PHEROMONES FROM MALE DOMESTIC MICE INFLUENCES THE SEXUAL FUNCTION AND GONADAL STEROIDS LEVELS OF FEMALE LABORATORY RATS

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SILVA, G. R. da; PALOZI, R. A. C.; BARBOZA, L. N.; PRANDO, T. B. L.; GASPAROTTO, F. M.; LOURENÇO, E. L. B.; GASPAROTTO JUNIOR, A. Pherormones from male domestic mice influences the sexual function and gonadal steroids levels of female laboratory rats. **Arq. Ciênc. Vet. Zool. UNIPAR**, Umuarama, v. 18, n. 2, p. 95-101-, abr./jun. 2015.

ABSTRACT: It is well established that the behavior of different mammalians, including rodents, may undergo profound changes in the presence of individuals of other species. Therefore, the aim of this study was to verify if the presence of pheromones from male mice could influence the reproductive parameters of female rats. Twenty Swiss (SW) male mice and 16 Long Evans (LE) female Rats were housed in separate rooms, with communication in the ceiling in its entire length. A continuous flow of air was planned to pass through the room of the mice before the room of the rats and finally be exchanged with the external environment. During 45 days, vaginal smears were collected. Relative weights of reproductive organs, estradiol, progesterone, and dehydroepiandrosterone levels were also measured. The total duration of the estrous cycle and relative organ weight remained unchanged. On the other hand, the proestrus and estrus phases, as well as the estradiol levels were increased, while the diestrus phase was significantly reduced. The results have shown that maintaining LE female rats in the presence of pheromones from male SW mice can significantly change the sexual function and gonadal steroid levels.
KEYWORDS: Estradiol. Estrous cycle. Ovulatory cycle. Pheromones. Rodents.

FEROMÔNIOS DE INFLUÊNCIAS MASCULINOS NOS RATOS DOMÉSTICOS A FUNÇÃO E OS NÍVEIS DE ESTERÓIDES SEXUAIS DOS RATOS DE LABORATÓRIO FÊMEAS

RESUMO: É bem conhecido que o comportamento de diferentes mamíferos, incluindo roedores podem sofrer alterações profundas na presença de indivíduos de outras espécies. Portanto, estudamos se a presença de feromônios de camundongos machos poderiam influenciar os parâmetros reprodutivos de ratas. Vinte suíços (SW) ratos do sexo masculino e 16 Long Evans (LE) ratas foram colocadas em locais distintos, com a comunicação no teto em toda a sua extensão. Um fluxo contínuo de ar foi planejado para passar através da sala de ratinhos antes da sala de ratos e, finalmente, ser trocado com o ambiente externo. Durante 45 dias, esfregaços vaginais foram recolhidas. Os pesos relativos dos órgãos reprodutores, o estradiol, progesterona e níveis de dehidroepiandrosterona também foram medidos. A duração total do ciclo estral e peso de órgãos em relação, manteve-se inalterada. Por outro lado, as fases de proestro e estro, bem como os níveis de estradiol foram aumentadas, enquanto que a fase diestro foi reduzida significativamente. Os resultados mostraram que a manutenção de ratas LE na presença de feromônios de camundongos SW do sexo masculino pode alterar significativamente a função sexual e os níveis de esteróides sexuais.
PALAVRAS-CHAVE: Estradiol. Ciclo estral. Ciclo ovulatório. Feromônios. Roedores.

FEROMONAS DE INFLUENCIAS MASCULINOS EN RATONES DOMÉSTICOS LA FUNCIÓN Y LOS NIVELES DE ESTEROIDES SEXUALES EN RATAS HEMBRAS DE LABORATORIO

RESUMEN: Es bien sabido que el comportamiento de diferentes mamíferos incluyendo roedores pueden sufrir cambios profundos en la presencia de individuos de otras especies. Por lo tanto, se estudió si la presencia de feromonas de ratones machos podrían influir en los parámetros reproductivos de las ratas hembra. Veinte suizos (SW) ratones machos y 16 Long Evans (LE) ratas hembras, fueron alojados en habitaciones separadas, con la comunicación en el techo en toda su extensión. Se planificó un flujo continuo de aire para pasar a través de la habitación de los ratones antes de la habitación de las ratas y, finalmente, ser intercambiado con el ambiente externo. Durante 45 días se recogieron frotes vaginales. También se midieron los pesos relativos de los órganos reproductivos, estradiol, progesterona, y los niveles de dehidroepiandrosterona. La duración total del ciclo estral y el peso relativo de órganos se mantuvo sin cambios. Por otro lado, las fases de proestro y de estro, así como los niveles de estradiol aumentaron, mientras que la fase de diestro redujo significativamente. Los resultados han

