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The absence of maternal pineal melatonin rhythm during pregnancy and lactation impairs offspring physical growth, neurodevelopment, and behavior

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ABSTRACT

Maternal melatonin provides photoperiodic information to the fetus and thus influences the regulation and timing of the offspring's internal rhythms and preparation for extra-uterine development. There is clinical evidence that melatonin deprivation of both mother and fetus during pregnancy, and of the neonate during lactation, results in negative long-term health outcomes. As a consequence, we hypothesized that the absence of maternal pineal melatonin might determine abnormal brain programming in the offspring, which would lead to long-lasting implications for behavior and brain function. To test our hypothesis, we investigated in rats the effects of maternal melatonin deprivation during gestation and lactation (MMD) to the offspring and the effects of its therapeutic replacement. The parameters evaluated were: (1) somatic, physical growth and neurobehavioral development of pups of both sexes; (2) hippocampal-dependent spatial learning and memory of the male offspring; (3) adult hippocampal neurogenesis of the male offspring. Our findings show that MMD significantly delayed male offspring's onset of fur development, pinna detachment, eyes opening, eruption of superior incisor teeth, testis descent and the time of maturation of palmar grasp, righting reflex, free-fall righting and walking. Conversely, female offspring neurodevelopment was not affected. Later on, male offspring show that MMD was able to disrupt both spatial reference and working memory in the Morris Water Maze paradigm and these deficits correlate with changes in the number of proliferative cells in the hippocampus. Importantly, all the observed impairments were reversed by maternal melatonin replacement therapy. In summary, we demonstrate that MMD delays the appearance of physical features, neurodevelopment and cognition in the male offspring, and points to putative public health implications for night shift working mothers.

1. Introduction

According to the European Foundation for the improvement of living and working conditions and the US Department of Labor, almost a fifth of the worldwide workforce is engaged in shift work, with 20% of European and American workers engaged in night shifts (Labor, 2005; Parent-Thirion, 2007). Recent data in the human and animal literature

suggest that chronodisruption (i.e., disturbance of temporal organization, mainly circadian, of endocrinology, physiology, metabolism, and behavior) during pregnancy has been associated with an increased risk of miscarriage, preterm delivery and low birth weight, in addition to higher incidence of sleep, metabolic and cardiovascular disturbances in the offspring (Knutsson, 2003; Navara and Nelson, 2007; Reiter et al., 2012; Zhu et al., 2004).

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Several social situations (such as work at night, shift work, artificial light exposure at night) cause a reduction in maternal melatonin production and a disturbance of central and peripheral clocks that control circadian rhythms (Dumont and Paquet, 2014). The maternal pineal gland and its secretory product melatonin have a pivotal role in providing photoperiodic information to the fetus, thus influencing the regulation and timing of the offspring's internal rhythms and their preparation for extrauterine life (Torres-Farfan et al., 2011; Velazquez et al., 1992).

In addition to its circadian rhythm modulatory role, maternal melatonin also acts as a neuroendocrine modifier of several physiological systems (e.g., neural, cardiovascular, energy metabolism, immunological and inflammatory responses). It is also a potent direct free radical scavenger and indirect antioxidant and cytoprotective agent at all levels in the maternal–placental–fetal unit. Considering all these data, maternal melatonin seems to be essential for a successful pregnancy (Carrillo-Vico et al., 2013; Cipolla-Neto et al., 2014; Ferreira et al., 2012; Manchester et al., 2015; Vilches et al., 2014) and may represent an important component of the intrauterine environment for perinatal and postnatal programming of brain and behavior (Bale, 2015).

This idea is reinforced by the presence of melatonin receptors in the fetal nervous system, especially in hypothalamic suprachiasmatic and arcuate nuclei and hippocampus (Lacoste et al., 2015). It should be stressed that, in rodents, significant development of hippocampal and hypothalamic neural circuits occurs postnatally during the lactation phase (Bouret et al., 2004; Markakis, 2002; Seress, 2007). One likely mechanism through which melatonin would influence brain development is via modulation of plastic events, such as neurogenesis and neuroprotection. Recent studies show that melatonin administration after birth increases cell proliferation, differentiation and survival of novel neurons in the hippocampus (Kim et al., 2004; Ramirez-Rodriguez et al., 2009; Ramirez-Rodriguez et al., 2011). No investigation of those parameters, however, has been performed to evaluate the intrauterine role of melatonin.

In the present study, we aimed to examine whether gestational and lactational maternal pineal melatonin absence could induce changes in the offspring somatic and physical growth, neurodevelopment and its effects on cognitive functions and neurogenesis in adulthood. We hypothesized that maternal absence of pineal melatonin during gestation and/or early post-natal period might have more severe consequences, as it may alter brain development and programming, and thereby have long-term consequences for behavior and brain function. To test our hypothesis, we used a model of gestational hypomelatoninemia during pregnancy and lactation resulting from the surgical removal of the pineal gland. Our specific aims were to investigate the effects of maternal pineal melatonin absence and its therapeutic replacement on: (1) somatic, physical growth and neurobehavioral development of offspring; (2) hippocampal-dependent spatial learning and memory of male adult offspring and (3) adult hippocampal neurogenesis.

2. Material and methods

2.1. Animals

Nursing female Wistar rats ($n = 78$) and their male and female offspring (Animal Facility of the Institute of Biomedical Sciences - São Paulo, Brazil) were kept in a temperature-controlled environment ($22 \pm 2^\circ\text{C}$) under a 12L:12D light–dark cycle (dark to light transition, Zeitgeber time 0 - ZT0 and light to dark transition, Zeitgeber time 12 - ZT12), with food and water ad libitum. All experimental procedures complied with the Brazilian Guidelines for Care and Use of Animals in Education or Scientific Research Activities determined by the National Council of Animal Experimentation Control (DBCA – CONCEA, 2016) and were approved by the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo (CEUA Protocol No.

