LISIANE DOS SANTOS OLIVEIRA

DESMAME PRECOCE: EFEITOS SOBRE A EVOLUÇÃO DO COMPORTAMENTO ALIMENTAR EM RATOS

Recife, 2010

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Tese apresentada ao Programa de Pós-graduação em Nutrição do Centro de Ciências da Saúde da Universidade Federal de Pernambuco para obtenção do título de Doutor em Nutrição

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Recife, 2010

Oliveira, Lisiane dos Santos

Desmame precoce: efeitos sobre a evolução do comportamento alimentar em ratos / Lisiane dos Santos Oliveira. – Recife : O Autor, 2010.
130 folhas: il., fig., tab.

Tese (doutorado) – Universidade Federal de Pernambuco. CCS. Nutrição, 2010.

Inclui bibliografia e anexos.

1. Desmame precoce. 2. Comportamento alimentar. I. Título.

613.953	CDU (2.ed.)	UFPE
649.33	CDD (20.ed.)	CCS2010-128

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Tese aprovada em 07 de maio de 2010

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Recife, 2010

A Deus,

Por todas as bênçãos e presença constante na minha vida,

Pelo auxílio em minhas escolhas,

Por me confortar nas horas difíceis,

E, sobretudo pelos anjos colocados no meu caminho,

Os quais eu chamo de Amigos!

Ao meu saudoso **Vovô Otávio** (*In memoriam*)

Você foi quem primeiro previu tudo isso que está acontecendo hoje.

Quando nasci, suas palavras foram: Ela vai ser uma doutora!

Estou chegando lá!

Ao meu Pai **José Francisco** (*in memoriam*)

Com toda saudade e carinho

Obrigada por todo apoio e carinho que em vida o senhor me proporcionou

Seu belo exemplo de vida simples de um homem trabalhador me incentivou a

sempre lutar pelos meus objetivos e a valorizar minhas conquistas.

Como eu queria que o senhor estivesse comigo neste momento!

Mas tenho certeza de que onde estiver, está feliz.

A minha Mãe **Severina Maria**, que tanto sonhou com este momento.

Obrigada por está sempre a meu lado me apoiando e me incentivando em todos os momentos.

Obrigada por tudo que a senhora fez e deixou de fazer para me dar uma boa educação.

Você é minha fonte de inspiração! Te amo!

Aos meus avós **Severino e Maria Francisca**,

Aos meus tios **Antônio e Jane**

E ao meu primo **Gleidson**,

Por todo apoio e carinho em todos os momentos da minha vida.

Vocês são meu porto seguro!

Agradecimentos

Agradeço ao meu orientador e amigo Prof. Raul Manhães de Castro, pela amizade, carinho, apoio e incentivo. Obrigada por sempre ter confiado e acreditado em mim, quando muitas vezes nem eu acreditava que fosse conseguir. Obrigada pelas conversas sérias que me renderam lições pra toda a vida. Tenho o maior orgulho de ter sido sua orientanda e fazer parte do seu grupo. Muito obrigada por tudo!

À minha co-orientadora Sandra Lopes de Souza, obrigada por toda ajuda ao longo da pesquisa e de minha vida profissional. Você é uma pessoa na qual tento me espelhar para ser uma pesquisadora melhor, uma professora de anatomia melhor e, sobretudo um ser humano melhor. Obrigada por sua amizade e por fazer parte marcante da minha trajetória.

Às companheiras Carol Peixoto, Matilde, Lígia, Manuela, Renata, Madge e Aline. A ajuda de vocês foi essencial para o êxito do meu trabalho. Obrigada por tudo!

Às estagiárias Deise, Lígia e Lívia, pela dedicação ao trabalho e pela amizade durante este período, jamais me esquecerei de vocês. Obrigada também a Larissa, Kássia, Tássia, Juliete, Priscila, Pétala e Eduarda. No início ou no fim, por maior ou menor tempo que convivemos, cada uma de vocês teve uma importante contribuição neste trabalho, tornando os dias mais alegres, compartilhando as conquistas. Muito obrigada!

Aos colegas de Doutorado Patrícia, Marco e Soninha, obrigada pelo apoio e companheirismo durante esse período.

Ao professor Eulálio, por sua contribuição. Obrigada por sua disponibilidade e atenção comigo em todos os momentos que precisei de sua ajuda.

A coordenação e aos professores da Pós-Graduação em Nutrição, pela excelente conduta na nossa formação

À querida Neci e a equipe da secretaria da Pós-Graduação pela dedicação, profissionalismo e amizade demonstrados durante todo esse período.

Aos amigos Bruno e Amanda, meus ex-estagiários e hoje pós-graduandos, obrigada pela amizade e pela torcida. Vocês são muito especiais na minha vida.

Aos amigos do NNI em especial aos que convivi mais próximo: Lúcia, Wylla, Rogério, Beth, Karla, Michele, Carol Leandro, Ana Elisa, Marcelos,

Ribas, Tereza, Keli, Raquel, Antônio, Adriano, Felipe, Wellington, Rowena. Obrigada pela colaboração, pelas palavras e gestos de carinho e pelo companheirismo.

Aos amigos do CAV (nem me arrisco a citar nomes), obrigada pela amizade, apoio e incentivo durante esses dois anos de convivência.

Aos meus amigos Padres Josenildo, Pedro, João e Luiz pelas orações e palavras de incentivo.

Enfim, a todas as pessoas amigas que de alguma forma contribuíram para realização desse trabalho, meus sinceros agradecimentos.

“Deus nos fez perfeitos e não escolhe os capacitados, capacita os escolhidos. Fazer ou não fazer algo só depende de nossa vontade e perseverança”

Albert Einstein

"A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável."

Galileu Galilei

“Um pouco de ciência nos afasta de Deus.
Muito, nos aproxima”

Louis Pasteur

Resumo

O período de lactação é considerado crítico para o desenvolvimento de sistemas orgânicos entre eles o sistema nervoso. Mudanças ambientais como a qualidade da nutrição ou o estresse são capazes de promover alterações estruturais no desenvolvimento e provocar alterações comportamentais em longo prazo. O presente trabalho teve como objetivo investigar os efeitos do desmame precoce por separação materna sobre o comportamento alimentar na vida adulta. No primeiro artigo original intitulado "Effects of the early weaning on the circadian rhythm and behavioral satiety sequence in rats" foi demonstrado que o desmame precoce não altera o peso corporal e o consumo alimentar basal, mas promove retardo no aparecimento da saciedade e alteração do ritmo circadiano de consumo alimentar na vida adulta. O segundo artigo original intitulado "Early weaning programs rats to have a dietary preference for fat and palatable foods in adulthood" demonstrou que o desmame precoce conduz a preferência alimentar por dieta lipídica e induz a hiperfagia por dieta palatável com alta densidade calórica. Em conclusão, a manipulação do período de desmame parece interferir com a programação do comportamento alimentar promovendo alterações perceptíveis no organismo adulto.

Palavras-chave: Programação, desmame precoce, comportamento alimentar, seqüência comportamental de saciedade, ritmo alimentar, preferência alimentar.

Abstract

The period of lactation is considered critical for development of organic systems between them the nervous system. Environmental changes as the type of nutrition or stress are capable to promote structural alterations in development and to provoke behavioral alteration in long term. The present work had as objective to investigate the effect of early weaning by maternal separation on feeding behavior in adult life. In the first original article entitled “Effects of the early weaning on the circadian rhythm and behavioral satiety sequence in rats” it was demonstrated that early weaning does not modify the body weight and food intake basal, but promotes delayed retardation in the appearance of the satiety and alteration of the circadian rhythm of food intake in the adult life. The second original article entitled “Early weaning programs rats to have a dietary preference for fat and palatable foods in adulthood” it demonstrated that early weaning leads the alimentary preference for fat diet and induces the hyperphagia for palatable diet with high caloric density. In conclusion, the manipulation of the period of weaning seems to intervene with the programming of the feeding behavior promoting perceivable alterations with the adult organism.

Keywords: Programming, early weaning, feeding behavior, behavioral satiety sequence, alimentary rhythm, alimentary preference.

SUMÁRIO

APRESENTAÇÃO

A presente tese intitulada “Desmame precoce: efeitos sobre a evolução do comportamento alimentar em ratos” teve como objetivo estudar os efeitos do desmame precoce sobre alguns parâmetros do comportamento alimentar em ratos em jovens e adultos.

A fim de abordar aspectos importantes relacionados ao tema a revisão bibliográfica foi organizada em cinco capítulos da seguinte forma: 1. Programação: no qual foi revisada a relação entre as interferências no início da vida e o estado fisiológico futuro; 2. Lactação e Programação: considerando as evidências do período de lactação para a programação do indivíduo; 3. Comportamento Alimentar: abordando alguns aspectos da regulação do comportamento alimentar, 4. Artigo de Revisão "Behavioral Satiety Sequence: na experimental model for studying feeding behavior: consistindo de uma revisão sistemática da literatura sobre a utilização do modelo experimental da sequência comportamental de saciedade para o estudo do comportamento alimentar e 5. Desmame Precoce e suas consequências: apontando as alterações provocadas pelo desmame precoce sobre alguns aspectos comportamentais e fisiológicos.

Delineados os métodos, os resultados foram apresentados na forma de dois artigos originais enviados a revista internacional indexada. O primeiro artigo “Effects of early weaning on the circadian rhythm and behavioral satiety sequence in rats” teve como objetivo investigar os efeitos do desmame precoce sobre a sequência comportamental de saciedade em ratos jovens e adultos e sobre o ritmo circadiano do consumo alimentar em ratos adultos. O segundo artigo “Early weaning programs rats to have a dietary preference for fat and palatable foods in adulthood” teve como objetivo estudar o efeito do desmame precoce sobre a preferência alimentar aos macronutrientes

carboidratos, proteínas e lipídios e verificar o consumo alimentar em resposta a dieta palatável.

O desmame precoce é um problema de saúde pública presente na população atual. Apesar do conhecimento acerca dos benefícios do leite materno para o filho, o aleitamento materno ainda encontra resistência por parte da população. No entanto os efeitos tardios do desmame precoce ainda são pouco explorados pela literatura. É esperado que essa tese, utilizando o rato como modelo experimental contribua de forma positiva, fornecendo subsídios para outros pesquisadores e profissionais de saúde em favor do aleitamento materno.

Objetivos

Geral:

Investigar os efeitos do desmame precoce sobre a evolução do perfil do comportamento alimentar em ratos.

Específicos:

Verificar em ratos jovens (35 dias de vida):

- Eventuais efeitos do desmame precoce sobre o peso corporal, consumo alimentar e sobre a sequência comportamental de saciedade.

Verificar em ratos adultos:

- Eventuais efeitos do desmame precoce sobre o peso corporal, consumo alimentar e sobre a sequência comportamental de saciedade;

- O ritmo do consumo alimentar;

- A preferência alimentar a macronutrientes;

- A resposta à dieta palatável.

Hipóteses

Ratos submetidos a desmame precoce:

- Quando jovens:

  - Não apresentam aumento no peso corporal e no consumo alimentar

  - Não apresentam atraso na saciedade.

- Quando adultos apresentam:

  - Aumento no peso corporal, porém sem alteração no consumo alimentar diário;

  - Atraso no disparo da saciedade;

  - Manutenção do perfil alimentar predominantemente noturno com alteração no ritmo circadiano do consumo alimentar em alguns períodos;

  - Aumento na preferência alimentar a lipídios;

  - Hiperfagia em resposta à dieta palatável.

REVISÃO DA LITERATURA

2.1. PROGRAMAÇÃO

No início da vida dos mamíferos há fases onde ocorrem intensas modificações funcionais e estruturais que são chamadas de períodos críticos (Matsui, Morimoto *et al.*, 2009). No primeiro período crítico ocorre a multiplicação e organização iniciais de neuroblastos que acontece na fase pré-natal (Morgane, Austin-Lafrance *et al.*, 1993). No segundo ocorre crescimento rápido do encéfalo, com migração e diferenciação neuronal, sinaptogênese, multiplicação glial e mielinização evidentes que no rato acontece durante a lactação (Dobbing, 1964; Winick e Noble, 1966).

Alguns estudos vêm demonstrando que fatores epigenéticos ao incidirem em períodos críticos da vida podem repercutir sobre o desenvolvimento de órgãos e tecidos provocando alterações estruturais e fisiológicas permanentes (Ravelli, Stein *et al.*, 1976; Hales e Barker, 2001; Orozco-Solis, Lopes De Souza *et al.*, 2009). Para uma possível explicação da associação entre agressões no período crítico do desenvolvimento e repercussões tardias, foi sugerido o modelo da influência fenotípica (Hales e Barker, 1992; , 2001), assumindo que o organismo se adapta favoravelmente a um ambiente hostil prévio. Estes autores, inicialmente relataram aspectos relacionados à desnutrição fetal e a incidência da diabetes tipo II na fase adulta (Hales e Barker, 1992). Neste estudo, foi observado que o organismo se adapta à desnutrição perinatal programando o metabolismo da insulina (Hales e Barker, 1992). Esta adaptação tende a aumentar a aptidão do organismo para um provável ambiente agressivo subseqüente (Barker, 1998). A constatação dos períodos críticos do desenvolvimento é englobada pela hipótese da influência fenotípica e permite a explicação das eventuais conseqüências tardias (Ozanne e Hales, 1999). Esse fenômeno biológico que estabelece a relação entre estes estímulos no período crítico de desenvolvimento, particularmente durante a gestação e

lactação, e o estado funcional futuro foi denominado “programming”, ou seja programação (Hales e Barker, 1992; Lucas, 1994; Hales e Barker, 2001; Barker, 2004; De Moura e Passos, 2005).

2.2. LACTAÇÃO E PROGRAMAÇÃO

A lactação é um período crítico porque é uma fase em que ocorre importante desenvolvimento neurocomportamental e a interação mãe-filho tem grande participação nesse processo (Plotsky e Meaney, 1993; Caldji, Francis *et al.*, 2000; Liu, Diorio *et al.*, 2000). Ao nascer, os ratos filhotes dependem da mãe para comer, manter a temperatura corporal, e evacuarem até por volta do final da segunda semana de vida (Plaut e Davis, 1972). Dessa forma, o aleitamento materno é o único responsável pela nutrição no período inicial após o nascimento.

A aquisição de leite materno pelo lactente mamífero apresenta um fenótipo comportamental característico, respeitando uma seqüência temporal de condutas iniciando pelo estabelecimento do contato materno seguido da fixação ao mamilo, sucção e ingestão de leite e cessação da sucção (Hall e Rosenblatt, 1978; Blass e Teicher, 1980). Cada uma das etapas da seqüência do aleitamento materno é regulada por diversos fatores de acordo com o estágio de desenvolvimento do filhote (Blass e Teicher, 1980). Nas duas primeiras semanas de vida os filhotes ainda não abrem os olhos (De Souza, Nogueira *et al.*, 2004; Deiro, Manhaes-De-Castro *et al.*, 2004) e o estabelecimento do contato materno e a fixação do mamilo parece depender do olfato (Singh e Tobach, 1975; Teicher e Blass, 1976). Nessa fase o controle da ingestão de leite depende da mãe (Friedman, 1975). Após a segunda semana de vida, o padrão do comportamento alimentar neonatal inicia um processo de transição até o desmame onde

o filhote já apresenta um padrão alimentar adulto (Hall e Rosenblatt, 1978; Blass e Teicher, 1980). A partir do 14º dia de vida, os ratos iniciam o consumo de alimentos sólidos, mas o aleitamento materno continua sendo a preferência alimentar dos filhotes até a terceira semana de vida (Hahn e Koldovsky, 1966). Ao final da terceira semana de vida inicia o desmame espontâneo e vai até por volta do 30 dias de vida quando cessa o consumo de leite (Krecek e Kreckova, 1957; Hahn e Koldovsky, 1966; Henning, 1981).

Ratos adultos apresentam um padrão noturno de alimentação. Em laboratórios com condições normais de luz alternando claridade e escuridão a cada 12 horas apresentam até 80% do seu consumo na fase escura (Mayer, Marshall *et al.*, 1954). Até por volta da segunda semana de vida os filhotes não exibem nenhum ritmo de alimentação expressado pelo consumo de leite ou pelo consumo de alimentos sólidos (Babicky, Ostadalova *et al.*, 1970). Esse padrão inicia por volta dos 19 dias de vida influenciado pela presença materna (Levin e Stern, 1975), a qual é capaz de induzir a escolha da dieta pelos filhotes (Galef e Clark, 1972). Uma influência é o leite materno, pois, os filhotes tendem a ingerir preferencialmente substâncias consumidas pela mãe, presentes no leite materno (Galef e Henderson, 1972). Outra influência é a interação da mãe com os filhotes na presença do alimento, induzindo-os a iniciarem o consumo de alimentos sólidos (Galef e Clark, 1972).

Durante a lactação, o fator ambiental parece ser importante para a programação do metabolismo, dentre esses fatores podemos destacar o tipo de nutrição, a incidência de estresse e o estado hormonal. Dentre as agressões nutricionais que podem ocasionar programação destacamos a desnutrição. Dois modelos experimentais de desnutrição vem sendo explorados pela literatura: a desnutrição calórica e a desnutrição protéica. Alguns trabalhos associaram ambos os modelos de desnutrição à alteração do peso corporal sem, no entanto, alterar o consumo alimentar basal na vida adulta (Vicente, De

Moura *et al.*, 2004; De Moura, Lisboa *et al.*, 2007; Fagundes, Moura *et al.*, 2007; Lisboa, Fagundes *et al.*, 2008). Além do peso corporal a desnutrição está associada a outras alterações persistentes na vida adulta. A desnutrição calórica durante a lactação provoca aumento do peso corporal, aumento da expressão de hormônio do crescimento (GH) mRNA (De Moura, Lisboa *et al.*, 2007), aumento nos níveis séricos de T3 e redução de hormônio estimulador da tireóide (TSH) (Vicente, De Moura *et al.*, 2004), aumento na expressão dos receptores para leptina na pituitária (Vicente, De Moura *et al.*, 2004) na vida adulta. Já animais que foram submetidos à desnutrição protéica na lactação permanecem com redução de peso até a vida adulta (Passos, Ramos *et al.*, 2000; Vicente, De Moura *et al.*, 2004; De Moura, Lisboa *et al.*, 2007), apresentam redução na expressão de GH mRNA (De Moura, Lisboa *et al.*, 2007), aumento de T3 e T4 e redução de TSH (Vicente, De Moura *et al.*, 2004). Ainda apresentam na vida adulta os níveis aumentados de corticosterona e catecolaminas (Fagundes, Moura *et al.*, 2007), em decorrência do aumento da atividade do eixo hipotalamo-pituitario-adrenal (HPA) (Lesage, Dufourny *et al.*, 2002).

