.5178
	15	20	3.90 ± 0.37	3.40 ± 0.37	0.9430	0.3581
Sand with cat urine odor	3	12	-	-	-	-
	7	19	0 (0-0)	0(0-0)	50	>0.9999
	11	20	0.60 ± 0.26	0.70 ± 0.26	0.2683	0.7915
	15	20	1.60 ± 0.33	2 ± 0.53	0.6290	0.5373

Data represent the median values (IQ) or mean ± SEM for time spent over the areas of own nest odor, juvenile rat odor, adult male rat odor, and sand with cat urine odor in male and female pups on PND 3, 7, 11, and 15. Mann-Whitney Test U or Unpaired *t* Test was performed to compare male and female pups in each age; p<0.05.

3.3.2 Development of olfactory aversive behavioral responses to cat urine odor

Data analysis showed that rat pups did not exhibit odor avoidance behaviors with adult male rats over the study period (PND 7, 11, and 15) as can be observed in (Figure 3A).

None of the male and female pups on PND 3 responded to the predator odor avoidance test. About 50% of males (5 of 10 tested) and 70% of females (7 of 10 tested) on PND 7 and, 90% (9 of 10 tested) of males and 100% of females (10 of 10 tested) on PND 11 responded to the test. Of the offspring at PND 15, all males (10 of

10 tested) and all females (10 out of 10 tested) also responded actively to the predator odor avoidance test.

Male offspring at PND 7 chose the maze side with the clean odor a median of times slightly higher when compared to the predator odor side or the escape area ($p < 0.05$), but the *post hoc* did not indicate a significant difference between areas. The responses of females at PND 7 were not significant for any of the areas of Y-maze. However, male and female offspring on PND 11 and females on PND 15 significantly avoided the area with predator odor ($p < 0.05$) when compared to the area with clean odor and the escape area. However, male offspring on PND 15 did not have significant responses, further details in (Table 2, Box 1).

We observed that the behavioral responses of the male and female offspring to the predator odor did not differ statistically according to sex (Table 3), in this way we combined the data of the pups in groups by postnatal age, PND 7, 11, and 15 and evaluated through the One-Way ANOVA the number of choices in the Y-maze during postnatal development.

The Friedman test did not identify a significant difference between the choices made by pups at PND 7 in the Y-maze [Friedman = 4,895; p = 0.0865] (Figure 3B). However, for pups at PND 11, One-Way ANOVA found a significant difference [F (2, 38) = 6.136; p = 0.0118), and *Post Hoc* showed that the pups on PND 11 significantly avoided predator odor (0.60 ± 0.18) when compared to clean odor (1.90 ± 0.33) and escape area (1.95 ± 0.35) (Figure 3B). One-Way ANOVA also found a significant difference in pup responses on PND 15 [F (2,38) = 7,278; p = 0.0022], and the *Post hoc* indicated that this difference is significantly greater between the clean odor (3.25 ± 0.29) and predator odor (1.80 ± 0.31) than with the escape area (2.40 ± 0.37) (Figure 3 B). Thus, the data showed that rat pups at PND 11 and 15 presented consistent avoidance responses to a predator odor, having more preference to a familiar odor like the clean shavings than to the escape area. These avoidance responses were not found in younger pups.

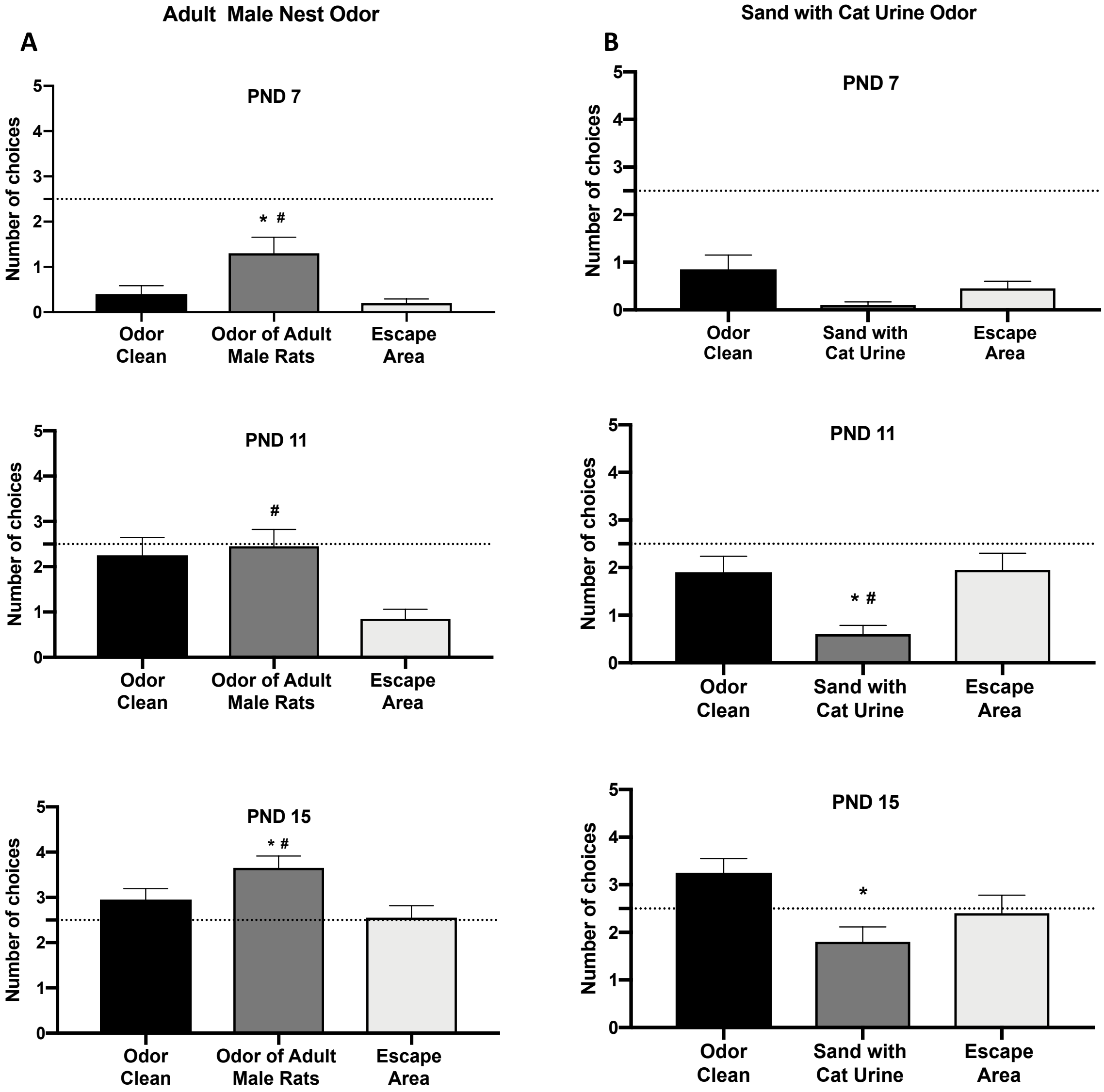

Figure 3: Profile of behavioral responses to adult male nest odor and sand with cat urine odor (predator odor) during early life. A) Number of choices to adult male nest odor, clean odor, and escape area in the Y-maze of pups with PND 7 (above, n=20), PND 11 (middle, n=20) and PND 15 (below, n=20). B) Number of choices to predator odor, clean odor, and escape area in the Y-maze of pups with PND 7 (above, n=14), PND 11 (middle, n=19), and PND 15 (below, n=20). Data correspond only to responsive pups in the avoidance test and data are expressed as mean ± SEM. One-way ANOVA was performed, followed by Tukey's *post hoc* test when appropriate. * Different from clean odor; $^{\#}$ Different from escape area. $p < 0.05$.

3.4 NATURAL VARIATION OF THE MATERNAL BEHAVIOR

The maternal behavior of lactating females was evaluated in postpartum days 2, 6, 10, and 14 with the aim of tracing a profile of the variation of these behaviors parallel to the development of the pup's behavioral responses to biologically relevant odors. Table 4 and Figure 4 show the pattern of maternal behavior of lactating females during the first two weeks of postnatal life of the pups. We observed that the number of events recorded for high crouch and for licking of the pups posture decreased significantly as the postpartum period progressed ($p<0.05$). Differently, the number of events recorded for posture supine was significantly higher on PPD 14 ($p <0.05$) when compared to PPD 2, 6, and 10.

Table 4: Cumulative maternal behavior of the four daily observations during postpartum period day 2, 6, 10, and 14.

Behavioral parameters	**Postpartum Day**	**Mean±S.E.M or Median (IQ)**	**F (3, 117) or X^2**	***P value***
High crouch posture	PPD 2	17.13±1.50	32.21	**<0.0001**
	PPD 6 *	12.28±1.26		
	PPD 10 * #	9.45±0.97		
	PPD 14 * #	7.20±1.05		
Low crouch posture	PPD 2	0(0-0)	5.982	0.1125
	PPD 6	0(0-0)		
	PPD 10	0(0-0.75)		
	PPD 14	0(0-1)		
Supine posture	PPD 2	0(0-0)	17.97	**0.0004**
	PPD 6	0(0-0)		
	PPD 10	0(0-1)		
	PPD 14 *	1(0-3)		
Licking	PPD 2	4(2-6.5)	34.33	**<0.0001**
	PPD 6 *	2(0.25-4)		
	PPD 10 *	1(0-3)		
	PPD 14 *	1(0-2)		
Dam off the nest	PPD 2	8.40±1.27	17.93	**<0.0001**
	PPD 6 *	12.10±1.11		
	PPD 10 *	14.08±1.14		
	PPD 14 * #	14.95±0,90		

Data are presented as mean ± SEM (parametric data) or median (IQ) (non-parametric data) and represent the accumulated occurrence of the behavior in postpartum days 2 (n=10), 6 (n=10), 10 (n=10), and 14 (n=10). One-way ANOVA or Friedman Test was performed, followed by Dunn's or Tukey's *post hoc* test when appropriate. * Different from PPD2; # Different from PPD 6. $p<0.05$.

When we analyzed the maternal care by time of day and the postpartum day, we observed that the mean number of events recorded for high crouch posture during the daylight period (09:00 h, 12:00 h, and 15:00 h) was significantly greater than the mean of events recorded in the dark period (18:00 h) on PPD days 2, 6, 10, and 14 (p <0.05). However, only on PPD 2 and 10 the mean of licking of the pups was higher in daylight than in the dark period (p <0.05), and curiously on PPD 6 and 14 this had no more significant difference between the daylight period and the dark period (p > 0.05). No significant changes were identified for the low crouch nursing posture between the PPD 2, 6, 10, and 14 but not for comparison among daytime schedules (p > 0.05), further details in Table 4, 5. The supine nursing posture had a slight increase as the postpartum period progressed, and this was significantly higher on PPD 14 compared to PPD 2, 6, and 10 (p <0.05), (Table 4), however, there was no difference among the time of the day (p> 0.05) (Table 5).

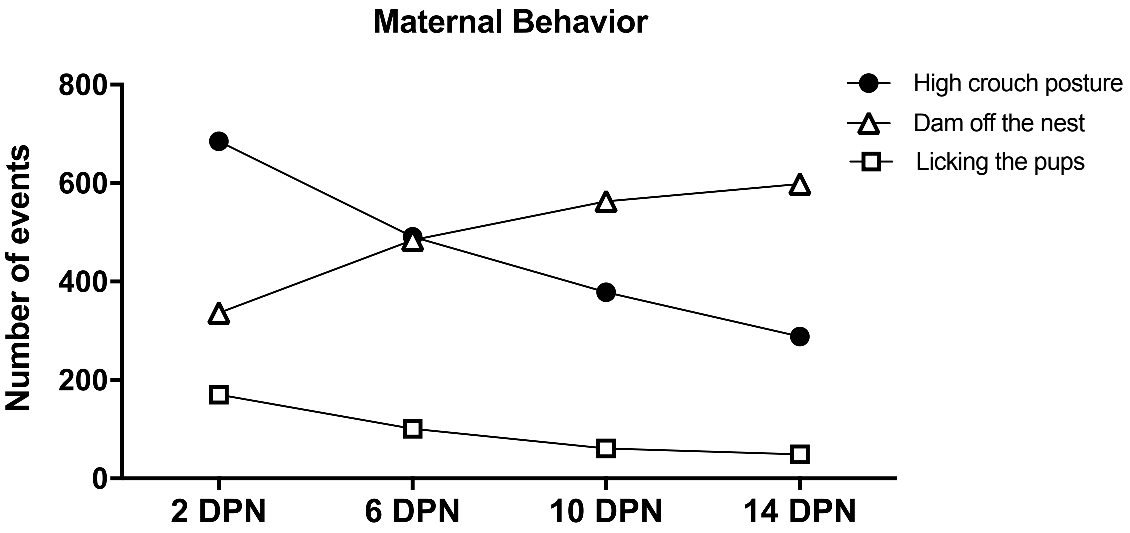

Figure 4: Naturally occurring variations in maternal behavior at PPD 2, 6, 10, and 14. Illustration of cumulative occurrence of maternal behavior during PPD 2 (n=10), PPD 6 (n=10), PPD 10 (n=10), and PPD 14 (n=10). High crouch posture (HG), Licking the pups (L) and Dam outside the nest (OFF).

Finally, Figure 4 and Table 4 show that as the postpartum period progresses the dam left the nest more frequently (p <0.05), and the period in which these absences occurred were significantly higher in the dark cycle (18:00 h) when compared to the light cycles (09:00 h, 12:00 h, and 15:00 h), (p <0.05). On PPD 10, the number of events the mother was observed outside the nest was not significantly different between the dark cycle and the light cycles, more details are presented in Table 5.

Table 5: Differences in maternal behavior during the 4 periods of observation in postpartum day 2, 6, 10, and 14.

Behavioral parameters	**PPD**	**Period of observation**				**F (3.27) or X^2**	***P value***
		Mean± S.E.M or Median (IQ)					
		09:00 h	**12:00 h**	**15:00 h**	**18:00 h**		
High crouch posture	**2**	24.10±1.75	22.80±1.63	18.30±1.29	**3.30±1.11**$^{*\# a}$	53.07	**<0.0001**
	6	18.20±2.26	14.90±1.40	14.60±1.24	**1.40±0.73**$^{*\# a}$	22.95	**<0.0001**
	10	13.90±1.68	11.30±1.52	11.10±1.00	**1.5±0.58**$^{*\# a}$	14.78	**0.0001**
	14	12.30±2.75	7.20±1.22	8.30±1.52	**1.00±0.59**$^{*\# a}$	11.69	**0.0011**
Licking	**2**	7.10±1.0	4.50±1.01	3.80±0.41	**1.60±0.54**$^{* a}$	8.830	**0.0032**
	6	3.50±0.77	2.30±0.42	3.60±1.06	0.70±0.39	3.128	**0.0770**
	10	2.20±0.46	1.60±0.58	2.00±0.42	**0.30±0.21**^{a}	3.288	**0.0499**
	14	2.10±0.76	1±0.36	1.5±0.45	0.30±0.15	2.795	**0.0856**
Low crouch posture	2	0(0-0.25)	0(0-0)	0(0-0)	0(0-0)	2.000	0.5724
	6	0(0-0.75)	0(0-0)	0(0-0)	0(0-0)	1.435	0.6974
	10	0(0-1)	0(0-1)	0(0-0.25)	0(0-0.25)	1.023	0.7958
	14	0(0-0)	0(0-1.25)	0(0-2.25)	0(0-1)	4.071	0.2539
Supine posture	2	0(0-0)	0(0-0.25)	0(0-0.25)	0(0-1)	2.167	0.5385
	6	0(0-0.25)	0(0-1.5)	0(0-0)	0(0-0)	3.000	0.3916
	10	0.5(0-1)	0(0-1)	0(0-0.25)	0(0-1)	1.345	0.7185
	14	0(0-5.25)	1(0-3.25)	1.50(0.75-3)	0(0-1)	2.788	0.4254
Dam off the nest	**2**	3±0.66	3.6±0.49	5.7±1.19	**21.30±0.86**$^{*\# a}$	134.2	**<0.0001**
	6	8.1±1.32	9.2±1.52	10.60±1.10	**20.50±2.33**$^{*\# a}$	10.93	**0.0005**
	10	9.3±1.94	10.90±1.31	12.60±1.64	23.50±0.76	16.28	**<0.0001**
	14	9.6±1.12	**14±1.24***	**14.60±1.50***	**21.60±0.88**$^{*\# a}$	28.50	**<0.0001**

Data represent the maternal behavior (high crouch posture, low crouch posture, supine posture and licking the pups) and dam off the nest on 4 periods of observation in postpartum day 2, 6, 10, and 14. Data parametric are presented as mean ± SEM and data non-parametric as the median (IQ). One-way ANOVA or Friedman Test was performed, followed by Dunn's or Tukey's *post hoc* test when appropriate. * Different from 09:00 h; # Different from 12:00 h; ^{a} Different from 15:00 h. p<0.05.

4. DISCUSSION

This study provided a detailed analysis of the behavioral profile of rat pups in response to different biologically relevant odors (own nest odor, juvenile rat nest odor, adult male rat nest odor, and predator odor) on postnatal days 3, 7, 11, and 15, along with the natural variation in maternal behavior in the postpartum days 2, 6, 10, and 14. Our results showed that the olfactory preference responses to the own nest odor, juvenile rat nest odor, and even responses to the adult male rat nest odor were evident from PND 7 and not before. Although the responses remained unchanged between PND 7 and 11, pups at PND 15 showed an increase in olfactory preference for own nest odor and the adult male rat nest odor. For the juvenile rat nest odor, the preference remained stable between days PND 7 and PND 15, and for a predator odor rat pups showed avoidance responses from PND 11, not before (Box 1). Parallel to the postnatal development of pups, we also observed a significant decrease in the high crouch and licking posture and a significant increase in the number of offspring events between PND 2 and PND 6.

Box 1: Olfactory behavioral responses to own nest odor, juvenile rat nest odor, adult male rat nest odor or sand with cat urine (predator odor) in male and female infant rats on PND 3, 7, 11, and 15. (-) Pups not responsive; (↑) Trend preference with $0.05<p<0.1$; (↑↑*) Preference with $p<0.05$; (↓) Trend avoidance with $0.05<p<0.1$; (↓↓*) Avoidance with $p<0.05$

PND	Sex	Own nest Odor	Juvenile rat nest odor	Adult male nest odor	Sand with cat urine odor
3	M	↑	↑	-	-
	F	↑	↑	-	-
7	M	↑↑*	↑↑*	↑	↓
	F	↑↑*	↑↑*	↑	↓
11	M	↑↑*	↑↑*	↑	↓↓*
	F	↑↑*	↑↑*	↑↑*	↓↓*
15	M	↑↑*	↑↑*	↑↑*	↓
	F	↑↑*	↑	↑↑*	↓↓*

4.1 THE BEHAVIORAL PREFERENCE TO OWN NEST DEVELOPS ALONG WITH IMPORTANT VARIATIONS IN MATERNAL BEHAVIORS

Our results showed that pups of all ages studied presented olfactory responses to own nest odor (Table 1) and that this preference tended to increase, similarly in males and females, as the postnatal development progressed (Figure 2A). On PND 3 the pups already discriminated the odor of their own nest, however, it is from PND 7 that the own nest odor approximation responses became significantly more consistent. Between PND 7 and 11 these behavioral responses remained stable and increased again in PND 15.