199/116f, book 2).

2.2. Dams and surgical procedures

Eight-week-old female rats were anesthetized with an intraperitoneal injection of ketamine and xylazine (0,4 g/kg and 0,02 g/kg) and subjected to pinealectomy (PINX) as previously described (Ferreira et al., 2012). Briefly, anesthetized animals were placed in a stereotaxic apparatus (David Kopf Instruments, CA, USA) and a sagittal opening was made in the scalp. The skin was pulled apart to expose the lambda confluence, a disc-shaped opening was made around the lambda with a circular drill and the pineal gland was removed with a fine forceps. Meticulous care was taken to avoid injury to the nervous system adjacent to the pineal gland. The disc-shaped piece of bone was replaced and, after brief hemostasis, the scalp was sutured with cotton thread. Animals of the control group (CTL) were subjected to a similar surgical protocol, but their pineal gland was not removed. The integrity of the gland itself after the excision and the post-mortem analysis of the dams' brains allowed us to verify the success of the pinealectomy procedure and the integrity of the adjoining central nervous system.

Immediately after the surgical procedures, PINX rats were randomly assigned to two groups, one received vehicle (PINX) and the other received melatonin (PINX + MEL) diluted in the drinking water. Melatonin (Sigma-Aldrich; St. Louis, MO, USA) replacement therapy was based on a previous study (Ferreira et al., 2012), with the hormone being added to the drinking water exclusively during the dark phase (ZT 12 to ZT 0). CTL and PINX rats received the same concentration of vehicle (ethanol 10^{-8}v:v) in water during the dark phase. During the light phase, all the animals received tap water free of melatonin and vehicle. Melatonin dosage (0.5 mg/kg) was attained by daily correction of melatonin drinking solution concentration based on the previous day measurement of individual body weight and nocturnal water intake.

Thirty days after surgery, female rats were caged with experienced adult males (2:1) across two estrous cycles. The presence of vaginal plugs or sperm was considered indicative of pregnancy and designated as gestational day 0. Pregnant rats were immediately isolated and kept one per cage. Melatonin or vehicle administration was maintained throughout gestation and lactation.

Litters from 30 dams (PINX $n = 10$, PINX + MEL $n = 10$ and CTL $n = 10$), designated PINX-F1, PINX + MEL-F1, and CTL-F1 had their physical and neurobehavioral development (described below) evaluated up to postnatal day (PND) 35. From PND 60 to 77, they were tested for spatial learning memory. The experimental design of the present study is illustrated in Fig. 1.

2.3. Offspring procedures

On PND4, all litters were examined and culled to 8 pups (4 males and 4 females) that were uniquely identified within each litter with a small tattoo on one or more of the paws and the pups were allowed to nurse until PND21.

2.3.1. Neurobehavioral and developmental landmarks

Throughout the lactation period, pups were individually evaluated regarding neurological reflexes development and physical developmental characteristics. Subjects were individually weighed at PND4, 7, 17, 21 and 60.

2.3.2. Physical developmental parameters

The day of each of the following events was recorded for each animal: pinna detachment (PD) (the opening of ear channel), incisor eruption (IE) (observation of superior and inferior teeth), eye opening (EO) (opening of both eyes lids), testis descent (TD) (descent of both testes to scrotum), and vaginal opening (VO) (opening of vaginal channel). The mean day of occurrence of each event for each group was then calculated.

Fig. 1. Experimental design. Female Wistar rats were submitted to pinealectomy (PINX) or SHAM surgery (CTL). The PINX rats were assigned to two groups and received either melatonin (PINX + MEL) or vehicle (PINX). After four weeks, rats were allowed to mate and received melatonin/vehicle until the end of lactation. Somatic (body weight), physical (pinna detachment, auditory conduit opening, eruption of the incisors, eye opening, testis descendant, vaginal opening) development and the consolidation of reflex responses (palm grasp, righting, cliff avoidance, auditory startle response, free-fall righting and walking) of female and male offspring were determined during lactation. Spatial memory was tested on Morris Water Maze on day 61.

2.3.3. Reflexes development

Neurological reflexes ontogeny, described below, followed the experimental model established by Smart and Dobbie (1971) in rats. The following reflexes were assessed: (1) surface righting reflex (SR) - the pup was placed on its back and allowed to assume the normal upright position with all four feet on the table; (2) palmar grasp reflex (PG) - grasp of a paper clip with forepaws when stroked; (3) auditory startle response (AS) - the pup was exposed to a sudden loud, sharp noise and the expected response is a prompt extension of the head and the limbs followed by withdrawal of the limbs and a crouching posture; (4) cliff avoidance (CA) - the pup was placed on the edge of a table, with the forepaws and face just over the edge and the expected response is to move away from the “cliff” to avoid falling; (5) free-fall righting (FFR) - the pup was held with the back downwards 35 cm above a soft cotton pad and dropped; the expected response is to turn around in mid-fall to land on its four paws; (6) onset of walking (W) - each pup was placed on the table and observed. The task was considered completed when the pup walked freely for ≥ 5 s on three consecutive days.

Two male and two female pups from each litter were evaluated for the aforementioned parameters daily from PND1 to PND35 (between ZT2 and ZT6). After each evaluation, pups were returned to their home cage. A reflex response was considered to have occurred within a maximum observation period of 10 s. The first of a series of three consecutive days in which the reflex was exhibited was recorded as the day of consolidation of the reflex.