A desnutrição no início da vida também altera sensibilidade a agentes anorexígenos. Estudos demonstraram que animais que desnutridos durante a gestação e lactação ou apenas durante a lactação não se tornaram hipofágicos na presença de inibidores de recaptação de serotonina (Barreto Medeiros, Cabral Filho *et al.*, 2002; Lopes De Souza, Orozco-Solis *et al.*, 2008) provavelmente em decorrência da dessensibilização de receptores 5-HT-1B (Lopes De Souza, Orozco-Solis *et al.*, 2008). Além do comportamento alimentar, a desnutrição na lactação também tem alterado a resposta de agentes serotoninérgicos sobre o comportamento agressivo, não reduzindo a agressividade na presença de fluoxetina (Barreto-Medeiros, Feitoza *et al.*, 2004).

Estresse no início da vida também vem sendo associada à programação. Estudos sugerem que o estresse no início da vida promove a longo prazo mudanças em sistemas de neurotransmissores e em estruturas cerebrais (Fleming, O'day *et al.*, 1999; Francis e Meaney, 1999). Normalmente as respostas ao estresse estão associadas com à ativação do eixo HPA com aumento na liberação do hormônio liberador de corticotrofina (CRH), aumento na liberação das catecolaminas e aumento nos níveis de glicocorticóides como o hormônio adrenocorticotrófico (ACTH) e a corticosterona (Mcewen, 2002; Tsigos e Chrousos, 2002). Um dos modelos de estresse neonatal é a separação materna, nesse modelo os filhotes são diariamente separados da mãe por um breve período. Esse modelo produz em longo prazo aumento na resposta do eixo HPA, aumento nos níveis de mRNA CRF no núcleo hipotalâmico paraventricular (PVN) e aumento nos níveis basais de corticosterona e ACTH em ratos adultos (Plotsky e Meaney, 1993; Ladd, Huot *et al.*, 2000; Huot, Thrivikraman *et al.*, 2001; Aisa, Tordera *et al.*, 2008; Marais, Van Rensburg *et al.*, 2008). Além dessas a separação materna provoca alterações comportamentais como a maior capacidade de resposta ao estresse e aumento no comportamento de ansiedade na vida adulta (Plotsky e Meaney, 1993; Ladd, Owens *et al.*, 1996; Wigger e Neumann, 1999; Huot, Thrivikraman *et al.*, 2001). Outros estudos demonstraram que o estresse neonatal também aumenta a preferência por alimentos doces e palatáveis na vida adulta (Silveira, Portella *et al.*, 2004; Silveira, Portella *et al.*, 2005). Esse efeito sobre o comportamento alimentar parece ocorrer como uma resposta adaptativa ao estresse, atenuando a reatividade do eixo HPA (Pecoraro, Reyes *et al.*, 2004).

É possível que os efeitos da desnutrição e do estresse sobre a programação se devam a mudanças nos níveis hormonais encontrados nestas situações (Fagundes, Moura *et al.*, 2007). Foi demonstrado que o tratamento materno com corticosterona

durante a lactação está associado a altos níveis de receptores para corticosterona no hipocampo, porém, redução da secreção de corticosterona em resposta ao estresse e apresentam melhor aprendizado e melhores estratégias para lidar com situações estressantes (Catalani, Casolini *et al.*, 2000). Também foi observado que o hipertireoidismo neonatal está associado com hipertireoidismo na vida adulta demonstrando que alterações nos hormônios da tireóide, durante a lactação, afetam permanentemente o eixo HPA (Varma e Crawford, 1979; Dussault, Coulombe *et al.*, 1982; Walker e Courtin, 1985). Outro hormônio envolvido com a programação é a leptina. Durante a lactação esse hormônio programa sobrepeso, aumento do consumo alimentar, aumento nos níveis séricos de leptina, resistência a leptina, hiperinsulinemia, hipertrigliceridemia, aumento nos níveis de catecolaminas, hipertensão sistólica e aumento dos batimentos cardíacos (Lins, De Moura *et al.*, 2005; Toste, De Moura *et al.*, 2006; Trevenzoli, Valle *et al.*, 2007).

Essas evidências corroboram a importância do período de lactação para o desenvolvimento e programação do indivíduo. A adaptação dos sistemas fisiológicos ao ambiente inicial programa o metabolismo para se ajustar àquelas condições inicialmente encontradas. Quando o ambiente durante a lactação é adverso a adaptação metabólica é necessária para a sobrevivência naquelas condições, entretanto mudanças constantes as quais o organismo se expõe ao longo da vida pode ser a origem de distúrbios metabólicos encontrados na vida adulta.

2.3. COMPORTAMENTO ALIMENTAR

O comportamento alimentar é regulado através da interação complexa entre mecanismos periféricos e centrais que controlam a fome e a saciedade (Nagase,

Nakajima *et al.*, 2002). Diversas moléculas sinalizadoras participam desse processo, tais como os neurotransmissores, neuropeptídeos e hormônios (Valassi, Scacchi *et al.*, 2008). Entre os neurotransmissores clássicos destacam-se as catecolaminas (Wellman, Miller *et al.*, 2003; Maidel, Lucinda *et al.*, 2007), e a serotonina (Lopez-Alonso, Mancilla-Diaz *et al.*, 2007; Tanaka e Kido, 2008). Entre os neuropeptídeos destacam-se o neuropeptídeo Y (NPY) (Ramos, Meguid *et al.*, 2005; Mahaut, Dumont *et al.*, 2010), peptídio relacionado ao agouti (AGRP) (Katsuki, Sumida *et al.*, 2001; Ilnytska e Argyropoulos, 2008), Pro-opiomelanocortina (POMC) (Millington, 2007), transcrito regulado pela cocaína e anfetamina (CART) (Aja, Sahandy *et al.*, 2001), Orexina (Rodgers, Halford *et al.*, 2000; Rodgers, Halford *et al.*, 2001) e colecistoquinina (Geary, 2004). Entre os hormônios destacam-se a insulina (Vettor, Fabris *et al.*, 2002) e a leptina (Friedman, 1998).

Sinais gerados em resposta ao comportamento alimentar são integrados no córtex cerebral através de mecanismos envolvendo principalmente o hipotálamo e o tronco encefálico (Blevins, Schwartz *et al.*, 2002; Funahashi, Takenoya *et al.*, 2003; Blevins e Baskin, 2010). O hipotálamo possui uma rede de elaboração e emissão de sinais anorexigênicos e orexigênicos que ajustam os sinais relacionados ao apetite e a saciedade (Blundell, 1991).

Várias manipulações ambientais durante a lactação têm resultado em alterações no padrão alimentar adulto. Observou-se que a incidência de estresse durante a lactação aumenta o apetite por alimentos palatáveis (Silveira, Portella *et al.*, 2004), provavelmente em decorrência do aumento da ansiedade que aumenta o consumo de alimentos doce (Ely, Dapper *et al.*, 1997). Já o tratamento com leptina durante a lactação provoca maior consumo alimentar na vida adulta (Lins, De Moura *et al.*, 2005).

A desnutrição materna durante a lactação também é capaz de alterar o consumo alimentar e o balanço energético. O modelo experimental de desnutrição energética promoveu aumento do consumo alimentar no período pós-desmame como um mecanismo compensatório após a restrição durante a lactação e após 53º dias de vida o consumo foi estabilizado. Entretanto provocou sobrepeso comparado aos animais bem nutridos durante a lactação (De Moura, Lisboa *et al.*, 2007). Enquanto que a desnutrição protéica também promove um aumento inicial do consumo alimentar após a lactação e depois o consumo é estabilizado não havendo mais diferença em relação a animais bem nutridos, porém os animais permanecem com deficiência de peso até a vida adulta (Fagundes, Moura *et al.*, 2007; Lisboa, Fagundes *et al.*, 2008; Fagundes, Moura *et al.*, 2009). No entanto a análise da seqüência comportamental de saciedade após um período de 4 horas de jejum em animais submetidos à desnutrição protéica perinatal mostrou um discreto retardo na saciedade com aumento do consumo alimentar (Orozco-Solis, Lopes De Souza *et al.*, 2009). É possível que algumas alterações estruturais e hormonais provocadas pela desnutrição estejam latentes e se manifestem na presença de algum fator instigante.

Semelhante ao desmame precoce alguns estudos utilizaram o modelo da inibição materna da prolactina próximo ao final da lactação, a qual promove a inibição da produção de leite (Bonomo, Lisboa *et al.*, 2005). Esse modelo promoveu alterações bem semelhantes à desnutrição, não alterou o consumo alimentar basal e provocou sobrepeso na vida adulta, provavelmente em decorrência de hipotireoidismo (Bonomo, Lisboa *et al.*, 2007; Bonomo, Lisboa *et al.*, 2008). Apesar de não alterar o consumo alimentar basal esse tratamento provoca resistência a leptina que possui efeitos anoréxicos sobre o comportamento alimentar (Bonomo, Lisboa *et al.*, 2007).

Como foi observado, apenas o estudo do consumo alimentar é insuficiente para observar efeitos tardios de manipulações ambientais. Dessa forma o uso de outros métodos são necessários para uma melhor avaliação de parâmetros relacionados à fome e a saciedade. Desde a década de 70 o modelo experimental conhecido como seqüência comportamental de saciedade vem sendo utilizado como uma ferramenta eficaz para a avaliação do comportamento alimentar. Para melhor compreensão deste método foi realizada uma revisão sistemática da literatura intitulada "**Behavioral Satiety Sequence: a experimental model for studying feeding behavior",** a qual foi enviada para a revista de nutrição e encontra-se a seguir.

Behavioral Satiety Sequence: an experimental model for studying feeding behavior

Esse artigo de revisão é intitulado: **“Behavioral Satiety Sequence: an experimental model for studying feeding behavior”**. Foi submetido como artigo original à revista: **Revista de nutrição**. É classificada pela CAPES como qualis B4 e indexada pelo scielo. (ANEXO A)

Title: Behavioral Satiety Sequence: a experimental model for studying feeding behavior (Seqüência Comportamental de saciedade: um modelo experimental para o estudo do comportamento alimentar)

Short title: Behavioral Satiety Sequence (Seqüência Comportamental de Saciedade)

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ABSTRACT

Feeding behavior is controlled by interactions between psychobiological and physiological systems. In rats, there is a sequence in the feeding behavior that is characterized by similar movements in the beginning and in the finish of a meal, known as the behavioral satiety sequence. In the sequence, eating is followed by grooming and other activities, finishing with resting. The objective of this systematic review is to evaluate the use of the behavioral satiety sequence as an experimental model for the study of feeding behavior. A systematic search of the electronic databases Medline, Lilacs, SciELO, Biblioteca Cochrane and Pubmed was carried out, using combinations of the key words “behavioral”, “satiety” and “sequence”. Ninety articles were found and, of these, fifteen articles were selected for review. The studies showed the efficacy of using the behavioral satiety sequence to evaluate of the effects of some types of manipulations on feeding behavior. With this method of study, it is also possible to observe diverse factors that can intervene with the feeding behavior, such as sedation, malaise or inhibition of intake, by increasing the satiety. The behavioral satiety sequence offers solid tools to gain a better understanding of how treatment can influence feeding behavior.

Keywords: Behavioral satiety sequence, feeding behavior, eating, grooming, resting.

RESUMO

O Comportamento alimentar é controlado por interações entre sistemas psicobiológicos e fisiológicos. Em ratos, existe uma sequência no comportamento alimentar que é caracterizada por movimentos similares no início e ao término de uma refeição, conhecida como sequência comportamental de saciedade. Na seqüência o ato de comer é seguido pela limpeza e outra atividades, terminando com o descanso. O objetivo dessa revisão sistemática é avaliar o uso da seqüência comportamental de saciedade como um modelo experimental para o estudo do comportamento alimentar. Uma busca sistemática das bases de dados Medline, Lilacs, Scielo, Biblioteca Cochrane e Pubmed foi realizada, usando cominações das palavras chaves "behavioral", "satiety" e "sequence". Noventa artigos foram encontrados e, desses, quinze artigos foram selecionados para a revisão. Os estudos mostraram a eficácia do uso da seqüência comportamental de saciedade para a avaliação dos efeitos de alguns tipos de manipulações sobre o comportamento alimentar. Com esse método de estudo, também é possível observar diversos fatores que podem intervir no comportamento alimentar, assim como sedação, mal-estar ou inibição do consumo por aumento da saciedade. A sequência comportamental de saciedade oferece sólidas ferramentas para obter um entendimento melhor de como um tratamento pode influenciar o comportamento alimentar.

Palavras-chaves: Sequência comportamental de saciedade, comportamento alimentar, comer, limpeza, descanso.

1. Introduction

The control of appetite is based on a psychobiological system [1]. An interaction exists between psychological events (hunger perception, cravings, hedonic sensations), behavioral operations (intake of meals, snacks, energy and macronutrients), peripheral physiologic and metabolic events, and the levels of neurotransmitter and metabolic interactions in the brain [2]. Appetite reflects the synchronous operation of events and processes at all these levels [1].

The feeding behavior of animals is an adaptive response, arising from demands of the internal environmental and is modulated by limitations imposed by the external environment [3]. Neural events trigger and guide behavior, but each behavioral act involves a response in the peripheral physiological system [1]. These physiological responses are termed satiety signals, and can be represented by the satiety cascade [1.] Satiety are the events subsequent to food intake that suppress hunger and maintain an inhibition toward eating for a particular period of time [1]. Hunger can be regarded as the necessity to eat or a period in which satiety signals are absent [1]. Between hunger and satiety is satiation, a group of processes that determine meal termination [1]. The coordinated effects of satiation and satiety control the size and frequency of eating episodes, thereby causing the eating pattern [1].

Studies have demonstrated that some behaviors in animals follow specific patterns [4, 5]. Thus, after eating, an adult rat presents a period of grooming and locomotor activity [5]. After this period, the animal rests or sleeps [4]. Following these observations, a behavioral sequence was identified that is associated with satiety, because the cessation of eating is not a sufficient condition for the complete appearance of the behavioral sequence [6]. Thus the Behavioral Satiety Sequence (BSS) was denominated [6].

In 1975, the BSS was utilized for the first time as an experimental model for the study of the satiety [7]. This work confirmed the association of postingestive behavior with satiety, and it is still considered a landmark for the consolidation of the BSS as a technique for the study of feeding behavior.

In rodents, the BSS is characterized as an initial phase of eating, followed by grooming and locomotor activities and finishes with an eventual phase of resting behavior [7, 8]. The eating itself is characterized by biting, gnawing, or swallowing food directly from a dish or from the front paws [7, 8]. This action is one of the elements of feeding behavior that is related to the biological necessity of getting nutrients.

Grooming is characterized by the licking of the body, feet and genitals, by scratching the coat or head with the hind leg, by stroking whiskers with the paws and the biting of the tail [7, 8]. These actions normally occur after eating [7]. Locomotion involves movements with the participation of the four limbs and rearing (front paws raised from the cage floor, either supported against a wall or free standing) [7, 8]. The locomotor activity is related to exploratory behavior [9]. It consist of acts and postures that permit the animal to acquire information and to become familiar with its environment [9]. Alterations of activity related to the BSS can intervene in feeding behavior [8]. The increase in the duration and/or frequency of the non-feeding activities associated with the BSS can delay the start of the rest period and fragment the eating into numerous, short episodes [3, 7].

Resting is characterized by the inactivity of the animal, who sits or lies in a relaxed position with its head curled close to body or resting on the floor [7, 8]. Resting is the final posture assumed in the BSS [7, 8]. The appearance of the resting posture in the BSS is a condition caused by satiety [7]. This fact was demonstrated by changes of the palatability of the reduced food intake and because resting does not take place [10]. It has

also been verified that the start of resting can be anticipated by the prefeeding period [11]. Drugs-induced changes can alter the BSS and resting occur earlier than eating [8].

The objective of this systematic review of the BSS is to evaluate the usage of this experimental model in the study of the feeding behavior. Consideration was also paid to the methods that have been utilized in the study of the BSS.

2. Method

A systematic search of the literature was carried out from November 2007 to January 2008 in the electronic databases Medline (National Library of Medicine), Lilacs (Literatura Latino-americana e do Caribe em Ciências da Saúde), SciELO (Scientific Electronic Library Online), Biblioteca Cochrane and PubMed. This search prioritized studies that utilized the BSS in the period from 1975 until 2008. The bibliographical search used combinations of the key words "behavioral", "satiety" and "sequence". With the objective of clearly defining the adequacy of the literature found for this review, the following criteria of inclusion had been established: a) studies that used the BSS method; b) research with rats or mice; c) articles that consider the time when BSS was evaluated, such as duration of eating, grooming and/or resting behavior. Articles that did not present the duration of the eating, grooming and/or resting behaviors or that had problems in the statistical analyses, such as missing significance values or the absence of the confidence interval, were excluded.

3. Results

The initial search of the databases found fifteen articles in Medline, two articles at the Biblioteca Cochrane and ninety articles in PubMed. The two articles of the Biblioteca Cochrane were discarded because they did not concern the BSS. The fifteen articles found in Medline were also identified by PubMed. Of the ninety articles that were found via PubMed, thirty were eliminated after analysis of the abstracts because they did not concern the BSS. After reading the abstracts, sixty articles were selected and after complete analysis of each article, only fifteen articles passed the inclusion criteria mentioned in the Materials and Methods section. The results tabulated for this study were the murine species, the phase of the light/dark cycle, the duration of the evaluation of the BSS, the type of diet or treatment and the method of its administration, the amount of food consumed and the duration of the eating, and grooming and resting behaviors. The duration of the eating, grooming and resting behaviors were presented as mean or percentage ± standard deviation. The articles found were categorized in agreement with the type of manipulation: nutritional, pathological or pharmacological and are presented in summary in Tables 1, 2 and 3, respectively.

All the articles found had used the analysis of variance as the statistical method for identifying differences between the studied groups.

4. Discussion

4.1.Methodological considerations.

For the analysis of the BSS, all the examined studies relied on a standard procedure in which a food-deprived animal is placed in an observation arena with ad libitum access to food and water for a period of 45-60 min. Food deprivation is used to obtain a high feeding baseline while the objective of the observation arena is to provide a larger enclosure than the animal's house-cage to allow the expression of all the behavioral parameters of the BSS. The experimental protocol used differed, however, between the different laboratories in some respects. These included the phase of the light/dark cycle in which the test was performed, the duration of the fasting period and how the behavioral data were analyzed and presented.

Most living organisms exhibit behavioral and physiological rhythms including those associated with sleep, feeding and energy homeostasis. Therefore, the expression of the BSS can vary depending on the phase of the light/dark cycle in which the analysis is performed. Initially, the BSS was monitored essentially during the light phase [7], but more recently the analysis of the BSS during the dark phase has been preferred [12, 13]. In the first studies in which the BSS was analyzed during the light phase, the animals were submitted to a fasting period of 17-20 hours [3, 7]. The objective of this manipulation, was to reduce the latency period to start eating and to stimulate the appearance of the characteristic behaviors of the BSS. However, the rat is a nocturnal animal and, consequently, most of its active behaviors, including food consumption, occur during the dark cycle. During this phase, rats consume up to 80% of their daily food intake [14].