A similar pattern of responses was described in Meyer & Alberts (2016), who also identified that rodent pups (*Mus musculus*) at PND 5, 10, and 12 presented orientation and preference responses to the own nest odor in relation to clean odor and that preference also increased during postnatal development. The work of Professor Regina Sullivan's group consistently showed that the attachment to the mother's nest was through a learning process and consequently the memory formation of this initial filial relationship (Landers & Sullivan, 2012; Opendak & Sullivan, 2016; Sullivan & Wilson, 1991). In both humans and animals, attachment required a brief learning experience of the sensory characteristics of the maternal figure (odor, texture, color, sound) that occured during the infant's first interactions with its caregiver (Debiec & Sullivan, 2017; Raineki et al., 2010; Sullivan & Holman, 2010).

Specifically, maternal odor controlled the interactions of rat pups with the mother, including approach responses, social behavior, and attachment to the nipple (Raineki et al., 2010). In rats, the olfactory response of the pup to the odor of the mother was evaluated by the orientation and approaching behavior of the odor source and occured during the first 10 days of the pup's life. In this period, also called the attachment-sensitive period, pups could be easily conditioned to a different odor other than the maternal one, as long as it was paired with thermo-tactile stimuli (Debiec & Sullivan, 2017; Landers & Sullivan, 2012; Meyer & Alberts, 2016) indicating an associative learning process.

At the same time, we observed variations in the maternal behavior in relation to the pups from PPD 6, there was specifically a reduction in the posture of high crouch feeding and also in the frequency of the behavior of licking the pups. Decrease in these behaviors tended to decline as the postpartum period progressed (Figure 2). The high crouch posture was considered an active maternal care posture that the lactating rats performed mainly during the first days of postpartum and this gradually decreased as the pups aged (Cramer & Alberts, 1990). We also observed, from PND 6, increase in the frequency with which mothers were absent from the nest, especially during the dark period (Table 4, 5).

Mothers performed this nursing posture stimulated by the pups, which stimulated the maternal womb to gain access to their nipples. The behavioral repertoire presented by mothers in the first week of postnatal life was rhythmic and most of the nursing postures observed were in the first session of daily recording at 09:00 h and, throughout the day, these behaviors gradually diminished until the last recording at 18:00 h, already in the dark period. From that moment, the mothers began presenting behaviors of leaving the nest to explore the housing box.

During this period the pups' responses to the own nest odor were mediated and influenced by some associative process within the nest, we assumed that the stability and constant matching of the maternal odor (own nest odor) and the nest with repertoires of maternal behavior were the basis for associative learning that occured within the nest. From this natural pairing, the noradrenaline system was triggered in the offspring, which was necessary to form the odor association with contingencies of maternal behavior (Debiec & Sullivan, 2017; Opendak & Sullivan, 2016).

The pups’ abilities to detect, recognize, and discriminate among other odors were likely to increase with age. According to the literature, other changes in postnatal development might also contribute to the increase in the olfactory preference of the pup due to the smell of their own nest, including the expansion and differentiation of the olfactory system that occured throughout the postnatal period 5 - 12 (Pomeroy et al., 1990).

4.2 DEVELOPMENT OF OLFACTORY PREFERENCE TO THE JUVENILE RAT NEST ODOR

To better understand the development of olfactory preferences for non-maternal odors, and as these responses changed over the postnatal period, we tested the olfactory preference of pups at PND 3, 7, 11, and 15 for the odor of juvenile rats in relation to clean shavings. Only 30% of the pups tested on PND 3 responded to the juvenile rat nest odor, however from PND 7 the responses to the juvenile rat nest odor were evident indicating that the rat pups, still in the sensitive period of learning attachment, might also prefer non-maternal odors, probably due to postnatal experience within the nest. This olfactory preference for juvenile rat nest odor did not change between PND 7, 11, and 15 (Table 1) and there was no difference in responses between male and female pups.

Thus, our results showed that own nest odor and juvenile rat nest odor triggered similar behaviors in the pups during the first two weeks of postnatal life.

The identification of members belonging to the group or external to it and / or of biological and non-biological offspring was of evolutionary importance and was related to the survival behavior of the species (Lévy & Keller, 2009). In rodents, the mechanisms that allow the offspring to identify other members of the same species and of the same age were based on olfactory clues, emitted by feces, urine, and corporal excretions of the offspring. These olfactory cues were used by the offspring to recognize the mother, to find her in the nest, and to be directed to the nipple for nursing was essential for the establishment of affective bonds between pups and mothers (Perry et al., 2016).

Our data indicated that instead of losing interest in the maternal odor (own nest odor) as the postnatal age progressed, rat pups might discriminate and direct behaviors of preference toward juvenile rat nest odor, even though they were experiencing mother-infant interaction within the nest. Probably this olfactory preference for the juvenile rat nest odor related to the associative processes that occurred within the nest.

4.3 DEVELOPMENT OF BEHAVIORAL RESPONSE TO ODOR AVOIDANCE OF ADULT MALE RATS NEST ODOR

In order to continue to understand how olfactory preferences of rat pups develop to important odors and how these responses change with advancing age, we evaluated the preference or avoidance responses to the adult male rats nest odor, which is considered an aversive stimulus to rat pups. In order to do this, we submitted male and female rats on PND 3, 7, 11, and 15 to the Y-maze for adult male rats nest odor vs. clean shavings.

At PND 3, none of the pups tested was responsive to the odor test of adult male rats. From PND 7, the offspring surprisingly exhibited approximation and preference responses for the nest odor of adult male rats and as the postnatal age advanced the responses preferably also increased similarly in male and female pups. According to the study by Perry et al. (2016), the adult male rats nest odor for offspring exclusively raised with the mother was highly aversive. In our experiment the adult male rats nest odor was not an aversive odor for rat pups at PND 7, 11, and 15.

Studies have shown that adult male rats commit infanticide against rat pups, presumably to increase breeding opportunities (Mennella & Moltz, 1988). Thus, the adult male rats nest odor was considered an aversive odor for rat pups, as it interrupted the ultrasonic vocalizations during the first days of life and caused immobility and / or freezing in pups with more than 10 days of postnatal life (Hofer, Shair, & Brunelli, 2002; Perry et al., 2016; Takahashi, 1992). The work of Perry et al. (2016), showed that rat pups at PND 8, 14, and 23 strongly avoided the adult male rats nest odor.

Surprisingly the pups at PND 7, 11, and 15 showed behaviors of approach to this odor. We attributed this olfactory preference to the fact that adult male rats were bred in the same housing as neonate rats, but in separate housing boxes. We assumed that the fact that the tested pups shared the same physical environment as the adult males made the odor familiar and, consequently, that odor lost the aversive effects observed in other studies.

Perry's study showed that rat pups exposed to the odor of adult male rats in their nest from birth with both mother and father no longer exhibited aversive responses to the odor of male rats in a Y-maze. Instead, they showed preference for male odor (Perry et al., 2016).

Other studies also showed that pups raised in "co-breeding" with a familiar adult male, presented olfactory responses to the odor of this adult male on days PN 14 and 23 (Nacher et al., 2004; Roth et al., 2006; Roth & Sullivan, 2005).

Thus, our results indicated that early-life experiences, even when the pups were confined to the nest, might alter behavioral responses to preference and / or aversion to relevant odors.

4.4 DEVELOPMENT OF BEHAVIORAL AVOIDANCE RESPONSE TO PREDATOR ODOR

Finally, we evaluated the approach and avoidance responses of rat pups at PND 3, 7, 11, and 15 to a naturally aversive stimulus (cat urine odor). Pups at 3 and 7 days of postnatal life did not present avoidance responses to the predator odor. However, from PND 11 rat pups exhibited consistent avoidance responses to predator odor and these responses increased as the postnatal age progressed similarly in males and females.

Studies have shown that odor guided approximation and avoidance behaviors that began at birth in many mammals (Al Aïn et al., 2017; Moriceau, 2004). Olfactory cues were essential for newborn rodents to approach the mother and attach to the nipples, and also to suppress the ultrasonic vocalizations in the presence of a predator (Perry et al., 2016; Takahashi, 1992).

In the altricial species, very young pups confined to the nest depended entirely on the care of their caregiver for protection. The expression of fear changed during maturation in ways appropriate to the animal's developmental stage and ecological niche: the rat pup did not freeze to the predator's odor until it began to crawl out of the nest. For a rat, these brief exits started around postnatal day 10 (Bolles & Woods, 1964), when the amygdala functionally integrated to support species-specific innate defensive responses to predator odor such as freezing (Moriceau et al., 2004; Sullivan et al., 2000; Wiedenmayer & Barr, 2001).

The work of Professor Regina Sullivan's group showed that up to PND 9, pups were extremely dependent on maternal care. At this stage of life, mothers often mistreated their young, with bites, kicks, and similar behaviors. In this way, the formation of an aversive pairing between the mother and the pup would be disadvantageous for the survival of the animal. The group retook Bowlby's attachment theory, pointing out that one of the characteristics of the initial bond was that regardless of the physical abuse they suffered, infants remained attached to the caregiver. On the other hand, at 9 days of age the rats began to move quite a bit and the environment with which they had contact increased, thus, aversive learning was necessary for survival outside the maternal environment. And this coincided with the end of the sensitive period and with the pup's neurobiological maturation (Debiec & Sullivan, 2017; Moriceau et al., 2010; Opendak & Sullivan, 2016).

5 FINAL CONSIDERATIONS

The present study provided a detailed analysis of the behavioral profile of rat pups in responses to different biologically relevant odors (own nest odor, juvenile rat nest odor, adult male rat nest odor, and predator odor) on postnatal days 3, 7, 11, and 15, along with the natural variations in maternal behavior in postpartum days 2, 6, 10, and 14. Nowadays, most of the studies on olfactory preferences derive from studies involving diets, postnatal pups at more advanced ages, huddling, among other more complex forms of social affiliation.

In this study we showed that the olfactory preference responses to the own nest odor, juvenile rat nest odor, and even responses to the adult male rat nest odor were evident from PND 7 and not before. During this period the pups' responses to the own nest odor were being mediated and influenced by some associative process within the nest, we assumed that the stability and constant matching of the own nest odor with repertoires of maternal behavior were the basis for an associative learning within the nest. We also showed that instead of losing interest in the mother's scent as the postnatal age progressed, the rat pups presented behaviors that were preferential to the juvenile rat nest odor, even when they were experiencing mother-infant interaction within the nest. Probably, this olfactory preference for the juvenile rat nest odor in such young pups was also induced by the associative processes that occured during interaction with the mother.

For aversive odors, such as predator odor, our work indicated that pups presented avoidance responses from PND 11 and not before. Parallel to the postnatal development of olfactory preference and pup avoidance responses, we showed a significant decrease in maternal behavior at the end of the first postpartum week and a significant increase in other behavior not directed at the pups at the beginning of the second week of postnatal life.

According to the data presented in this study, we corroborated the view that the experiences lived in the first days of life through mother-pup interaction had strong relation with the relationship between caregiver and infant and, moreover, we concluded that these experiences modulated the behavioral responses to other non-maternal odors, such as congeners and predators.

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CAPÍTULO III

ARTIGO II – Este artigo foi enviado para a revista *"Developmental Psychobiology"*, conforme comprovante no Anexo B.

REPEATED CROSS-FOSTERING AFFECTS MATERNAL BEHAVIOR AND OLFACTORY PREFERENCES IN RAT PUPS

Running title: Cross-fostering and mother-infant relationship

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ABSTRACT

Disturbances in early mother-infant relationships are strongly associated with changes in the development of a number of physiological and behavioral systems in the neonate that can create a complex change in patterns of vulnerability throughout life. Institutionalized children, despite receiving adequate care, display important behavioral changes in adult life, and such changes are strongly associated with a lack of selective and consistent attachment to the maternal figure in early life. It is not yet clear how human adoption processes alter the mother-infant relationship early in life. Here, to mimic the situation of human adoption and explore the impact on the formation of attachment between the infant and a caregiver, we use a repeated cross-fostering (RCF) model in rodents in two postnatal periods, postnatal days (PND) 1 to 3 (early RCF) and 9 to 11 (late RCF). Early exposure but not late exposure to RCF reduces the pups' preference for the odor of the mother figure. Moreover, early exposure and slightly later exposure to RCF reduce maternal care and motivation in collecting the pups and bringing them to the nest. The RCF protocol creates an unstable environment for mother-pup interaction, but it seems to affect learned attachment to the mother only when it occurs during the corresponding sensitive period of development.

Keywords*:* Infant attachment behavior; mother-infant interactions; cross-fostering, olfactory preference, sensitive period, maternal behavior, rats.

1 INTRODUCTION

Several studies have consistently shown that mother-pup attachment is a learning process and consequently that this initial filial relationship depends on memory formation (Sullivan & Wilson, 1991; Landers & Sullivan, 2012; Opendak & Sullivan, 2016). In both humans and animals, attachment requires a brief learning experience of the sensory characteristics of the maternal figure (odor, texture, color, sound) that occurs during the infant's first interactions with its caregiver (Debiec & Sullivan, 2017; Raineki et al., 2010; Sullivan & Holman, 2010).

Altricial neonates must learn their mother's odor and use it to guide their movements. As the mother is the only source of food, warmth and protection, learning this odor is critical to the survival of the newborn (Sullivan, 2003; Landers & Sullivan, 2012). Specifically, the maternal odor controls the interactions of rat pups with the mother, including approach responses, social behavior and attachment to the nipple (Raineki et al., 2010). In rats, the olfactory response of the pup to the odor of the mother is evaluated by orientation and approach behavior toward the odor source and is formed during the first 10 days of the pup's life. In this period, also called the attachment sensitive period, pups can easily be conditioned to a different odor than the maternal one, as long as it is paired with thermal and tactile stimuli (Debiec & Sullivan, 2017; Landers & Sullivan, 2012; Meyer & Alberts, 2016), indicating an associative learning process.

In addition to the importance of the study of the mother-child relationship early in life, there is evidence that this link establishes the basic model of future social behavior (Moriceau et al., 2010; Sullivan et al., 2015), impacting the development of adult sociability (Callaghan et al., 2014). Thus, in this study, we used the protocol of repeated cross-fostering (RCF) by lactating females as an environmental variable in the formation of the attachment between the pup and its mother at the beginning of development, and we analyzed how postnatal environment interventions that tend to disrupt mother-offspring attachment alter these behavioral patterns in rats.

Maternal adoption is an area that has not yet been explored in depth by neuroscience. The rationale of the present work is to mimic the human situation of adoption. Institutionalized children, despite receiving adequate care, exhibit important behavioral changes in adult life, sometimes quite serious (Bruce et al., 2009). For this purpose, we used an experimental model that consists of performing successive daily transfers of pups among lactating female caregivers during their synchronized postnatal period, similar to a previously described experimental protocol (Di Segni et al., 2015). Pup exchange between lactating females occurred during 2 postnatal time windows: during the attachment sensitive period on postnatal days (PND) 1, 2 and 3 and in the transition from the sensitive to the postsensitive period on PND 9, 10 and 11. Using these interventions in the mother-pup relationship, we evaluated the olfactory preference of the pups for the odor of the maternal figure through olfactory preference tests on PND 7 and 15, and to evaluate the pups' ability to target the mother's nipple, we used the nipple attachment test on PND 8 and 16. In addition, we observed the maternal care behavior of the lactating females as well as their motivation to collect the pups and take them to the nest from PND 1 to PND 6 or from PND 9 to

PND 14, respectively. Finally, we studied the effect of the repeated exchange of lactating female caregivers on the behavioral inhibition system of olfactory preference of the pups through approach and avoidance responses to a naturally aversive stimulus on PND 7 and 15.

Our hypothesis is that the RCF procedure affects the formation of the developing mother-pup attachment, considering the instability that the procedure triggers. The RCF in an animal model differs from the adoption of children because, among other aspects, the rat pups are cared for and nursed by lactating females in the same postpartum period. However, the procedure of fostering by the exchange of the mother creates an unstable neonatal environment for the pups by disrupting the bond with the specific caregiver.

2 MATERIALS AND METHODS

2.1 SUBJECTS AND HOUSING CONDITIONS

Forty-eight females and twelve male Wistar rats, all adults (PND 70), were obtained from the Center for Reproduction and Experimentation of Laboratory Animals (CREAL), UFRGS, Porto Alegre, and were group housed in transparent acrylic cages (4 animals per 45 × 31 × 21 cm cage) with food (Nutrilab, Colombo, Brazil) and filtered water freely available. The colony room was maintained at constant humidity (38%) and temperature (22 ± 1˚C) with a 12:12-h light/dark cycle (lights on at 06h00).

After two weeks of habituation, virgin females were mated with males on the evening of proestrus. The presence of spermatozoids in the vaginal smear the following morning was considered an indicator of pregnancy, and this day was designated gestational day (GD) 0. On GD 16, the pregnant rats were individually housed in transparent acrylic cages (dimensions described above) and were left undisturbed. All litters were born within 12 h of each other and were examined until 11h00 of each day (from GD 21 to GD 22). The day of birth was considered day 0. A group of litters remained for 24 h (until PND 1) with their biological mothers, and other litters remained with their biological mothers until PND 9. On the morning of PND 1, all litters were standardized to 8 pups (4 male and 4 females) and randomly assigned to RCF or control conditions. Animal care and all behavioral experiments were performed according to the guidelines of the National Institutes of Health (NIH) and the domestic National Council for Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee in Use of Animals (CEUA) of the Universidade Federal do Rio Grande do Sul (No. 31562/2016) (Appendix B).

2.2 RCF MODEL

The RCF protocol used in this work was based on previous studies (Bartolomucci et al., 2004; D'Amato et al., 2011; Luchetti et al., 2015). Litters (8 pups) were exchanged between dams on PND 1 or PND 9, and this procedure was repeated every 24 h for 3 consecutive days, from PND 1 to PND 4 (early RCF group) or from PND 9 to 11 (late RCF group). The dams were removed from their home cages and temporarily placed in empty cages with clean wood shavings as bedding. Next, the litters were carefully removed from the nest and placed in the cages of their foster mothers, and the dams were returned to their home cages. The procedure occurred between 10h30 and 11h00 and took no longer than 3 min. After the last change, on PND 3 or PND 11, the pups remained with their last foster mother until PND 6 or PND 14. Therefore, each litter was cared for by four different mothers: one biological and three foster mothers. The same procedure was applied to the control group dams on the same days and at the same times as the RFC groups except that they were returned to their own litters. During the days of the RCF procedure, before returning to their foster nest or home nest, each pup was weighed. For each behavioral measurement, one male and one female sibling per litter was used. A total of 384 pups (192 males and 192 females) were used for behavioral measurements, half on PN7 and the other half on PND 15. The timing of the experimental procedures and the total numbers of litters and pups used are reported in Figure 1.