DOI: https://doi.org/10.25110/arqvet.v18i2.2015.5379
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demostrado que el mantenimiento de LE en ratas hembras, con presencia de feromonas de ratones SW macho puede cambiar significativamente la función sexual y los niveles de esteroides sexuales.
PALABRAS CLAVE: Estradiol. Ciclo estral. Ciclo ovulatorio. Feromonas. Roedores.

Introduction

It is well established that the behavior of different mammalians including rodents may undergo profound changes in the presence of others individuals (LI.; LIBERLES, 2015). In rodents, particularly domestic mouse (*Mus musculus*) and laboratory rats (*Rattus norvegicus*), these behavior changes and physiological aspects are quite evident when different species are kept in the same environment (HARKNESS; WAGNER, 1995). This is due to the presence of pheromones that can influence the sexual behavior of different species kept in the same physical environment. Furthermore, many data suggest that pheromones may stimulate the gonadotropic function of the pituitary gland in rodents, changing the duration of the estrous cycle and the secretion of several hormones (MAFFATT, 2003; WACKER; LUDWIG, 2012; SCHANK; ALBERTS, 2000).

Female rodents are poliestric, have spontaneous ovulation and regular and successive estrous cycles that may vary with age and species. These cycles are also influenced by light, seasons of the year and life circumstances. On the other hand, estrous cycles occur without seasonal influence in rodents submitted to environmental control under laboratory conditions (MAEDA; MURR; COOPER, 2000; GOLDMAN; MURR; COOPER, 2007).

Evidence of the influence of male and female pheromones on the sexual function and gonadal steroids levels is less conclusive in rats than in many other rodents. Rats are generally much less sensitive than mice and under normal laboratory conditions, there is little evidence of the synchronization of cycles with cage mates, which would normally be apparent in females of many other mammalian species living in close proximity. Similarly, there is no clear evidence of cycle changes after pairing, as seen in the “Whitten effect” in mice, although it has been reported that regular cycles are better maintained in the long term in female rats if males are kept in the same room (MAEDA; MURR; COOPER, 2000; LOHMILLER; SWING, 2006).

Detailed knowledge of the estrous cycle and gonadal steroids levels in laboratory rats are very important for several pharmacological studies and/or reproductive toxicity evaluation. When incorporated with other measures, the determination of the cycling status of the rats can provide important information about the nature of the agent that cause damage to the reproductive system. Thus, it could help integrating the date into a more comprehensive mechanistic portrait of the effect, and in terms of risk assessment, it may provide some indication of the toxicant capacity to Influence reproductive physiology of humans (GOLDMAN; MURR; COOPER, 2007). In this sense, knowing the reproductive parameters of female laboratory rats is essential to guarantee reproducibility and safety of results.

Thus, the aim of the present study was to determine whether the presence of male domestic mice, partially separated by a physical barrier, could directly influence the sexual function and gonadal steroids levels of female laboratory rats.

Material and Methods

Animals

Maintenance and use of animals were in accordance with the Brazilian Animal Welfare Legislation. All experimental procedures adopted in this study were previously approved by the Institutional Ethics Committee of the “Universidade Paranaense” (UNIPAR, Brazil; authorization number 22932/2012). Twenty Long Evans (LE) female rats were purchased from Universidade Estadual Paulista "Júlio de Mesquita Filho", Araraquara, Brazil, and 20 Swiss (SW) male mice were purchased from “Universidade Federal do Paraná”, Curitiba, Brazil at the age of 16 weeks. The animals were allowed to adapt to the new conditions for two weeks. At the research vivarium of the “Universidade Paranaense”, 4 animals per cage were kept under standard conditions (mean temperature of 22 ± 2°C, 50 ± 20% relative humidity, 12:12 h light–dark cycle) on sterilized aspen wood granulate bedding. Animals received commercial pelletized diet (Nuvilab CR1, Curitiba, Brazil) and water *ad libitum*. The animals had been tested according to the FELASA recommendations (NICKLAS et al., 2002) and were free of the listed micro-organisms.