Thanks to the development of monitoring technology, it became possible to document the BSS during the dark phase using specialized video recorders and red light. The use of this methodology allows not only the analysis the BSS under more natural conditions, but has also the advantage to reduce the period of fasting. Actually, the studies carried out in the dark phase usually use a four-hour period of food deprivation [8].

Also, the data gathered in these studies were analyzed in diverse ways. Some authors calculated the frequency of each behaviors, e.g., the number of episodes of each behavioral category per time bin (usually 5 min), whereas other authors expressed the duration of each behavior in relation to the total length of the observation period (45-60 min).

Few works, only six articles of the fifteen articles studied, presented data about the latency to start eating (Tables 1, 2 and 3). That is, the interval of time between the presentation of food and the moment at which the animal actually starts eating. Pre-satiated rats present longer latency periods to begin feeding [10]. Since food ingestion reduces the stimulation to start a new meal, latency to start feeding can be a measure of the animal's motivation to eat.

Food intake is also an important factor for the interpretation of the BSS. By the measurement of food intake and the duration of eating, that is, the time the animal actually spends consuming food, the rate of feeding can be determined by calculating the ratio between food intake (g) and the duration of eating (min). It is also possible to quantify the mean intake per eating episode through quantification of the relationship between food intake (g) and the number of eating episodes.

4.2.Bibliographical analysis.

The studies examined, demonstrated the ample applicability and usefulness of the BSS for the evaluation of feeding behavior. In particular, these studies show that the analysis of the BSS is a simple method to establish the microstructure of feeding. That is, to define for each behavior associated with food intake its duration and/or its frequency.

In the first category of studies in which the effects of nutritional manipulations on the temporal pattern of feeding were examined (Table 1), it was readily observable how hunger and satiety states [11], the palatability of the diet [10], and the satiation power of the ingested diet [15] affect feeding behavior. Thus, the adulteration of food with quinine [10], a substance with bitter taste, induced a reduction in food intake without affecting the duration of eating and abolished the resting behavior usually observed at the end of the BSS. Since satiety is associated with sleeping or inactive behavior, the absence of resting indicates the lack of satiation. This observation underlies the fact that the sapid properties of the food are a crucial determinant of feeding and that this factor must be considered when the anorexic or orexigenic properties of a drug are studied or when the characterization of a drug response involves its administration through the drinking water.

The BBS has been also used to examine the effect of a high-protein diet on satiety [15], and in particular on food intake, the rate of feeding and the relation between food intake and the duration of eating. In this study, animals were fed either standard chow or a high-protein diet and their feeding behavior was evaluated daily. On the first day, the animals fed the high-protein diet exhibited a reduction in food intake and the

rate of feeding, as well as an increased duration of eating and a decreased duration of resting. From the second to the fourteenth day, no differences between the two groups in the temporal pattern of the BSS were observed. The reduction in duration of resting in the first day indicates that a high-protein diet delays the appearance of satiety but this initial aversion to the high-protein diet is followed by adaptation.

Finally, it has been demonstrated that the interval between meals can affect food intake and the appearance of satiation [10]. Specifically, it was observed that the larger the period of fasting, the greater the food intake and the duration of eating, and that the larger the duration of the pre-feeding period, the smaller the latency to begin eating and the duration of feeding. These results indicate that a smaller interval of time between meals can reduce the motivation to eat the following meal. Using this experimental approach, one can obtain information about the temporal display of feeding behavior, and identify the level of control at which individual behaviors are affected by nutritional manipulations.

In the second category of studies (Table 2), the BSS was used to analyze how a pathological state can interfere with food intake. In one of these studies [16], the effects of muramyl dipeptide on feeding behavior were analyzed. Muramyl dipeptide is the minimally active subunit of bacterial peptidoglycan, which is released at significant levels during infections by gram-positive bacteria [17, 18], and has been associated with a reduction in food intake during infection [19]. In this study, none of the parameters of the BSS including the amount of ingested food, the duration of eating and the rate of feeding were altered within the first two hours after the administration of muramyl dipeptide. However, three hours after the administration of muramyl dipeptide there was a reduction in the duration of grooming and an increase in the length time of resting. These behavioral changes are similar to those that appear during illness. This study also

evaluated the cumulative food intake over a 24-hour period. This analysis demonstrated that the inhibitory effects of muramyl dipeptide on food intake, extend from the third to the tenth hour after its administration. Collectively, these data suggest that the hypophagic effect of the illness induced by a bacterial infection, results from an alteration of the physiological mechanisms involved in the regulation of satiety.

In relation to the changes in feeding behavior associated with a pathological state, it has also been observed that the discomfort caused by the administration of lithium chloride (LiCl) is related to a diminished food intake, a reduced duration of grooming and an enhanced length of eating [20]. The reduction in food intake was correlated with the rise in the duration of eating, thereby resulting in reduction in the rate of feeding. LiCl-induced anorexia is associated with behavioral signs of malaise such as a reduction in active behaviors, a low rate of food intake and the disruption of the BSS. These observations corroborate previous studies [16], indicating that anorexia is related to the discomfort provoked by sickness.

The BSS has been extensively used in pharmacological studies aimed to identify new therapeutic targets for the treatment of eating disorders and its consequence such as obesity, as well as to get insight into the mechanism involved in the control of feeding behavior both at the central and peripheral level (Table 3). In relation to the first point, it has been recently demonstrated that orexin participates in the regulation of feeding behavior by stimulating food intake [20]. Orexin-A and orexin-B are neuropeptides derived from prepro-orexin. Both peptides exert their actions through the activation of the orexin-1 and orexin-2 receptors but orexin-A binds with a higher affinity to the orexin-1 receptor [21]. The intracerebroventricular administration of orexin-A leads to an

increase of food intake. The fact that this hyperphagic effect is not associated with an enhanced duration of the meal, indicates that the orexigenic properties of orexin-A are due to its capacity to increase the rate of feeding [12]. On the other hand, the intraperitoneal administration of SB-334867, an antagonist of the orexin-1 receptor, reduces food intake and eating duration and enhances the length of rest [13, 20]. These results indicate that orexin stimulates food intake through its interaction with orexin-1 receptors.

The intestinal hormone cholecystokinin is a satiety signal and has anorexic effects [6, 7, 22, 23]. The administration of an equianoretic dose of the natural satiety-related signal cholecystokinin octapeptide (CCK-8S) induced a reduction in food intake, along with an increase in the latency to begin eating, an increase in the duration of eating and resting, and a reduction in the duration of grooming. Collectively, these observations indicate that cholecystokinin reduces satiety before the beginning of food intake and stimulates satiety after the initiation of the meal. The analysis of the effects of other manipulations, pharmacological or nutritional, on the pattern of the BSS induced by the administration of cholecystokinin, might be of interest for the dissection of other variables related to satiety.

Endogenous opioids are also involved in the regulation of appetite. The systemic or central administration of these peptides induces hyperphagia [24]. The physiological mechanisms underlying the orexigenic effects of opiods were investigated by the use of naloxone, an antagonist of opioid receptors [12]. The administration of this compound was shown to reduce both food intake and the duration of eating in control rats, without affecting the latency to start eating. Thus, opioids clearly regulate the satiety process but, in disagreement with a generally accepted idea, they do not seem to be involved in the motivation to eat.

The BSS has also been used to analyze the anorexic effects of the YY3-36 peptide [25, 26]. This peptide is released into the gastrointestinal tract during the postprandial period [27].The examination of the BSS pattern after the administration of the YY3-36 peptide, showed significant reductions in food intake and in the duration of eating. In addition, there was as an increase in the duration of resting which is consistent with an advance in the appearance of satiety. These results indicate that the YY3-36 peptide reduces food intake because it promotes satiety [28]. The stimulation of type 2 pre-synaptic receptors for neuropeptide Y (NPY), inhibits the release of NPY and GABA from hypothalamic arcuate nucleus neurons. Given that NPY is a potent orexigenic peptide and that YY3-36 peptide stimulates also NPY type 2 receptors [25], the possibility exists that the inhibitory effects of YY3-36 on food intake are mediated by NPY. In agreement with this hypothesis, the administration of a Y2 receptor antagonist did not alter by itself the BSS but prevented the anorexic effect of YY3-36 [28].

The BSS has been extensively used for the study of the effects of serotonin on food intake. Although all the studies performed so far agree that the inhibitory effects of serotonine on food intake are related to its capacity to advance the appearance of satiety, some discordant results exist in relation to how serotonin affects the other behavioral components of the microstructure of feeding. For example, the administration of fluoxetine, a selective serotonin reuptake inhibitor, reduces food intake, but does not alter the duration of eating nor it increases the length of resting [8]. In contrast, the administration of fenfluramine, a serotonin reuptake inhibitor which stimulates also the release of serotonin, leads to an inhibition of food intake which is associated with a reduction in the duration of eating and an increase in the period of resting [29]. Moreover, metergoline, a serotoninergic receptor antagonist, does not alter food intake or the duration of eating, but does increase time spent resting [8]. When fluoxetine was applied

together with metergoline, the duration of time spent eating and resting increased but food intake did not change [8], indicating that metergoline inhibited the effect of fluoxetine on the inhibition of food intake.

These controversial results can be explained by the fact that serotonin interacts with 14 different receptors which have been classified into different families according to their pharmacological, molecular and functional properties. Among these, serotonin 5-HT1B and 5-HT2C receptors have been specifically recognized as mediators of serotonin-induced satiety [8]. Thus, RU-24969, an agonist of the 5-HT-1A and 5-HT-1B receptors, reduces food intake and the duration of eating without altering the length of resting [8]. Similarly, the administration of CP-94,253, a selective 5-HT1B receptor agonist, reduced food intake and the duration of eating and grooming but, in contrast to the administration of RU-24969, increased the duration of resting [30]. These findings clearly demonstrate that 5-HT-1B receptors modulate the inhibitory effects of serotonin on food intake.

On the other hand, initial studies indicated that the administration of MK-212, a 5-HT-2 receptor agonist, reduces food intake and increases the duration of resting but does not alter the length of eating [8]. These data indicate that serotonin 5-HT-2 receptors regulate feeding behavior through the stimulation of satiety. Subsequent studies with RO- 60-0175 and VER23779, two selective 5-HT-2C receptor agonists, confirmed that the specific stimulation of 5-HT2C receptors inhibits food intake through a reduction in the duration of feeding time and increase in the length of resting [29, 31]. Collectively, these and other analysis of the BSS in association with pharmacological studies using selective serotonin receptor compounds, indicate that the anorexic action of serotonin is mediated by separate receptors subtypes. Thus, while the reduced meal size consecutive to the administration of serotonin would depend on the stimulation 5-HT1B receptors,

the reduction in feeding rate would result from the selective stimulation of 5-HT2C receptors.

Conclusion.

The BSS is a non-invasive, low expensive and highly efficient method for the analysis of feeding behavior. The effects of pharmacological and nutritional manipulations on the natural physiological regulation of food intake can be evaluated using the BSS. It is regrettable that a big number of studies aimed to characterize the orexigenic or anorexigenic properties of a drug utilize the amount of ingested food as the only variable of feeding behavior. Although a reduction or an increase in food ingestion reflects an effect on appetite, the measurement of food intake alone does not allow to determine whether these changes are due to an alteration of the physiological mechanisms regulating food intake, or are due to non specific effect such as sedation, hyperactivity, malaise or enhance of the satiety. The BSS is a solid experimental tool for improving our understanding of the complex psychological and physiological process involved in the regulation of feeding behavior.

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Table 1. Effects of nutritional manipulations on the Behavioral Satiety Sequence

Authors	Species	Phase of the light/dark cycle	Time	Treatment/ diet	n	Food intake	Latency (s) or (%)	Duration of eating (s) or (%)	Duration of grooming (s) or (%)	Duration of rest (s) or (%)
(10)	Rat Lister hooded	light	1 h	control	10	n/a	31±8 s	725±30s	233±30s	279±119s
				quinine 0.015%		n/i	35±8s	786±100s	239±32s	113±68s
				quinine 0.04%		reduction	25±8s	603±77s	273±19s	5±4s*
				saccharine 0.2%		n/i	15±2s	704±25s	236±30s	240±111s
				saccharine 0.3%		n/i	21±5s	702±33s	259±32s	277±145s
(15)	Rat Wistar	light	1h	AIN-93/P 14	8	n/i	n/i	18.9±1.8%	12.4±1.4%	54.9±4.2%
				AIN-93/ P50 (1º dia)				32.2±5.5%*	10±2.2%	39.2±8.7%*
				AIN-93/ P50 (2º dia)				18.3±2.2%	13.7±1.3%	50.5±2.8%
				AIN-93/ P50 (14ª dia)				13.9±1.2%	13.7±2%	60.4±3.2%
(11)	Rat Lister hooded	light	1h	Control A	12	n/i	19±4s	828±44s	293±38s	511±114s
				Prefeeding 3 min		n/i	30±6s	729±41s[a]	316±40s	606±144s
				Prefeeding 6 min		n/i	44±7s[a]	637±50s[a]	331±32s	617±137s
				Prefeeding 9 min		n/i	48±22s[a]	598±54s[a]	305±35s	588±161s
				Control B		16.9±1.3	21±4s	585±68s	346±53s	426±143s
				Fasting 3 h		17.7±1.2	26±8s	677±51s	317±38s	365±110s
				Fasting 6 h		21.7±1.0g*	24±6s	788±50s[b]	345±69s	232±66s
				Fasting 12 h		22.2±1.0g*	21±7s	778±63s[b]	374±73s	332±135s

Data are means of the duration in seconds or mean of the percent duration ± sd; (AIN-93)= diet formulated for rodents by the American Institute of Nutrition in 1993 (32, 33); (P14) = Diet with 14% protein; (P50) = diet with 50% protein; n/a = not applicable to the group, n/i = data not informative in the original reference, (*) = significant difference (p>0.05) in relation to the control group, (a) = significant difference (p>0.05) in relation to the control group A, (b) = significant difference (p>0.05) in relation to the control group B.

Table 2. Effects of pathological state on the Behavioral Satiety Sequence

Authors	Species	Phase of the light/dark cycle	Time	Treatment/ diet	n	Food intake	via	Food intake	Latency (s) or (%)	Duration of eating (s) or (%)	Duration of grooming (s) or (%)	Duration of rest (s) or (%)
(16)	Rat Sprague -Dawley	light	1 h	Saline 1st hour	8	0,9%	i.p	5.5±1.2g	n/i	10±1%	10±3%	52±4%
				Saline 3rd hour				3.3±0.3g		7±1%	13±3%	58±9%
				MDP 1st hour	8	1.5 mg/Kg		6.3±1.0g		10±2%	7±3%	54±4%
				MDP 3rd hour				2.2±0.6g		4±1%	5±1%*	82±3%*

Data are means of the duration in seconds or the mean percentage of the duration ± sd; n/i = data not informative in the original reference, (*) = significant difference (p>0,05) in relation to the saline group in the respective schedule.

Table 3. Effects of pharmacological manipulations on theBehavioral Satiety Sequence

Authors	Species	Phase of the light/dark cycle	Time	Treatment/ diet	n	Dose	Via	Food intake	Latency (s) or (%)	Duration of eating (s) or (%)	Duration of grooming (s) or (%)	Duration of rest (s) or (%)
(12)	Rat Lister hooded	light	1 h	Control	12	n/a	i.c.v	14.02±0.96g	23(15-32)s	804±49s	305±51s	700±159s
				orexina-A	12	3.33 µg		19.99±1.15g*	12(6-19)s	905±62s	429±52s	298±63s
					12	10 µg		18.92±1.11g*	12(6-30s	823±45s	461±49s	417±79s
					12	30 µg		19.26±1.09g*	16(8-50)s	928±69s	474±42s	464±135s
(20)	Rat Lister hooded	light	1 h	Control	10	n/a	i.p	21.25±0.8g	25±4s	659±32s	263±24s	593±106s
				LiCl	10	90 mg/Kg		12.36±0.98g*	22±3s	991±66s*	138±20s*	828±119s
				SB-334867	10	10 mg/Kg		16.41±1.46g*	26±4s	575±33s	236±41s	858±144s
					10	30 mg/Kg		12.06±1.35g*	37±7s	467±44s	154±36s	1251±185s*
(13)	Rat Lister hooded	light	1 h	Control	10	n/a	i.p	n/a	21±5s	705±43s	194±29s	1007±105s
				SB-334867	10	30 mg/Kg		reduction	15±4s	418±41s*	160±31s	1634±129s*
				Control	10	n/a		n/a	23±4s	719±50s	278±30s	480±146s
				CCK-8S	10	5 µg/Kg		reduction	199±65s*	1151±87s*	154±21s*	855±107s*
(12)	Rat Lister hooded	dark	1 h	Control	10	n/a	i.p	n/i	38±12s	n/i	n/i	n/i
				naloxone	10	1 mg/Kg		reduction, 54%	58±12s	reduction	not altered	not altered
					10	2.5 mg/Kg		reduction, 61%	37±11s	reduction	not altered	reduction
					10	5.0 mg/Kg		reduction, 65%	41±12s	reduction	not altered	not altered
(28)	rat Sprague-Dawley	light	90 min	Control	8		i.v.	5.41±0.46	n/i	33±3%	n/a	34±7%
				Antagonist Y2	8	5 mg/Kg		5.49±0.44		31±3%	not altered	38±5%
				PYY 3-36	8	50 mg/Kg		3.58±0.35*		22±2%*	not altered	63±4%*
				Antagonist Y2 + PYY 3-36	8	5+50 mg/Kg		6.24±0.64		34±5%	not altered	35±9%
(8)	Rat Lister hooded	dark	40 min	Control	12	n/a	i.p.	8.0±0.4g	n/i	360±31s	207±46s	628±114s
				Fluoxetine (ISRS)	12	10 mg/Kg		4.1±0.9g*	n/i	279±57s	176±32s	1127±162s*
				Metergoline	12	1 mg/Kg		7.9±0.7g	n/i	419±72s	99±20s	1129±142s*
				Fluoxetine + metergoline	12	10+1 mg/Kg		7.3±0.8g	n/i	506±77s*	105±20s	1051±114s*
(8)	Rat Lister hooded	dark	40 min	Control	12	n/a	i.p	8.92±1.31g	n/i	405±61s	371±47s	511±62s
				MK-212	12	5 mg/Kg		4.33±0.81g*	n/i	320±48s	371±69s	1042±129s*
(8)	Rat Lister hooded	dark	40 min	Control	12	n/a	i.p.	7.2±0.9g	n/i	318±38s	298±46s	802±149s
				CP-94.253	12	5 mg/Kg		3.1±06g*	n/i	160±26s*	173±33s	1274±175s
				Control	12	n/a		9.1±0.94g	n/i	530±39s	248±42s	398±137s
				RU-24969	12	1 mg/Kg		6.22±0.79g*	n/i	440±42s*	264±44s	305±107s
(29)	Mice	light	40 min	Control	12	n/a	i.p.	1.82±0.18g	n/i	n/a	n/a	n/a
				RO-60-0175	12	3 mg/Kg		1.55±0.31g	n/i	reduction	not altered	increase
					12	10 mg/Kg		1.18±0.23g*	n/i	reduction	not altered	increase
				Fenfluramine	12	3 mg/Kg		1.00±0.19g*	n/i	reduction	not altered	increase
(30)	Rat Lister hooded	dark	40 min	Control	12	n/a		n/a	n/i	n/a	n/a	n/a
				CP-94.253	12	1.25 mg/Kg		not altered	n/i	reduction	not altered	not altered
					12	2.5 mg/Kg		reduction 37%	n/i	reduction	not altered	increase
					12	5 mg/Kg		reduction 78%	n/i	reduction	reduction	increase
(31)	Mice	light	40 min	Control	12	n/a	s.c.	n/a	n/i	n/a	n/a	n/a
				VER23779	12	3 mg/Kg		reduction		reduction	reduction	reduction
					12	10 mg/Kg		reduction		reduction	reduction	reduction

Dates presented are the means of the duration in seconds or mean percent of duration ± sd; n/a = not applicable to the group; n/i = the original reference is not informative; (*) = significant difference (p>0.05) in relation to the control group; (reduction) = reduction of the duration of the behavior in relation to the control group [values not available in the original article]; (not altered) = no significant differences in relation to the control group [values not present in the original article].