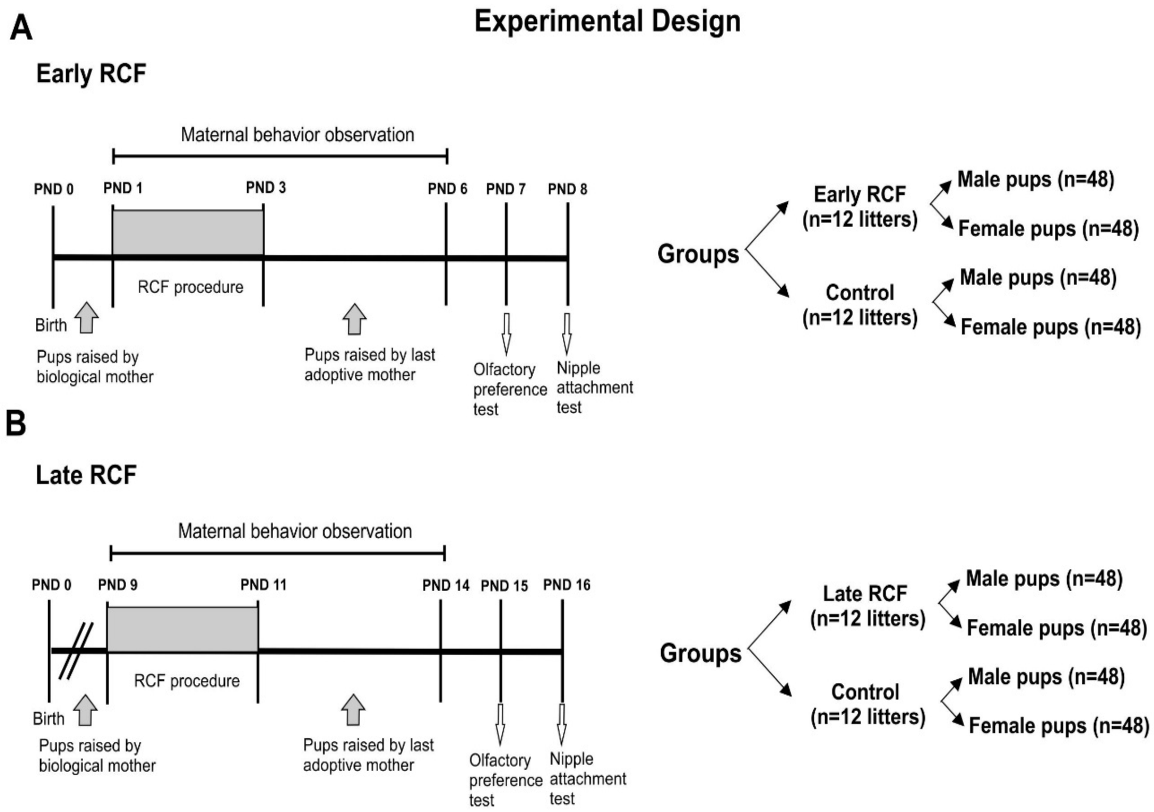

Figure 1. Design of the cross-fostering experiment. Offspring rats changed caregivers every day, from one day after birth (PND 1) until PND 3 (early repeated cross-fostering) (A) or from PND 9 until PND 11 (late repeated cross-fostering) (B), experiencing four different caregivers (1 biological mother and 3 foster mothers). After the fostering period, the last foster mother raised the pups until PND 8 or until PND 16. Maternal behavior was observed during the cross-fostering period and three days after. On PND 7 or PND 15, the pup was tested for olfactory preferences in a two-choice test, and they were tested for maternal nipple attachment on PND 8 or PND 16. The distribution of experimental groups and the number of pups tested are described in the right.

2.3 BEHAVIORAL PROCEDURES

2.3.1 Maternal behavior

Video cameras were positioned lateral to the housing cages for maternal behavior recording from PND 1 to PND 6 or from PND 9 to PND 14. A discrete observation was made every 3 min within each observation period during four periods per day: 3 during the light phase (09h00, 12h00, 15h00) and one at the beginning of the dark phase (18h00) (with 25 observations in 4 periods per day = 100 total observations/mother/day) (Pardo et al., 2016). For each observation, the location of the mother (in or out the nest) was noted. If the mother was in the nest, the following nursing postures were noted: high crouch posture (mother nursing pups in an arched-back posture); low crouch posture (mother nursing pups in a "blanket" low arched-back posture); supine posture (a passive posture in which the mother is lying on her back or side while the pups nurse). We also recorded licking of the pups (licking the surface of their bodies and their anogenital regions), nest building, and self-grooming behavior.

The total nursing behavior was calculated by adding the scores for nursing postures and licking pups during the recording periods (12h00, 15h00 and 18h00). Recordings from the 09h00 period are presented separately because the RCF procedure was performed after. To evaluate the variation in the nursing behavior in the RCF and control groups, we separately compared the cumulative mean scores of the two groups during the period of the RCF procedure on PND 3 or 11 (corresponding to PND 1, 2 and 3 or PND 9, 10 and 11, respectively) and after the period of the RCF procedure on PND 6 or 14 (corresponding to PND 4, 5, and 6 or PND 12, 13, and 14, respectively).

2.3.2 Pup-retrieval test

Control and early RCF dams were tested for pup-retrieval behavior from PND 1 to 4, between 10h30 and 11h00 (twenty-four hours after the last change of litters). After the litter was removed from the home nest, they were weighed and then distributed across the floor of their own home cage or that of their foster mother. The dam was placed back in her home cage, and the number of retrieved pups and the latency to retrieve each of them were recorded. The test ended when the mother collected the 5th pup or when 15 min of observation had been completed. This test was not performed in the late RCF group because older pups exit and enter the nest alone, and dams have reduced retrieval behavior. To assess any variation in maternal motivation between control and early RCF dams, we compared the cumulative latency to retrieve the first and fifth pups during the four postpartum days.

2.3.3 Olfactory preference test

Pups from the control and RCF groups were subjected to olfactory preference tests using a Y-maze apparatus on PND 7 and PND 15. The Y-maze consisted of three arms constructed from transparent acrylic (L 12 × W 12 × H 20 cm). One of the arms was the starting zone (L 8.5 × W 8 × H 20 cm), where the pup was placed at the beginning of the test. The other two arms (L 8.5 × W 8 × H 20 cm) were connected to small acrylic boxes with several holes in the walls (L 12 × W 12 × H 20 cm), with each of the two boxes containing the stimuli for the tests. These small acrylic boxes were attached to an air pump (aquarium air pump, Master Brand, Super Type II) that propelled air into the chamber at a flow rate of 2 L/min. The purpose of this air pump was to move the bedding odors or test odors toward the arms of the maze through the perforated walls. We tested the following stimuli combinations: clean bedding vs home bedding, pregnant female bedding (collected from on GD 20 and stored in refrigerator at -5º C until the day of test) or sand with cat urine. A portion of home bedding (300 ml) or sand with cat urine (60 grs) was placed in one of the Y-maze arms, and a portion of clean bedding (300 ml) was placed in the other arm. On postnatal day 7, one male and one female pup from each litter were removed from the nest (the order of removal was always randomized), when placed in a small box equipped with paper towels and transported to the behavior test room (maintained at a temperature of 28º-29º C). Each pup was placed in the starting area of the Y-maze, and after 5 s, the two doors dividing the maze arms were removed. Pup behavior was videotaped (Sony Handycam). The test was performed in five consecutive trials of 1 min each with 30-second intervals between each trial. At the end of each session, the pup was placed in the same box used for transportation, the Y-maze was cleaned with water, and the positions of the stimuli in the two arms were switched. After the test, the pups were returned to their nests, and each tested pup was given a mark (with a nontoxic permanent marker) on one of their legs.

The videos were analyzed using the software EthoWatcher (Crispim Junior et al., 2012). We recorded the time the pups spent over one of the areas of tested stimuli. The criterion defining the entry of the pup into one of the arms of the maze was when its whole body passed the line that divided that arm from the starting zone. Animals that did not enter either of the odor areas during the 5 test trials were considered nonresponsive pups. The results are expressed as the percentage of time the pup remained in each arm of the maze during the 5 trials of the test. The sum of the total time spent over one of the areas in the 5 trials was divided by the total duration of the test (300 s) and multiplied by 100. From there, the mean ± SEM or the median (IQ) for each experimental group was calculated and then used to compare the RCF and control groups.

2.3.4 Nipple attachment test

On PND 8 or PND 16, pups from each litter were tested for nipple attachment on their anesthetized biological mother (control and RCF group) or last foster mother (RCF group). On the test, the dams were removed from their nests, anesthetized with ketamine (35 mg/kg i.p) and xylazine (3 mg/kg i.p), placed on her side on the floor of a holding cage (25 × 40 × 20 cm) and transported to the behavior test room (maintained at 28º-29º C temperature). The dams were anesthetized to eliminate any active role in the pups' performance during the test and prevent milk let-down (Raineki, et al., 2010). Two minutes later, pups from each litter were removed from their nest, placed in small holding cages covered with paper towel and transported to the behavior test room. For the test, the anesthetized dam was placed on her side in a transparent acrylic cage (25 × 40 × 20 cm) heated to a standard nest temperature (28º-29º C). The pups were placed in the acrylic cage with the anesthetized dam, approximately 15 cm away. A 3-min observation period was allowed to each pup. The latency to reach the dam’s ventrum and suckle at her nipple was recorded. Pups from the control group were tested to attach to their biological mother’s nipple (1 male and 1 female), and pups from the RCF group were tested in two ways: to attach to their biological mother’s nipple (1 male and 1 female) or to attach their last foster mother’s nipple (1 male and 1 female). Pups used in the nipple attachment test were those tested in the olfactory preference test on PND 7 to home bedding (biological or last foster mother) or pregnant nest bedding (biological mother with GD 20). After a 3-min test, the pups were returned to the holding cage, remained there until the other siblings were tested and then returned together to their home nests. Before returning to the nests, the dams were allowed to recover from the anesthesia (approximately 30-40 min) in the behavior test room. All tests were recorded on video for further analysis. The mean latency to attach the dam’s nipples was calculated and compared between the control and RCF groups.

2.4 BODY WEIGHT GAIN OF PUPS

From the daily recording of the pup’s body weight from PND 1 to PND 4, the variation in body weight gain (delta weight, g) was calculated using the following formula: delta weight = (final weight on the PND 4 –initial weight on PND 1). The variation in weight gain was calculated separately for male and female pups, and the mean of the delta weight was compared between the control and RCF groups for each sex.

2.5 STATISTICAL ANALYSIS

For each experimental group, data were tested for normality using the Kolmogorov-Smirnov normality test. The results are presented as the mean ± SEM or median (IQ), and the number of animals per group is expressed as "*n*". All data were analyzed using GraphPad Prism 8 Software (San Diego, CA). Data with normal distribution were analyzed through a parametric Student’s *t*-test or two-way ANOVA, and data that did not meet the requirements of normality were analyzed by nonparametric tests, such as the Kruskal-Wallis test, Wilcoxon test or Mann-Whitney U test. For all tests, statistical significance was defined as $p < 0.05$. Significant results are indicated by asterisks (*) in the figures and in bold in the associated tables.

3 RESULTS

3.1 EFFECT OF EARLY RCF ON MATERNAL BEHAVIOR OF LACTATING FEMALES

3.2 NURSING BEHAVIOR

To identify any variation in the maternal care induced by the RCF protocol application, we recorded the maternal behavior during the period of the RCF procedure

(PND 1-PND 3) and during the next two days (PND 4-PND 6). Two-way ANOVA was applied to the maternal behavior data set to assess the relative effect of the early RCF procedure and the progress of the postpartum period on the total nursing behavior pattern. Lactating females of both groups gradually reduced their nursing behavior from PND 1 to 6, and this pattern was significantly affected by the early RCF procedure [Interaction postpartum period × RCF procedure: F (5,110) = 6.09; $p < 0.0001$]. Bonferroni's post hoc analysis showed that lactating females that experienced the RCF procedure significantly reduced their nursing behavior at PND 2 [early RCF: 25.67 ± 3.50, n=12 vs Control: 53.75 ± 2.34, n= 12; $p < 0.0001$] and at PND 3 [early RCF: 21.83 ± 2.91, n=12 vs Control: 37.17 ± 2.10, n= 12; $p < 0.05$] (Figure 2A).

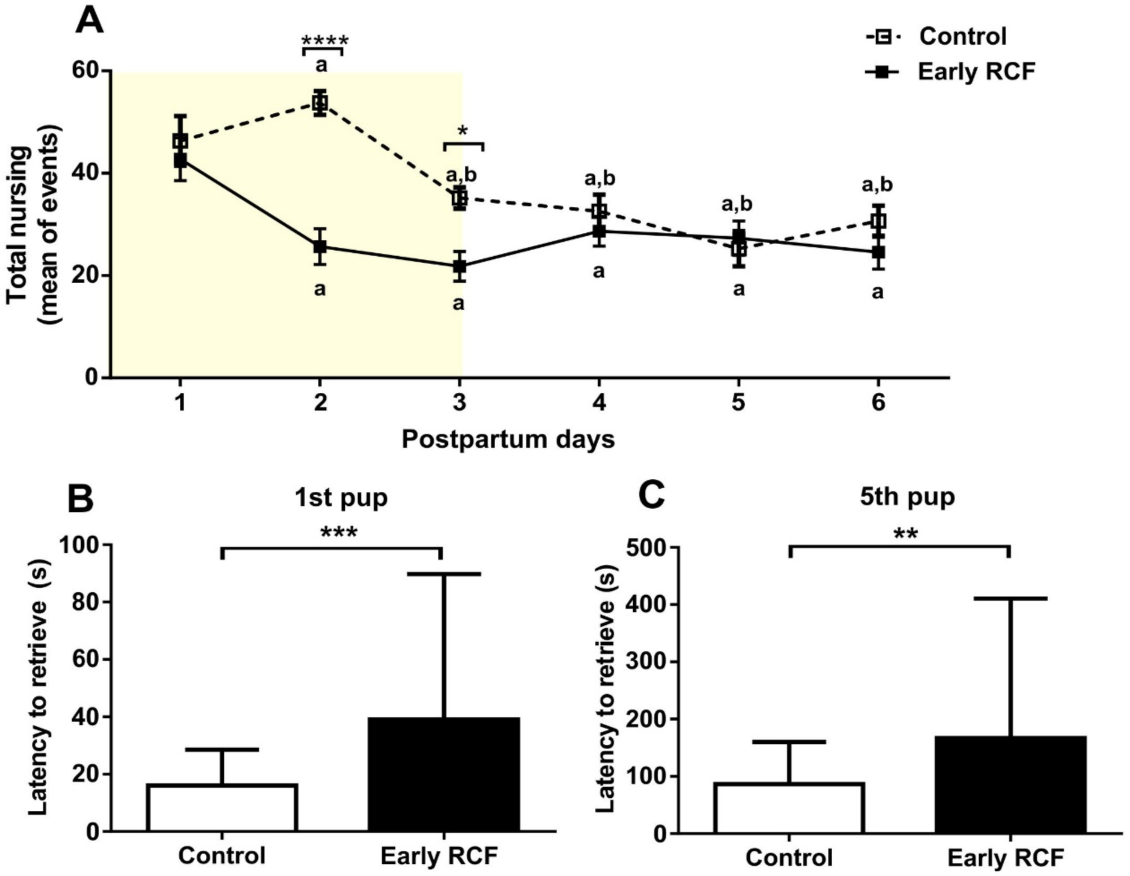

Figure 2. Effects of early repeated cross-fostering on nursing behavior and maternal motivation. (A) Comparison of the number of total cumulative events of nursing behavior (high crouch+ low crouch licking + supine +high crouch licking postures), recorded at 12 h, 15 h, and 18 h between the control and early RCF groups from PND 1 to 6. Comparison of cumulative latency to retrieval the first (B) and five pups (C) between control and early RCF group lactating females from PND 1 to 4. Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ control vs early RCF group. ^{a} A

significant change in comparison with PND 1. [b] A significant change in comparison with PND 2. For all groups, n=12 litters. RCF: repeated cross-fostering group.

Table 1 shows the total nursing score, which was the sum of high crouching, low crouching, supine and licking behaviors. The score was discriminated by period of the day and postpartum day. On day 2, at the three timepoints after the RCF procedure (12h00, 15h00 and 18h00), the early RCF group showed a significant decrease in the score in comparison with the control group. This was also observed on day 3 and day 6, at 15h00.

Table 1: Effects of the early RCF procedure on the total nursing behavior score (sum of nursing behaviors) during 4 periods per day.

		Groups			
PND	Period of observation	Control (n=12)	Early RCF (n=12)	*t* (22)	*P* value
Day 1	09h00	20.25± 2.00	18.33 ±1.87	0.70	0.49
	12h00	20.00 ± 2.07	18.83 ±1.65	0.44	0.66
	15h00	16.92 ± 2.18	15.67 ± 2.11	0.41	0.68
	18h00	9.42 ± 1.91	8.25 ±1.54	0.48	0.64
Day 2	09h00	22.00 ± 1.17	20.83 ±0.84	0.81	0.43
	12h00	**25.50 ± 1.18**	**14.58 ± 2.12**	**4.51**	**0.0002**
	15h00	**20.92 ± 1.67**	**6.75 ±1.07**	**7.14**	**<0.0001**
	18h00	**7.33 ± 1.05**	**4.33 ± 1.18**	**1.91**	**0.07**
Day 3	09h00	17.50 ± 3.52	18.25 ±1.91	0.19	0.85
	12h00	16.25 ± 1.73	12.08 ± 1.74	1.69	0.10
	15h00	**15.50 ± 1.73**	**6.75 ±1.26**	**4.09**	**0.0005**
	18h00	3.42 ± 1.33	3.00 ± 0.98	0.25	0.80
Day 4	09h00	21.00 ± 1.39	19.08 ± 1.65	0.88	0.38
	12h00	15.83 ± 2.02	14.25 ± 1.94	0.57	0.58
	15h00	12.33 ± 1.79	11.17 ± 2.17	0.41	0.68
	18h00	4.42 ± 1.18	3.25 ± 0.59	0.88	0.39
Day 5	09h00	15.33 ± 2.39	17.58 ± 1.42	0.81	0.43
	12h00	12.33 ± 1.68	15.67 ±1.64	1.42	0.17
	15h00	9.92 ± 1.66	7.92 ± 1.86	0.80	0.43
	18h00	3.00 ± 0.83	3.75 ±1.19	0.52	0.61
Day 6	09h00	16.75 ± 2.19	16.25 ± 2.06	0.17	0.87
	12h00	15.25 ± 2.53	13.83 ± 1.79	0.46	0.65
	15h00	**13.25 ± 1.29**	**7.25 ± 1.48**	**3.06**	**0.006**
	18h00	2.17 ± 0.63	3.50 ± 1.57	0.79	0.44

Note. Data are presented as the mean ± SEM and represent the cumulative maternal nursing score (number of high crouch posture + low crouch posture + supine posture + licking) on 4 periods of observation from PND 1 to 6. The early RCF procedure was performed between the observation periods at 09h00 and 12h00. Parentheses contain the number of litters. Significant differences ($p<0.05$) according to Student's *t*-test are indicated in bold.

Table 2 shows the cumulative score of maternal behavior parameters at PND 3 and at PND 6. At PND 3, immediately after the RCF procedure (from PND 1 to PND 3), the early RCF group showed a significant decrease in high crouching posture but an increase in self-grooming behavior and exits from the nest. Furthermore, after finishing the RCF procedure, the early RCF group showed a significant increase in low crouch posture and a significant increase in nest building behavior as well as self-grooming behavior.

Table 2. Cumulative maternal behavior during the early RCF procedure period (PND 1- PND 3) and during the first six postpartum days (PND 1 – PND 6).

		Groups			
Behavioral parameters (number of observations)	**PND**	**Control (n=12)**	**Early RCF (n=12)**	***t* (22)**	***P* value**
Total Nursing	**PND 3**	**135.3 ± 6.98**	**90.25 ± 6.10**	**4.85**	**<0.0001**
	PND 6	88.50 ± 6.37	80.58 ±7.04	0.83	0.41
High crouch posture	**PND 3**	**101 ± 5.59**	**58.17 ± 4.75**	**5.83**	**<0.0001**
	PND 6	53.08 ± 5.74	47.42 ± 5.71	0.70	0.49
Low crouch posture	PND 3	8.75 ± 2.54	10.67 ± 2.33	0.56	0.58
	PND 6	**7.67 ± 1.79**	**21.67 ± 3.49**	**3.57**	**0.002**
Supine Posture	PND 3	7.25 ± 2.29	5.58 ± 1.15	0.65	0.52
	PND 6	15.83 ± 3.37	7.67 ± 2.43	1.96	0.06
Licking	PND 3	0.75 ± 0.35	1.50 ± 0.53	1.18	0.25
	PND 6	0.83 ± 0.29	2.17 ± 0.65	1.87	0.07
High crouch posture and licking	PND 3	17.50 ± 1.79	14.33 ± 2.10	1.15	0.26
	PND 6	16.50 ± 1.57	12.33 ± 1.77	1.76	0.09
Nest building	PND 3	8.33 ± 3.05	8.08 ± 1.64	0.07	0.94
	PND 6	**2.75 ± 0.91**	**7.83 ± 1.81**	**2.51**	**0.02**
Dam off-nest	**PND 3**	**76.25 ± 6.67**	**122.9 ± 4.78**	**5.69**	**<0.0001**
	PND 6	125.6 ± 5.39	137.3 ± 7.53	1.27	0.22
Self-grooming	**PND 3**	**16.08 ± 1.57**	**23.50 ± 2.07**	**2.85**	**0.009**
	PND 6	**21.67 ± 2.69**	**29.08 ± 1.07**	**2.56**	**0.02**

Note. Data are presented as the mean ± SEM and represent the accumulated occurrence of the behavior from PND 1 to 3 (reported at PND 3) and from postpartum days 1 to 6 (reported at PND 6) recorded at 12h00, 15h00and 18h00. Parentheses contain the number of litters. Student's *t*-test indicated significant differences (*p*<0.05) in bold.