After the neurodevelopmental evaluation on PND35, the animal housing cages were moved to different facilities under identical environmental conditions as the pre-weaning housing room described above to perform the behavioral tests (described below).

2.3.4. Offspring behavioral tests

Long-term effects of maternal melatonin deprivation during pregnancy and lactation on spatial memory were studied only on male offspring by submitting the animals to the Morris Water Maze (MWM) starting at PND61. A male pup from each litter was randomly selected to undergo this experiment, totaling 8 animals per group. The behavioral tests were run during the dark phase of the light-dark cycle

(ZT14) and the light intensity was the minimum required for the operation of the recording system (< 10 lx). Light intensity was measured with a digital light meter Model-LD 204 (Instrutherm Measuring Instruments Ltda., São Paulo, Brazil).

2.3.4.1. Assessment of spatial learning and memory in the Morris Water Maze. Hippocampal-dependent spatial learning and memory of male offspring at PND61 were assessed using the MWM. The MWM apparatus and procedures were similar to those described in previous studies (Motta-Teixeira et al., 2016). The pool was a round black fiberglass tank, 200 cm in diameter and 50 cm in height, filled to a depth of 25 cm with water (26 ± 1 °C). A movable circular plastic platform 9 cm in diameter, mounted on a plastic column, was placed in the center of one of the quadrants of the pool, about 2 cm below the water surface. The MWM was located within a 3.13×3.5 m room with several salient cues hanging on the walls.

Each trial consisted of placing the rat near the border of the pool facing the wall and allowing it to swim until the platform was found. If the rat did not find the platform within 120 s, it was then manually guided to it, where it remained for a 10-s period. The trials were recorded using a computerized video-tracking system (Ethovision Pro; Noldus Information Technologies, Wageningen, The Netherlands) that allowed to measure latency, path length, time spent and frequency across the critical counter.

2.3.4.2. Reference memory. In the water maze spatial reference memory-training task, the platform was located in a single, fixed position in the center of the critical quadrant for the entire training period. Each animal was subjected to three trials per session, one session per day, with an intertrial interval (ITI) of 10 min, for 8 consecutive sessions (subdivided in 3 blocks: 1 = 1–3 sessions, 2 = 4–6 sessions and 3 = 7–8 sessions). Acquisition of the spatial reference memory task was assessed by decreases in latency and path length. The percentage of time spent within a 33-cm wide ring containing the platform (the critical ring), which external border was 33 cm distant from the pool border, helped to analyze the spatial bias of the animals in the pool. Training proceeded until the subjects reached

an asymptotic level of performance, which was demonstrated to occur in the 8th session.

A probe test, with the platform removed, was conducted 24 h after the end of the training phase. During this test, the animals were allowed to freely swim in the pool for 3 min. The percentage of time the animal spent within the critical quadrant relative to the other quadrants, the amount of time the animal spent within the counter, and the number of times the animal swam across that counter (counter frequency), in three time bins of 60 s each, allowed assessment of long-term memory for platform location through the extent of spatial bias in the first time bin, and evaluation of the rate of extinction of this behavior by comparing the same parameter over the three consecutive time bins.

2.3.4.3. Working memory. In the water maze working memory training task, the platform location was changed in each of the sessions, being positioned in the center of the critical quadrant along trials of the same session. Each animal was subjected to three trials per session, one session per day, with four sessions with an ITI of 10 min and four sessions with an ITI of 0 min, totaling eight sessions. Acquisition of the spatial working memory task was assessed by the reduction in latency and path length, and by the increasing percentage of time spent within the critical counter where the platform was in the previous session.

2.4. Immunolabeling

Immunohistochemistry was used to identify proliferating cells (Ki-67⁺) and newborn neurons (DCX⁺) in the hippocampus of PND60–77 male offspring to determine the lasting effects of maternal melatonin deprivation during gestation and lactation (MMD) on adult neurogenesis. Again, only male offspring were used in this experiment. The rats were anesthetized with a lethal dose of ketamine and xylazine (100 and 7 mg/kg, respectively), perfused via the ascending aorta with cold 0.9% saline, and then fixed with a freshly prepared solution of 4% formaldehyde dissolved in 100 mM phosphate buffer (PB, pH 7.4). After removal from the skull, brains were postfixed overnight using the same fixation solution and then transferred to a 30% sucrose solution for cryoprotection. The brains were then sliced in a freezing microtome (Leica Biosystems, Nussloch, Germany), in 1-in-10 series of 40- μ m frontal sections that were stored in antifreeze solution (ethylene glycol/glycerol; Sigma Chemical, St. Louis, MO, USA) at -30°C until processing.