2.4. DESMAME PRECOCE E SUAS CONSEQUÊNCIAS

O período de lactação finaliza com o desmame. O desmame espontâneo inicia-se por volta do 21º dias de vida e vai até aproximadamente o 30º dia de vida quando cessa completamente a ingestão de leite (Krecek e Kreckova, 1957; Hahn e Koldovsky, 1966; Henning, 1981). Apesar dos filhotes conseguirem sobreviver sem a presença materna a partir do 14º dia de vida, o desmame nessa idade é considerado precoce.

O leite materno é responsável pela nutrição do neonato. Cerca de 69,8% de sua calorias são provenientes dos lipídios e apenas 6,8% das calorias são provenientes dos carboidratos (Azara, Maia *et al.*, 2008). Já as dietas comerciais, a exemplo da AIN-93, apresentam maior percentual de carboidratos (64%) e menor percentual de gordura (16,7%) (Reeves, Nielsen *et al.*, 1993). No desmame precoce o leite materno é retirado e a alimentação é imposta como única fonte de nutrientes. Dessa forma o desmame precoce pode ser considerado uma agressão nutricional.

Além do fator nutricional o desmame precoce por separação da mãe pode ser um fator estressante, pois priva o filhote do cuidado materno (Kikusui, Isaka *et al.*, 2005). Os efeitos do desmame precoce sobre o eixo HPA foram investigados logo após o desmame e foi demonstrado o aumento nos os níveis de corticosterona 48 horas após a separação materna (Kikusui, Ichikawa *et al.*, 2009).

Muitos estudos encontraram alterações comportamentais na vida adulta associadas ao desmame precoce (Back e Angel, 1982; Kikusui, Takeuchi *et al.*, 2004; Kanari, Kikusui *et al.*, 2005; Kikusui, Isaka *et al.*, 2005; Ito, Kikusui *et al.*, 2006). O teste do labirinto em cruz elevado, um teste clássico que mede parâmetros relacionados ao comportamento ansioso, demonstrou que ratos desmamados precocemente entram menos vezes e passam menos tempo nos braços abertos do labirinto, indicando aumento

nos níveis de ansiedade (Ito, Kikusui *et al.*, 2006; Kikusui, Kiyokawa *et al.*, 2007). Além do labirinto, outros testes como hole-board e open-field também demonstraram aumento do comportamento de ansiedade (Kanari, Kikusui *et al.*, 2005). No entanto ambiente estimulante e enriquecido ameniza os efeitos do desmame precoce sobre a ansiedade, realçando a importância da interação mãe-filho durante o desenvolvimento (Iwata, Kikusui *et al.*, 2007). Além da ansiedade, o desmame precoce também provoca aumento da agressividade (Kikusui, Takeuchi *et al.*, 2004; Nakamura, Kikusui *et al.*, 2008).

Foi demonstrado que curtos períodos diários de separação materna induz em longo prazo hiperatividade do eixo HPA evidenciado pelo aumento nos níveis basais de corticosterona em ratos machos adultos desmamados precocemente (Plotsky e Meaney, 1993; Ladd, Huot *et al.*, 2000; Kikusui, Nakamura *et al.*, 2006). Também é possível observar que o desmame precoce provoca respostas neuroendócrinas e autônomas mais intensas na presença de agentes estressores em ratos e camundongos adultos (Ito, Kikusui *et al.*, 2006; Kikusui, Nakamura *et al.*, 2006). Essas evidências demonstram que a carência materna ao final do período de aleitamento torna os indivíduos mais susceptíveis ao estresse.

É possível que tais alterações comportamentais e neuroendócrinas causadas pelo desmame precoce sejam devidas a alterações estruturais. Estudos observaram que camundongos desmamados precocemente apresentam na 8ª semana de vida mielinização alterada e menor peso cerebral que os normalmente desmamados (Kikusui, Kiyokawa *et al.*, 2007). É provável que essas alterações sejam consequências da diminuição da neurogênese e da expressão de fator neurotrópico cérebro-derivado brain-derived neurotrophic factor (BDNF) no hipocampo em resposta aos níveis aumentados de glicocorticóides na terceira semana de vida (Kikusui, Ichikawa *et al.*, 2009). Além

disso, o desmame precoce provoca baixa expressão de receptores 5HT-1B no hipocampo (Nakamura, Kikusui *et al.*, 2008). A ativação desses receptores facilita a saciedade (Lee, Kennett *et al.*, 2002) e o aumento da agressividade após algumas horas de privação alimentar foi associado à redução na expressão desses receptores provocadas pelo desmame precoce (Nakamura, Kikusui *et al.*, 2008).

Outro modelo de desmame precoce é a inibição materna da prolactina ao final da lactação, a qual promove a inibição da produção de leite (Bonomo, Lisboa *et al.*, 2005). Esse tratamento durante o final da lactação programou síndrome metabólica em longo prazo apresentando quadros de sobrepeso; aumento da gordura corporal, visceral e subcutânea; hiperglicemia e resistência a insulina; hipercolesterolemia com aumento de LDL-colesterol e redução de HDL-colesterol; e hipertrigliceridemia (De Moura, Bonomo *et al.*, 2009). Esses achados ressaltam a importância do fator ambiental durante período de lactação para o desenvolvimento dos sistemas e da interação mãe-filho ao final da gestação como agente programador.

O leite materno é considerado o melhor alimento para o recém-nascido devido ao seu teor adequado de nutrientes e por conter imunoglobulinas que fortalecem o sistema imunológico. Apesar das recomendações clínicas para o aleitamento materno exclusivo até os 6 meses de vida e de forma complementar até por volta dos dois anos de idade o desmame precoce ainda tem sido uma prática comum na população brasileira causada por diversos fatores (Araujo, Da Cunha *et al.*, 2008). Aliado a uma alimentação inadequada no primeiro ano de vida, o desmame precoce tem sido associado a quadros de obesidade ainda na infância (Sigulem, Taddei *et al.*, 2001) . Em humanos, as consequências tardias do desmame precoce, em particular, são muito pouco conhecidas. Esse trabalho experimental tem como objetivo estudar possíveis

repercussões do desmame precoce sobre o comportamento alimentar e dessa forma subsídios para as pesquisas epidemiológicas que abordem esse tema.

MÉTODOS

3.1. ANIMAIS E DESMAME

Foram utilizados ratos da linhagem Wistar com idades entre zero e 150 dias, provenientes da colônia do Biotério do departamento de nutrição da Universidade Federal de Pernambuco. As fêmeas nulíparas com peso entre 250 e 300g foram obtidas do Biotério do Departamento de Nutrição e mantidas em Biotério de experimentação com ciclo claro/escuro de 12 horas invertido (luz acesa as 18h00), temperatura de 25 ± 1ºC, durante 15 dias para adaptação, com livre acesso à água e a dieta padrão do biotério (Purina do Brazil S/A). Após o período de adaptação as fêmeas foram acasaladas na proporção de duas fêmeas para um macho. Um dia após o nascimento, todos os filhotes nascidos no mesmo dia foram randomizados e foram separados os filhotes machos com 6-8g de peso. Após a sexagem os filhotes machos foram distribuídos na proporção de 8 filhotes por mãe. Quando a quantidade de filhotes machos não foi suficiente, a ninhada foi completada com fêmeas. Os grupos experimentais foram delineados de acordo com o período do desmame por separação da mãe em: grupo desmamado no 15º dia (D15); grupo desmamado no 21º dia (D21) e o grupo desmamado no 30º dia (D30) (Figura 1). Todos os experimentos foram realizados de acordo com as recomendações do Comitê Brasileiro de Experimentação Animal - COBEA, e aprovado pelo comitê de ética em experimentação animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (Anexo B).

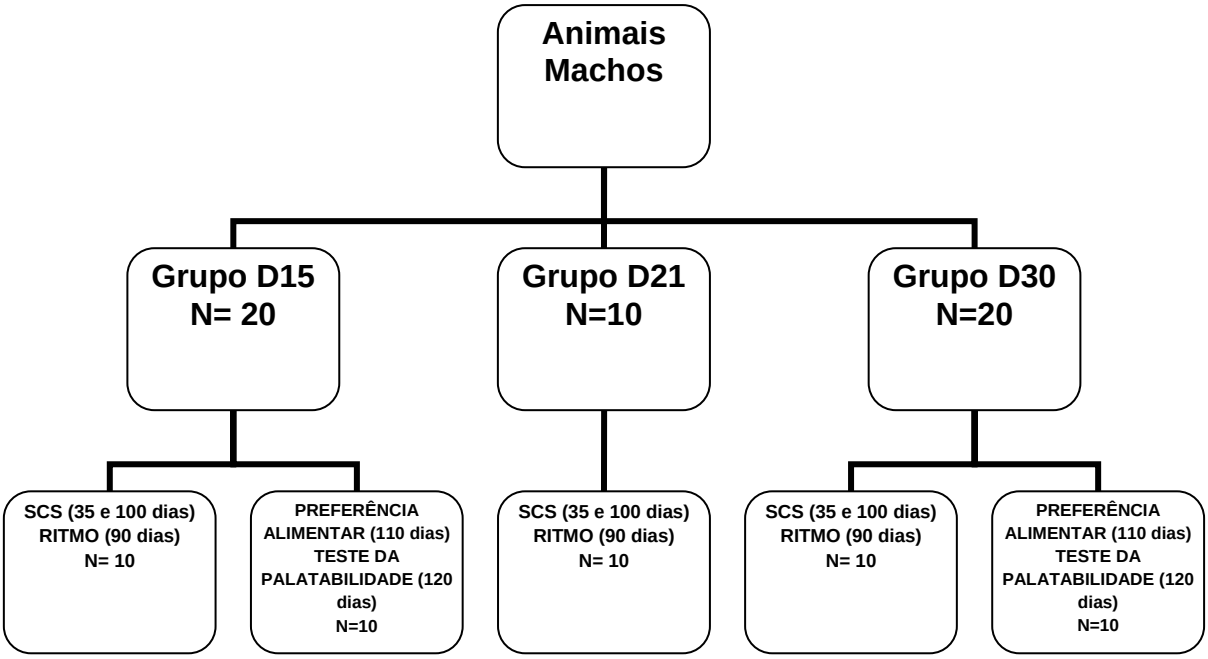

Figura 1. Fluxograma representando o delineamento experimental.

3.2. PESO CORPORAL E CONSUMO ALIMENTAR

O peso corporal (g) dos animais de todos os grupos foi aferido diariamente do 15º ao 30º dia pós-natal. Após o 30º dia de vida, os animasi foram mantidos em caixas individuais e o peso corporal de cada animal foi aferido a cada 30 dias até os 150 dias de vida.

O consumo alimentar de 24 horas foi realizado em animais com 35, 90, 120 e 150 dias de vida. As medidas de consumo foram tomadas através da diferença entre a quota oferecida (g) e quota rejeitada (g) após 24 horas

3.3. SEQUÊNCIA COMPORTAMENTAL DE SACIEDADE

O estudo da sequência comportamental de saciedade (SCS) ocorreu no 36º e no 100º dia de vida no horário de 16h30 as 17h30. A análise da SCS foi precedida por privação alimentar de três horas e realizada como descrita por Halford *et al.* 1998. Ao início do teste foi oferecido a cada animal 50g de ração e ao final da observação foi

aferido o rejeito. O consumo foi quantificado através da diferença entre a quota oferecida e a quota rejeitada. Os comportamentos característicos da seqüência comportamental de saciedade e o consumo de ração foram observados durante 60 minutos por experimentadores treinados. Os Comportamentos foram categorizados como: comportamento de alimentação (caracterizado por mordida, roedura ou deglutição de ração disposta no comedouro), comportamento de limpeza (caracterizado por lamber o corpo, as patas e a genitália, por arranhar o pêlo ou a cabeça com as patas, por afagar o pêlo com as patas e morder a cauda) e comportamento de descanso (caracterizado pela inatividade do animal, por sua posição relaxada, tendo a cabeça curvada sobre o corpo, ou o repouso do animal sobre o fundo da gaiola, estendido de lado ou sobre o ventre). A duração de cada comportamento foi quantificada com cronômetro digital e anotada em protocolo apropriado. Com os dados obtidos durante a análise da SCS foram calculados outros parâmetros: Taxa local de alimentação – razão entre o consumo de ração (g) e a duração do comportamento de alimentação (min); Taxa global de alimentação – razão entre o consumo de ração (g) e a duração total da observação da seqüência comportamental de saciedade (min).

3.4. RITMO CIRCADIANO DO CONSUMO ALIMENTAR

Para investigação da ritmicidade circadiana de ingestão alimentar, foram avaliados ratos com 90 dias de idade. O ritmo alimentar foi avaliado através da quantificação do consumo de ração padrão a cada 4 horas durante o ciclo claro/escuro . Os horários das aferições foram: 6h00, 10h00, 14h00, 18h00, 22h00 e 2h00. Dessa forma foi obtido o consumo em três pontos do ciclo escuro, e três do ciclo claro. O

consumo foi avaliado por três dias consecutivos e foi calculada a média de consumo por período de 4 horas para cada grupo.

3.5. PREFERÊNCIA ALIMENTAR A MACRONUTRIENTES

Para o estudo da preferência alimentar a macronutrientes foram confeccionadas três dietas: protéica, lipídica e glicídica, cuja única fonte de calorias foi proteína, lipídios e carboidratos, respectivamente. Os componentes presentes em cada dieta estão dispostos na tabela 1 e foram baseadas no modelo experimental de Tanaka e Kido, 2008. O estudo iniciou aos 110 dias de vida, sendo realizado em três dias consecutivos. Antes da análise de preferência, foi realizada uma adaptação dos animais as diferentes dietas. Esta consistiu da colocação das três dietas concomitantemente por 1 hora após 6 horas de privação alimentar no mesmo horário da realização do teste. No dia do teste, após 6 horas de privação alimentar, foram inseridos nas gaiolas de cada animal três recipientes, cada um contendo 30 g de uma das dietas experimentais. Após uma hora os recipientes foram retirados e o rejeito foi aferido. A quota ingerida (g) foi quantificada através da diferença entre a quota oferecida e a quota rejeitada. Foi realizada uma média de consumo de cada dieta nos três dias do experimento.

Tabela 1. Composição das dietas utilizadas no estudo da preferência alimentar.

Composição	Protéica	Lipídica	Glicídica
Caseína	93,5%	-	-
A. Graxo de cadeia curta	-	77%	-
Óleo vegetal	-	10%	-
Amido de milho	-	-	62,3%
Sacarose	-	-	31,2%
Mix Mineral	3,5%	7%	3,5%
Mix vitamina	1%	2%	1%
Celulose	2%	4%	2%

3.6. TESTE DA RESPOSTA A DIETA PALATÁVEL

Para o estudo da resposta à dieta palatável foi confeccionada uma dieta hipercalórica e palatável (tabelas 2 e 3). O estudo foi realizado nos animais com 120 dias de vida. Três dias antes do experimento os animais foram adaptados às dietas. No dia do experimento foi colocado um recipiente contendo 50g da dieta palatável e 50g da dieta padrão. Após 24 horas foi quantificado o rejeito. O estudo ocorreu em três dias consecutivos e foi realizada uma média de consumo dos três dias para cada animal.

Tabela 2: Composição da dieta hipercalórica e palatável

Composição	Proporção
Creme de avelã com chocolate (Nutella®)	55%
Dieta padrão (Labina®)	33%
Água	12%

Tabela 3: Composição de macronutrientes da dieta hipercalórica e palatável

Composição de Macronutrientes	Teor em 100g
Proteína	12,3g
Carboidrato	49g
Lipídio	17,5g
Calorias	402,5Kcal

3.7. ANÁLISE ESTATÍSTICA

Todos os dados foram expressos em média ± erro padrão da média (SEM), com exceção das tabelas que foram expressas em média ± desvio padrão. Na comparação entre os grupos, foi utilizado, quando apropriado, teste "t" de Student ou ANOVA one way, seguida do teste de Tukey. Um valor de $P < 0,05$ foi considerado significante.

RESULTADOS

4 ARTIGOS ORIGINAIS

No presente trabalho de tese, foram examinadas as conseqüências do desmame precoce sobre a programação do comportamento alimentar, em ratos adultos. Dois artigos científicos originais foram submetidos a revistas internacionais. Doravante, serão apresentados em ordem cronológica os artigos em suas versões originais.

Effects of early weaning on the circadian rhythm and behavioral satiety sequence in rats

O primeiro artigo original deste estudo é intitulado: **"Effects of early weaning on the circadian rhythm and behavioral satiety sequence"**. Foi submetido como artigo original à revista: **Behavioural Processes**. É classificada pela CAPES como qualis B1 para área de medicina II e possui fator de impacto igual 1,441. (ANEXO C)

Em resumo, neste artigo, foi verificado o efeito do desmame precoce ou tardio sobre padrões comportamentais relacionados a saciedade e ao ritmo alimentar em ratos adultos. Foi demonstrado que a manipulação na idade do desmame modifica de forma duradoura a seqüência comportamental de saciedade e o ritmo do consumo alimentar. No entanto, o peso corporal e o consumo alimentar basal não sofreram variações. Considerando o desmame precoce uma agressão, o mesmo parece interferir na programação de mecanismos funcionais de controle do comportamento alimentar, provocando alterações duradouras, detectáveis no organismo adulto.