3.2.1 Pup retrieval

From PND 1 to PND 4, in the period immediately after performing the RCF protocol, we recorded the latency of lactating females to collect the first and fifth pups to the nest in their own home cages. Analysis of the cumulative data from the four days shows that lactating females from the early RCF group take significantly longer time to collect the first pup [Control: Mdn 16 (8.25-28.50) vs Early RCF: Mdn 39 (18-89.75);

U=672, *p*= 0.0004, Mann-Whitney Test] (Figure 2 B) and the fifth pup [Control: Mdn. 86.5 (61.75-160.3) vs Early RCF: Mdn 167 (79-410.5); U=790, *p*= 0.0076, Mann-Whitney Test] (Figure 2 C) in comparison with the latencies from control group lactating females.

3.3 EFFECT OF EARLY RCF ON THE OLFACTORY PREFERENCE OF RAT PUPS ON POSTNATAL DAY 7

3.3.1 Biological or adoptive mother nest bedding vs clean bedding

To understand how RCF at early postnatal periods might affect the capacity of rat pups to form attachment to their caregiver, we submitted infant rats to the RCF protocol for 3 days (from PND 1 to PND 3). On PDN 7, early RCF (to the last adoptive mother nest bedding) and control rats (biological mother nest material) were tested based on the amount of time they spent in the nest bedding area. In the olfactory preference test, early RCF group pups were less responsive to the stimuli presented than the control group pups. From 24 pups tested in the early RCF group, only 4 pups (male: 1 of 12 tested; female: 3 of 12 tested) were entered at least one of the arms of the Y-maze. In the control group, from 24 pups, 20 were actively responsive to at least one stimulus presented (male: 9 of 12 tested; female: 11 of 12 tested).

Male (median of 25.67 s) and female pups (median of 14.17 s) from the control group spent significantly more time in the biological mother's nest area compared to their time in the clean bedding area (0 s) [male: W=45, *p*=0.0039; n=9; female: W = 66, *p* = 0.0010; n = 11; Wilcoxon rank test] (Figure 3 A). On the other hand, male and female pups from the early RCF group spent a median of 0 s in the adoptive mother bedding area as well as in the clean bedding area (Figure 3 B). Comparison of the time spent over the area of home bedding between control and early RCF pups show that male [early RCF. Mdn: 0 (0-0) vs. control, Mdn: 25.67 (3.33-33.58); U=19.50; p=0.0003, n=12, Mann-Whitney test] and female pups [early RCF. Mdn: 0 (0-2.75) vs. Control, Mdn 14.17 (9.5-26.58); U=14.50; p=0.0006, n=12, Mann-Whitney test] from the early RCF group spent a significantly less time over the area of home bedding than male and female pups from the control group (Figure 3 C).

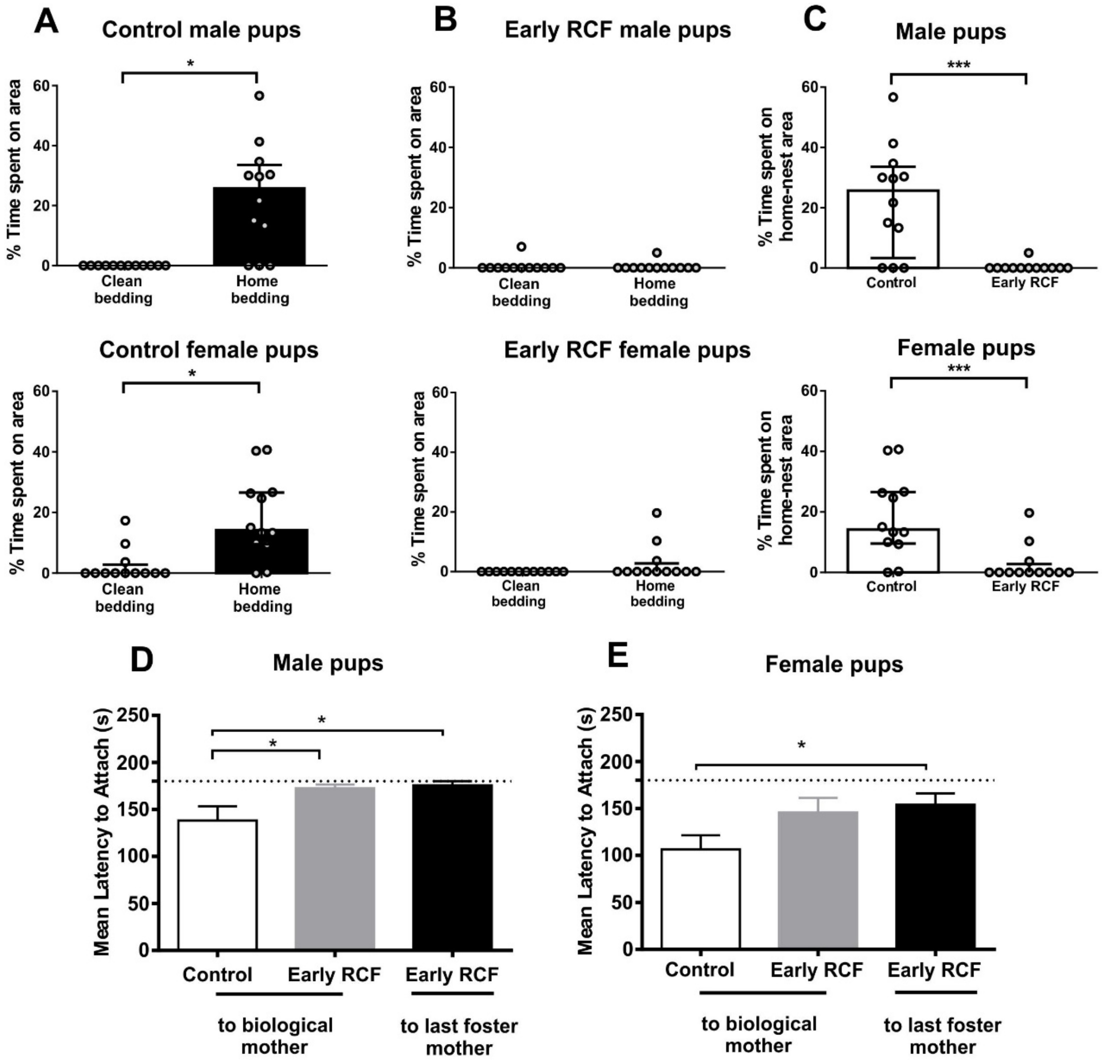

Figure 3. Effect of early repeated cross-fostering on the olfactory preference to home bedding odor on postnatal day 7 and nipple attachment on postnatal day 8. (**A**) Comparison of the percentage of time over the area of home bedding odor of male (above) and female pups (below) from the control group. (**B**) Comparison of percentage over the area of home bedding odor (corresponding to the last foster mother) of male (above) and female pups (below) from the early RCF group. (**C**) Comparison of percentage of time over the area of home bedding odor between control and early RCF group male (above) and female pups (below). Data are expressed as the median (IQ). **p< 0.05* clean bedding vs home bedding and *** *p<0.001* controls vs early RCF group. Comparison of the latency to attach to biological or last foster mother's nipple in male (**D**) a female pups (**E**) from the control and early RCF groups. **p< 0.05*, significantly different from the control group. For all groups, n=12 pups. RCF: repeated cross-fostering group.

3.3.2 GD 20 Biological mother nest-odor vs clean bedding

To evaluate the behavior of the pups in response to the odor of their biological mother, we used nest material from GD 20 pregnant rats (biological mothers) after the pups had undergone the RCF procedure. It was thought as a control group through which we could evaluate the behavior of pups in response to the odor of their biological mother after having been submitted to the RCF procedure. We expected that pups could express the memory of their biological mother. The drawback in the group is mainly the preservation of the stimulus. Sawdust with mother's feces was preserved at -5º C of GD 20 until the olfactory preference test day.

Similar to the biological or adoptive mother preference test, from 24 pups tested in the early RCF group, only 4 pups (male: 3 of 12 tested; female: 2 of 12 tested) were entered at least one of the arms of the Y-maze. In the control group, from 24 pups, 17 were actively responsive to at least one stimulus presented (male: 9 of 12 tested; female: 8 of 12 tested). Table 3 shows that male and female responsive pups from the early RCF group spent a reduced time over the area of pregnant rat nest material or clean bedding. On the other hand, male and female pups from the control group spent slightly longer time in the GD pregnant rat nest material area than in the clean bedding area.

Table 3: Olfactory behavioral responses to biologically relevant odors in male and female pups on PND 7 and PND 15 that experienced early or late cross-fostering procedure.

Group	Sex	N	Clean bedding	Tested odor	W or *t* (11)	*P* value
PND 7			**Clean bedding vs Pregnant rat nest bedding**			
Control	M	9	0 (0-11.08)	9 (0-24.17)	29	0.09
	F	8	0 (0-4.25)	5.5 (0-10.75)	22	0.15
Early RCF	M	3	0 (0-9.75)	0 (0-0)	-6	0.25
	F	2	0 (0-0)	0 (0-0)	-3	0.50
			Clean bedding vs Predator odor			
Control	M	7	1.61 (0-14.25)	0 (0-0)	-16	0.20
	F	4	0 (0-8.67)	0 (0-0)	-10	0.13
Early RCF	M	0	-	-	-	-
	F	0	-	-	-	-
PND 15			**Clean bedding vs Pregnant rat nest bedding**			
Control	M	11	14.42 ± 4.48	23.97 ± 4.55	1.45	0.17
	F	**11**	**12.36 ± 2.42**	**32.14 ± 4.85**	**4.36**	**0.001**
Late RCF	M	10	29.78 ± 5.84	18.19 ± 4.64	1.69	0.12
	F	12	27.03 ± 6.35	21.19 ± 4.83	0.61	0.55
			Clean bedding vs Predator odor			
Control	**M**	**10**	**13.19 ± 3.95**	**6.08 ± 2.25**	**2.64**	**0.02**
	F	**9**	**21.75 ± 5.42**	**6.83 ± 2.48**	**2.86**	**0.02**
Late RCF	M	7	16.67 ± 6.93	3.38 ± 1.21	1.87	0.08
	F	8	13.75 ± 5.67	7.22 ± 2.24	1.43	0.18

Note. Data represent the percentage of time that responsive pups spent over the areas of the Y-maze during five trials of the olfactory preference test. Parametric data are represented as the mean ± SEM, and nonparametric data are represented as the median (IQ). Parametric and nonparametric data were analyzed using Student's *t*-test and Wilcoxon's test, respectively. Significant differences (*p*<0.05) are indicated in bold.

3.3.3 Predator odor vs clean bedding

To evaluate the response to predator, pups were exposed to sand with cat urine vs clean bedding and measured the behavioral responsive and avoidance behavior to this stimulus. Male and female pups from early RCF group pups were less responsive to the stimuli presented than the control group pups. None of the 24 pups (male and female) tested in the early RCF group were entered into the arms of the Y-maze. On the other hand, in the control group, from 24 pups, 11 were actively responsive to at least one stimulus presented (male: 7 of 12 tested; female: 4 of 12 tested). These responsive pups from the control group spent more time in the clean bedding area than in the predator odor area (Table 3).

3.3.4 Effect of early RCF on nipple attachment behavior of pups at PND 8

To understand how RCF at early postnatal periods can affect the ability of pups to use the maternal odor to locate the mother nipples, we evaluated the latency to reach the nipple of the caregiver in pups with PND 8 that experienced an early RCF protocol during PND 1-3. Control pups remained with their biological mother until the test day. Early RCF group pups were tested to their anesthetized last adoptive mother or to their biological mother, and control group pups were tested to their biological mother. When early RCF group pups were tested to their adoptive mother, only 7 of 24 pups (1 male and 6 female) attached the nipple of the lactating female. A similar pattern was observed when the pups were tested to their biological mother, only 7 of 24 pups (3 male and 4 female) attached to the biological mother's nipple. On the other hand, 16 of 24 pups from the control group attached to their biological mother (6 males and 10 females). All pups in the three tested groups that failed to attach were observed to remain in the stating zone when they were placed at the beginning of the test.

Male (Figure 3 D) and female pups (Figure 3 E) from the early RCF group had a similar mean latency to attach to their biological mother's nipple (male: 172.5 ± 4.11; female pups: 145.8 ±15.69) or to their last foster mother (male pups: 175.8 ± 4.17; female pups: 154 ± 12.03) [male pups: *t* (22) = 0.57; *p*=0.57 and female pups: *t* (22) = 0.42; *p*=0.68, unpaired Student's *t*-test]. The mean latency to attach to the biological mother was higher in male pups from the early RCF group than in male pups from the control group [Control: 138.2 ±15.17 vs early RCF: 172.5 ± 4.11; *t* (22) = 2.18, *p*=0.04, unpaired Student's *t*-test].

Furthermore, male pups from the early RCF group showed a higher latency to attach to their last foster mother compared to the latency to attach to biological mother in male pups from the control group [Control: 138.2 ±15.17 vs early RCF: 175.8 ± 4.17; *t* (22) = 2.39, *p*=0.025, unpaired Student's *t*-test] (Figure 3 D). Similarly, female pups from the early RCF group showed slightly higher latencies to attach to their biological mother [control: 106.6 ±14.88 vs. early RCF: 145.8 ± 15.69; *t* (22)= 1.81, *p*= 0.08, unpaired Student's *t*-test] or their last foster mother [control: 106.6 ±14.88 vs. early RCF: 154 ± 12.03; *t* (22)= 2.48, *p*= 0.02, unpaired Student's *t*-test] when compared to female pups from the control group (Figure 3 E).

3.4 EFFECT OF EARLY RCF ON PUPS' WEIGHT GAIN

To verify that the RCF procedure did not affect the nutrition of the pups, we measured the pup's weight during each day of the RCF protocol procedure (PND 1-4) and calculated the total weight gain variation during 3 days of the RCF procedure. There was no difference in the variation in gain weight of the early RCF and control groups for both males [RCF: 20.57 ± 0.50 g vs Control: 21.11 ± 1.56; *t* (0.94) = 22, *p* = 0.35; n= 12; unpaired Student's *t*-test] and female pups [RCF: 20.57 ± 0.50 g vs Control: 22.62 ± 1.57 g; *t* (1.50) = 22, *p* = 0.14; n= 12; unpaired Student's *t*-test].

3.5 EFFECT OF LATE RCF ON THE MATERNAL BEHAVIOR OF LACTATING FEMALES

3.5.1 Nursing behavior

Lactating females in both the late RCF and control groups gradually reduced their nursing behavior from PND 9 to 14, and this pattern was significantly affected by the late RCF procedure [Interaction postpartum period × RCF procedure: F (5,110) = 5.16; *p*=0.0003, two-way ANOVA]. Bonferroni's post hoc analysis showed that lactating females that experienced the RCF procedure significantly decreased their nursing behavior at PND 9 [late RCF: 23.33 ± 2.49, n=12 vs Control: 37.17 ± 5.21, n= 12; *p* = 0.02] in relation to their pairs from the control group (Figure 4).

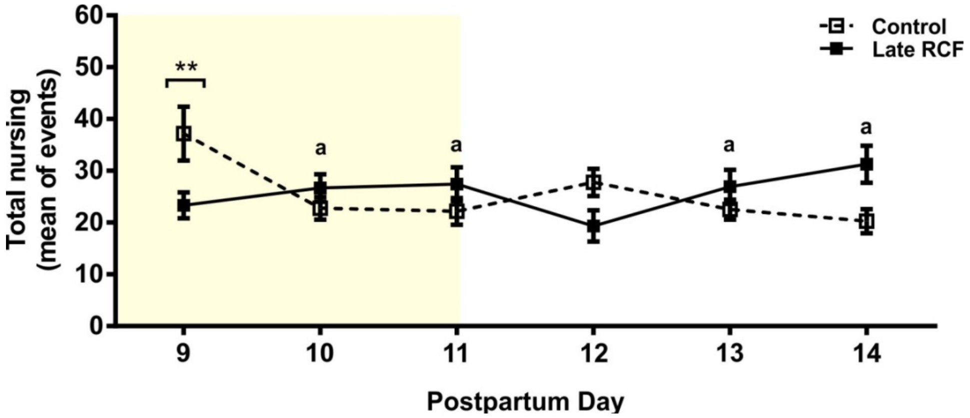

Figure 4. Effects of late repeated cross-fostering on nursing behavior. Comparison of the number of total cumulative events of nursing behavior (high crouch+ low crouch licking + supine +high crouch-licking postures), recorded at 12 h, 15 h, and 18 h between the control and early RCF groups from PND 9 to 14. Data are presented as the mean ± SEM. ***p*< 0.01 control vs early RCF group. ^{a} A significant change in comparison with PND 1. For all groups, n=12 litters. RCF: repeated cross-fostering group.

Table 4 shows the total nursing score discriminated by the period of the day and postpartum day. On day 9 at 15h00, the late RCF group showed a significant decrease in the score. This was also observed on day 12 at 12h00. On the other hand, on day 13 at 12h00 was observed a significant increase in the score was observed.

Table 4. Effects of late RCF procedure on the total nursing behavior (sum of nursing behaviors) during 4 periods of day

		Groups			
PND	**Period of observation**	**Control (n=12)**	**Late RCF (n=12)**	***t* (22)**	***P* value**
Day 9	09h00	13.42± 1.85	13.33 ±1.84	0.03	0.97
	12h00	14.50 ±2.17	12.25 ± 1.59	0.84	0.41
	15h00	**13.67 ±0.92**	**8.17 ± 1.52**	**3.09**	**0.01**
	18h00	9.00 ±3.09	2.92 ± 3.20	1.90	0.07
Day 10	09h00	12.33 ±2.41	13.25 ±2.17	0.28	0.78
	12h00	9.25 ± 1.81	11.92 ±1.68	1.08	0.29
	15h00	7.75 ± 0.79	7.42 ± 1.96	0.16	0.87
	18h00	5.75 ± 1.50	7.33 ± 1.38	0.78	0.45
Day 11	09h00	15.17 ± 1.80	16.83 ± 1.36	0.74	0.47
	12h00	9.92 ±1.82	13.42 ±1.67	1.42	0.17
	15h00	9.33 ± 1.42	10.00 ±1.66	0.31	0.76
	18h00	2.92 ± 0.71	4.00 ± 1.28	0.85	0.41
Day 12	09h00	14.00 ±2.42	12.00 ±1.84	0.66	0.52
	12h00	**14.50 ±2.17**	**7.17 ±1.68**	**2.67**	**0.01**
	15h00	6.67 ± 1.35	7.50 ±1.48	0.42	0.68
	18h00	6.58 ± 1.29	4.67 ± 1.33	1.03	0.31
Day 13	09h00	10.17 ±1.64	9.92 ± 1.28	0.12	0.91
	12h00	**7.00 ±1.36**	**12.67 ±1.88**	**2.45**	**0.02**
	15h00	8.52 ± 1.69	9.25 ± 1.97	0.39	0.70
	18h00	7.25 ± 1.58	5.00 ± 0.84	1.26	0.22
Day 14	09h00	12.50 ± 1.89	12.83 ± 1.06	0.15	0.88
	12h00	6.50 ± 1.39	11.08 ± 1.88	1.96	0.06
	15h00	9.50 ±2.03	2.08 ± 2.49	0.84	0.41
	18h00	6.33 ± 1.29	10.67 ± 1.70	2.03	0.05

Note. Data are presented as the mean ± SEM and represent the cumulative maternal nursing score (number of high crouch posture + low crouch posture+ supine posture + licking) on 4 periods of observation from PND 9 to 14. A late RCF procedure was performed between the observation periods at of 09h00 and 12h00. Parentheses contain the number of litters. Student's *t*-test indicated significant differences ($p<0.05$) in bold.