Proliferating cells were immunostained for Ki-67 (a protein expressed during all phases of the cell cycle) localization, as described by Naninck et al. (2015). Briefly, brain sections were mounted on glass slides (Superfrost Plus slides; Menzel, Braunschweig, Germany) and, after 24 h drying, washed with 0.05 M TBS pH 7.6 (3×5 min) and then subjected to antigen retrieval in citrate buffer (0.01 M, pH 6.0) by microwave exposure for 15 min (5 min at 800 W, 5 min at 400 W and 5 min at 200 W). After cooling for 20 min, the sections were washed in TBS (3×5 min) and incubated with 0.3% H_2O_2 for 15 min to block endogenous peroxidase activity, followed by 3×5 min rinses in TBS, incubation in blocking buffer (2% milk powder in TBS) and subsequently incubated with primary antibody anti-Ki67 (1:2000; Novocastra Laboratories, Newcastle upon Tyne, UK, catalog # NCL-Ki-67-MM1) in Supermix (0.5% Triton X-100, 0.25% gelatin in TBS) for 1 h at room temperature followed by overnight incubation at 4°C . Then, sections were rinsed 3×5 min in TBS and incubated for 2 h at room temperature in biotinylated rabbit anti-goat (1:200; Vector Laboratories, Burlingame, CA, USA) followed by a 90 min incubation with avidin–biotin–peroxidase complex solutions (ABC Kit, Elite Vectastain; Brunschwig Chemie, Amsterdam) diluted 1:800 in TBS. Followed by amplification with tyramine (1:500, 0.01% H_2O_2 , for 30 min), the sections were rinsed and stained with 3,3'-diaminobenzidine substrate (DAB; 0.02% Sigma; 0.01% H_2O_2 in 0.05 M Tris). The sections were then counterstained with hematoxylin.

Newborn neurons were revealed by microtubule binding protein doublecortin (DCX) immunohistochemistry. For DCX, staining was conducted using free-floating sections. One free-floating series of sections from each animal was pretreated with 0.3% H_2O_2 (SigmaChemical) for 15 min, followed by 6×5 min rinses in KPBS and incubation in primary anti-DCX antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 3% normal rabbit serum diluted in KPBS + 0.3% Triton X-100, for ~ 16 h at room temperature. Subsequently, the sections were rinsed in KPBS (6×5 min) and incubated for 1 h at room temperature in biotinylated rabbit anti-goat immunoglobulin G (IgG; Vector Laboratories, Burlingame, CA, USA, catalog #BA5000) diluted 1:800 in KPBS with 0.3% Triton X-100, and further incubated for 1 h at room temperature using avidin–biotin–peroxidase complex solutions (Vector) diluted 1:333 in KPBS. The sections were then rinsed and subjected to immunoperoxidase reactions using DAB (0.02%; Sigma) as chromogen with 0.003% H_2O_2 as reaction substrate and nickel ammonium sulfate (NAS) at 2.5% diluted in sodium acetate buffer 0.2 M, pH 6.5. The reaction was terminated after ~ 10 min with successive rinses in KPBS. Sections were finally mounted onto gelatin-coated slides, air-dried overnight at room temperature and coverslipped with DPX (Fisher Scientific).

Ki-67 immunoreactive cells (Ki-67-ir) counting was performed using an Olympus BH2 light microscope under $400\times$ magnification and was conducted by an experimenter that was blind to the subject's treatments. Ki-67-labeled cells were counted in the entire subgranular zone (SGZ) to obtain an estimate of the total number of labeled cells. The SGZ was defined as a 20- μ m zone between the GCL and the hilus. The total number of Ki-67 labeled cells was estimated by multiplying the total number counted cells by 10.

DCX-immunoreactive cells (DCX-ir) were estimated in the five subjects of each group using 40 μ m-thick serial coronal sections spaced 400 μ m apart. The thickness was chosen based on the minimum thickness accepted for Optical Fractionator probes considering tissue shrinkage after processing. An optical microscope coupled to a camera (Nikon Instruments Inc., Melville, NY, USA), connected to a motorized stage (Ludl Electronic Products, Hawthorne, NY, USA) controlled by Stereo Investigator software (MBF Biosciences, Williston, VT, USA) was employed. A contour of the dentate gyrus was delineated at $4\times$ magnification using Nissl counterstaining. Immunoreactive cells presenting robust and visible labeling were counted at final $400\times$ magnification. The counting frame size and the sampling grid were established based on pilot studies, as previously described (Takada et al., 2015).

2.5. Statistical analysis

Data were assessed for normality and homogeneity of variance to determine whether to use parametric or non-parametric statistical tests. All statistical analyses were performed using STATISTICA software version 7 (Stat Soft Inc.; Tulsa, Oklahoma, USA) and GraphPad Prism version 6 (Graph Pad Software Inc.; San Diego, California, USA).

For statistical analysis, data related to the somatic growth (body weight) were analyzed by two-way repeated-measures ANOVA. Bonferroni's post hoc test was used. Physical features and reflex maturation were analyzed by factorial ANOVA (Tukey–Kramer post hoc). Scores on the water maze memory test were analyzed using a repeated-measures ANOVA using group as the between-subjects factor and sessions, ITIs and trials as within-subjects factors. Probe-test scores were analyzed using group as the between-subject factor and time bin as the within-subject factor. An independent ANOVA was run for each score. Differences were considered significant when $p < 0.05$. Tukey–Kramer post hoc analyses were conducted when p values reached significance. Effect sizes were recorded for any significant effects (η^2 or Cohen's d). Ki-67-ir cells' counting was analyzed using one-way analysis of variance followed, when appropriated, by Bonferroni's multiple comparisons test. DCX-ir cells' counting was evaluated through a Kruskal–Wallis nonparametric one-way ANOVA. Statistical significance was set at

Physical development

Reflex development

Fig. 2. Effects of maternal melatonin deprivation during gestation and lactation on physical and neurodevelopment of the offspring. Body weight of pups during lactation and adult life male (A) and female (B) pups born to CTL, PINX and PINX + MEL dams. The weight of every pup was measured and an average was calculated per litter per postnatal day indicated in the x-axis, $n = 10$ litters per group. Mean $\pm$ SEM. Two-way ANOVA followed by Bonferroni post hoc test. $*p < 0.05$. Day of appearance (mean $\pm$ SEM) of physical milestones (C) fur development; (D) pinna detachment; (E) eye opening; (F) superior incisor eruption; (G) testis descent; (H) vaginal opening. Mean $\pm$ SEM. The bar indicates sex differences and letters indicate significant group differences according to ANOVA analyses. $***p < 0.001$. Reflex appearance age (days - mean $\pm$ SEM.) (A) palmar grasp; (B) righting reflex; (C) free-fall righting; (D) walking (E) Z-score neurodevelopment. Mean $\pm$ SEM. The bar indicates sex differences, letters indicate significant group differences according to two-way ANOVA analysis. $***p < 0.001$.