Effects of early weaning on the circadian rhythm and behavioral satiety sequence in rats

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Abstract:

The objective of this work was to study the effect of early weaning on circadian rhythm and the behavioral satiety sequence in adult rats. Male Wistar rat pups were weaned for separation from the mother at 15 (D15), 21 (D21) and 30 (D30) days old. Body weight and food intake was measured every 30 days until pups were 150 days old. At 90 days of age, the circadian rhythm of food intake was evaluated every 4 hours for three days. Behavioral satiety was evaluated at 35 and 100 days of age. This work demonstrated that body weight and food intake were not altered, but the behavioral satiety sequence demonstrated that the D15 group delayed satiety compared with the D30 group at 100 days of age. In the circadian rhythm of the food intake study, early weaning (D15) changed food intake in the intermediary period of the light phase and in the intermediary period of the dark phase. In conclusion, our study showed that early weaning may alter the feeding behavior mainly in relation to satiety and the circadian rhythm of feeding. It is possible that the presence of other environmental stimuli during early weaning can cause hyperphagia and deregulate the mechanisms of homeostasis and body weight control. This study supports theories that depict insults during early life as determinants of chronic diseases and can be the basis for strategies to encourage breastfeeding.

Key words: Behavioral satiety sequence; Circadian rhythm; Early weaning; Feeding behavior.

1. Introduction

Some studies demonstrate that epigenetic factors occurring in critical periods of life can affect the development of organs and tissues, provoking permanent structural and physiological alterations (Hales and Barker, 2001; Orozco-Solis et al., 2009; Ravelli et al., 1976). The biological phenomenon that establishes the relationship between these stimulations in the critical period of development, particularly during pregnancy and lactation, and the future functional state is called programming (Barker, 2004; de Moura and Passos, 2005; Lucas, 1994).

Lactation is a critical period because in this period important neurobehavioral development occurs and mother-pup interaction is strong during this process (Caldji et al., 2000; Liu et al., 2000; Plotsky and Meaney, 1993). Environmental changes, malnutrition (Orozco-Solis et al., 2009), hormonal alterations (Passos et al., 2007), or the neonatal stress of maternal separation (Hancock and Grant, 2009) during lactation can provoke permanent functional alterations. Early weaning is one of the most important events in the beginning of life for mammals (Nakamura et al., 2008). After weaning, the young mammals become nutritionally and behaviorally independent from their mothers. In rats, spontaneous weaning begins around the third week of life and continues until 30 days of age when the pups cease milk intake completely (Henning, 1981; Krecek and Kreckova, 1957).

Early weaning is considered a model of neonatal stress, which increases the autonomic response to stressor agents (Ito et al., 2006), probably as a result of hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis (Ladd et al., 2000; Plotsky and Meaney, 1993). In the long term, neonatal stress provokes behavioral alterations such as an increase of anxiety in rats (Ito et al., 2006) and an increase of aggressiveness in mice (Kikusui et al., 2004). This suggests that interactions between the mother and the pup during the end of the lactational period are important for behavioral development in rodents (Kanari et al., 2005; Kikusui et al., 2005). The reduced maternal care in this phase also has been associated with alterations in the behavioral and physiological responses to stress in adult progeny (Liu et al., 2000; Liu et al., 1997).

Another behavior that can be programmed by nutrition and stress at the beginning of the life is feeding behavior (Orozco-Solis et al., 2009). Malnutrition at the beginning of life can modify the Behavioral Satiety Sequence (BSS) (Orozco-Solis et al., 2009) and can lead to the development of a greater preference for high fat foods (Cambraia et al., 2001). Conversely, stress from maternal separation in the lactation period increases palatable food intake (Silveira et al., 2004; Silveira et al., 2005).

In different mammalian species, mechanisms that control feeding behavior are influenced by circadian rhythm. The light cycle (light-dark) is an effective signal that synchronizes the biological rhythm with the environment (Ohta et al., 2008) and is directly related with feeding behavior (Tallett et al., 2009). It is possible that, similar to malnutrition, aggression in the initial periods of life can result in the alteration of the circadian rhythm of the feeding behavior (Orozco-Solis et al., 2009).

Although an important paper has shown that aggression in the neonatal period can exert changes on the programming of feeding behavior, there are few studies concerning the effect of precocious weaning on the programming of this behavior. The objectives of this work are to investigate the effect the manipulation of the weaning period exerts on feeding behavior through the study of the BSS and to investigate if this manipulation can interfere with the circadian rhythm of food intake.

2. Materials and Methods:

2.1. Animals:

Wistar rats, aged between 0 and 150 days old, originated from the Department of Nutrition's colony from the University Federal de Pernambuco – Brazil. Virgin female Wistar rats weighing 250-300g were obtained and maintained in the laboratory with an inverted light/dark cycle of 12 hours (lights on at 6:00 p.m.) for 15 days for adaptation, with water and a standard diet (Purina do Brasil S/A) ad libitum. After the adaptation period, females were assigned in a proportion of two females for one male. The day of the birth was considered day zero. Day one after birth, pups were divided into male and female groups and eight male pups were assigned per dam. The experimental groups were classified in accordance with the weaning period. The pups from the D15 group (early weaning) were separated from the mother on the 15th postnatal day. The pups from the D21 group (control 21 days) were separated from the mother on the 21st postnatal day. The pups from the D30 group (control 30 days) were separated from the mother on the 30th postnatal day. On the 30th postnatal day, animals from all groups were housed in individual acrylic cages (54 x 30 x 20 cm) for realization of the experimental procedures. All experiments were performed in accordance with recommendations from the Comitê Brasileiro de Experimentação Animal – COBEA, and were approved by the Comissão de Ética em Experimentação Animal from Centro de Ciências Biológicas from the Universidade Federal de Pernambuco.

2.2. Measurement of body weight and food intake

The body weight (g) of each pup was recorded daily from the 15th until the 30th postnatal day. After the 30th postnatal day, the body weight was recorded every 30 days until pups reached 150 days of life. Food intake was measured on the 35th, 90th, 120th and 150th days of life. For the circadian rhythm study, food intake was measured, beginning on the 90th day, for three consecutive days at 4 hour intervals.

2.3. Behavioral Satiety Sequence

The BSS study occurred on the 36th and 100th day of life. The analysis of the behavioral satiety sequence was performed essentially as described by Halford et al. (Halford et al., 1998). Feeding and non-feeding behaviors during a 60 min test meal were continuously scored by a highly trained experimenter, blind to the nutritional status of the animals, and recorded on a videotape to be re-examined by a second skilled observer. Behaviors were categorized as: eating (ingesting food, gnawing, chewing or holding food in paws), grooming (body care movements with the mouth or forelimbs), and resting (sitting or lying in a resting position or sleeping). Other measures scored from the behavioral observation of feeding were: food intake (food consumed (g) during the time of observation of the BSS), meal duration (time (s) over the entire monitoring period the animal was actually eating food), local feeding rate (amount of food consumed (g)/ meal duration (min)) and global feeding rate (amount of food consumed (g)/ analysis of BSS duration (min)). To promote feeding, food was removed from home cages 3 h before the onset of the test and the presentation of food took place 1 h before the onset of the dark cycle. Food was weighed at the beginning and end of each session.

2.4. Data analysis

Experimental results are expressed as means ± S.E.M. All data were analyzed using a SigmaStat 2.03 demo program. Body weight, feeding behavior and data from the BSS were analyzed using a one-way ANOVA followed by the Tukey test for multiple comparisons between groups. Before using the ANOVA test, data were submitted to variance and normality tests with 5% tolerance.

3. Results

3.1. Body Weight and Food intake

In the period after weaning, a reduction ($p < 0.05$) of body weight of animals from the D15 group was observed. Between the 16th and 18th day of life, the D15 group presented a minor body weight reduction in relation to the D21 and D30 groups. In the 19th day of life, the body weight of the D15 group was less than the D30 group. Beginning on the 20th day of life, the body weight recovered and did not differ between groups (Figure 1A and 1B). No difference between groups in food intake was observed until 150 days of life (Fig. 2).

3.2. Behavioral Satiety Sequence

Behavioral satiety sequence in the progression of feeding behavior, cleaning and resting place without interruption of behavioral sequence was observed. Each 5 min period was quantified for the duration of each behavior and there was no difference between the experimental groups in the 12 evaluation periods (Figure 3).

The point of satiety occurred at 41 minutes in the D15 group, at 39 minutes in the D21 group and at 36 minutes in the D30 group We observed a delay in triggering satiety in the D15 group compared with the D30 group (Figure 3).

The rate of feeding (food intake/duration of feeding) was lower ($p < 0.05$, one-way ANOVA followed by Tukey test) in the D15 group compared with the D21 group. The D30 group showed no difference ($p > 0.05$, One Way ANOVA) compared to the D15 and D21 groups (Table 1).

At 100 days, we repeated the evaluation of the SCS and the progression of behavioral feeding, cleaning and rest was again without interruption of the behavioral sequence in the groups. The point of satiety occurred at 43 minutes in the D15 group, at 35 minutes in the D21 group and at 22 minutes in the D30 group. Thus, a delay in

triggering satiety in the group D15 was observed compared with the D30 group (Figure 4).

During the total period of BSS, the D15 group had a longer duration ($p < 0.05$, one-way ANOVA) of feeding behavior than the group of late weaning (D30). The cleaning behavior did not differ ($p > 0.05$, One Way ANOVA) between groups. Regarding the resting behavior, the D15 group and D21 group had a longer duration ($p < 0.05$, One Way ANOVA) than the D30 group (Table 2).

Regarding the parameters involved, we observed that food consumption in the D15 and D21 groups was higher ($p < 0.05$, One Way ANOVA) than in the D30 group, but the feeding rate did not differ ($p > 0.05$, One Way ANOVA) between groups (Table 2).

3.3. Circadian rhythm of food intake

In the light phase between 11:00 PM – 2:00 AM, it was observed that the D15 group (1.45 ± 0.67) showed a lower food intake ($p < 0.05$, One Way ANOVA) relative to the D30 group (2.56 ± 1.37). In the dark phase between 10:00 AM-2:00 PM, it was observed that the D15 (8.86 ± 1.79) and D21 (8.93 ± 1.25) groups had a higher food intake ($p < 0.05$, One Way ANOVA) than the D30 group (7.19 ± 1.82) (Figure 5).

4. Discussion

The present study investigated the effect of early or late weaning on behavioral patterns related to satiety and feeding rhythm in adult rats. Manipulation of weaning age prompted a lasting change in the behavioral satiety sequence and the rate of food consumption. However, body weight and baseline food intake did not vary.

The ideal age for weaning rats kept in the laboratory is still controversial. Weaning at 21 days of life is the most frequent practice; however, spontaneous weaning begins around the third week of life and continues until 30 days when pups cease to consume milk (Hahn and Koldovsky, 1966). From the 14th day of life, rats begin the consumption of solid food (Hahn and Koldovsky, 1966) and are able to eat and maintain body temperature, and they are able to evacuate themselves from the 13th day of life (Plaut and Davis, 1972). In our work, early-weaned animals were separated from their mothers at 15 days of life. At that age, pups can still breastfeed, but they can survive independently of their mothers. Previous studies used pups aged between 14 and 16 days of life for models of early weaning (Hahn and Kirby, 1973; Ito et al., 2006; Kikusui et al., 2009). However, the weaning age used as controls in these studies was variable. Thus, there are studies that use control animals weaned at 21 days of age (Kikusui et al., 2007; Kikusui et al., 2006; Kikusui et al., 2004; Nakamura et al., 2008), and others use the default of 30 days (Angel and Back, 1981; Back and Angel, 1982; Hahn and Kirby, 1973; Ito et al., 2006). In this paper we studied the effects of early weaning on some aspects of feeding behavior and controlled weaning at 21 days of age. We also investigated the effects of weaning at 30 days of life to ascertain whether the difference between these two ages of weaning caused changes in eating behavior. However, we observed that all aspects of the group D21 were very similar to the D15 group and the differences only appeared when comparing the D15 group to the D30 group.

Early weaning led to weight loss immediately after maternal separation, although the animals' weight recovered soon after. Breast milk is responsible for the nutrition of the neonate. About 69.8% of a neonate's calories come from fat and only 6.8% of calories come from carbohydrates (Azara et al., 2008). Diets trade for rats show, including the AIN-93, a higher percentage of carbohydrates (64%) and less fat

(16.7%) (Reeves et al., 1993). At the end of lactation, rats initiate food intake but still consume milk until complete weaning. The abrupt change of milk consumption to the exclusive consumption of solid foods with different nutritional composition from breast milk may have caused the initial weight loss. Until adulthood, early weaning did not alter body weight gain. Other studies found that early weaning by inhibition of lactation with maternal bromoergocriptin caused an increase in body weight (Bonomo, Lisboa et al. 2007; Bonomo, Lisboa et al. 2008; de Moura, Bonomo et al. 2009). However, our results are consistent with the dietary baseline, which also was not changed by early weaning.

We studied the behavioral satiety sequence at 35 and 100 days of life. The study of the behavioral satiety sequence for 35 days showed that early weaning slowed the firing of satiety and reduced the feeding rate, although not significantly. The feeding rate is a parameter widely used in the literature as a sensitive indicator of drug action on feeding behavior (Halford et al., 1998). This rate indicates the speed at which the animal feeds. There were no differences in food consumption, but animals that underwent early weaning showed a tendency to reduce food intake without altering the duration of feeding behavior, which is the cause of the reduced feeding rate.

When the behavioral satiety sequence was reassessed in adulthood, we found that the animals that underwent later weaning were quicker to reach satiety. We also observed that the longest period of lactation caused less consumption without changing the rate of local food, and therefore less time for eating and for the onset of satiety was needed. Because there was no change in cleaning behavior, periods of rest also lasted longer when early satiety occurred. This advance in the firing of satiety caused by the longer period of breastfeeding was very similar to the pattern found in studies of the behavioral satiety sequence in rats treated with acute doses of CP-4,253, a selective agonist of serotonin 5HT-1B receptors. The administration of CP-4,253 significantly decreases food intake and duration of feeding behavior without changing the feeding rate or increasing the duration of rest (Lee, Kennett et al. 2002). Studies have shown that early weaning causes a low expression of 5HT-1B receptor mRNA in the hippocampus (Nakamura, Kikusui et al. 2008), but little is known about the effects of early weaning on the levels of these receptors in the hypothalamic nuclei responsible for satiety. However, the incidence of stress in the third week of life reduces serotonin

levels in the hippocampus, amygdala and hypothalamus (Matsui, Morimoto et al., 2009).

Several experimental studies have shown that early weaning causes increased aggression and anxiety behavior in adulthood (Kanari et al., 2005; Kikusui et al., 2004; Nakamura et al., 2008). These changes may be due to hyperactivity of the HPA axis and the increase in circulating levels of glucocorticoids in the home cage and in response to stress agents (Ladd et al., 2000; Plotsky and Meaney, 1993). It has been shown that increased levels of glucocorticoids increase the consumption of sucrose and this effect was considered a form of compensation to reduce the signs of stress (Dallman et al., 2003). Fasting is considered a stressor in rats weaned early and increases aggressiveness (Nakamura, Kikusui et al. 2008). Thus, the period of fasting that preceded the study of the behavioral satiety sequence may have increased food intake and delayed satiety.

Adult rats exhibit a pronounced nocturnal feeding rhythm. In a laboratory with a normal light cycle with light / dark alternating every 12 hours, about 75 to 80% of food intake occurrs during the dark period (Mayer, Marshall et al. 1954). In this study, the consumption pattern was preserved overnight; all the peak power occurred during the dark period. However, in adulthood early weaning changed food intake during periods in the middle of the dark phase and the middle of the light phase. The ontogeny of the feeding rhythm is influenced by endogenous factors and also by the influence of maternal behavior (Levin and Stern, 1975). During the first two weeks of life, no feeding rhythm is expressed through the consumption of milk or by eating solid foods (Babicky et al., 1970). By the 14^{th} day, the pups still have their eyes closed (de Souza et al., 2004; Deiro et al., 2004) and therefore the feeding rhythm is not influenced by the light cycle. Until the 18^{th} day, the pattern of milk consumption, expressed by weight gain, is predominantly diurnal and is imposed by the mother (Levin and Stern, 1975). When the pups begin consuming solid foods, this behavior is imposed by the mother because the pups do well and repeat the behavior (Galef and Henderson, 1972). Between the 15^{th} and 18^{th} day of life, there is rhythm in the consumption of solid food (Levin and Stern, 1975). Only around the 19^{th} day of life does it appear that the nocturnal pattern of feeding and the control of this pattern is influenced by endogenous factors (Levin and Stern, 1975). Thus, maternal absence from the 15^{th} day of life in this work may have affected this learning and contributed to the initial detection of a difference in the feeding rhythm.

In conclusion, our study showed that early weaning altered the feeding behavior mainly in relation to satiety and the circadian rhythm of feeding. It is possible that the presence of other environmental stimuli during early weaning can cause hyperphagia and deregulate the mechanisms of homeostasis and body weight control. Currently, the world population is suffering with a high incidence of obesity, a disease of multifactorial origin (Bruce and Byrne, 2009; Grundy, 1998). Early weaning has been associated with the presence of increased weight and obesity in children (Sigulem et al., 2001). This study supports theories that link the insults occurring during periods of early life as determinants of chronic diseases and can be the basis for strategies to encourage breastfeeding. However, knowledge about the structural and physiological changes that may be involved in the regulation of feeding behavior is still scarce and more studies are needed.