Table 5 shows the cumulative score of maternal behavior parameters at PND 11 and PND 14. At PND 11, immediately after the RCP procedure (from PND 9 to PND 11), the late RCF group showed a significant increase in high crouch, high crouch +

licking posture and self-grooming behavior but showed a significant decrease in low crouch and supine posture. Furthermore, days after the RCF procedure, the late RCF group showed an increase in high crouch posture and nest building behavior.

Table 5: Cumulative maternal behavior during the late RCF procedure period (PND 9-PND 11) and during PND 9 to 14.

		Groups			
Behavioral parameters (number of observations)	**PND**	**Control (n=12)**	**Late RCF (n=12)**	***t* (22)**	***P* value**
Total Nursing	PND 11	82.08 ±6.61	77.42 ± 3.98	0.60	0.55
	PND 14	70.50 ±2.88	77.50 ± 7.58	0.86	0.39
High crouch posture	**PND 11**	**31.42 ±3.66**	**44.08 ±3.49**	**2.50**	**0.02**
	PND 14	**19.75 ±2.15**	**36.25 ± 3.34**	**4.15**	**0.0004**
Low crouch posture	**PND 11**	**25.33 ±4.51**	**9.42 ±1.71**	**3.30**	**0.0033**
	PND 14	18.67 ±2.51	13.00 ± 3.28	1.37	0.18
Supine Posture	**PND 11**	**13.00 ±2.48**	**7.25 ± 1.43**	**2.01**	**0.05**
	PND 14	8.00 ± 1.72	9.83 ± 1.54	0.79	0.44
Licking	PND 11	1.83 ± 0.74	2.17 ± 0.52	0.37	0.72
	PND 14	3.75 ± 1.07	3.25 ±0.98	0.34	0.73
High crouch posture and licking	**PND 11**	**10.50 ± 1.10**	**14.50 ±0.99**	**2.70**	**0.01**
	PND 14	12.67 ±1.31	15.17 ± 1.83	1.11	0.28
Nest building	PND 11	1.92 ±1.10	4.33 ±0.89	1.70	0.10
	PND 14	**1.58 ±0.59**	**5.66 ± 1.08**	**3.32**	**0.003**
Dam off-nest	PND 11	139.1 ±7.25	138.8 ±3.34	0.03	0.97
	PND 14	156.5 ±4.29	140.3 ±7.52	1.87	0.07
Self-grooming	**PND 11**	**9.50 ± 1.49**	**16.42 ± 2.12**	**2.67**	**0.013**
	PND 14	11.25 ± 2.11	14.25 ± 1.84	1.07	0.29

Note. Data are presented as the mean ± SEM and represent the cumulative occurrence of the behavior from PND 9 to 11 (reported at PND 11) and from PND 9 to 14 (reported at PND 14). Parentheses contain the number of litters. Student's *t*-test indicated significant differences ($p<0.05$) in bold.

3.6 EFFECT OF LATE RCF ON THE OLFACTORY PREFERENCE OF PUPS FROM RATS AT PND 15

3.6.1 Biological or adoptive mother nest-odor vs clean bedding

In the olfactory preference test, pups from the control and late RCF groups were equally responsive. In the control group, 100% of males (12 of 12 tested) and 91.66% of female's pups (11 of 12 tested) were responsive. Similarly, in the RCF group, 100% of male and female pups (12 of 12 tested) actively responded at least to one of the stimuli in the Y-maze.

Figure 5 shows that pups from both groups showed a significant olfactory preference for the biological or adoptive mother nest odor. Male pups from the control group spent significantly more time in the area of their own-nest bedding in relation to the clean bedding [own-nest bedding: mean 36.12 ± 3.84 vs clean bedding: mean 14.86 ± 3.15; *t* (11) = 3.96, *p* = 0.0022; paired Student's *t*-test] (Figure 5 A, above). A similar result was observed for female pups from the control group [Adoptive mother nest material: mean 31.03 ± 5.71 vs. clean bedding: mean 13.22 ± 3.11; *t* (11) = 2.96, *p* = 0.0013; paired Student's *t*-test] (Figure 5 A, below). Similarly, male [own-nest material: mean 32.53 ± 4.14 vs. clean bedding: mean 12.67 ± 2.69; *t* (11) = 3.70; *p* = 0.0035, paired Student's *t*-test] (Figure 5 B, above) and female pups [own-nest material: mean 37.11 ± 5.21 vs. clean bedding: mean 19.19 ± 4.18, *t* (11) = 2.56, *p* = 0.0263, paired Student's *t*-test] (Figure 5 B, below) from late RCF group spent significantly more time over their own-nest material area than over clean bedding area.

Male pups from the late RCF group spent similar time over the home-bedding area as male pups from the control group [late RCF: 32.53 ± 4.14 vs. Control: 36.12 ± 3.84; *t* (22) =0.64, p=0.53, n=12, unpaired Student's *t*-test] (Figure 5 C, above). Similarly, female pups from the early RCF group spent similar time over the home-bedding area as female pups from control pups [late RCF: 37.11 ± 5.28 vs. Control: 31.03 ± 5.72, *t* (22) = 0.78, p=0.44, n=12, unpaired Student's *t*-test] (Figure 5 C, below).

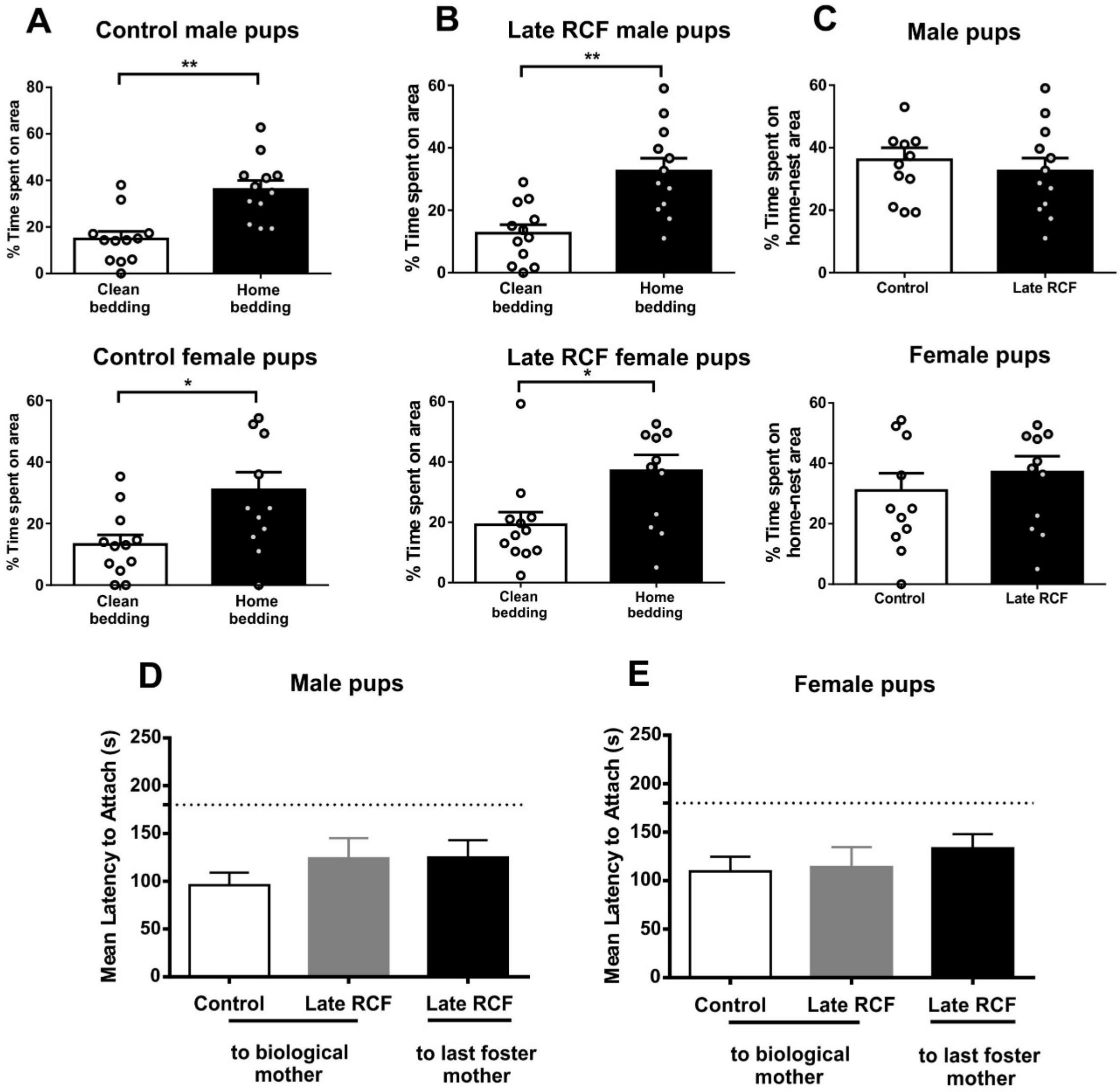

Figure 5. Late cross-fostering has no effects on olfactory preference on home bedding on postnatal day 15 or on nipple attachment on postnatal day 16. (A) Comparison of percentage of time over the area of home bedding odor of male (above) and female pups (below) from control group. B) Comparison of percentage over the area of home bedding odor (corresponding to the last foster mother) of male (above) and female pups (below) from late RCF group. C) Comparison of percentage of time over the area of home bedding odor between control and late RCF group male (above) and female pups (below). Comparison of the latency to attach to biological or last foster mother's nipple in male (D) a female pups (E) from control and late RCF group. Data are expressed as the mean ± SEM. **p< 0.05* and ** *p<0.01* clean bedding vs home bedding. For all group n=12 pups. RCF: repeated cross-fostering group.

3.6.2 GD 20 Biological mother nest-odor vs clean bedding.

At PND 15, 22 pups from the late RCF group (10 males and 12 females from 24 tested) were responsive to at least one of the stimuli in the Y-maze test. Table 3 shows that female pups from the control group spent significantly more time over the pregnant rat’s bedding area than in the clean bedding area. Male and female pups from the late RCF group showed no significant difference in their time spent over the clean bedding or over the pregnant rat’s bedding area.

3.6.3 Predator odor vs clean bedding

We also evaluated avoidance response to predator odor in control and late RCF pups at PND 15. Approximately 83% of the male offspring (10 of 12 tested) and 75% of the females (9 of 12 tested) of the control group were responsive to the odors presented (sand with cat urine or clean bedding). In the late RCF group, 58% of male pups (7 of 12 tested) and 66% of female pups (8 of 12 tested) were responsive. Table 3 shows that male and female pups from the control group spent more time in the clean bedding area than in the cat urine area. Similarly, male and female pups from the late RCF group also spent more time in the clean bedding area than in the cat urine sand area, although without statistical significance.

3.6.4 Effect of late RCF on nipple attachment of rat pups

In PND 16, the latency to reach the nipple of lactating females was evaluated. The following tests were performed: Control group pups were tested to attach to their biological mother, and pups from the late RCF group were tested to attach to their biological and to their last adoptive mother. Figures 5 D-E show that male and female pups from the late RCF group show a similar mean latency to attach to their biological or their last adoptive mother [Male pups: biological mother: 124.1 ±21.24 vs. last adoptive mother: 124.8 ± 18.26; *t* (22) = 0.024, *p*= 0.98. Female pups: biological mother: 114.2 ± 20.46 vs last adoptive mother 133.5 ± 14.57; *t* (22) = 0.76, *p*= 0.45, unpaired Student’s *t*-test]. Male pups from the late RCF group showed no significant difference in their latency to attach to their biological mother [Control: 95.90 ± 13.14 vs. late RCF: 124.20 ± 21.24; *t* (22) = 1.13, p= 0.27, unpaired Student's *t*-test] or their last adoptive mother compared with the latency to attach in the control group [Control: 95.90 ± 13.14 vs late RCF group to last adoptive mother: 124.8 ± 18.26; *t* (22) =1.29, p=0.212, unpaired Student’s *t*-test] (Figure 5 D). Similarly, female pups from the late RCF group showed no difference in their latency to attach to their biological mother

[Control: 109.7 ± 14.98 vs. late RCF group: 114.2 ± 20.46; *t* (22) = 0.178, p= 0.86, unpaired Student's *t*-test] or their last adoptive mother compared with the latency to attach in the control group [Control: 109.7 ± 14.98 vs late RCF group: 133.5 ± 14.57; *t* (22) =1.139, p=0.267, unpaired Student's *t*-test] (Figure 5 E).

4 DISCUSSION

This study analyzes the effects of RCF of lactating females on the formation of rat-mother attachment in two important periods of postnatal development: sensitive and postsensitive periods for attachment learning. The hypothesis of this research was that the RCF of caregiving lactating females, performed in the first days of the pup's life (PND 1-3), changes the neonatal environment of the pups, compromising the formation of mother-infant attachment, while RCF in the transition period from the attachment sensitive period to the postsensitive attachment (PND 9-11), although it alters environmental stability, is no longer able to affect the formation of mother-infant attachment, since this bonding is already established. Here, we provide the first evidence that RCF during the sensitive period for infant attachment learning reduces the daily maternal care behavior toward the pups and delays the developmental behavioral response to maternal odor and other biologically relevant odors. On the other hand, the RCF procedure during the postsensitive period for infant attachment slightly reduces the daily maternal care toward older pups, and although it did not alter the behavioral response to maternal odor, the response to other biologically relevant odors was altered.

4.1 EFFECTS OF RCF ON MATERNAL CARE AND MATERNAL MOTIVATION

Our results show that the RCF procedure performed during the first postpartum days significantly alters the maternal nursing behavior pattern toward the pups. Specifically, reducing high crouch posture and increasing low crouch posture. Furthermore, the maternal motivation to retrieve the pups was significantly altered during those days. These effects were restricted to the second and third day of the

RCF procedure, and after the mothers were allowed to care their adoptive pups without any disturbance, the maternal behavior recovered to control levels. Interestingly, the first day of the RCF procedure (one day after delivery) did not alter the total nursing behavior of the adoptive mothers, suggesting that in this day, maternal behavior is quite stable and less sensitive to environmental influences. Other studies using the same protocol have reported no significant effects on the maternal behavior pattern during or after the days of RCF procedure (D'Amato et al., 2011; Luchetti et al., 2015). This discrepancy is likely due to differences in the protocol of maternal behavior observation. D'Amato and Luchetti recorded in two daily sessions of 30 min each, while in our protocol, maternal behavior was recorded in four daily sessions of 72 min each. Another possible explanation for the discrepancy could be the implementation of the RCF protocol itself. In the protocol of D'Amato and Luchetti, the pups are scattered over the floor of the foster dam cage and then immediately covered with the foster nest bedding. In our procedure, we did not cover the pups with foster mother bedding, possibly the odor of the alien pups was an important factor to rapidly display any maternal behavior toward the pups.

High crouch posture is considered an active nursing posture the lactating rats perform mostly during the early postpartum days, and this gradually decreases as the pups grow older (Cramer & Alberts, 1990). The dams perform this nursing posture stimulated by the pups, which bouts the mother's ventrum to access her nipple. In our study, during the days in which this repertoire was high, the RCF mother was observed mostly outside the nest, suggesting that this active nursing posture was reduced because the mother was absent from the nest. Interestingly, after the procedure was end, the frequency of this posture increased similarly to the control dams, but the less active low crouch posture increased.

This suggests that the RCF procedure on the early postpartum day significantly interrupts maternal behavior. The procedure removing the mother from her nest, replacing her pups from another litter and scattering over the floor of her nest seems to create a stressed environment for pair mother-infant, reducing the opportunity for the mother-infant interaction and the loss of other hidden functions (Hofer, 1984) important for pup`s development. Consistent with the idea that the RCF procedure was a stressful situation, we found an increased score in self-grooming behavior during and after the days of the RCF procedure. It has been proposed that self-grooming is an important adaptive behavior that rats display in stressful situations to reduce anxiety and that lactating female rats exhibit increased self-grooming behavior when they are exposed to stressed environments (Nephew & Bridges, 2011). This suggests that the RCF protocol in our study was an important stressor for the dam that spent more time exploring the cage and less time caring the pups.

During the early postpartum day, the lactating rats have an increased motivation to retrieve the pups, and this motivation is stimulated by the pup`s stimuli (Numan & Woodside, 2010; Olazábal et al., 2013). Using the pup-retrieval test, we assessed the motivation to retrieve the pups in control and RCF dams. The test was performed in the same dam’s residence cage and consisted in that the RCF and control females recovered their own or foster pups. Immediately after the RCF procedure, the pups were placed on the floor of the home cage, and then the dam was placed. We observed the latency for the dam to retrieval the first 5 pups. In this condition, the RCF dams show a increased latency to retrieve the pups and group them before display any nursing posture. Mother rats use the odor and ultravocalizations of their pups as a cue to retrieve them to the nest (Brouette-Lahlou et al., 1991; Moore & Samonte, 1986), and another study has shown that mice pups exposed to the RCF procedure during PND 1-4 show increased ultravocalizations compared with control pups or handled pups (Luchetti et al., 2015). This suggests that the prolonged time to retrieve the pups that we observed during the RCF procedure might reflect the reduced responsiveness for the pup’s stimuli, possibly mediated by odor. This idea is consistent with Farrell and Alberts, who have suggested that lactating rats with 6-8 PND pups use the pup’s odor as a cue to respond to a vocalizing pup (Farrell & Alberts, 2002). However, the RCF protocol causes stress to both mothers and offspring, so it is difficult to determine whether maternal behaviors are altered by changes in the pups' behaviors relative to the mother or if the mother changes her own behavior due to possible hormonal changes related to the stress that the intervention causes. More studies need to be performed to elucidate these issues.

Maternal nursing behavior naturally reduces as the pups become older, gain better motor control and explore the cage (Thiels, Alberts, & Cramer, 1990). For example, at PND 10, high postures nursing events reduced almost 50% since PND 1; however, passive nursing posture eventually increased in those days, and the mother spent less time licking the pups and spent more time outside the nest exploring the cage along with the pups. The RCF procedure performed during this period (PND 9-11) slightly alters maternal behavior care. Only during the first day did we observed a significant alteration in total nursing care toward the pups. Although the next days of the RCF procedure, we did not find important variation in the total nursing behavior, we observed interesting variations in specific nursing postures. Specifically, we observed an increase in high crouch and high crouch-licking posture and a reducing score in supine nursing posture. These effects were observed during the period of the RCF procedure, and the effects on the high crouch posture continued to be observed after the RCF procedure was finished. Maternal behavior reflects the dynamic interactions between a nursing female caregiver and her offspring (Rilling & Young, 2014), so if cross-fostering occurs, it is possible that the characteristics of the adopted pups alter the behavior of the adoptive mother or that the maternal environment has a considerable impact on the offspring (Yamamuro, 2008).