$p < 0.05$.

3. Results

3.1. Growth measurements

Fig. 2(A–B) illustrates the weight gain during the 4th, 7th, 14th, 21th, 45th e 60th days of life of the offspring born to CTL, PINX and PINX + MEL dams. In relation to weight gain, the ANOVA with repeated-measures revealed **sex** ($F_{(1,392)} = 64.26, p < 0.05; \eta^2 = 0.968$), **group** ($F_{(2,392)} = 52.63, p < 0.05; \eta^2 = 0.641$), **time** ($F_{(3,392)} = 3151, p < 0.05; \eta^2 = 0.998$) and **group * sex * time** interaction ($F_{(6,392)} = 3.95, p < 0.05, \eta^2 = 0.254$) effects on weight gain in the offspring born to CTL, PINX and PINX + MEL dams.

Post hoc analysis revealed that male PINX-F1 and PINX + MEL-F1 weighed significantly less than CTL-F1 on PND14 and PND21 ($p < 0.05$; PND14: $d_{\text{PINX/CTL}} = 9.36$; $d_{\text{PINX+MEL/CTL}} = 7.882$; PND21: $d_{\text{PINX/CTL}} = 6.419$; $d_{\text{PINX+MEL/CTL}} = 7.307$). Moreover, female PINX-F1 and PINX + MEL-F1 weighed significantly less than CTL-F1 on PND14 ($p < 0.05$; $d_{\text{PINX/CTL}} = 5.533$; $d_{\text{PINX+MEL/CTL}} = 3.706$). Post hoc analysis also revealed that male PINX-F1 weighed significantly more than both CTL-F1 and PINX + MEL-F1 on PND45 and PND60 ($p < 0.05$; PND45: $d_{\text{PINX/CTL}} = 14.101$; $d_{\text{PINX/PINX+MEL}} = 11.304$; PND60: $d_{\text{PINX/CTL}} = 6.320$; $d_{\text{PINX/PINX+MEL}} = 7.650$). PINX-F1 females weighed significantly more than PINX + MEL-F1 and CTL-F1 on PND45 and PND60 ($p < 0.05$; PND45: $d_{\text{PINX/PINX+MEL}} = 5.665$, $d_{\text{PINX/CTL}} = 3.980$; PND60: $d_{\text{PINX/PINX+MEL}} = 4.125$, $d_{\text{PINX/CTL}} = 4.521$).

3.2. Developmental milestones

Fig. 2 presents mean $\pm$ SEM for age at fur development (C), pinna detachment (D), eyes opening (E), eruption of incisor teeth (IE), testis descent (G) and vaginal opening (H). ANOVA revealed significant effects on fur development, pinna detachment, and superior incisor teeth for **group** (FD: $F_{(2,98)} = 5.72, p < 0.05; \eta^2 = 0.087$; PD: $F_{(2,98)} = 6.48, p < 0.05; \eta^2 = 0.104$; IE: $F_{(2,98)} = 4.14, p < 0.05; \eta^2 = 0.074$), **sex** (FD: $F_{(1,98)} = 8.68, p < 0.05; \eta^2 = 0.066$; PD: $F_{(1,98)} = 5.71, p < 0.05; \eta^2 = 0.046$) and also significant interaction effects for **group * sex** (FD: $F_{(2,98)} = 5.72, p < 0.05; \eta^2 = 0.087$; PD: $F_{(2,98)} = 3.52, p < 0.05; \eta^2 = 0.056$). There is also a **group** significant effect for testis descent ($F_{(2,49)} = 27.30, p < 0.05; \eta^2 = 0.942$), but a lack of significance for eruption of inferior incisor teeth ($F_{(2,98)} = 0.414, p = 0.66; \eta^2 = 0.008$). Post hoc analysis indicates that PINX-F1 males delayed the appearance of these physical milestones compared to CTL-F1 and PINX + MEL-F1 ($p < 0.05$, FD: $d_{\text{PINX/CTL}} = 0.745$, $d_{\text{PINX/PINX+MEL}} = 0.373$; PD: $d_{\text{PINX/CTL}} = 0.863$, $d_{\text{PINX/PINX+MEL}} = 0.518$; EO: $d_{\text{PINX/CTL}} = 1.425$, $d_{\text{PINX/PINX+MEL}} = 1.569$; IE: $d_{\text{PINX/CTL}} = 0.241$, $d_{\text{PINX/PINX+MEL}} = 0.696$, TD: $d_{\text{PINX/CTL}} = 11.459$, $d_{\text{PINX/PINX+MEL}} = 5.382$).

On the other hand, these physical milestones were not affected in female offspring (post hoc analysis $p > 0.05$), except for eye opening, that was delayed in PINX-F1 and PINX + MEL-F1 pups in comparison to CTL-F1 pups ($p < 0.05$, $d_{\text{PINX/CTL}} = 1.051$, $d_{\text{PINX+MEL/CTL}} = 0.901$). There is no significant effect for vaginal opening ($F_{(2,49)} = 0.62, p > 0.05, \eta^2 = 0.011$).