Experimental procedure

Before the beginning of the experiment, blood samples were collected via tail vein to assess the different biochemical and hematological parameters as health indicators. One week after this procedure, the animals were housed in separate rooms (4 m^2 each), with communication on the ceiling (opening of 30 cm) in its entire length. A continuous flow of air was planned to pass through the room of mice before the room of rats and finally be exchanged with the external environment. A 12:12 h light-dark photoperiod was controlled by time switches. The temperature was daily recorded and varied from 22°C to 25°C. Two LE female rats were housed per cage (to prevent Lee-Boot effect), and 2 weeks prior to use, all animals underwent daily vaginal smears to evaluate cycle stage based on cytology (control). Although 20% of LE female rats did not show regular cycle, only rats that had completed two consecutive estrous cycles were used in the following studies.

During 45 days, to assess the progression of the estrous cycle, vaginal smears were performed twice daily, one in the morning (9:00 am) and one in the afternoon (05:00 pm). Considering the short time span of the estrous phases, daily collections were performed with an interval of 8 h to obtain a more precise classification of each phase. Vaginal secretion was collected with a plastic pipette filled with 10 µL of normal saline (0.9% NaCl) by inserting the tip into the vagina, but not deeply. Vaginal fluid was placed on glass slides. A different glass slide was used for each cage of animals. One drop was collected with a clean tip from each rat. Unstained material was observed under light microscope without the use of condenser lens, with 10 and 40 x objective lenses.

Three types of cells could be recognized: epithelial cells are round and nucleated; cornified cells are irregular and without nucleus; and leukocytes are little round cells. The proportion among them was used for the determination of the estrous cycle phases (WESTWOOD, 2008). Only one slide was used for each cage. At the end of experimental period, rats were euthanized by decapitation and autopsied. The reproductive tract, liver and adrenals were removed through laparotomy and weighed. The relative organ weight of each animal was calculated as follows. Relative organ weight: (absolute organ weight × 100%)/ body weight of rats on the day of sacrifice. For statistical comparison of the relative weight of target organs, another group of LE female rats not exposed to pheromones from SW male mice was used. Additionally, serum dehydroepiandrosterone (DHEA), estradiol and progesterone levels were measured using a microparticle enzyme immunoassay (AxSYM DHEA, estradiol and progesterone assay). Kits were purchased from Abbott laboratories (Abbott Park, IL, USA).

Results

Indicator of health and welfare in laboratory female rats

When compared with published data, significant alterations in hematological parameters and erythrocyte indices could not be observed. Biochemical profile in relation to analytes and enzymes measured showed no discrepancy in relation to data previously published (MAEDA; MURR; COOPER, 2000; LOHMILLER; SWING, 2006) (Table 1 and 2)**.**

Table 1: hematological data for LE female rats before exposure to SW male mice pheromones

Parameters	Values
RBC (106/mL)	7.32 ± 0.24
Hemoglobin (g/mL)	15 ± 0.15
Hematocrit (%)	47 ± 1.50
MCV (fl)	64 ± 3.16
MCH (pg)	20 ± 0.11
MCHC (%)	32 ± 0.82
Platelets (103/mm3)	710 ± 43
WBC (103/mm3)	7.27 ± 0.43
Leucocytes (103/mm3)	7.12 ± 0.61
Neutrophils (103/mm3)	1.59 ± 0.15
Lymphocytes (103/mm3)	5.15 ± 0.37
Monocytes (103/mm3)	0.14 ± 0.02
Eosinophilis (103/mm3)	0.17 ± 0.03

Values are expressed as mean ± S. E. M. of sixteen rats.

Table 2: Additional biochemical data for LE female rats before exposure to SW male mice pheromones

Parameters	Values
Glucose (mg/dL)	125 ± 7
Total cholesterol (mg/dL)	137 ± 18
Triglycerides (mg/dL)	347 ± 139
Urea (mg/dL)	32 ± 0.82
Creatinine (mg/dL)	0.51 ± 0.01
Total protein (g/dL)	6.64 ± 0.31
Albumin (g/dL)	4.74 ± 0.33
Globulin (g/dL)	1.89 ± 0.23
Total bilirubin (mg/dL)	0.17 ± 0.01
Direct bilirubin (mg/dL)	0.10 ± 0.01
Indirect bilirubin (mg/dL)	0.07 ± 0.01

Values are expressed as mean ± S. E. M. of sixteen rats.