In our study, we showed that repeated cross-fostering in the sensitive period for attachment learning reduces maternal behavior in relation to the pups in the first postpartum days and reduces the olfactory responses of the pups to the maternal odor and to other relevant odors. However, RCF in the postsensitive period of attachment learning has little impact on maternal behavior, altering only the first day of care in relation to older pups, and does not alter olfactory preference responses for maternal odor but undermines responses to other relevant odors in this phase of transition from dependence on maternal care to independent living.

4.2 EARLY RCF ALTERS OLFACTORY BEHAVIOR RESPONSE TO THE HOME-NEST ODOR

Our results demonstrate that the repeated exchange of lactating female caregivers alters the process of establishing the mother-infant relationship of developing rats. During the first postpartum period (characterized as the learning attachment period), the repeated exchange of lactating female caregivers abolished the acquisition olfactory preference for the odor of the last adoptive mother (Figures 3B-C) and for the odor of the biological mother (Table 3) analyzed in the PND 7.

The pup's attachment to its mother can be operationally defined as the orientation and approach behavior the pup displays for the mother's scent and the odors associated with it. This behavior is expressed and can be measured by the olfactory preference responses of the pup to the odor of the mother in tests of two choices as presented in this paper. This initial olfactory preference of the pup is formed during interactions with the mother in the nest through associative learning processes and occurs in a time window for its formation, called the attachment sensitive period (Debiec & Sullivan, 2017; Opendak et al., 2017), which corresponds to the first 10 days of a rat's life. This learning is also strongly dependent on the behavioral repertoires that the mother directs to the pup and on the responsiveness of the pup's nervous system to maternal tactile stimuli.

Data from the present study suggest that although lactating females remained with offspring exhibiting a stable behavioral repertoire on PND 4, on PND 2 and 3, they had a significant change in maternal behavior, which was reestablished immediately after the end of the intervention. Thus, the changes of mothers performed in the first days of the pups' postnatal development signified a change in the important neonatal environment that impacted the formation of the mother-child attachment.

The work of Professor Regina Sullivan's laboratory suggests that the mother-infant attachment is formed through the temporal association of maternal odor and mother's tactile stimulation (licking) the offspring. The pairing of these two stimuli is quickly learned by pups and results in search behavior and approach to conditioned odor. From this learning process, the maternal odor assumes the ability to regulate the physiological processes and behaviors of the pups (Al Aïn et al., 2017; Debiec & Sullivan, 2017; Polan & Hofer, 1998; Sullivan et al., 1986).

In this study, the adopted pups were exposed to maternal stimuli from 4 different mothers, a condition that may have negatively interfered in the formation of the mother-infant attachment in PND 7. The changes in maternal information, both olfactory, gustatory, tactile and thermal, caused by the RCF procedure probably disrupted the associative learning process necessary to establish the link between the adoptive mother and the developing infant (Landers & Sullivan, 2012; Luchetti et al., 2015). In addition, instability in the postnatal environment induced by RCF also interfered with the ability of the adopted pups to seek and approach the mother's nipple in PND 8 (Figure 3 D-E) to suckle, but this reduced capacity did not affect the body weight gain of the pups, which on average was the same as the pups that remained with their biological mothers.

Leon (1992) described that the origin of the pup's attraction to the mother's odor is in the feces of the lactating rat, since the pup prefers the feces of a lactating rat when compared to the feces of a non-lactating rat. The reason for that preference is presence of cecotrope, which is extremely attractive to the pups. Cecotrope is produced in the cecum of all adult rats, however, only lactating rats are able to excrete enough cecotrope to attract the pups (Leon, 1974; Leon, 1992). Most likely, this is due to the increase in feed consumption during lactation (Leon, 1975). In addition to dietary intake, other factors, such as hormonal, act to regulate excretion of cecotrope by lactating rats (Leon, 1974). In present study, the odor of females on GD 20 was used to evaluate the behavior of the offspring in response to the odor of their biological mother after being submitted to the RCF protocol. However, results did not confirm our hypothesis, since pups did not show preference for the GD20 bedding of their mother. Considering that cecotrope increases after delivery, it is possible that the amount of maternal odor on the stimuli used was low. Another possible reason for the absence of preference could be the reduction in odoriferous molecules during the time of conservation of the material used as stimuli (sawdust and feces).

In short, the total nursing in RCF dams reduces importantly compared to control dams and also reduced maternal motivation. The behavioral olfactory preference to foster mother nest material was also reduced, and the latency to attach the mother’s nipples was delayed. This period has been proposed as a sensitive period to learn attachment to the mother, which is acquired in interaction with the mother into the nest. Therefore, the repertoire of the mother is important and the rhythmicity of how this behavior is expressed to the pups. The mother rats display a rhythmic maternal behavior repertory. Most nursing postures were observed in the first session of the day (9h00) and gradually reduced at the final session (18h00) when the mother displayed behavior outside the nest (nest building, self-grooming). In supporting the associative learning that occurs in the nest, the constant and repetitive pairing of maternal and nest odor with maternal behavior repertories is probably critical instead of the quantity of maternal behavior. The absence of the mother may reduce the opportunity to provide stimuli to the pups (warm, licking, skin contact) and reduce the opportunity for these stimuli to be paired with the maternal odor or the nest. In addition, the reduced maternal stimulation might reduce the pup's arousal, which has been proposed that the maternal stimuli trigger the noradrenaline system in the pups, which is necessary to form the association of odor with maternal behavior contingencies (Debiec & Sullivan, 2017; Opendak & Sullivan, 2016).

4.3 LATE RCF DOES NOT ALTER THE OLFACTORY BEHAVIOR RESPONSE TO THE HOME-NEST ODOR

We also evaluated the behavioral responses of the offspring that were submitted to the RCF protocol during the transitional sensitive period for the postsensitive period, and our results show that repeated cross-fostering of caregiving female animals during PND 9-11 does not interfere with responses (Figures 5 B-C) and does not affect the search behavior and approach to odor of the biological mother (Table 3). It is important to note that the last lactating female caregivers remained with the offspring adopted in the PND 12-15 period and the biological mothers had the litters before the RCF protocol, that is, in the PND period 1-9. Pups who were adopted late also sought the nipple of both the adoptive mother and the biological mother in the PND 16 to suckle. In addition, the body weight gain of the adopted pups is on average the same as the pups that remained with their biological mothers and never went through any environmental intervention. These data suggest that RCF during the transition from the sensitive to postsensitive period does not alter the behaviors required by the pups to ensure their survival.

The mother's odor is critical for the survival of rat pups, as it is through it that the pup seeks and approaches the mother to protect itself, warm up and feed itself. In addition, the maternal odor also regulates mother-infant interactions early in life, when attachment learning is fully favored and aversive learning is attenuated to prevent pups from avoiding their caregiver (Raineki et. al., 2010; Perry, Al Aïn et al., 2016; Debiec & Sullivan, 2017). It is noteworthy that the mother's odor has special control over the pups in the postnatal period 10 to 15. Specifically, at this age, the pups are crossing the transitional sensitive period, in which the maternal stimuli have the unique ability to prevent the learning of threats through suppression of amygdala activity mediated by the presence of the mother in the nest (Debiec & Sullivan, 2017; Opendak &

Sullivan, 2016; Sullivan & Holman, 2010; Upton and Sullivan, 2010). Therefore, it is not uncommon for our results to show a strong olfactory preference for the odor of the adoptive and biological mother of the offspring who were submitted to the RCF protocol or by the biological mother of the control pups on day PND 15 of the postsensitive period.

4.4 RCF PERFORMED DURING THE ATTACHMENT SENSITIVE AND POSTSENSITIVE PERIODS DOES NOT ALTER AVOIDANCE BEHAVIOR TOWARD BIOLOGICALLY AVERSIVE ODORS

Finally, this study also analyzed the effect of repeated exchange of lactating female caregivers on the olfactory preference-inhibition system through approach and/or avoidance responses to a naturally aversive stimulus (predator odor) on PND 7 and 15. The analysis of the responses showed that both control and adopted pups suppress predator odor approaching behavior (Table 3) in the two periods studied. The odor guides the approach and avoidance behaviors that begin at birth in many mammals, including humans (Al Aïn et al., 2017; Moriceau, 2004). Olfactory cues are essential for newborn rodents to approach the mother and attach to the nipples and to suppress the ultrasonic vocalizations in the presence of a predator (Perry et al., 2016; Takahashi, 1992). In rat pups, the odor of an adult male rat is an aversive odor, as inferred by the fact that it interrupts ultrasonic vocalizations during the first days of life and causes immobility/freezing in older pups from PND 10 (Hofer et al., 2002; Moriceau, 2004; Perry et al., 2016; Takahashi, 1992). Our results show behavioral avoidance responses to a biologically aversive odor already in pups at 7 days of postnatal life. It is important to note that none of the tested animals had experience with this type of odor.

Finally, we can conclude that RCF by caregiving lactating females during the attachment sensitive period alters the olfactory preference for the maternal odor of in rat pups at PND 7. This olfactory learning is necessary for the formation of attachment mother-pup during the attachment sensitive period; however, once this learning is consolidated, RCF by caregiving nursing females does not cause further damage to the olfactory learning that is necessary for the survival of the pups. We can also conclude that this environmental intervention does not interfere with the ability of lactating females to care for offspring other than their biological ones. Together, these results suggest that the same postnatal environmental intervention (cross-fostering) at different times in development may induce different or even opposing effects on maternal and filial behavioral characteristics.

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CAPÍTULO IV

4. DISCUSSÃO GERAL

No primeiro estudo desta tese, apresentado no capítulo 2, exploramos o desenvolvimento das respostas comportamentais para odores biologicamente relevantes durante as três primeiras semanas do período pós-natal de filhotes de ratos e caracterizamos o perfil do comportamento maternal durante esse período. Primeiramente, avaliamos as respostas comportamentais ao próprio odor de ninho e ao odor de ninho de rato juvenil em filhotes machos e fêmeas nos dias DPN 3, 7, 11 e 15. Procuramos determinar o momento em que os filhotes mostram preferência para o odor do próprio ninho e para o odor de ninho de rato juvenil em relação a um odor neutro. Até onde sabemos, as respostas ao odor do ninho de rato juvenil não foram estudadas em filhotes de ratos. A seguir, avaliamos as respostas ao odor de ninho de rato adulto e para urina de gato também nos DPN 3, 7, 11 e 15 para identificar quando os filhotes mostram aversão ao odor de ninho de rato adulto e odor de predador em relação a um odor neutro. Por fim, avaliamos o comportamento materno nos dias DPP 2, 6, 10 e 14 para identificar quais características do comportamento materno que contribuiriam para o desenvolvimento normal da prole.

Nossos resultados mostraram que as respostas de preferência olfatória para o odor do próprio ninho; para o odor de ratos juvenis; e para o odor de ratos machos adultos foram evidentes a partir do DPN 7 e não antes. Embora tenhamos observado que as respostas se mantiveram sem mudanças entre o DPN 7 e 11, os filhotes com DPN 15 apresentaram um aumento na preferência olfatória para o odor do ninho e para o odor de ratos machos. Para o odor de congêneres a preferência se manteve estável entre os dias DPN 7 e DPN 15. E para um odor de predador os filhotes de ratos apresentaram respostas de evitação a partir do DPN 11, não antes. Paralelamente com o aparecimento dessas respostas comportamentais dos infantes para os odores biologicamente relevantes nós também mostramos que o repertório de comportamentos materno é intenso durante a primeira semana pós-natal, onde a postura de *high crouch* e *licking* foram significativamente maiores e à medida que o período pós-natal avança os comportamentos maternos diminuem e gradualmente outros comportamentos aumentam como por exemplo a *supine posture* e também o número de vezes que a mãe sai do ninho (*dam off the nest*).

O aprendizado do odor da mãe e do ninho é coincidente com o período no qual o cuidado materno é intenso, ao qual o filhote está exposto dentro do ninho. Enquanto que, o aprendizado de preferência por congêneres ocorre em uma janela temporal maior. Possivelmente, certos padrões de comportamento materno após o dia pós-natal 10 sejam importantes para induzir essas preferências. Kojima e Alberts (2009) sugeriram que as experiências com a mãe dentro do ninho, tanto antes como depois do dia pós-natal 10 são capazes de induzir respostas comportamentais de preferências para os congêneres.

Além dessa caracterização inicial, esta tese teve como foco principal estudar, do ponto de vista experimental, os efeitos comportamentais da adoção de ratas lactantes cuidadoras sobre a formação do vínculo (apego) mãe-filhote em dois períodos importantes do desenvolvimento pós-natal: durante o período sensível e no período pós-sensível a aprendizagem de apego. Uma de nossas hipóteses era que a troca repetida de fêmeas lactantes cuidadoras (adoção cruzada) realizada nos primeiros dias após o nascimento dos filhotes (DPN 1 a 3) comprometeria a formação do vínculo mãe-filhote, enquanto que a adoção realizada mais tardiamente (DPN 9 a 11), embora altere a estabilidade ambiental, não seria mais capaz de afetar a formação do apego mãe-filhote, uma vez que esse vínculo já está estabelecido. De outro lado, a troca repetida de filhotes, embora de mesma idade, afetaria negativamente o comportamento de cuidado materno para os filhotes que não fossem os seus biologicamente. Em essência, o estudo analisou a especificidade da relação mãe-filhote, ou seja, se haveria uma certa identidade específica dos estímulos dos filhotes e da mãe, de forma recíproca.

Esta tese forneceu evidências de que a adoção cruzada realizada durante o período sensível para a aprendizagem do apego do filhote pela fêmea cuidadora lactante reduziu o comportamento de cuidado materno diário, especificamente nos dias pós-natal 2 e 3. Essa redução no cuidado maternal foi observada durante o período em que o protocolo de RCF estava sendo realizado, no entanto, imediatamente após o término do RCF, ou seja, a partir do dia 4 pós-natal, as mães estabilizaram o cuidado maternal em relação aos filhotes adotados. Além disso, este estudo mostrou que a adoção atrasou o desenvolvimento da resposta comportamental ao odor materno e a outros odores biologicamente relevantes, confirmando a hipótese de que a adoção realizada no período sensível ao apego compromete a formação do vínculo mãe-filhote.

Por outro lado, o procedimento de adoção tardia realizado durante o período de transição do sensível para o pós-sensível ao apego não interferiu nas respostas de busca e aproximação dos filhotes ao odor da última mãe adotiva. No entanto, os filhotes adotados tardiamente não mostraram comportamentos de preferência para o odor da mãe biológica (odor da mãe biológica na condição de gestante). Nossa expectativa era de que os filhotes pudessem expressar a memória de sua mãe biológica. Uma explicação alternativa para esse resultado é de que o odor materno utilizado como estímulos (maravalha e fezes) possa ter sido fraco para desencadear o comportamento de aproximação dos filhotes. Com o armazenamento relativamente prolongado, os odores da mãe presentes no material podem ter se dissipado. Outra possibilidade também relacionada à densidade do odor presente no estímulo utilizado é o fato de que as fezes das fêmeas gestantes têm menos cecótropos (onde se encontra o odor) do que das fêmeas lactantes. Assim, não podemos afirmar que procedimento de RCF realizado durante o período pós-sensível ao apego tenha interferido na preferência pelo odor da mãe biológica. Com relação ao comportamento maternal, a adoção tardia reduziu o cuidado materno diário, em relação aos filhotes mais velhos, especificamente no dia 9 pós-natal. Esse resultado confirma a hipótese inicial de que a troca repetida de filhotes, embora de mesma idade, afetaria negativamente o comportamento de cuidado materno para os filhotes que não fossem os seus biologicamente.

Nos próximos itens, discutimos mais detalhadas os resultados obtidos nesta tese.

4.1 EFEITOS DO RCF PRECOCE SOBRE O CUIDADO E A MOTIVAÇÃO MATERNA

O modelo de adoção realizado através da troca repetida de fêmeas lactantes durante o período sensível para a aprendizagem de apego reduziu o comportamento materno em relação aos filhotes nos dias 2 e 3 pós-natal. A postura de agachamento com dorso elevado é considerada uma postura de amamentação ativa que a fêmeas lactantes realizam com alta frequência, principalmente durante os primeiros dias do pós-parto, e esse comportamento diminui naturalmente à medida que a idade dos filhotes avança (Cramer & Alberts, 1990). Em nosso estudo, durante os dias em que esse repertório de comportamento materno foi alto, as mães submetidas ao protocolo de RCF foram observadas principalmente fora do ninho, indicando que a postura de amamentação com dorso elevado (*high crouch*) foi reduzida, uma vez que elas estavam ausente do ninho. Curiosamente, após o término do procedimento de RCF, a frequência da postura de amamentação com dorso elevado aumentou de forma semelhante às mães do grupo controle.

As mães realizam essa postura de amamentação incentivadas pelos filhotes, que estimulam o ventre materno para ter acesso ao seu mamilo (Alberts, 2008; Sullivan, 2017). O odor materno regula as interações mãe-filhote no início da vida, quando a aprendizagem de apego é totalmente favorecida e a aprendizagem aversiva é atenuada para evitar que os filhotes aprendam a evitar a mãe ou cuidador (Debiec & Sullivan, 2017; Perry et a., 2016; Raineki et al., 2010).

Em nosso primeiro estudo (artigo 1) observamos variações no comportamento maternal em relação aos filhotes a partir do dia pós-natal 6. Especificamente a partir desse período a postura de amamentação com o dorso elevado (*high crouch),* e a frequência do comportamento de lamber os filhotes (*licking*) começaram a diminuir gradualmente conforme o período pós-parto progrediu. Também observamos que a partir do dia 6 pós-natal aumentou a frequência com que as mães se ausentam do ninho, principalmente durante o período escuro. O repertório comportamental apresentado pelas mães na primeira semana de vida pós-natal foi rítmico e a maioria das posturas de amamentação observadas foram na primeira sessão de registro diário às 9h e ao longo do dia esses comportamentos diminuíram gradualmente até o último registro às 18h, já no período escuro. A partir desse momento as mães passaram a apresentar comportamentos de deixar o ninho para explorar a caixa moradia (artigo 1). Este intenso cuidado materno fornecido pelas mães nos primeiros dias vida é considerado um fator crítico para o desenvolvimento adequado dos mamíferos, e alterações nessas interações podem modificar a fisiologia e o comportamento da prole a longo prazo (Meaney, 2001).

Durante o período de lactação, as mães apresentam comportamento de cuidar dos filhotes e esses comportamentos são manifestados através da busca dos mesmos quando estes se afastam do ninho, pela estimulação da micção por meio da lambida anogenital, pelo posicionamento sobre os filhotes para provê-los de nutrição e calor, pela construção do ninho e por defesa contra intrusos (Albert & Walsh, 1995; Lee et al., 1969).

O trabalho de Maccari et al (1995) mostrou que as mães que passaram pelo procedimento de adoção permaneceram mais tempo lambendo e, apresentaram uma menor latência para o recolhimento dos filhotes do que as mães biológicas. No presente estudo, as mães adotivas apresentaram uma maior latência para o recolhimento dos filhotes do que as mães biológicas. O procedimento de remover a mãe de seu ninho, substituir seus filhotes por filhotes de outra ninhada e espalha-los pelo chão da caixa moradia, provavelmente criou um ambiente de estresse significativo tanto para a mãe quanto para a prole, e, como consequência reduziu a oportunidade para que ocorresse a interação mãe-filhote (Hofer, 1984).

Outro grupo, verificou que mães adotivas apresentaram maior frequência de comportamento exploratório e maior pontuação no teste de construção de ninho do que as mães biológicas (Lerch et al., 2014). Da mesma forma, Barbazanges et al (1996) mostraram que o procedimento de adoção, quando realizado nas primeiras 3-6 horas de vida dos filhotes, aumentou o comportamento de lamber da mãe, mas quando realizado nos dias pós-natal 5 ou 12 não alterou o perfil do comportamento materno, revelando que houve um período em que as mães foram mais sensíveis à separação de seus filhotes.