3.3. Reflex development

The effects of the absence of maternal melatonin and its replacement therapy could be seen on the development of different neurological reflexes. Data on reflex development is displayed as mean $\pm$ SEM in Fig. 2.

ANOVA revealed significant effects on palmar grasp, cliff avoidance, free fall righting and walking for **group** (PG: $F_{(2,98)} = 10.54, p < 0.05; \eta^2 = 0.145$; CA: $F_{(2,98)} = 3.64, p < 0.05; \eta^2 = 0.062$; FFR: $F_{(2,98)} = 23.31, p < 0.05; \eta^2 = 0.273$; W: $F_{(2,98)} = 35.22, p < 0.05$;

$\eta^2 = 0.353$) and **sex** (PG: $F_{(1,98)} = 19.92, p < 0.05; \eta^2 = 0.098$; CA: $F_{(1,98)} = 7.56, p < 0.05; \eta^2 = 0.064$; FFR: $F_{(1,98)} = 16.61, p < 0.05; \eta^2 = 0.097$; W: $F_{(1,98)} = 19.92, p < 0.05; \eta^2 = 0.100$) and also significant interaction effects for **group * sex** (PG: $F_{(2,98)} = 5.06, p < 0.05; \eta^2 = 0.070$; FFR: $F_{(2,98)} = 3.84, p < 0.05; \eta^2 = 0.045$; W: $F_{(2,98)} = 4.06, p < 0.05; \eta^2 = 0.041$; except for CA: $F_{(2,98)} = 1.88, p > 0.05$). Post hoc analysis indicates that PINX-F1 males presented delayed onset of those neurological reflexes when compared to CTL-F1 and PINX + MEL-F1 animals ($p < 0.05$, PG: $d_{\text{PINX/CTL}} = 3.278$, $d_{\text{PINX/PINX+MEL}} = 1.318$; CA: $d_{\text{PINX/CTL}} = 0.506$, $d_{\text{PINX/PINX+MEL}} = 0.436$; FFR: $d_{\text{PINX/CTL}} = 2.344$, $d_{\text{PINX/PINX+MEL}} = 2.772$; W: $d_{\text{PINX/CTL}} = 2.361$, $d_{\text{PINX/PINX+MEL}} = 1.275$).

No group effects were found for righting reflex and auditory startle (RR: $F_{(2,98)} = 2.09, p > 0.05$; AS: $F_{(2,98)} = 0.05, p > 0.05$) but significant effects for **sex** (RR: $F_{(1,98)} = 18.23, p < 0.05; \eta^2 = 0.140$; AS: $F_{(1,98)} = 13.78, p < 0.05; \eta^2 = 0.114$) and interaction effects **group * sex**. (RR: $F_{(2,98)} = 3.51, p < 0.05; \eta^2 = 0.054$; AS: $F_{(2,98)} = 3.49, p < 0.05; \eta^2 = 0.058$). Post hoc analysis indicates a trend for PINX-F1 males to present delayed maturation of the righting reflex in relation to CTL-F1 and PINX + MEL-F1 ($p = 0.06$).

Palmar grasp, cliff avoidance, righting reflex and auditory startle neurological reflexes were not affected in female offspring. However, we found a significant advance on the day of appearance of free-fall righting in PINX + MEL group in relation to CTL-F1 and PINX-F1 pups and delay for walking ($p < 0.05$; FFR: $d_{\text{PINX/PINX+MEL}} = 1.491$, $d_{\text{PINX+MEL/CTL}} = 1.416$; W: $d_{\text{PINX+MEL/CTL}} = 1.067$).

In a subsequent analysis, a z-normalization was applied according to (Guilloux et al., 2011). For each neurological reflex and physical development, a Z-score for day of appearance of physical milestones and reflex maturation was calculated as follows: $Z = ((x - \mu)/\sigma)$ where X represents the individual data for the observed parameter, and μ and σ the mean and standard deviation for the control group, respectively. Then, a Z-developmental score (Fig. 2E) compiling all days of physical development and neurodevelopment was calculated as a whole. These analyses revealed statistical significance for **group** ($F_{(2,90)} = 23.01, p < 0.05; \eta^2 = 0.222$), **sex** ($F_{(1,90)} = 34.43, p < 0.05; \eta^2 = 0.166$) and for the interaction **group * sex** ($F_{(2,90)} = 18.34, p < 0.05; \eta^2 = 0.177$). Therefore our results show that the absence of maternal melatonin delays the neurodevelopment in a sex-dependent manner.

3.4. Behavioral data in the MWM task

This experiment was performed only with male offspring to pinealectomized dams. The subjects were exposed to 8 days of testing in the reference-memory version of the MWM task (with four trials per day and an ITI of 10 min) starting on PND61, followed by a Probe Test. This allowed the evaluation of spatial memory acquisition and retention.

3.4.1. Spatial reference memory

During the test, all animals of all groups significantly improved their performance by reducing their latency and path length to reach the hidden platform (Fig. 3A), as revealed by repeated-measures ANOVA: effect of **sessions** (Latency: $F_{(5,503)} = 5.47, p < 0.05; \eta^2 = 0.063$; Path length: $F_{(5,503)} = 20.97, p < 0.05; \eta^2 = 0.014$), **trial** (Latency: $F_{(2,548)} = 19.23, p < 0.05; \eta^2 = 0.036$; Path length: $F_{(2,548)} = 16.13, p < 0.05; \eta^2 = 0.037$) and **block** (Latency: $F_{(2,503)} = 180.0, p < 0.05; \eta^2 = 0.368$; Path length: $F_{(5,503)} = 119.23, p < 0.05; \eta^2 = 0.277$).