Pheromones from male mice can affect estrous cycle of female rats

The duration of each phase of the estrous cycle of female rats at the beginning of the experimental period and after 45 days of exposure to pheromones from SW mice is shown in Table 3. Moreover, Figure 1 shows the total duration of the estrous cycle (A) and the dispersion between animals (B) after 45 days of exposure to pheromones of male mice. The total duration of the estrous cycle was approximately 4.9 days with an average growth of 2.1 days between different animals, showing no statistically significant difference from data obtained at the beginning of the experimental period.

The proestrus phase (Figure 2A-C and Table 3), characterized by the large number of epithelial cells, had an average duration of approximately 32.8 hours (A), with standard error of approximately 3 hours (control: 12 ± 1.54 hours; $p<0.05$). Some animals showed significant internal variation at this stage of the cycle, with a variation that reached 45.3 hours among animals with the greatest discrepancy (Figure 2B).

Figure 2 and Table 3 illustrates the duration of the estrus phase (2D) and the internal variation among animals (2E). It was observed that estrus phase had an average duration of 35.5 hours with standard error of approximately of 3.3 hours (control: 12 ± 2.20 hours; $p<0.05$). At this stage, primarily cornified cells are observed (2F).

The metaestrus (Figure 3A-C and Table 3) had an average duration of approximately 27 hours, with standard error of approximately 2.1 hours (control: 21 ± 4.06 hours). At metaestrus, the presence of epithelial cells, leukocytes and some cornified cells can be observed (3C).

The diestrus phase (Figure 3D-F and Table 3) was identified as the shortest stage of the estrous cycle, with an average duration of approximately 22.9 hours and standard error of approximately 4.3 hours (control: 56 ± 8.12 hours; $p<0.05$). It was also observed that some animals showed significant variation at this stage of the cycle, with internal

variation that reached 55.9 hours among animals with the greatest discrepancy. This phase was characterized by the presence of large amounts of leukocytes.

Pheromones from male mice influences the gonadal steroids levels of female rats

The serum DHEA, estradiol and progesterone levels of LE female rats at the beginning of the experimental period and after 45 days of exposure to pheromones from SW male mice are shown in Table 4. The serum estradiol values of LE female rats were significantly increased after 45 days of exposure to pheromones from SW male mice, with an increase of approximately 40%. DHEA and progesterone levels were not affected by exposure.

Final body weights and relative organ weight of female rats was not affected by pheromones from male mice

Final body weights and organ weights were measured at the end of the experimental period in all animals. No statistically significant differences were observed in absolute (g) and relative weights (%) of almost all isolated organs from the beginning of the experimental period and after 45 days of exposure (Table 5).

Figure 1. Effects of pheromones from SW male mice on estrous cycle of LE female rats. Results are expressed as mean ± standard error of the mean (A) and dispersion (B) of 16 animals.

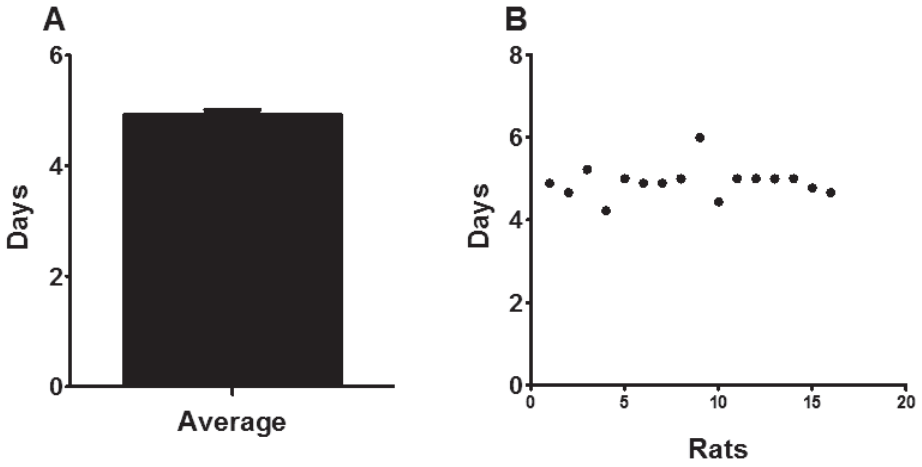

Figure 2. Effects of pheromones from SW male mice on proestrus (A-C) and estrus (D-F) phases of LE female rats. Results are expressed as mean ± standard error of the mean (A) and dispersion (B) of 16 animals.