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Figure 1

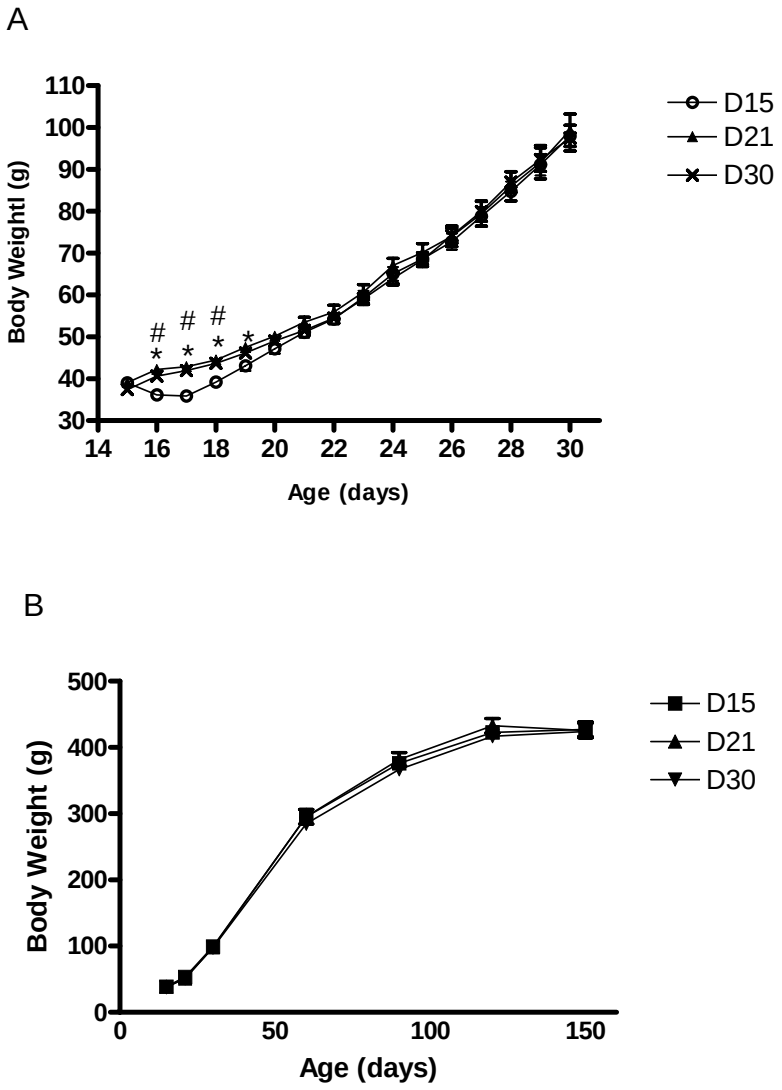

Figure 1 - Effect of the manipulation of the weaning period on the body weight of young animals (a) until the adult age (b). The animals were weaned at 15 days (D15), 21 days (D21) or 30 days (D30). The data are expressed as means ± S.E.M. (*) difference between D15 and D21, (#) difference between D15 and D30 (One-way ANOVA followed by Tukey test, $p > 0.05$).

Figure 2

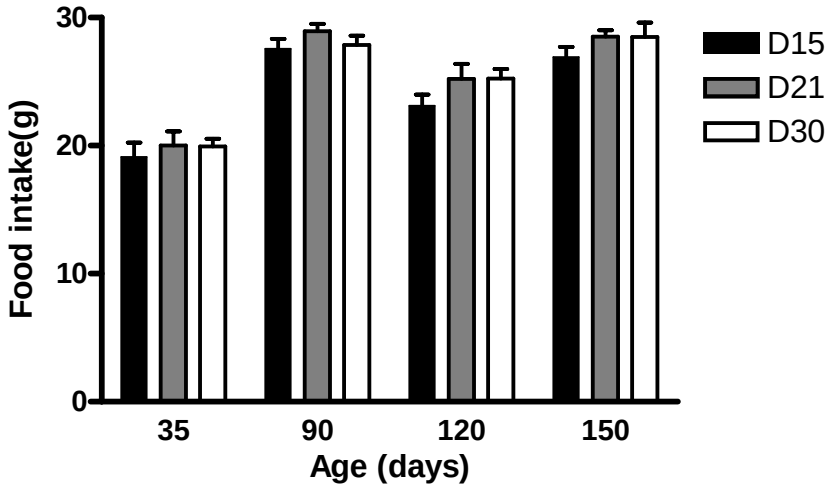

Figure 2 - Effect of the manipulation of weaning period on food intake (g). The pups were weaned at 15 days (D15), 21 days (D21) or 30 days (D30) and at 35, 90, 120 and 150 days of life the food intake was evaluated. The data are expressed as means ± S.E.M. There was no difference between groups (ANOVA, $p< 0.05$).

Figure 3

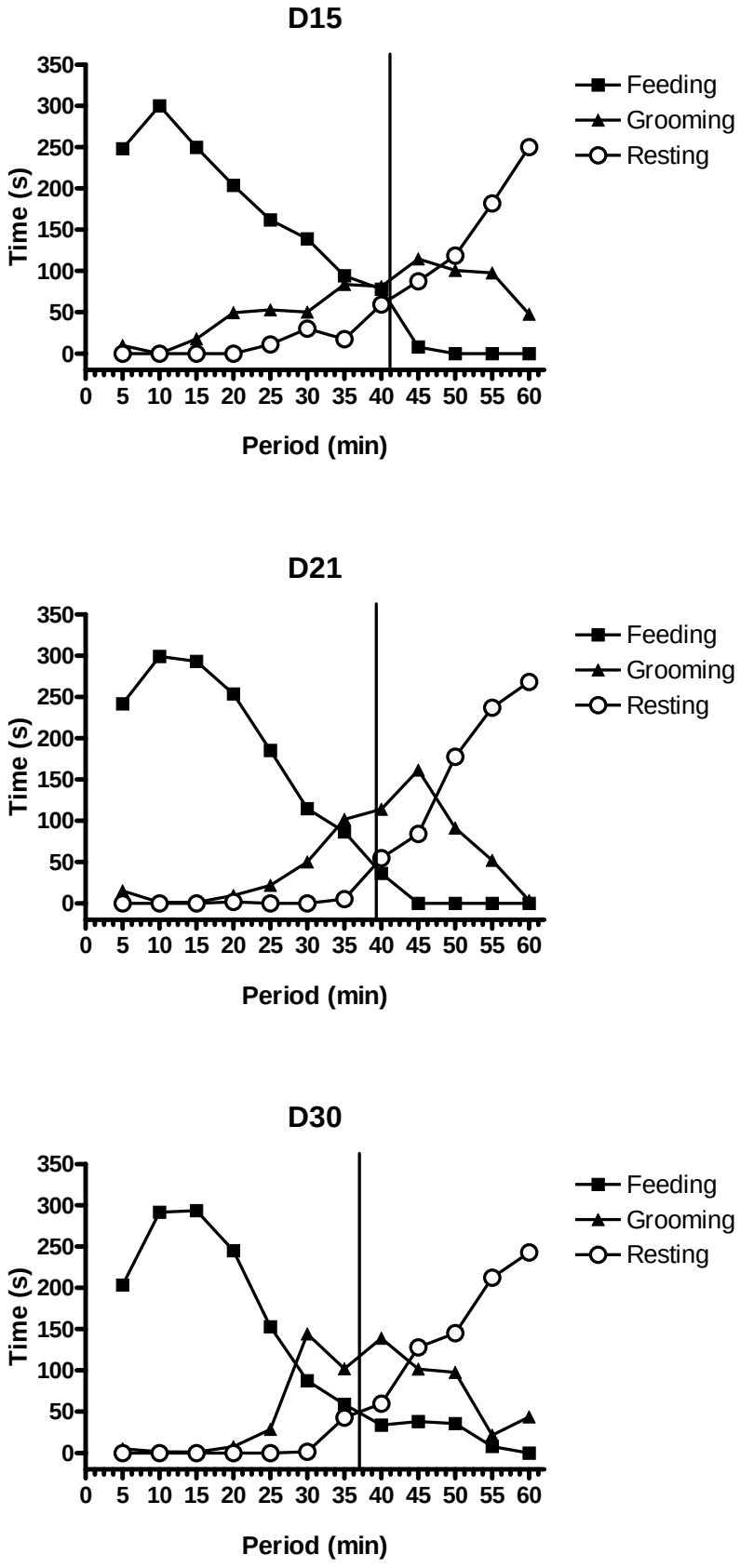
D15
Time (s)
Period (min)
Feeding
Grooming
Resting
D21
Time (s)
Period (min)
Feeding
Grooming
Resting
D30
Time (s)
Period (min)
Feeding
Grooming
Resting

Figure 3 - Effect of manipulation of the weaning period on the behavioral satiety sequence in rats 36 days of age. The animals were weaned at 15 days (D15, n = 10), 21 days (D21, n = 10) or 30 days (D30, n = 10). At 36 days, animals were subjected to a period of 3 hours of fasting. After fasting, 25 g of food was offered for 1 hour and feeding behavior, cleaning and rest were evaluated. The transition point between the feeding behavior and rest is shown. Data are expressed as means. There was no difference between groups ($p < 0.05$, ANOVA).

Figure 4.

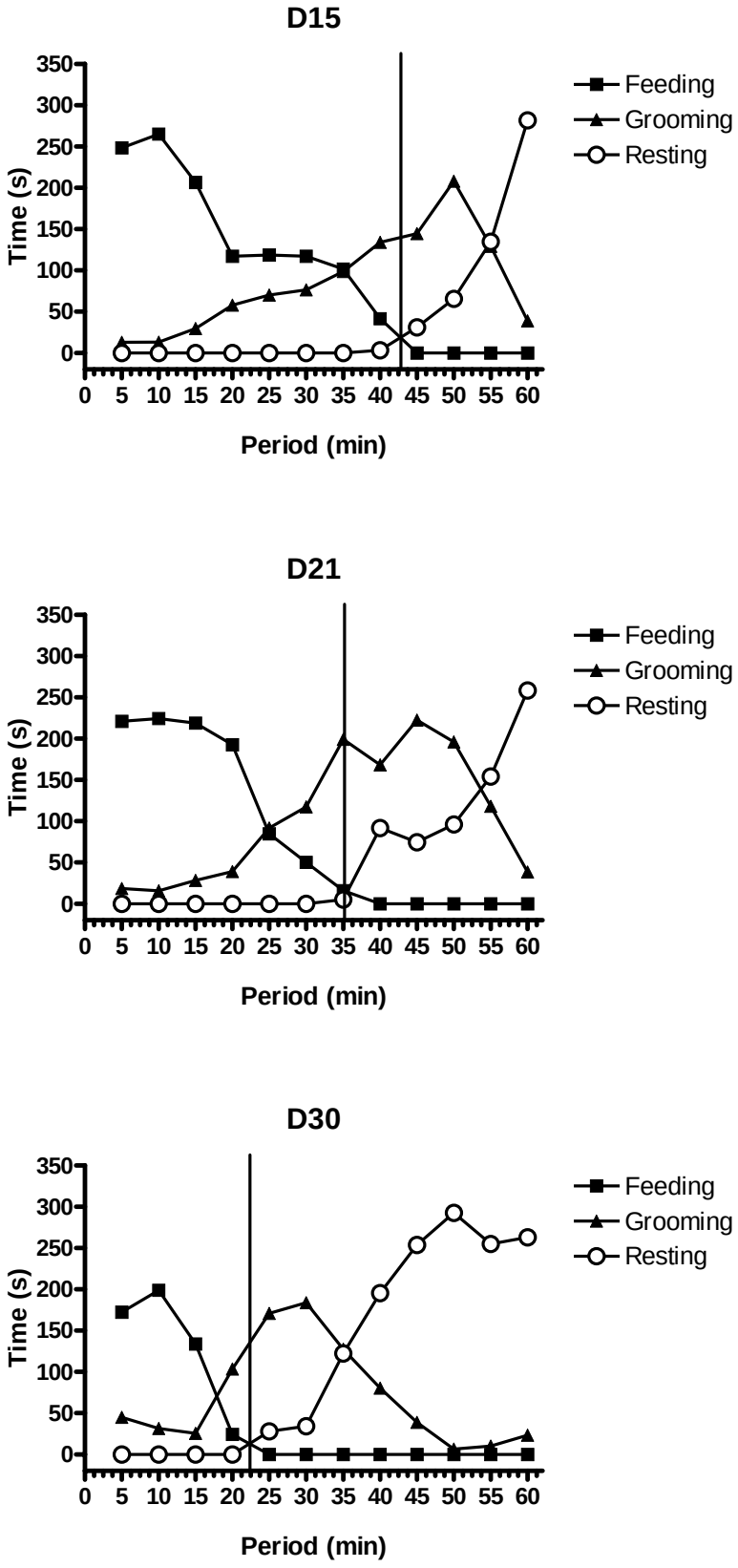
D15
Time (s)
Period (min)
Feeding
Grooming
Resting
D21
Time (s)
Period (min)
Feeding
Grooming
Resting
D30
Time (s)
Period (min)
Feeding
Grooming
Resting

Figure 4 - Effect of manipulation of the weaning period on the behavioral satiety sequence in rats 100 days of age. The animals were weaned at 15 days (D15, n = 10), 21 days (D21, n = 10) or 30 days (D30, n = 10). At 100 days the animals underwent a period of 3 hours of fasting. After fasting, 25 g of food was offered for 1 hour and feeding behavior, cleaning and rest were evaluated. The transition point between the feeding behavior and rest is shown. Data are expressed as means.

Figure 5.

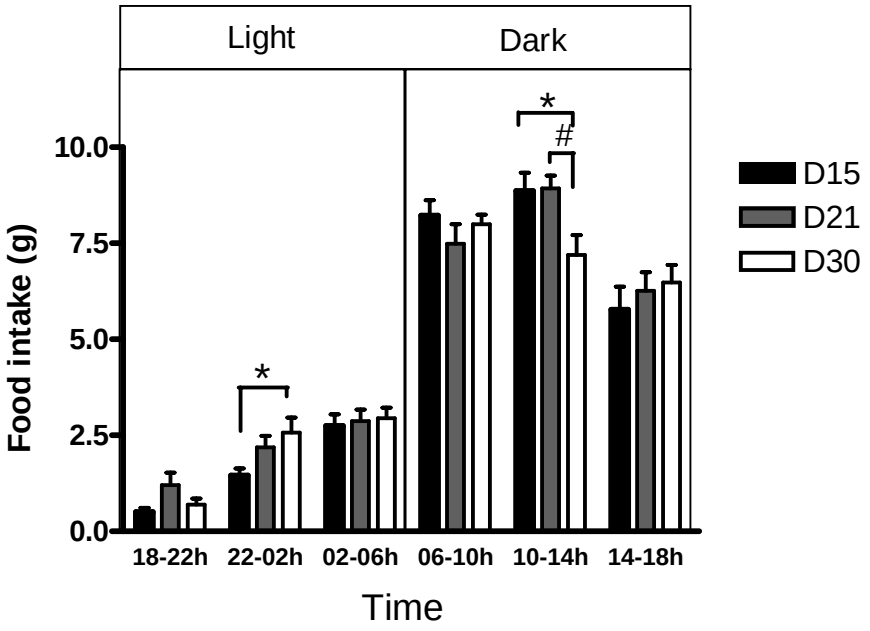

Figure 5 - Effect of manipulation of the weaning period on the circadian rhythm of food consumption of adult rats. (*) Difference between D15 and D30. (#) Difference between D21 and D30.

Table 1 - Effect of early weaning on the parameters assessed during the behavioral satiety sequence in rats at 36 days of age.

	D15	D21	D30
Food intake (g)	4,42 ± 1,56	6,22 ± 2,45	5,32 ± 1,00
Feeding duration (s)	1482 ± 486	1509 ± 292	1448 ± 480
Grooming duration (s)	706 ± 266	622± 330	694 ± 382
Resting duration(s)	755± 591	752± 456	832± 600
Local feeding rate (g/min)	0,17± 0,02 *	0,24± 0,07	0,22± 0,06
Global feeding rate (g/min)	0,07± 0,02	0,10± 0,04	0,08± 0,02

Data are expressed as the mean ± SD (*) difference between D15 and D21 ($p < 0.05$, ANOVA).

Table 2 - Effect of early weaning on the parameters assessed during the behavioral satiety sequence in adult rats.

	D15	D21	D30
Food intake (g)	6,57 ± 1,26	6,65 ± 1,29	5,12 ± 0,66 # *
Feeding duration (s)	1124 ± 383	1007 ± 390	712 ± 242 #
Grooming duration (s)	1013 ± 402	1253 ± 352	845 ± 304
Resting duration(s)	516 ± 276	680 ± 519	1443 ± 390 # *
Local feeding rate (g/min)	0,36 ± 0,08	0,36 ± 0,04	0,40 ± 0,09
Global feeding rate (g/min)	0,11 ± 0,02	0,11 ± 0,02	0,08 ± 0,01# *

Data are expressed as the mean ± SD. (#) Difference between 15 and 30 (*) difference between 21 and 30 ($p < 0.05$, ANOVA).

Early weaning programs rats to have a dietary preference for fat and palatable foods in adulthood

O segundo artigo deste estudo é intitulado: **"Early weaning programs rats to have a dietary preference for fat and palatable foods in adulthood"**. Foi submetido como artigo original à revista: **Behavioural Processes**. É classificada pela CAPES como qualis B1 para área de medicina II e possui fator de impacto igual 1,441. (ANEXO D)

Nesse artigo foram estudados os efeitos do desmame precoce sobre a preferência alimentar pelos macronutrientes carboidratos, proteínas e lipídios. Também foi verificado a presença de hiperfagia em resposta a oferta de dieta hiperlipídica palatável. O presente trabalho demonstrou que o desmame precoce por separação materna não provoca alteração de peso corporal e do consumo alimentar basal em ratos adultos até 150 dias de vida. No entanto na presença de outros fatores como a livre escolha de nutrientes ou a oferta de dieta hiperpalatável e hipercalórica tornaram os animais desmamados precocemente mais susceptíveis a consumirem mais gordura e tornarem-se mais hiperfágicos. Uma maior duração dessas condições pode possibilitar o aumento constante da ingestão calórica tornando o desmame precoce um fator predisponente para a obesidade.

Title: Early weaning programs rats to have a dietary preference for fat and palatable foods in adulthood

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Abstract

The objective of this work was to study the effect of early weaning on alimentary preference to the macronutrients protein, carbohydrate and fat in adult rats. Male Wistar rat pups were weaned by separation from the mother at 15 (D15) or 30 (D30) days old. Body weight and food intake were measured every 30 days until pups were 150 days old. At 110 days of age, the alimentary preference was evaluated for 1 hour on 3 consecutive days. At 120 days of age, the palatable diet test was conducted during 3 consecutive 24-hour periods. Body weight and food intake were not altered, but early weaning in rats induced an alimentary preference to fat and hyperphagia of a palatable diet. In conclusion, early weaning, although it did not modify body weight or basal food intake, promoted an increased preference for palatable and fatty foods. This demonstrates that early weaning is not capable of promoting perceptible alterations of alimentary behavior under normal laboratory conditions. However, in the presence of a stimulating factor such as a choice of nutrients or a palatable diet, a possible latent effect on dietary preferences may become apparent. Over the long term, this preference for foods with high caloric density can lead to obesity and metabolic perturbations.

Key words: Early weaning, feeding behavior, macronutrients preference, palatable food

1. Introduction

Programming is a biological phenomenon in which ambient factors act in an initial critical period of life, affecting the development of the organism (Barker, 2004; de Moura and Passos, 2005; Lucas, 1994). Pregnancy and lactation are considered critical periods because in these periods the incidence of environmental factors, such as the nutritional and hormonal state or stress can promote permanent structural and physiological alterations (Hales and Barker, 2001; Lins et al., 2005; Matsui et al., 2009; Orozco-Solis et al., 2009; Ravelli et al., 1976).