The lack of maternal melatonin during gestation and lactation disrupted the acquisition of a spatial reference memory task in the water maze and its replacement therapy was able to overcome these outcome, as revealed by the significant group effects for latency ($F_{(2,548)} = 7.40, p < 0.05; \eta^2 = 0.015$) and path length ($F_{(2,548)} = 3.03, p < 0.05; \eta^2 = 0.007$). Post hoc analyses show both poorer acquisition and performance by the PINX-F1 animals relative to CTL-F1 and PINX + MEL-F1 ($p < 0.05$; Latency: $d_{\text{PINX/CTL}} = 2.367$, $d_{\text{PINX/PINX+MEL}} = 3.729$; Path: $d_{\text{PINX/CTL}} = 2.005$, $d_{\text{PINX/PINX+MEL}} = 3.255$).

Spatial reference memory

Spatial working memory

Fig. 3. Effect of maternal melatonin deprivation on acquisition of spatial memory. Acquisition of reference memory in the Morris Water Maze task (mean of 3 trials along 3 sessions per block) as revealed by latency (A) and frequency across the counter (B). Acquisition of spatial working memory in the water maze task over 3 trials along sessions (4 sessions per ITI), as revealed by (C) latency and (D) path length. Data presented as mean $\pm$ SEM were analyzed using repeated-measures analysis of variance (ANOVA) with the groups (CTL-F1, PINX-F1 and PINX + MEL-F1) as the between-subject factor and the ITI or trial as the within-subject factor. Post hoc analyses with the Tukey–Kramer test is indicated as follows: *group significant difference. The significance level adopted was $p < 0.05$.

Swimming speed (data not shown) was also significantly different between groups ($F_{(2,548)} = 7.59$, $p < 0.05$; $\eta^2 = 0.010$) and along sessions ($F_{(7,503)} = 6.89$, $p < 0.05$; $\eta^2 = 0.06$). Post hoc analysis indicates that PINX-F1 swam slower than CTL-F1 and PINX + MEL-F1 ($p < 0.05$).

During the probe test (used to assess memory retention), ANOVA revealed a lack of significant differences in percentage of time in the platform quadrant ($F_{(4,57)} = 0.27$, $p > 0.05$; $\eta^2 = 0.010$) and percentage of entries in the platform quadrant ($F_{(4,57)} = 0.51$, $p > 0.05$; $\eta^2 = 0.020$ – Fig. 3B), indicating that all groups were able to remember the location of the platform.

3.4.2. Spatial working memory

On the first trial, when the critical information about the platform location was unknown, the latency (Fig. 3C) and path length (Fig. 3D) scores were relatively longer. However, on trial 2, when the animals could use the information about the platform location obtained on trial 1, the subjects exhibited latencies and path lengths decrements that were, as expected, stronger when the ITI was zero as compared with when the ITI was 10 min (Latency: $F_{(1,503)} = 8.13$, $p < 0.05$; $\eta^2 = 0.010$; Path length: $F_{(1,503)} = 10.40$, $p < 0.05$; $\eta^2 = 0.013$ – Fig. 3C and D). These decrements in latency and path length scores reached asymptotic levels on trial 3 (Trial: Latency: $F_{(2,558)} = 47.51$, $p < 0.05$; $\eta^2 = 0.059$; Path length: $F_{(2,558)} = 61.98$, $p < 0.05$; $\eta^2 = 0.075$).

The distance traveled to the platform from trial 1 to 3 did not show any improvement for the PINX-F1 group showing the effect of the absence of maternal melatonin. On the other hand, the traveled distance was reduced, from trial 1 to 3, for CTL-F1 and PINX + MEL-F1 groups in Phase 1 (10-min ITI), showing that melatonin replacement to the mothers recovered the function in the offspring comparing them to the offspring of the control group (Group: Latency: $F_{(2,558)} = 17.65$, $p < 0.05$; $\eta^2 = 0.022$; $d_{\text{PINX/CTL}} = 2.420$, $d_{\text{PINX/PINX + MEL}} = 1.368$; Path length: $F_{(2,558)} = 9.23$, $p < 0.05$; $\eta^2 = 0.011$; $d_{\text{PINX/CTL}} = 0.857$, $d_{\text{PINX + MEL/CTL}} = 0.725$). In contrast, in Phase 2 (0-min ITI) there were no differences between groups (Fig. 3C and D). Swim speed of each group did not differ significantly, which discards the possibility that the deficits in memory performance observed in Phase 1 are due to sensory or motor deficits.

3.5. Immunohistochemistry for Ki-67 and doublecortin (DCX)

The number of Ki-67-ir cells was measured to assess whether the absence of maternal melatonin during gestation and lactation altered the number of proliferative cells in the adult male offspring (PND60 – Fig. 4A). ANOVA revealed significant main Group effect ($F_{(2,24)} = 6.15$, $p < 0.05$; $\eta^2 = 0.338$) and PINX-MEL-F1 animals exhibited a significantly greater number of Ki-67 cells in the adult SGZ, as compared to CTL-F1 and PINX-F1 (Bonferroni post hoc, $p < 0.05$; $d_{\text{PINX + MEL/PINX}} = 1.522$, $d_{\text{PINX + MEL/CTL}} = 0.799$). This increase in PINX + MEL-F1

Fig. 4. Number of Ki-67 immunoreactive (Ki-67-ir) cells in the subgranular zone (SGZ) of the dentate gyrus (DG) of adult rats born from the CTL, PINX and PINX + MEL dams. (A) The amount of proliferating cells in the SGZ was significantly increased in male rats that were exposed to maternal melatonin replacement (PINX + MEL-F1). (B) and (C) Significant changes were also observed in the number of Ki-67-ir cells in the rostral-caudal axis. Data are presented as mean $\pm$ SEM. ($n = 8-9$ per group).