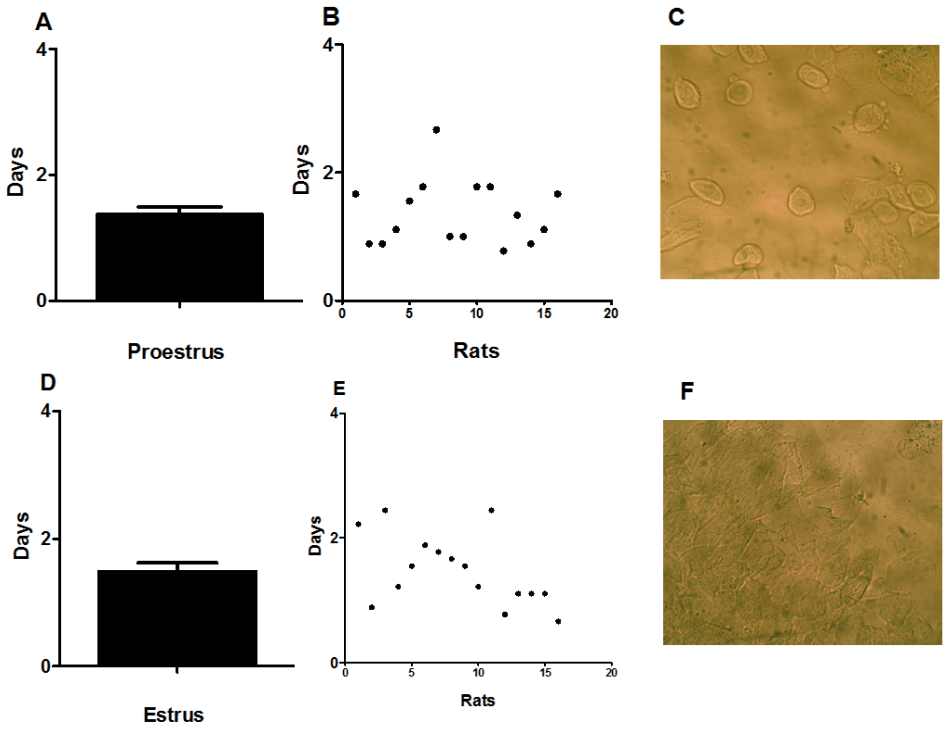

Figure 3: Effects of pheromones from SW male mice on metaestrus (A-C) and diestrus (D-F) phases of LE female rats. Results are expressed as mean ± standard error of the mean (A) and dispersion (B) of 16 animals.

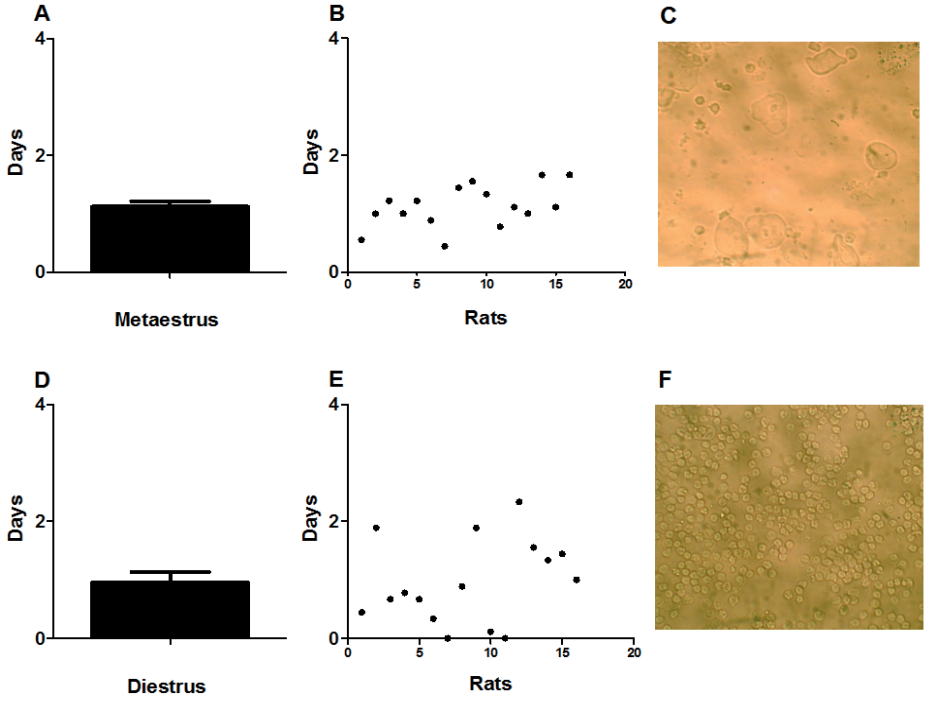

Table 3: Effects of exposure to SW male mice pheromones on the estrous cycle of LE female rats.