Lactation is a critical period in which important developmental events that are decisive for future neurologic and behavioral state occur. During this period, adverse environmental alterations can cause important physiological modifications and predispose the individual to some imbalances in adult life (Barker, 2004; de Moura and Passos, 2005; Dobbing, 1964; Lucas, 1994), and hormonal state or stresses can program permanent physiological alterations (Liu et al., 1997; Orozco-Solis et al., 2009; Passos et al., 2007). In this suckling period, the interaction between mother and pup has major effects on developmental processes because the sole nutritional source is the mother's milk. Moreover, the young rats also depend on their mother to maintain their body temperature and to defecate (Caldji et al., 2000; Liu et al., 2000; Plaut and Davis, 1972; Plotsky and Meaney, 1993). Therefore, maternal absence can be considered a harmful agent, as it deprives the younglings of maternal milk and care, causing perceptible alterations in adulthood (Hancock and Grant, 2009; Liu et al., 2000; Marais et al., 2008).

Weaning is one of the most important events of the early life of mammals (Nakamura et al., 2008). After weaning, the younglings become nutritionally and behaviorally independent from the mother. In rats, spontaneous weaning starts early, around the third week of life, and continues until approximately the 30th day of life, when the younglings cease milk intake (Henning, 1981; Krecek and Kreckova, 1957). Early weaning is also considered a neonatal stress model because it increases the autonomic response to stressors in adulthood (Ito et al., 2006), probably as a result of hyperactivity of the hypothalamic–pituitary–adrenal axis (Ladd et al., 2000; Plotsky and Meaney, 1993). In the long term, early weaning provokes behavioral alterations, such as

increased anxiety in rats (Ito et al., 2006) and increased aggressiveness in mice (Kikusui et al., 2004). This suggests that interactions between mother and pup during pre-weaning or the end of the lactation period are important for behavioral development in rodents (Kanari et al., 2005; Kikusui et al., 2005). The degree of maternal need in pups in this phase is associated with alterations in behavioral and physiological responses to stress in adulthood (Liu et al., 2000; Liu et al., 1997).

Another behavior that can be programmed by nutrition and stress in early life is feeding (Orozco-Solis et al., 2009). Animals malnourished in the perinatal period demonstrate alterations in satiety and exhibit a stronger preference for fatty foods (Cambraia et al., 2001; Orozco-Solis et al., 2009). Additionally, a brief daily episode of maternal separation during the neonatal period increases the appetite for palatable foods (Silveira et al., 2004; Silveira et al., 2005).

Despite the important finding that stresses in the neonatal period can program adult feeding behavior, studies are scarce concerning the effects of early weaning on programming with respect to alimentary preference. The objectives of this study were to investigate the effects exerted by manipulation of the weaning period on feeding behavior in terms of alimentary preference to carbohydrate, protein and fat and to evaluate food intake in response to a palatable diet.

2. Materials and Methods:

2.1. Animals:

Wistar rats aged 0–150 days from the Department of Nutrition, University Federal de Pernambuco, Brazil, were used. Virgin female Wistar rats weighting 250–300 g were obtained and maintained in a laboratory with an inverted light/dark cycle of 12 hours (lights on at 18:00) for a 15-day adaptation period, with water and standard diet (Purina do Brasil S/A) ad libitum. After the adaptation period, the rats were housed

at two females per male for mating. The day of birth of each litter was considered day 0. On day 1, male and female pups were separated and adjusted to eight male pups per dam. The experimental groups were delineated in accordance with the weaning period. The younglings from the D15 group (early weaning, n=10) were separated from their mothers on postnatal day 15, and the younglings from the D30 group (control, n=10) were separated from their mothers on day 30. On day 30, the animals from all groups were accommodated in individual acrylic cages (54 x 30 x 20 cm) for experimental procedures. All experiments were conducted in accordance with the recommendations of the Comitê Brasileiro de Experimentação Animal (COBEA) and were approved by the Comissão de Ética em Experimentação Animal from the Centro de Ciências Biológicas, Universidade Federal de Pernambuco.

2.2. Measurement of body weight and food intake:

The body weight of each pup was recorded daily from day 15 until day 30. After day 30, the body weight was recorded every 30 days until day 150. After 5 days of adaptation in individual cages, 24-hour food intake was measured on days 35, 90, 120 and 150.

2.3. Study of the alimentary preference to macronutrients

To study the alimentary preference to the three classes of macronutrients, three diets of similar consistencies were used, with the major caloric source as protein, fat or carbohydrate. The composition of each diet is presented in Table 1. Alimentary preference was evaluated on 3 consecutive days starting at day 110. Before the test, the animals adapted to their assigned diets for 3 days. The ingestion of different types of diets was measured during 1 hour after 6 hours of fasting.

2.4. Study of palatable diet preference

At day 120, two receptacles, containing a palatable diet and a standard diet, were placed in each animal cage. At day 117, Each animal had a 3-day adaptation period

consisting of introduction to the palatable diet. The palatable and standard diets ingested were measured after 24 hours. The study occurred over 3 consecutive days. The mean ingestion of each animal over the 3 days was calculated. The palatable diet contained 55% (by weight) hazel-nut cream with chocolate (Nutella®), 33% standard diet (Labina®) and 12% water. The nutritional overview of the palatable diet is given in Table 2.

2.5. Data analysis

Results are expressed as means ± S.E.M. All data were analyzed in the SigmaStat 2.03 demo program. The data of body weight and feeding behavior were analyzed utilizing Student's t test. Before Student's t test was used, the data were submitted to variance and normality tests with 5% tolerance. The data that did not pass the tests of normality and variance were square-root–transformed before the application of Student's t test.

3. Results

3.1. Body weight and food intake

In the post-weaning period, a reduction ($p<0.05$, Student's t-test) in body weight of the D15 group was observed compared to D30. Between days 16 and 18, the D15 group presented a lower mean body weight than the D30 group. From day 19 on, the body weight of the D15 group recovered and remained similar to that of D30 until day 30 (Figure 1A). No difference was observed in body weight between the groups from day 30 to day 150 ($p>0.05$, Student's t test) (Figure 1B).

There was no difference ($p>0.05$, Student's t-test) in the absolute (Figure 2A) or relative to body weight (Figure 2B) food intake between the D30 and D15 groups by day 150.

3.2. Alimentary preference to macronutrients

The D15 rats consumed more protein diet (D15: 2.50 ± 1.58; D30: 0.67 ± 0.91), carbohydrate diet (D15: 12.06 ± 2.33; D30: 6.38 ± 2.58) and fat diet (D15: 4.12 ± 1.48; D30: 0.82 ± 1.13) than the D30 group ($p< 0.05$, Student's t test) (Figure 3). In terms of the percentage of calories offered by each class of nutrients, the D15 group ingested 7.45% of its calories from protein, 33.39% from carbohydrate and 59% from fat. The D30 group consumed 12.96% of its calories from protein, 46.10% from carbohydrate and 40.95% from fat. Animals of the D15 group (59.16% ± 4.3%) consumed a higher proportion of calories from fat than the D30 group (40.95% ± 4.8%). The percentage of calories from protein and carbohydrate did not differ between the groups (Figure 4).

3.3. Palatable diet intake

The intake of the standard diet was evaluated before the beginning of the palatable diet intake test, and no differences ($p>0.05$, Student's t test) between the D30 group (25.24 ± 2.57 g) and the D15 group (23.10 ± 3.32 g) were observed (Figure 5A).

During the palatable diet test, intake of the standard and palatable diets was measured over 3 consecutive days. The standard diet intake was reduced ($p<0.05$, Student's t test) in both groups compared with the before the test. The palatable diet intake was increased in both groups, but this increase was greater in the D15 group (35.98 ± 3.51) than in the D30 group (29.34 ± 2.27) ($p>0.05$, Student's t test) (Figure 5).

When total caloric ingestion was calculated, we found that the D15 group consumed more calories than D30, whereas before the test, there were no differences between the groups (Figure 6).

4. Discussion

In the present study, early weaning in rats induced an alimentary preference to a high-fat diet and hyperphagia of a palatable diet, although it did not provoke alterations in body weight gain or basal food intake, in adult rats.

The ideal age for the weaning of rats maintained in a laboratory is still debated. Weaning within 21 days of life is more frequent and practical, but spontaneously, weaning starts around the third week of life and continues until pups are 30 days old, when they cease milk intake (Hahn and Koldovsky, 1966). At day 14, rats initiate solid food intake (Hahn and Koldovsky, 1966) and are capable of eating, maintaining body temperature and defecating without maternal assistance (Plaut and Davis, 1972). In this work, Wistar rats considered early-weaned were separated from their mothers at 15 days of age. At this time, they still may suckle, but they can already survive independently from the mother. Days 14 to 16 are often used for models of early weaning (Hahn and Kirby, 1973; Ito et al., 2006; Kikusui et al., 2009). However, the age of weaning used as the control in these studies is variable. There are studies that began weaning at 21 days as control (Kikusui et al., 2007; Kikusui et al., 2006; Kikusui et al., 2004; Nakamura et al., 2008), and others used the age of 30 days as standard (Angel and Back, 1981; Back and Angel, 1982; Hahn and Kirby, 1973; Ito et al., 2006). Here we studied the effects of early weaning on some parameters of adulthood feeding behavior, considering early weaning as beginning on day 15 and control weaning on day 30, with the goal of augmenting any behavioral alterations related to feeding.

Early weaning provoked a reduction of body weight immediately after the maternal separation, which recovered soon afterwards. In the early neonatal period, milk is the sole nutrient source for pups. At the end of the lactation period, rats initiate food intake but still ingest maternal milk until completely weaned. The abrupt change from milk to exclusively solid foods with nutritional composition different from the milk can provoke an initial reduction of body weight. About 69.8% of the calories of maternal milk come from fat, and only 6.8% come from carbohydrate (Azara et al., 2008). The commercial diet AIN-93 is high in carbohydrate (64%) and low in fat (16.7%) (Reeves et al., 1993). It is possible that this change in nutritional profile provoked a brief nutritional disequilibrium causing loss of body weight. Upon adulthood, the weaning

showed no effect on body weight. Other studies have observed that early weaning by inhibiting lactation with bromoergocriptine provokes increased body weight (Bonomo et al., 2008; Bonomo et al., 2007; de Moura et al., 2009). However, our results are consistent with the similar basal food intake observed between early-weaned and control rats. This observation is also in accord with studies using protein malnutrition in rats during the perinatal period, which found no alterations in food intake until adulthood (Fagundes et al., 2007; Orozco-Solis et al., 2009). Aside from malnutrition, inhibition of maternal prolactin, which has a similar effect as early weaning, also does not modify basal food intake in younglings (Bonomo et al., 2008; Bonomo et al., 2007; de Moura et al., 2009).

Although there was no difference in basal food intake, early weaning induced increased consumption of all three diets used in the test of alimentary preference in adulthood. The proportion of calories consumed from fat was higher in the D15 group than in the D30 group, suggesting that early weaning increases the preference for fat. Exposure to stress is involved in the selection of macronutrients and has been shown to increase the preference for fat (Teegarden and Bale, 2008). It has been proposed that this increase of fat intake, with consequent increased caloric density, is a method of attenuating the physiological effects of chronic stress (Dallman et al., 2003; Pecoraro et al., 2004). Early weaning is considered a chronic stressor because it induces hyperactivity of the hypothalamic–pituitary–adrenal axis in the long term (Ladd et al., 2000; Plotsky and Meaney, 1993), increasing glucocorticoid levels in adult animals (Ladd et al., 2000; Plotsky and Meaney, 1993), such as corticosterone (Kikusui et al., 2006). In addition, the administration of corticosterone modulates the selection of macronutrients, promoting greater fat intake (Bligh et al., 1993). The effects observed here of the earlier nutritional shift from maternal milk to solid food, which is a shift to a lower-fat diet, are similar to previous findings that maternal exposure to a low-fat diet during gestation and lactation promotes a preference among pups for a high-fat diet after weaning (Nakashima et al., 2008).

In this study, we demonstrated that early weaning increased palatable diet intake. Although the hyperphagic potential of the palatable diet was observed in both groups, early weaning induced a greater consumption of calories from the palatable diet. In addition, the increase in caloric intake was not accompanied by greater standard diet intake. Previous studies have demonstrated that neonatal stress caused by maternal

separation also increases the consumption of palatable diets, especially those with sweet flavor (McIntosh et al., 1999; Silveira et al., 2004; Silveira et al., 2005).

Early weaning reduces the expression of 5HT-1B receptor in the hippocampus (Nakamura et al., 2008). This receptor mediates the inhibitory effect of serotonin on food intake and ingestive behavior (Dourish, 1995; Samanin and Garattini, 1996; Simansky, 1996). Administration of an agonist of this receptor reduces palatable food intake (Lee et al., 2002). The repercussions of early weaning on the level of this receptor in the hypothalamus are not known, but the increased palatable food intake suggests the possibility of reduced levels of this receptor.

Early weaning alters the formation of myelin in the brain during development, particularly between the third and fifth week of life in male mice, and it reduces encephalic weigh at 8 weeks of age (Kikusui et al., 2007). There is an association between a deficit of myelin and increased anxiety behaviors (Ono et al., 2008). The increases in the preference for fat and the palatable diet provoked by early weaning observed in this study are in accordance with the finding that increased sweet food intake is provoked by stress (Ely et al., 1997). It has been proposed that foods with high energy density attenuate the negative effects associated with chronic stress (Dallman et al., 2003).

In conclusion, early weaning, although it does not modify body weight or basal food intake, promotes an increased preference for palatable and fatty foods. This demonstrates that early weaning is not capable of promoting alterations to alimentary behavior perceptible under normal laboratory conditions. However, in the presence of a stimulatory factor, such as the choice of nutrients or the presence of a palatable diet, a possible latent effect may become apparent. In the long term, this preference for foods with high caloric density can lead to obesity and metabolic perturbations.

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Table 1. Composition of experimental diets utilized in the alimentary preference study.

Component	Protein diet	Fat diet	Carbohydrate diet
Casein	93.5%	-	-
Shortening	-	77%	-
Salad oil	-	10%	-
α-Starch	-	-	62.3%
Sucrose	-	-	31.2%
Mineral mixture	3.5%	7%	3.5%
Vitamin mixture	1%	2%	1%
Cellulose	2%	4%	2%

Source: (Tanaka and Kido, 2008)

Table 2: Composition of macronutrients in the palatable diet

Component	Content/100 g
Protein	12.3g
Carbohydrate	49g
Fat	17.5g
Energy	402 5 kcal

Figure 1

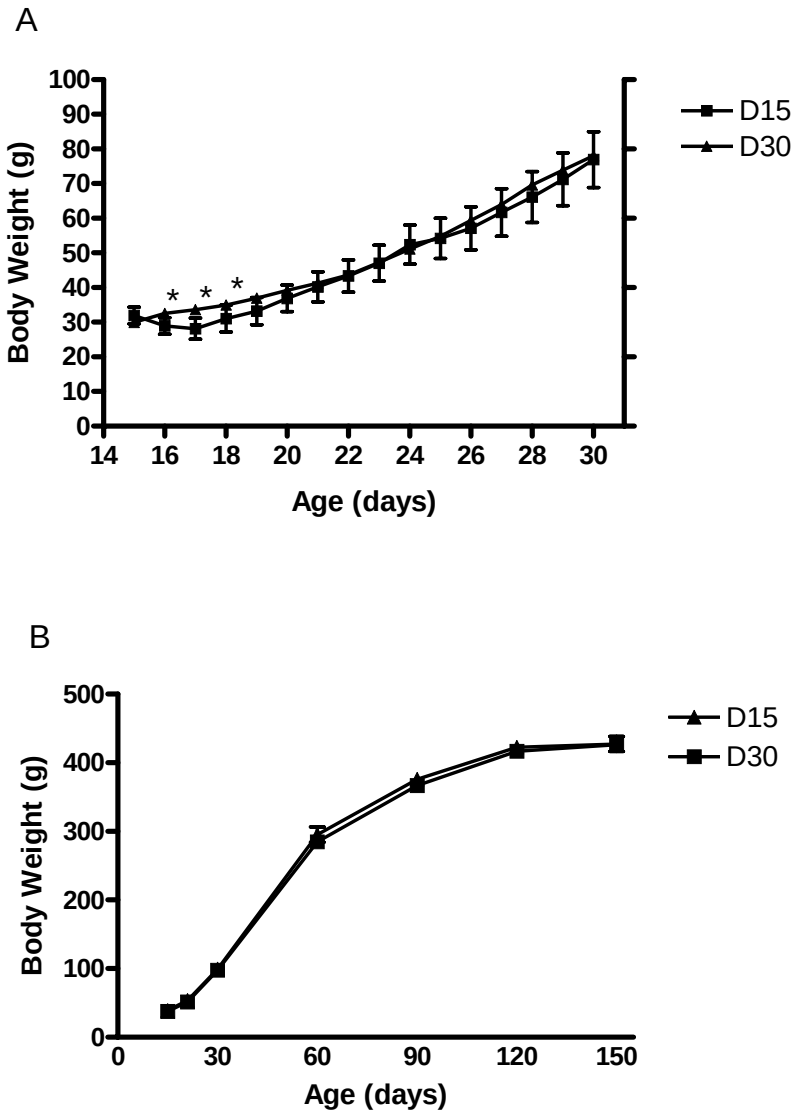

Figure 1. Effect of early weaning on body weight in young rats (A) until adulthood (B). Wistar rats were weaned at 15 (D15) or 30 (D30) days old. The data are expressed as mean ± S.E.M. *, $p<0.05$ between the D15 and D30 groups (Student's t test).

Figure 2.