Luchetti et al (2015) e D’Amato et al (2011), mostraram que o protocolo de adoção cruzada repetida por si só não alterou a frequência de lamber, *grooming* ou qualquer variação do cuidado maternal. Porém, é importante salientar que no protocolo de D'Amato e Luchetti, os filhotes eram colocados dentro do ninho e imediatamente cobertos com a maravalha do ninho adotivo. Em nosso procedimento, os filhotes eram espalhados pela caixa moradia da mãe adotiva e não eram cobertos com maravalha da mãe adotiva ou do ninho adotivo, possivelmente o odor dos filhotes adotivos tenha sido um fator importante para induzir rapidamente qualquer comportamento materno em relação aos filhotes.

Ainda assim, há uma inconsistência nos dados entre os diversos trabalhos disponíveis na literatura e esse fato pode ser devido a diferentes versões do protocolo de RCF, diferentes formas de registrar o comportamento maternal ou às diferentes cepas utilizadas por esses grupos de pesquisa.

Consistente com a ideia de que o procedimento de RCF é uma situação estressante para a mãe, encontramos um aumento no escore no comportamento de autolimpeza durante e após os dias do procedimento do RCF. Tem sido proposto que a autolimpeza é um comportamento adaptativo importante que os ratos exibem em situações estressantes para reduzir a ansiedade e que as ratas lactantes exibem o comportamento de autolimpeza aumentado quando elas são expostas a ambientes estressantes (Nephew & Bridges, 2011). Isso sugere que o protocolo RCF em nosso estudo foi um importante estressor para as mães, pois vimos que elas realizaram mais comportamentos de autolimpeza e também passaram mais tempo explorando a gaiola e consequentemente menos tempo cuidando dos filhotes.

4.2 EFEITOS DO RCF PRECOCE SOBRE OS COMPORTAMENTOS DOS FILHOTES

4.2.1 Preferência olfatória para o odor materno

Nosso estudo mostrou que a troca repetida de fêmeas cuidadoras lactantes durante o período inicial do pós-parto, afetou a preferência olfatória dos filhotes de ratos com 7 dias de vida para a mãe adotiva (última fêmea lactante) e também para a mãe biológica. Vale ressaltar que o estímulo das mães biológicas (maravalha no estado gestacional) foi utilizado para avaliar o comportamento da prole em resposta aos estímulos dessa mesma mãe após os filhotes terem sido submetido ao protocolo de RCF. Esperávamos que as crias pudessem expressar a memória de sua mãe biológica. Entretanto, os resultados não confirmaram nossa hipótese, uma vez que os filhotes com 7 dias de vida pós-natal não mostraram preferência pela maravalha de sua mãe biológica. Considerando que o cecótropo aumenta após o parto Leon (1975), é possível que a quantidade de odor materno nos estímulos utilizados tenha sido baixa. Outra possível razão para a ausência de preferência poderia ser a redução de moléculas odoríferas durante o tempo de conservação do material usado como estímulo (maravalha e fezes).

O apego do filhote à mãe pode ser operacionalmente definido como os comportamentos de orientação e aproximação que o filhote exibe para o cheiro da mãe e para odores associados a ela. Esse comportamento é expresso e pode ser medido pelas respostas de preferência olfatória do filhote ao odor da mãe em testes de duas escolhas, conforme apresentado neste trabalho. Essa preferência inicial do filhote é formada durante as interações com a mãe no ninho por meio de processos associativos de aprendizagem e ocorre em uma janela temporal para sua formação, denominada período sensível ao apego (Debiec & Sullivan, 2017; Opendak et al., 2017), o qual, corresponde aos primeiros 10 dias de vida de um rato. Os dados do nosso primeiro estudo, mostraram que os filhotes de todas as faixas etárias estudadas (3, 7, 11 e 15 DPN) apresentaram respostas de preferência olfatória ao odor materno e essa preferência aumentou conforme o desenvolvimento pós-natal avançava, de forma semelhante em filhotes machos e fêmeas. No DPN 3 os filhotes já discriminaram o odor materno, porém foi a partir do dia 7 DPN que as respostas de busca e aproximação se tornaram significativamente mais consistentes (artigo 1). Um padrão de respostas semelhante foi descrito no trabalho de Meyer e Alberts (2016), no qual filhotes de camundongos (*Mus musculus*) com 5, 10 e 12 DPN apresentaram respostas de orientação e preferência para o odor do próprio ninho em relação ao odor de maravalha limpa e essa preferência também aumentou durante o desenvolvimento pós-natal.

Além disso, nossos dados também indicaram que os filhotes adotados precocemente tiveram dificuldades em buscar e se aproximar do mamilo da mãe adotiva e da mãe biológica para a lactação. Provavelmente a instabilidade do ambiente pós-natal induzida pelo protocolo do RCF afetou essa capacidade. Para garantir a sobrevivência os filhotes de ratos exibem comportamentos de busca e aproximação persistentes em direção ao ventre materno, ligam-se ao mamilo e sugam o leite materno. Esses comportamentos que os filhotes direcionam a mãe além de serem importantes para a sobrevivência dos filhotes eles também são sinais importantes e necessários para receber o cuidado maternal (Alberts, 2008; Sullivan, 2017).

Os trabalhos do grupo da professora Regina Sullivan mostraram de maneira consistente que o vínculo (apego) filhote-mãe se dá por um processo de aprendizado e formação de memória desta relação filial inicial (Sullivan & Wilson, 1991; Landers & Sullivan, 2012; Opendak & Sullivan, 2016). Tanto em humanos como em animais, o apego requer uma breve experiência de aprendizado das caraterísticas sensoriais da figura materna (odor, textura, cor, som), que se produz durante as primeiras interações do neonato com seu cuidador (Debiec & Sullivan, 2017; Raineki et al., 2010; Sullivan & Holman, 2010). Neste período, também chamado de período sensível ao apego, os filhotes podem ser facilmente condicionados a outro odor que não seja o materno, desde que seja pareado com estímulos termo táteis (Debiec & Sullivan, 2017; Landers & Sullivan, 2012; Meyer & Alberts, 2016) indicando um processo de aprendizado associativo.

As alterações que encontramos no desenvolvimento das respostas de preferência olfatória em filhotes de ratos com 7 DPN sugerem que a adoção precoce provavelmente interrompeu o processo de aprendizagem associativa necessário para estabelecer a ligação entre a mãe adotiva e os filhotes em desenvolvimento (Landers & Sullivan, 2012; Luchetti et al., 2015). Os animais que passaram pelo processo de adoção precoce apresentaram dificuldade em reconhecer e preferir o odor da mãe biológica e também da mãe adotiva, o que indica que uma interferência ambiental, como a adoção, nesse período, teve consequências importantes na relação mãe-filhote.

Neste estudo, os filhotes adotados foram expostos a estímulos maternos de 4 mães diferentes, condição que pode ter interferido negativamente na aprendizagem do odor materno e consequentemente na formação do apego mãe-filhote no DPN 7. Tudo indica que o pareamento que deveria ocorrer dentro do ninho foi prejudicado pela diminuição dos comportamentos maternos diários e, consequentemente os filhotes apresentaram menos respostas de busca e aproximação pelo odor materno, e maior latência para as respostas de ligação ao mamilo. O aprendizado de preferência olfatória é fortemente dependente dos repertórios comportamentais que a mãe direciona para o filhote, e da responsividade do sistema nervoso do filhote para com os estímulos táteis maternos (Sullivan, 2017).

Provavelmente, as alterações no ambiente neonatal precoce, provocadas pelas trocas repetidas de fêmeas lactantes, impactaram a formação do apego mãe-filhote. É importante salientar que nos dias 2 e 3 pós-natal, as cuidadoras lactantes apresentaram uma mudança significativa no comportamento direcionado aos filhotes, o qual, foi restabelecido imediatamente após o término da intervenção.

4.2.2 Preferência Olfatória / evitação para o odor de predador

Também analisamos o efeito da troca repetida de cuidadores lactantes no sistema de inibição de preferência olfativa por meio de respostas de aproximação e / ou evitação de um estímulo naturalmente aversivo no dia 7 pós-natal. Observamos que a adoção precoce não alterou as respostas aversivas, pois os filhotes adotados com 7 DPN não apresentaram comportamento diferentes para o odor de predador quando comparado com os filhotes controles. É importante notar que nenhum dos animais testados tinha experiência com esse tipo de odor.

A atenuação do medo e da evitação foram sugeridas como características extremamente importantes do processo de apego, pois aprender a temer ou a evitar a fonte de alimento e proteção (mãe) não seria ideal para a sobrevivência dos filhotes. Assim Hofer & Sullivan, 2008 sugerem que é melhor formar um repertório de comportamentos de busca e de aproximação da mãe, independente da qualidade dos cuidados recebidos. Esta atenuação da aprendizagem aversiva e da evitação foi demonstrada em filhotes de ratos durante o período sensível para a aprendizagem de apego. Especificamente, o condicionamento de um novo odor com um estímulo doloroso, tal como choque de 0,5mA na cauda ou na pata traseira, mostrou que os filhotes que foram submetidos a este paradigma se aproximam do odor condicionado quando este é reapresentado (Camp & Rudy, 1988; Sullivan et al., 1986).

No primeiro estudo dessa tese fizemos uma caracterização das respostas de aproximação e evitação de filhotes de rato com diferentes idades pós-natal para o odor de predador (odor de urina gato) e observamos que filhotes com 3 e 7 dias de vida pós-natal não apresentaram respostas de evitação para o odor de urina de gato (artigo 1). A incapacidade dos filhotes de ratos em apresentarem comportamentos de medo e evitação em resposta a combinação dor – odor durante o período sensível ao apego pode ser devido ao fato de que o filhote de rato naturalmente experimenta um pouco de dor provocado pela sua mãe, por exemplo, quando a mãe deixa o ninho para cuidar de suas necessidades ela pode arrastar os filhotes ainda ligados aos seus mamilos ou então ela pode carregá-los pelo pescoço e machucá-los. Por isso aprender uma aversão a odores maternos associados a estas possíveis manipulações ásperas prejudicaria as respostas de procura dos filhotes por calor, nutrição, segurança e consequentemente a sobrevivência (Hofer & Shair, 1978).

4.3 Efeitos do RCF tardio sobre o cuidado materno

A adoção tardia alterou o comportamento materno em relação aos filhotes mais velhos. Especificamente os cuidados maternos que as mães adotivas direcionaram aos filhotes adotivos diminuiu significativamente no dia 9 pós-natal em relação as mães controles. Além disso, identificamos algumas características interessantes no comportamento maternal dessas fêmeas lactantes cuidadoras, como um aumento da postura *high crouch* e *high crouch* e *licking* e uma redução da postura supina durante o protocolo de RCF e aumento da postura *high crouch* após o término do procedimento de RCF*.* Até aproximadamente o dia 12 de vida pós-natal é a mãe quem toma a iniciativa de se aproximar dos filhotes. Sendo assim, a variação no cuidado maternal pode ser considerada o diferencial para as experiências sensoriais no desenvolvimento dos filhotes. Após esse período, os filhotes estão aptos a se locomover e a deixar o ninho, a partir de então passa a serem eles que se aproximam da mãe para requerer cuidados (Grota & Ader, 1974).

Os padrões de comportamento de apego mãe-filhote são dinâmicos, na qual a mãe contribui com padrões e níveis específicos de cuidado maternal, os quais variam conforme o filhote se desenvolve, enquanto que aparecem novos repertórios comportamentais nos filhotes através da interação mãe-filhote. A alteração dos padrões ou níveis específicos de cuidado materno impactam o desenvolvimento emocional e cognitivo do infante (Sullivan et al., 2011).

A intensidade e a frequência do cuidado materno variam principalmente com o avanço dos dias pós-parto, com o período do dia e com o número de filhotes na ninhada (Champagne et al., 2003; Cramer & Alberts, 1990). A observação pontual a cada 3 minutos durante 72 minutos em 4 períodos do dia (3 no ciclo claro e 1 no ciclo escuro) mostrou que conforme o período pós-parto avança as mães permaneceram menos tempo dentro do ninho cuidando dos filhotes, principalmente no período escuro e, curiosamente no DPN 10 as mães incrementaram a frequência de saída do ninho tanto nos período claro como no escuro (artigo 1).

Durante o tempo que os filhotes permanecem dentro do ninho, exclusivamente sob os cuidados maternos, a única variação ambiental que ocorre é o comportamento da própria mãe. Conforme o filhote se desenvolve a mãe apresenta naturalmente mudanças dinâmicas adequadas ao período de desenvolvimento do filhote. Assim, se o comportamento materno refletiu as interações dinâmicas entre uma fêmea cuidadora e sua prole (Rilling & Young, 2014), no momento em que a adoção cruzada repetida ocorreu, é possível que as características dos filhotes adotados alteraram o comportamento da mãe adotiva ou que o ambiente materno tenha tido um impacto considerável na prole (Yamamuro, 2008).

Nossos dados sugerem que o procedimento de adoção tardia é uma situação estressante para as mães, pois observamos um aumento no escore no comportamento de autolimpeza, o qual foi caracterizado como um comportamento relacionado à ansiedade em uma variedade de espécies (Spruijt et al., 1992). Tem sido sugerido que esse comportamento de autolimpeza é um comportamento ideal para estudar o desenvolvimento de transtornos depressivos e ansiosos e além disso, avaliar potenciais tratamentos para esses transtornos (Kalueff et al., 2007). Após o término do protocolo de RCF tardio, observamos um ligeiro aumento no comportamento de autolimpeza, porém, sem significância. Dessa forma, concluímos que o protocolo de adoção tardia foi um importante estressor para as mães, no entanto, depois que as mães permaneceram com os filhotes, sem interferências, esse comportamento tendeu a diminuir.

4.4 EFEITOS DO RCF TARDIO SOBRE OS COMPORTAMENTOS DOS FILHOTES

4.4.1 Preferência olfatória para o odor materno

O procedimento de adoção tardia realizado durante o período de transição do sensível para o pós-sensível ao apego não interferiu nas respostas de busca e aproximação dos filhotes ao odor da última mãe adotiva. Porém os filhotes adotados tardiamente não mostraram comportamentos de preferência para o odor da mãe biológica (odor da mãe biológica na condição de gestante). Nossa expectativa era de que os filhotes pudessem expressar a memória de sua mãe biológica. Entretanto, os resultados não confirmaram nossa hipótese. Conforme explicado anteriormente, é possível que a quantidade de odor materno nos estímulos utilizados (maravalha e fezes) tenha sido baixa. É importante ressaltar que as últimas fêmeas lactantes cuidadoras estiveram com a prole adotada no período de 12 a 15 DPN e as mães biológicas permaneceram com as ninhadas antes do protocolo de RCF, o qual correspondeu ao no período de 1 a 9 DPN. No primeiro estudo (artigo 1) evidenciamos que as respostas de preferência olfatória para o odor materno foram evidentes a partir do dia 7 DPN e, aumentaram conforme a idade dos filhotes avançou, sendo que no dia 15 DPN a resposta de preferência olfatória para o odor da mãe ainda foi alta e se manteve estável.

Vale ressaltar que o odor materno possui controle especial sobre os filhotes no período de 10 a 15 dias pós-natal, momento em que os filhotes estão atravessando o período de transição do período sensível para o pós-sensível a aprendizagem do apego. Nesse período os estímulos maternos têm a capacidade única de prevenir a aprendizagem aversiva dos filhotes a ameaças através da supressão da atividade da amígdala mediada pela presença da mãe no ninho (Debiec & Sullivan, 2017; Opendak & Sullivan, 2016; Sullivan & Holman, 2010; Upton & Sullivan, 2010).

Nossos dados forneceram evidências de que as adoções realizadas no período sensível e no período de transição para o pós-sensível, tiveram diferentes efeitos comportamentais na prole. Portanto, não é inusitado que nossos resultados tenham mostrado uma forte preferência olfativa pelo odor da mãe adotiva na prole que foi submetida ao protocolo de RCF ou pela mãe biológica nos filhotes controles no dia15 DPN (período pós-sensível a aprendizagem de apego).

Os filhotes que foram adotados tardiamente também procuraram o mamilo tanto da mãe adotiva quanto da mãe biológica no PND 16 para mamar. Consequentemente o ganho de peso corporal dos filhotes adotados foi semelhante ao dos filhotes que permaneceram com suas mães biológicas e que nunca passaram por nenhuma intervenção ambiental. O odor orienta os comportamentos de aproximação e evitação que começam no nascimento em muitos mamíferos (Al Aïn et al., 2017; Moriceau, 2004). As pistas olfativas são essenciais para roedores recém-nascidos se aproximarem da mãe, se fixarem aos mamilos e suprimirem as vocalizações ultrassônicas na presença de um predador (Perry et al., 2016; Takahashi, 1992).

4.4.2 Preferência Olfatória / evitação para o odor de predador

Este estudo também analisou o efeito do RCF durante o período de transição para o período pós-sensível ao apego sobre o sistema de inibição de preferência olfatórias por meio de respostas de aproximação e/ou evitação de estímulos considerados naturalmente aversivo no dia pós-natal 15. Os resultados mostraram que os filhotes que passaram pelo protocolo de RCF tardio, não apresentaram respostas de esquiva significativas para o odor aversivo no dia pós-natal 15, indicando que a adoção tardia alterou a capacidade de evitação a um odor biologicamente aversivo nessa idade, o que pode ser extremamente ariscado para os animais que estão começando a se aventurar fora do ninho.

Na caracterização que fizemos das respostas de aproximação e evitação de filhotes de rato com diferentes idades pós-natal para o odor de predador (odor de urina gato), no primeiro estudo dessa tese (artigo 1), observamos que filhotes com 11 dias de vida pós-natal apresentaram respostas consistentes de evitação ao odor de predador e essas respostas aumentaram conforme a idade pós-natal avança, de forma semelhante em machos e fêmeas.

Em filhotes de ratos, o odor de um rato macho adulto é um odor aversivo, inferido pelo fato de interromper as vocalizações ultrassônicas durante os primeiros dias de vida e causar imobilidade / congelamento em filhotes mais velhos no dia 10 pós-natal (Moriceau et al., 2004; Perry et al., 2016; Takahashi, 1992). Além disso, próximo ao dia pós-natal 10, os filhotes de ratos começam a se aventurar fora do ninho (Bolles & Woods, 1964). Com a finalidade de reduzir a aprendizagem de apego e permitir que o filhote comece a aprender a evitar os estímulos aversivos, os níveis de corticosterona aumentam gradualmente, embora um nível crítico seja alcançado naturalmente somente a partir do dia 10 pós-natal (Moriceau et al., 2004) quando a amígdala se integra funcionalmente para suportar respostas defensivas inatas específicas da espécie ao odor de predador, como o congelamento (Moriceau & Sullivan, 2004; Sullivan et al., 2000; Wiedenmayer & Barr, 2001). Isso coincide com o término do período sensível ao apego e com a maturação neurobiológica do filhote (Debiec & Sullivan, 2017; Moriceau et al., 2010; Opendak & Sullivan, 2016).

4.5 RELAÇÃO COM ESTUDOS EM HUMANOS

Esta tese apontou, do ponto de vista experimental, que a instabilidade do ambiente neonatal causado pelas trocas sucessivas de mães adotivas induziu alterações significativas na formação do vínculo (apego) mãe-filhote, dependendo da fase do desenvolvimento em que ocorreu a adoção. Estes resultados são importantes pois podem auxiliar a compreender, de forma indireta, os efeitos da adoção de crianças em diferentes idades após o nascimento sobre o apego por seus pais adotivos e a consequente a relação entre pais adotivos e filho adotado.