Phase of the cycle	Rats	
	Outset	After 45 days of exposure
Proestrus (h)	12.2 ± 1.54	32.8 ± 2.88[a]
Estrus (h)	13.3 ± 2.20	35.3 ± 3.36[a]
Metaestrus (h)	21.6 ± 4.06	26.8 ± 2.16
Diestrus (h)	56.9 ± 8.12	22.9 ± 4.32[a]
Total cycle (days)	4.3 ± 0.22	4.9 ± 0.35

Values are expressed as mean ± S. E. M. of sixteen rats in each group in comparison to the control (outset) using Student's t-test ([a]p<0.05).

Table 4: Effects of exposure to SW male mice pheromones on the estradiol, progesterone and DHEA levels of LE female rats

Organ	Rats	
	Outset	After 45 days of exposure
Estradiol (pg/mL)	35.1 ± 5.44	50.2 ± 4.23a
Progesterone (ng/mL)	20.1 ± 5.14	16.1 ± 5.14
DHEAa (ng/mL)	0.90 ± 0.19	0.75 ± 0.23

Values are expressed as mean ± S. E. M. of sixteen rats in each group in comparison to the control (outset) using Student's t-test ([a]p<0.05).

Table 5: Effects of exposure to SW male mice pheromones on the relative organs weight of LE female rats

Organ	Rats	
	Control	After 45 days of exposure
Uterus (%)	0,12 ± 0,01	0,13 ± 0,01
Ovaries (%)	0,02 ± 0,01	0,02 ± 0,01
Liver (%)	3,13 ± 0,07	3,33 ± 0,09
Adrenal (%)	0,02 ± 0,01	0,02 ± 0,01

Values are expressed as mean ± S. E. M. of sixteen rats rats in each group in comparison to the control using Student's t-test.

Discussion

Social cognition for many mammals, including rodents, begins at the main and accessory olfactory systems, where pheromones play a central role. Pheromones are chemical substances that through olfactory stimuli can play an important role in communication of different species (WACKER; LUDWIG, 2012; BUCK, 2000). In general, rodents can secrete two types of pheromones, signaling and priming. Priming pheromones include estrus-inducer, estrus-inhibitor, and adrenocortical activator, and can affect the estrous cycle of female mice or other rodents. At least three effects from pheromones are observed in mice, the Bruce effect, the Lee-Boot effect, and the Whitten effect. The Bruce effect occurs when the pheromones from a strange male prevent embryo implantation in females. The Whitten effect is characterized by synchronization of estrus in females following the introduction of a male. On the other hand, anestrous induction in some members of a group of females housed together is known as the Lee-Boot effect (HARKNESS; WAGNER, 1995; MAFFATT, 2003; WACKER; LUDWIG, 2012; SCHANK;

ALBERTS, 2000; MAEDA; MURR; COOPER, 2000; GOLDMAN; MURR; COOPER, 2007).

Although pheromones play an important role in the sexual behavior of mice, the role of male and female pheromones on the estrous cycle of rats is less conclusive. Rats are generally much less sensitive than mice, and under normal laboratory conditions, there is little evidence of the synchronization of cycles with cage mates. Additionally, there is no clear evidence of cycle changes after pairing, as observed in the "Whitten effect" in mice, although it has been reported that regular cycles are better maintained in the long term in female rats if males are kept in the same research vivarium (MAEDA; MURR; COOPER, 2000; LOHMILLER; SWING, 2006; KIYOKAWA et al., 2004).

In fact, researchers frequently maintain a research vivarium attached to their experimental facilities. Although practical, these environments do not have always fully controlled conditions of micro and macro environmental factors. Thus, micro and macro environmental conditions, especially the presence of males of other species or strains can lead to significant physiological and/or behavioral changes (BEYNON; HURST, 2004; LARSEN; KOKAY; GRATTAN, 2008; POHORECKY et al, 2008). It has been well established that the estrous cycle is light sensitive, and constant light results in persistent estrus and polycystic ovaries (KLÖTING et al, 2013). Nevertheless, there are few studies that investigate the influence of the presence of strange males of other rodent species on the estrous cycle of rats. The results obtained in this study show for the first time that the prolonged permanence of SW male mice near by LE female rats in research vivarium could directly influence the hormone secretion and duration of the different phases of the estrous cycle.