A

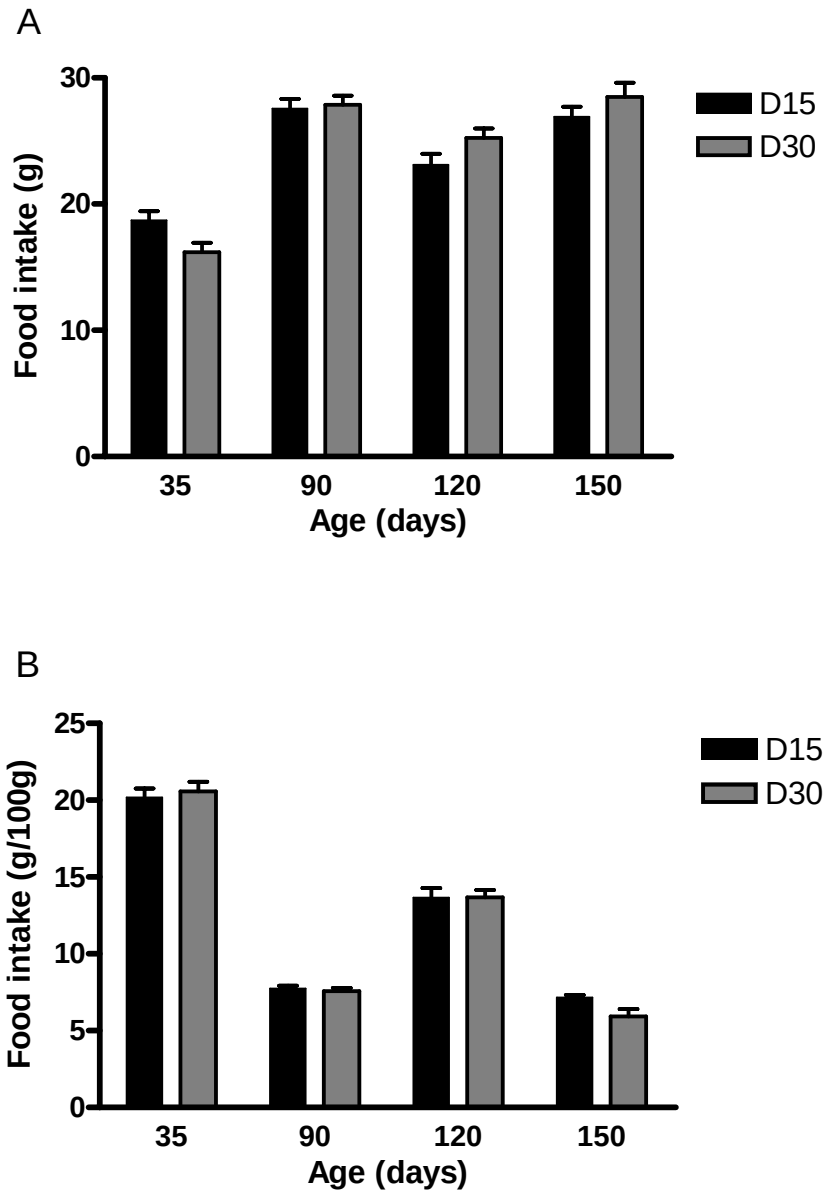

Figure 2. Effect of early weaning on absolute (g) (A) and relative (g/100 g of body weight) (B) in rats at days 35, 90, 120 and 150. Wistar rats were weaned at 15 (D15) or 30 (D30) days old. The data are expressed as mean ± S.E.M. There was no difference between the groups (Student's t test).

Figure 3.

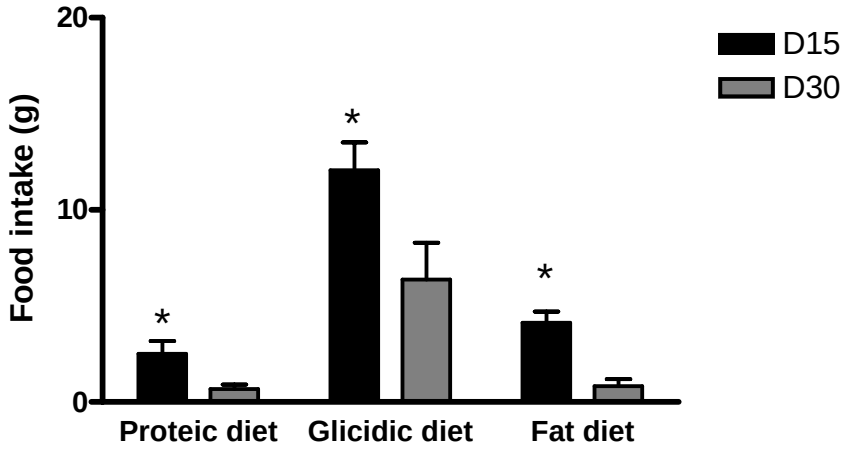

Figure 3. Effect of early weaning on the consumption of protein, carbohydrate and fat in adult rats. Wistar rats were weaned at 15 (D15) or 30 (D30) days old. At day 110, the animals were submitted to the 1-hour macronutrient preference test (protein, carbohydrate and fat) after 6 hours of fasting. The data are expressed as mean ± S.E.M. *, $p<0.05$ between the D15 and D30 groups (Student's t test).

Figure 4.

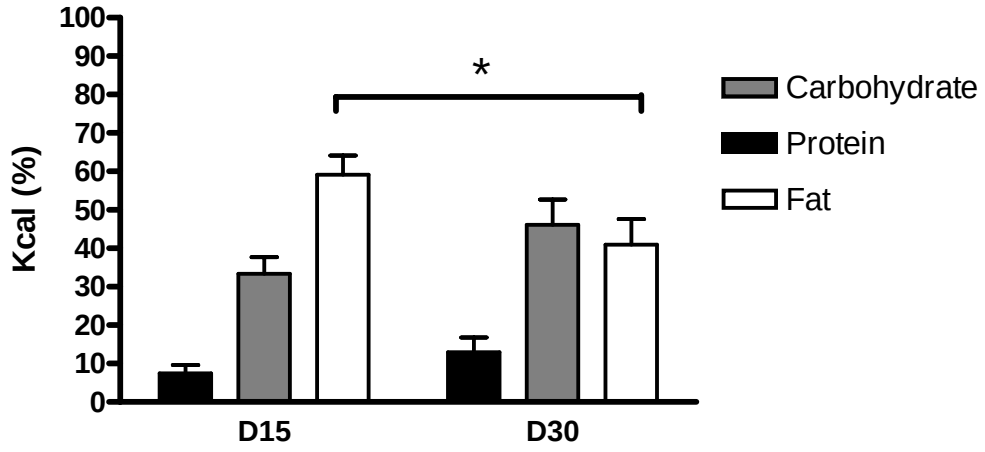

Figure 4. Effect of early weaning on caloric intake from protein, carbohydrate and fat in adult rats. Wistar rats were weaned at 15 (D15) or 30 (D30) days old. At day 110, the animals were submitted to the 1-hour macronutrient preference test (protein, carbohydrate and fat) after 6 hours of fasting. The data are expressed as mean ± S.E.M. *, $p<0.05$ between the D15 and D30 groups (Student's t test).

Figure 5.

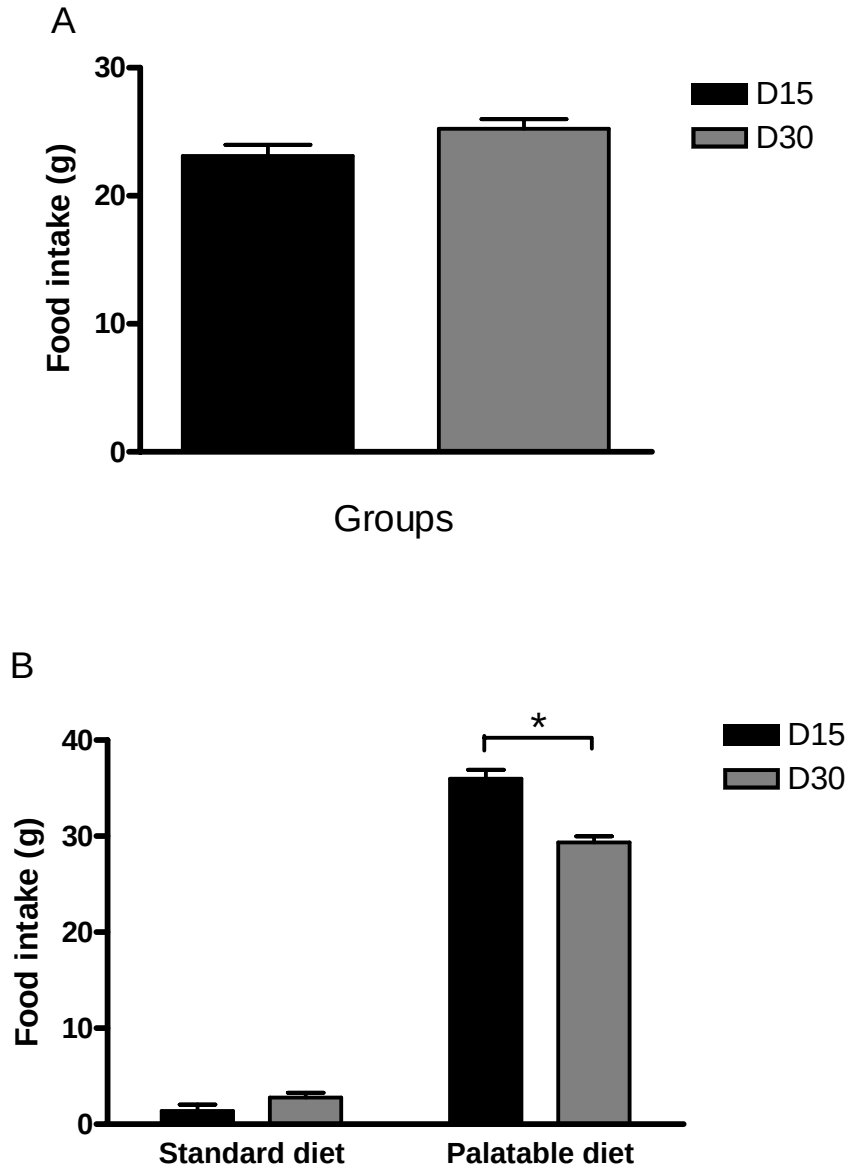

Figure 5. Effect of early weaning on standard diet intake before the palatable diet test (A) and on palatable diet intake during the test (B) in adult rats. Wistar rats were weaned at 15 (D15) or 30 (D30) days old. At day 120, the animals were submitted to the palatable diet test over 3 consecutive days. The data are expressed as mean ± S.E.M. *, $p<0.05$ between the D15 and D30 groups (Student's t test).

Figure 6.

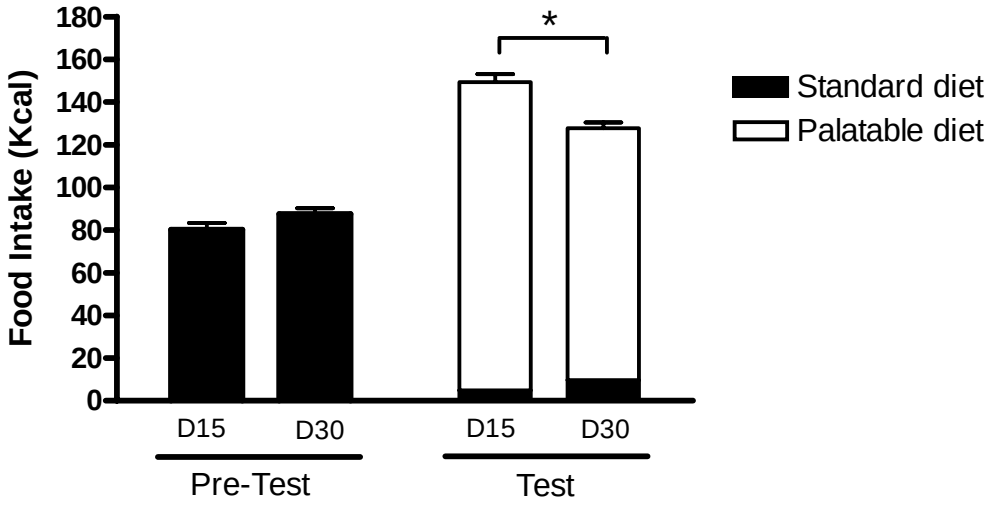

Figure 6. Effect of early weaning on caloric intake before and during the palatable diet test. Wistar rats were weaned at 15 (D15) or 30 (D30) days old. At day 120, the animals were submitted to the palatable diet test over 3 consecutive days. The data are expressed as mean ± S.E.M. *, $p<0.05$ between the D15 and D30 groups (Student's t test).

CONSIDERAÇÕES FINAIS

Esse trabalho demonstrou que o desmame precoce é capaz de alterar a programação do comportamento alimentar promovendo atraso no disparo da saciedade, alteração do ritmo circadiano do consumo alimentar e aumento do apetite por alimentos palatáveis e com alta densidade calórica. Esses resultados confirmaram todas as hipóteses iniciais com exceção do peso corporal que se manteve sem alterações até a vida adulta. Apesar do desmame precoce não alterar o peso corporal e o consumo alimentar basal, a presença de fatores estimulantes, como o jejum, a possibilidade de escolha entre carboidratos, proteínas e gorduras ou a disponibilidade de alimentos palatáveis, elevaram a ingestão calórica. Dessa forma, acredita-se que a presença contínua desses fatores poderia causar um desequilíbrio metabólico levando ao sobrepeso.

Apesar de alguns estudos utilizarem o modelo experimental do desmame precoce, as repercussões do desmame precoce sobre o comportamento alimentar ainda não haviam sido exploradas. No entanto ainda são necessários mais estudos relacionados às alterações hormonais e estruturais que ajudem a explicar às alterações relacionadas a esse comportamento.

A preocupação com os efeitos deletérios do desmame precoce é manifestada pelas políticas de saúde pública. Entre os vários fatores identificados na população que levam ao desmame precoce destacam-se os condicionantes sociais, econômicos e culturais. A constatação de efeitos possivelmente prejudiciais provocados pelo desmame precoce em estudos experimentais servem de base para as pesquisas epidemiológicas e clinicas e fortalecem as campanhas de incentivo ao aleitamento materno.

Diante dos resultados obtidos é possível delinear as seguintes perspectivas: estudar alterações hormonais e de estruturas relacionadas ao controle do comportamento

alimentar provocadas pela manipulação do período de desmame e investigar possíveis mudanças epigenéticas promovidas pelo desmame precoce, capazes de promover alterações metabólicas e/ou comportamentais em longo prazo.

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ANEXOS

Anexo A

YAHOO! MAIL BRASIL Classic

Acusa_recebimento_ protocolo_2178_Revista_de_Nutrição Quinta-feira, 22 de Abril de 2010 9:48

De: "Revistas CCV" <ccv.revistas@puc-campinas.edu.br>
Para: lisianenutricao@yahoo.com.br
Cc: mcmatoso@puc-campinas.edu.br

Campinas, 22 de abril de 2010 **CA/NE/RN nº 468/2010**

Ilustríssima Senhora

Venho pelo presente agradecer o envio do manuscrito **Behavioral Satelity sequence: a experimental...** para publicação na Revista de Nutrição. Para toda a correspondência futura relativa a este trabalho, por favor, refira-se ao protocolo número **2178.**

O Conselho Editorial procederá a tramitação regular do processo de aprovação do manuscrito, entendendo que o mesmo não foi publicado anteriormente e que não será submetido a outro periódico durante o período de revisão.

Tão logo quanto possível, V.Sa será notificado(a) a respeito do processo para consideração de eventuais sugestões dos revisores ou sobre a aprovação do trabalho.

Informamos que a partir da submissão do manuscrito aos revisores técnicos, serão aceitas somente duas novas versões a partir do original.

Novamente grata por seu interesse na Revista de Nutrição, expresso nossas saudações.

É com muita alegria que comunicamos a indexação da Revista de Nutrição na base Scopus e Web of Science!

Atenciosamente

Maria Cristina Matoso

Revista de Nutrição
Editora Gerente

Ilustríssima Senhora
Profa. Lisiane dos Santos Oliveira

Atenciosamente

Anexo B

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

Av. Prof. Nelson Chaves, s/n
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Recife, 27 de agosto de 2008

Ofício nº 54/08

Da Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Prof$_o$. Raul Manhaes de Castro**
Departamento de Nutrição – CCS
Universidade Federal de Pernambuco
Processo nº 23076. 005299/2008 - 76

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram seu projeto de pesquisa intitulado ***"Desmame precoce: Efeitos sobre o comportamento alimentar em ratos jovens e adultos".***

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEEA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais realizados.

Atenciosamente,

Profa. Maria Teresa Jansem
Presidente do CEEA

Observação:
Origem dos animais: Biotério do Departamento de Nutrição
Animais: Ratos; Wistar; machos; com 1 - 150 dias de vida
Número de animais previsto no protocolo: 48 animais
Número de animais previsto por grupo: 16 animais

Anexo C

Elsevier Editorial System(tm) for Behavioural Processes
Manuscript Draft

Manuscript Number:

Title: Effects of early weaning on the circadian rhythm and behavioral satiety sequence in rats

Article Type: Research Paper

Keywords: Behavioral satiety sequence; Circadian rhythm; Early weaning; Feeding behavior.

Corresponding Author: Sra Lisiane dos Santos Oliveira, M.D.

Corresponding Author's Institution: Universidade Federal de Pernambuco

First Author: Lisiane dos Santos Oliveira, M.D.

Order of Authors: Lisiane dos Santos Oliveira, M.D.; Sandra Lopes-de-Souza; Raul Manhães-de-Castro

Abstract: The objective of this work was to study the effect of early weaning on circadian rhythm and the behavioral satiety sequence in adult rats. Male Wistar rat pups were weaned for separation from the mother at 15 (D15), 21 (D21) and 30 (D30) days old. Body weight and food intake was measured every 30 days until pups were 150 days old. At 90 days of age, the circadian rhythm of food intake was evaluated every 4 hours for three days. Behavioral satiety was evaluated at 35 and 100 days of age. This work demonstrated that body weight and food intake were not altered, but the behavioral satiety sequence demonstrated that the D15 group delayed satiety compared with the D30 group at 100 days of age. In the circadian rhythm of the food intake study, early weaning (D15) changed food intake in the intermediary period of the light phase and in the intermediary period of the dark phase. In conclusion, our study showed that early weaning may alter the feeding behavior mainly in relation to satiety and the circadian rhythm of feeding. It is possible that the presence of other environmental stimuli during early weaning can cause hyperphagia and deregulate the mechanisms of homeostasis and body weight control. This study supports theories that depict insults during early life as determinants of chronic diseases and can be the basis for strategies to encourage breastfeeding.

Suggested Reviewers:

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Submission Confirmation Quinta-feira, 22 de Abril de 2010 11:12

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Anexo D

Elsevier Editorial System(tm) for Behavioural Processes
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Manuscript Number:

Title: Early weaning programs rats to have a dietary preference for fat and palatable foods in adulthood

Article Type: Research Paper

Keywords: Early weaning, Feeding behavior, Macronutrients preference, Palatable food

Corresponding Author: Sra Lisiane dos Santos Oliveira, M.D.

Corresponding Author's Institution: Universidade Federal de Pernambuco

First Author: Lisiane dos Santos Oliveira, M.D.

Order of Authors: Lisiane dos Santos Oliveira, M.D.; Sandra Lopes-de-Souza

Abstract: The objective of this work was to study the effect of early weaning on alimentary preference to the macronutrients protein, carbohydrate and fat in adult rats. Male Wistar rat pups were weaned by separation from the mother at 15 (D15) or 30 (D30) days old. Body weight and food intake were measured every 30 days until pups were 150 days old. At 110 days of age, the alimentary preference was evaluated for 1 hour on 3 consecutive days. At 120 days of age, the palatable diet test was conducted during 3 consecutive 24-hour periods. Body weight and food intake were not altered, but early weaning in rats induced an alimentary preference to fat and hyperphagia of a palatable diet. In conclusion, early weaning, although it did not modify body weight or basal food intake, promoted an increased preference for palatable and fatty foods. This demonstrates that early weaning is not capable of promoting perceptible alterations of alimentary behavior under normal laboratory conditions. However, in the presence of a stimulating factor such as a choice of nutrients or a palatable diet, a possible latent effect on dietary preferences may become apparent. Over the long term, this preference for foods with high caloric density can lead to obesity and metabolic perturbations.

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