Os bebês humanos se ligam rapidamente a seus cuidadores. Como originalmente sugerido por (Bowlby, 1965), a criança busca proximidade com a mãe ou com outro cuidador permanente para ter uma sensação de segurança. O apego é um vínculo forte que pode ser formado em qualquer estágio do desenvolvimento pós-natal, tanto em humanos como em outros animais sociais. Cabe ressaltar que o vínculo entre mãe e filho é um dos exemplos mais robustos de apego. Dentro desta dupla mãe-filho, o bebê está aprendendo e memorizando características do cuidador, o que permite que fortes ligações sejam formadas com cuidadores não-biológicos de ambos os sexos. Uma vez que as características principais do cuidador são aprendidas, um apego/vínculo rapidamente se forma e assim, o cuidador assume um papel importante ao influenciar o processamento dos estímulos sensoriais pelo bebê, o que altera o comportamento infantil.

Um aspecto importante, é que as experiências iniciais da criança com a sua mãe ou cuidador fornecem o modelo básico para as relações sócio afetivas na vida adulta (Moriceau et al., 2010; Sullivan et al., 2015). Da mesma forma, muitos trabalhos evidenciam as consequências da instabilidade no ambiente neonatal, entre elas, de ordem física, intelectual e social, podendo inclusive desencadear patologias de ordem físicas e mentais (Oppenheim et al., 2001). Dentro desta perspectiva, a estabilidade do ambiente neonatal, durante o período de formação do apego pela figura materna (cuidador) é crucial para o desenvolvimento saudável da criança e para o estabelecimento seguro do vínculo cuidador-criança ou pais adotivos-filhos adotados. Isso nos deixa um alerta para que sejam intensificadas medidas públicas nas instituições de acolhimento para que o ambiente neonatal seja o mais estável possível, tanto dentro das instituições quanto nos novos lares para onde as crianças adotadas forem encaminhadas.

CAPÍTULO V

5 CONCLUSÕES

1. A adoção cruzada repetida de fêmeas lactantes cuidadoras realizadas durante o período sensível a aprendizagem do apego mãe-filhote (DPN 1 a 3) afeta a formação do vínculo mãe-filhote e reduz o cuidado materno das fêmeas lactantes em relação aos filhotes recém-nascidos.

2. A adoção cruzada repetida de fêmeas lactantes cuidadoras realizadas durante o período de transição do sensível para o pós-sensível a aprendizagem do apego mãe-filhote (DPN 9 a 11) não altera o vínculo mãe-filhote

6 CONSIDERAÇÕES FINAIS

Como sempre, a extrapolação direta de pesquisas de qualquer espécie para seres humanos requer cautela. No entanto, o nosso modelo experimental, de adoção repetida de fêmeas lactantes em ratos (*repetead cross-fostering*), se mostrou um bom modelo para mimetizar o complexo fenômeno que é a adoção humana.

Dessa forma, apesar da adoção maternal ainda ser uma área pouco explorada de forma experimental pela neurociência, acreditamos que os resultados dessa tese contribuíram para elucidar os efeitos do rompimento do vínculo mãe-filhote (apego) no início do desenvolvimento pós-natal sobre os comportamentos filiais.

7 PERSPECTIVAS

Dando continuidade a este estudo, seria importante investigar, dentro deste modelo de RCF, a participação da corticosterona e da ocitocina periféricas, nos efeitos da adoção sobre a formação do apego. Assim como a expressão de receptores centrais de ocitocina em animais que foram expostos ao modelo de adoção RCF. Para a realização destes novos estudos já foram coletados o plasma e o encéfalo dos animais utilizados no presente estudo.

Além disso, uma outra abordagem interessante seria a realização de estudos epigenéticos em animais que foram expostos ao modelo de adoção RCF.

Tais abordagens poderiam fornecer mais informações sobre os fatores envolvidos na formação e a manutenção do apego mãe-infante, a qual, é importante para o entendimento de como os eventos ambientais no início da vida impactam o desenvolvimento de processos sócio emocionais, cognitivos e na saúde mental do infante.

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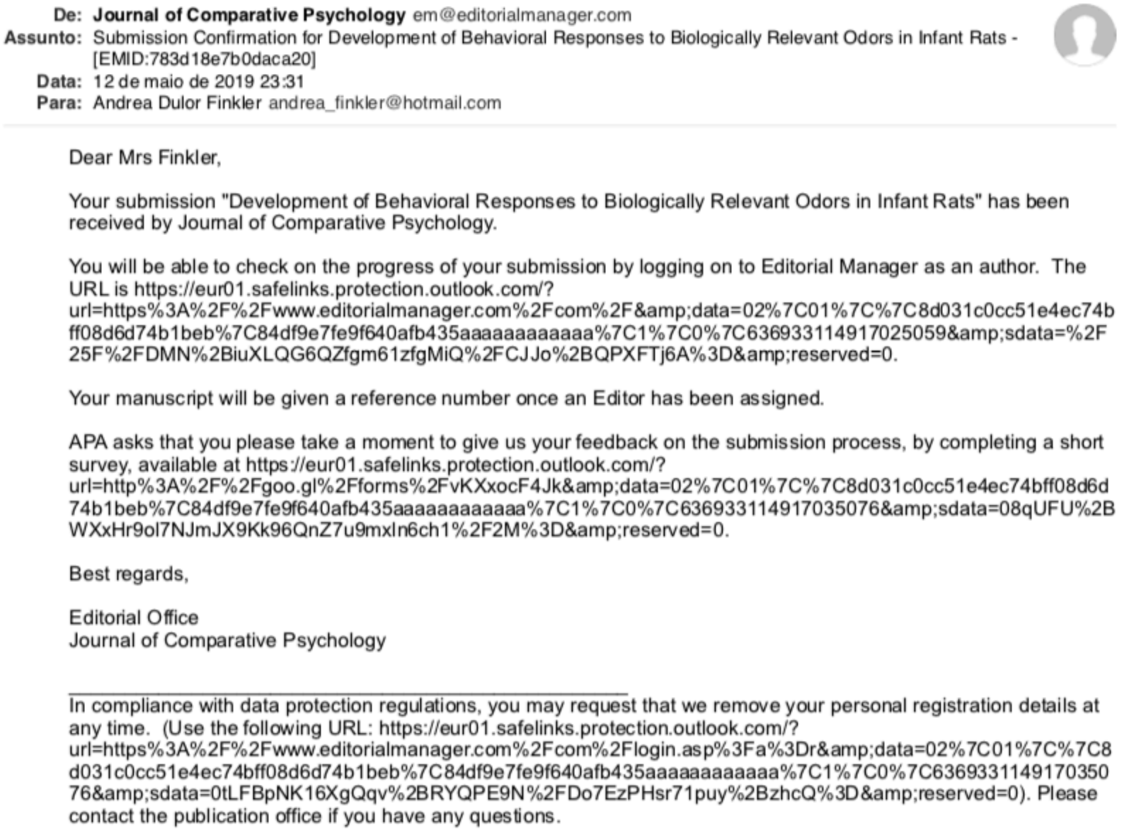
ANEXO A

Comprovante de envio do Artigo I *“Development of Behavioral Responses to Biologically Relevant Odors in Infant Rats”* para a revista *Journal Comparative Psychology.*



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Data: 12 de maio de 2019 23:31
Para: Andrea Dulor Finkler andrea_finkler@hotmail.com

Dear Mrs Finkler,

Your submission "Development of Behavioral Responses to Biologically Relevant Odors in Infant Rats" has been received by Journal of Comparative Psychology.

You will be able to check on the progress of your submission by logging on to Editorial Manager as an author. The URL is https://eur01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.editorialmanager.com%2Fcom%2F&data=02%7C01%7C%7C8d031c0cc51e4ec74bff08d6d74b1beb%7C84df9e7fe9f640afb435aaaaaaaaaaa%7C1%7C0%7C636933114917025059&sdata=%2F25F%2FDMN%2BiuXLQG6QZfgm61zfgMiQ%2FCJJo%2BQPXFTj6A%3D&reserved=0.

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Best regards,

Editorial Office
Journal of Comparative Psychology

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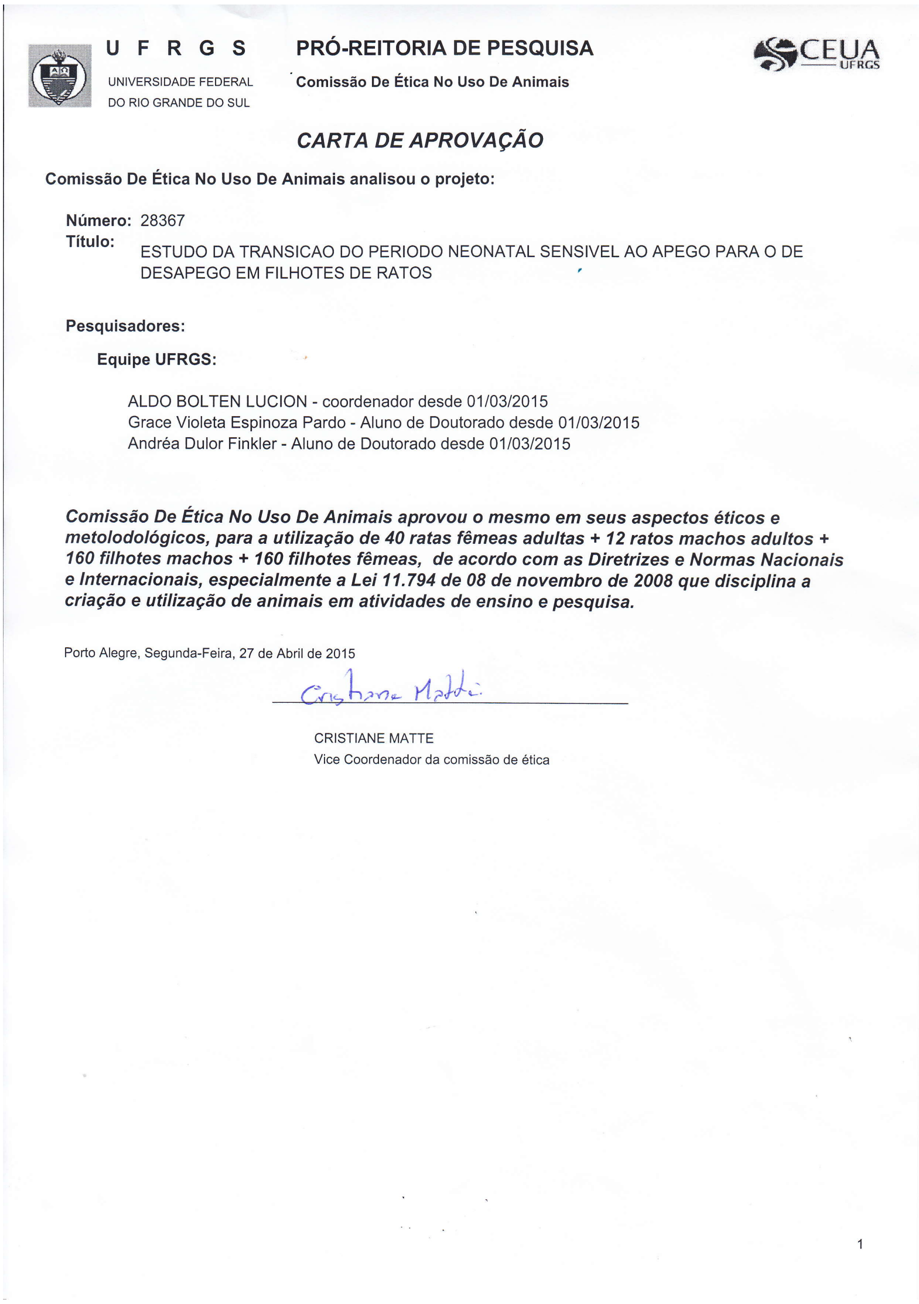
ANEXO B



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PRÓ-REITORIA DE PESQUISA
Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 28367
Título: ESTUDO DA TRANSICAO DO PERIODO NEONATAL SENSIVEL AO APEGO PARA O DE DESAPEGO EM FILHOTES DE RATOS

Pesquisadores:

Equipe UFRGS:

ALDO BOLTEN LUCION - coordenador desde 01/03/2015
Grace Violeta Espinoza Pardo - Aluno de Doutorado desde 01/03/2015
Andréa Dulor Finkler - Aluno de Doutorado desde 01/03/2015

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metolodológicos, para a utilização de 40 ratas fêmeas adultas + 12 ratos machos adultos + 160 filhotes machos + 160 filhotes fêmeas, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

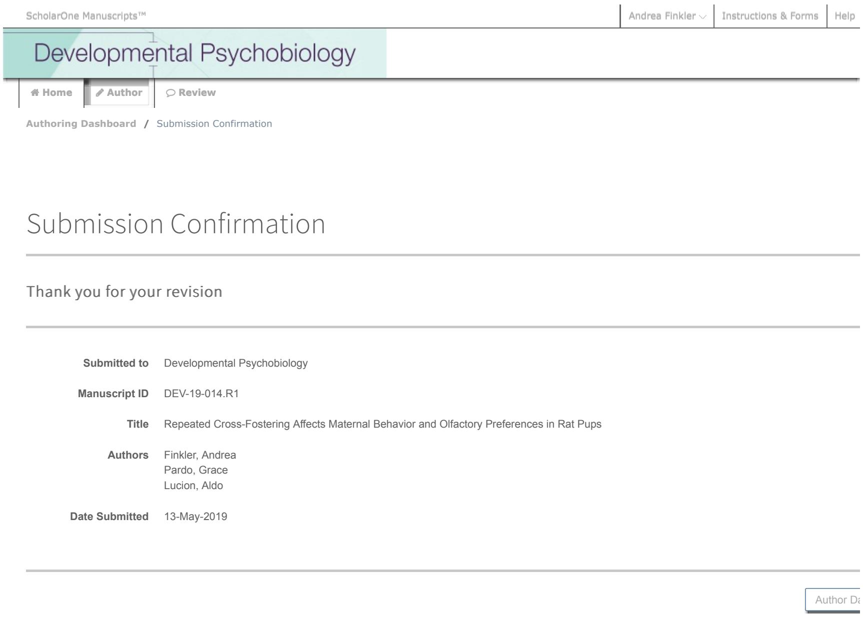
Porto Alegre, Segunda-Feira, 27 de Abril de 2015

Cristiane Matte

CRISTIANE MATTE
Vice Coordenador da comissão de ética

ANEXO C

Comprovante de envio do Artigo II *“Repeated Cross-Fostering Affects Maternal Behavior and Olfactory Preferences in Rats Pups”* para a revista *Developmental Psychobiology*.

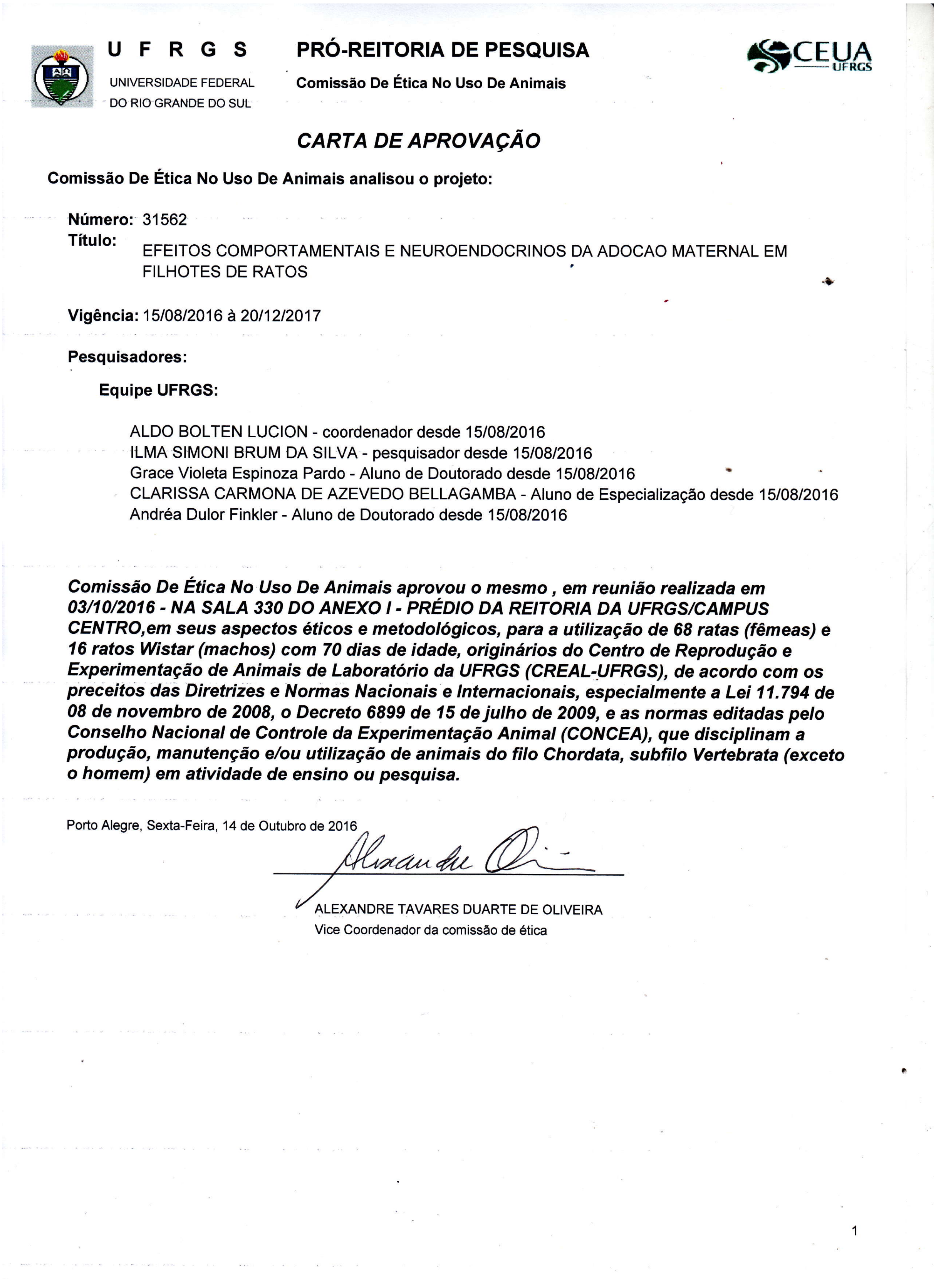


Submission Confirmation

Thank you for your revision

Submitted to	Developmental Psychobiology
Manuscript ID	DEV-19-014.R1
Title	Repeated Cross-Fostering Affects Maternal Behavior and Olfactory Preferences in Rat Pups
Authors	Finkler, Andrea Pardo, Grace Lucion, Aldo
Date Submitted	13-May-2019

ANEXO D



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CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 31562
Título: EFEITOS COMPORTAMENTAIS E NEUROENDOCRINOS DA ADOCAO MATERNAL EM FILHOTES DE RATOS

Vigência: 15/08/2016 à 20/12/2017

Pesquisadores:

Equipe UFRGS:

ALDO BOLTEN LUCION - coordenador desde 15/08/2016
ILMA SIMONI BRUM DA SILVA - pesquisador desde 15/08/2016
Grace Violeta Espinoza Pardo - Aluno de Doutorado desde 15/08/2016
CLARISSA CARMONA DE AZEVEDO BELLAGAMBA - Aluno de Especialização desde 15/08/2016
Andréa Dulor Finkler - Aluno de Doutorado desde 15/08/2016

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 03/10/2016 - NA SALA 330 DO ANEXO I - PRÉDIO DA REITORIA DA UFRGS/CAMPUS CENTRO,em seus aspectos éticos e metodológicos, para a utilização de 68 ratas (fêmeas) e 16 ratos Wistar (machos) com 70 dias de idade, originários do Centro de Reprodução e Experimentação de Animais de Laboratório da UFRGS (CREAL-UFRGS), de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 14 de Outubro de 2016

Alexandre Oli

ALEXANDRE TAVARES DUARTE DE OLIVEIRA
Vice Coordenador da comissão de ética

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