The estrous cycles of rats are characterized by morphological changes in ovaries, uterus and vagina that occur during different phases called proestrus, estrus, metaestrus and diestrus. The stages of the vaginal cell cycle will normally correlate with changes in the sexual hormone secretions and female reproductive organs, e.g. ovary and uterus. The proestrus and estrus phases of female rats last for 12 h each, while the metaestrus phase lasts for 21 h and the diestrus phase lasts for 57 h ((MAEDA; MURR; COOPER, 2000; LOHMILLER; SWING, 2006). In our study, before exposure to mice pheromones, these values are within normal levels. However, after exposure, the proestrus and estrus phases were increased and the diestrous phase was significantly shorter. Encouraged by these results, we measure estradiol, progesterone and DHEA levels in all LE female rats after 45 days of exposure. Our results showed that estrogen concentration was significantly increased, with a direct correlation between estradiol levels and proestrus/estrus cycle phases. Several studies have shown that estradiol levels can regulate the estradiol secretion by gonads. In the proestrus e estrus phases, a progressive increase in the estradiol level may occur, inducing sexual receptivity and ovulation (GOLDMAN; MURR; COOPER, 2007).

Based on these results, a question can be raised: can pheromones from SW male mice influence the sexual behavior of LE female rats? It is known that the odors have great importance since it is through innate smell of each species that animals of the same group recognize each other, mainly in the sexual behavior of mice. In mice and others rodents the preputial glands are one of the major sources of pheromones. These volatile chemo signaling compounds are known to elicit specific behavioral and physiological effects in their conspecifics (MAFFATT, 2003; POHORECKY et al, 2008). When male urine was added to female nares or female mice caged downwind from male mice, they developed synchronized estrus cycling, initiate prolactin-induced neurogenesis, and advance maternal behavior (SCHANK; ALBERTS, 2000; LARSEN; KOKAY; GRATTAN, 2008). Moreover, exposure of a male mouse to a female mouse separated from it by a holed partition induced specific behavior and an increase in blood testosterone in the male (AMSTISLAVSKAYA; POPOVA, 2004).

As can be seen, pheromones can be a powerful stimulus for behavior and must be considered when breeding mice or other rodents. It is noteworthy that in a vivarium, whether in research, breeding or education, there is the presence of many odors such as ammonia (resulting from animal urine), or others from the laboratory (perfumes, cleaning materials, etc.). Such factors can alter the physiological and pharmacological responses inherent to animals (LI; LIBERLES, 2015; BUCK, 2000). In fact, we believe that the presence of odors from male rodents from other species may also directly affect the behavior of rats or mice. Furthermore, the presence or absence of males of the same species and/or different species can directly affect hormone production with important changes in the estrous cycle and receptivity of females (MAFFATT, 2003). Thus, unlike results previously found, we found that odors from SW male mice, even in the absence of eye contact, can cause profound changes in hormonal concentration and regularity of the estrous cycle of LE female rats.

Despite the clear indication of the involvement of pheromones from SW male mice, we do not rule out, despite the tightly controlled environment, other macro or micro environmental factors that may also be involved in these changes. Environmental, dietary and health factors may also directly affect animal welfare, and consequently influence the duration of the estrous cycle phases of rodents. Sudden changes in temperature and humidity often cause stress, decrease reproduction rates and increase susceptibility to infections. Furthermore, photoperiod is undoubtedly one of the most important items that influence the circadian cycle, and may directly influence the behavior of animals. Abrupt changes in photoperiod affect the metabolism of animals, with consequent changes in hormone production, leading to anomalous behavior including reproduction (HARKNESS; WAGNER, 1995). Further studies should be conducted to investigate the interrelationship of micro and macro-environmental factors in the changes observed.

Conclusions

The results of this study showed that maintaining LE female rats in a research vivarium in the presence of pheromones from male SW mice can significantly change the hormone concentration and duration of each individual phase of the estrous cycle. These changes are very relevant in reproductive toxicology studies and/or reproduction physiology where the regularity of the estrous cycle is crucial for the reliability and reproducibility of results.

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Recebido em: 15.07.2015
Aceito em: 27.08.2015