Numbers of DCX immunoreactive (DCX-ir) cells were counted in the subgranular zone (SGZ) of the DG as depicted in (D). No significant changes were observed in the number (D) or density (E) of DCX-ir cells in the subgranular zone (SGZ) of DG. Data are presented as mean $\pm$ SEM. ($n = 4-5$ per group).

male adult offspring was also present in the rostral-caudal axis (Dorsal: $F_{(2,24)} = 4.52$, $p < 0.05$; $\eta^2 = 0.273$; $d_{\text{PINX+MEL/PINX}} = 1.260$, $d_{\text{PINX+MEL/CTL}} = 1.135$; Ventral: $F_{(2,24)} = 5.59$, $p < 0.05$; $\eta^2 = 0.318$; $d_{\text{PINX+MEL/PINX}} = 1.378$, $d_{\text{PINX+MEL/CTL}} = 0.296$ – Fig. 4B and C).

Both the number (Fig. 4C) and density (Fig. 4D) of neuronal progenitors in the dentate gyrus were measured using anti-DCX specific antibody to assess whether MMD altered the number of newborn neurons in the adult male offspring (PND77). No significant effect was observed between groups (Number: $F_{(2,10)} = 0.26$, $p > 0.05$; $\eta^2 = 0.05$; Density: $F_{(2,10)} = 1.48$, $p > 0.05$; $\eta^2 = 0.22$), indicating no effect on the number of young neurons.

4. Discussion

Our results show that maternal melatonin deprivation during gestation and lactation led to delayed physical maturation and neurobehavioral development with long-term effects on cognitive functions. The therapeutic replacement of the maternal plasma melatonin by adding melatonin to the drinking water during the escotophase prevented these developmental delays and cognitive disruption.

The development of regulatory systems underlying behavior and physiology in the neonatal rat is primarily determined by the dam (Huot et al., 2004). It is well known that maternal melatonin rhythm, through the placenta or maternal milk transference, provides photoperiodic information to the fetus/neonate and, through that, induces/synchronizes the circadian and seasonal rhythmicity in the offspring,

that is essential for environmental adaptation (Simonneaux, 2011).

Reflex ontology is a sensitive indicator of the adjustment of the neonate to extrauterine life in the early stages of the development. The delayed onset of reflexes could be a result of neurological disorder that might be later reflected in impaired activity or the lack of adaptive behavior to the new environment (Khalki et al., 2012). The maturation of physical characteristics and neurological reflexes are of vital importance to altricial animals such as rats.

Among the maturation of physical parameters assessed in this study, maternal melatonin deprivation significantly delayed the male offspring's onset of fur development, pinna detachment, eyes opening, eruption of superior incisor teeth and testis descent. Maternal melatonin replacement fully prevented these effects. On the other hand, these physical milestones were not affected in the female offspring. Our results are consistent with the evidences in the literature, which show that sex differences in response to early adverse life events, such as hypoxia, infection, trauma and growth restriction, is a prevalent phenomenon (for a review, see Bilbo, 2017). Our results suggest, therefore, that intrauterine melatonin not only works as a pacemaker of the circadian rhythm, but also acts as an important factor to be considered in the intrauterine/neonatal neural development. Furthermore, this role of melatonin may work in concert with other factors, such as sex-differences in epigenetic modulation in response to stress, different interactions between melatonin and steroid hormones and differences in microglial composition (Schwarz et al., 2012). Some of these possibilities are being investigated by our group.

Melatonin is a pleiotropic molecule with several mechanisms of action mediated or not by melatonin receptors, in both the mother and the fetus, and in consequence, it plays a role in fetal programming (Reiter et al., 2016; Reiter et al., 2014; Ferreira et al., 2012). Maternal melatonin during the perinatal period could modulate the expression of transcription factors or growth factors (e.g. epidermal growth factor - EGF, alpha transforming growth factor - TGF α , fibroblast growth factor - FGF) with critical roles in epithelial development, eye maturation and testicular descent (Calamandrei and Alleva, 1989; Cirulli and Alleva, 1994; Dono, 2003). We presume these physical developmental delays are due to alterations in maternal melatonin production that might alter the expression of transcriptional factors or growth factors levels in the fetus development, but that remains to be further studied.

Perinatal maternal melatonin, therefore, can work as an important neurodevelopmental and neuroprotector factor during fetal and neonatal brain development. The PINX-F1 male showed a significant delay in time maturation of palmar grasp, righting reflex, free-fall righting and walking. These sensorimotor responses indicate maturation of vestibular function and development of orientation, motor coordination and motor functions which are mediated by several structures involving neural structures such as the cortex, cerebellum, basal ganglia, brain stem and spinal cord, besides the skeletal muscle (Secher et al., 2006). Our results suggest a delayed maturation (neurogenesis and/or myelination) of these systems or a hampered functional organization of the complex synaptic circuitry that underlies these reflexes (Cecon et al., 2017; Chang et al., 2014).

The developing fetal brain is particularly susceptible to oxidative stress and lipid peroxidation that can lead to neuronal and white matter development disruption (Allen and Venkatraj, 1992). Melatonin provides a fundamental neuroprotective mechanism for normal neurodevelopment and embryonic growth via anti-oxidant, anti-apoptotic and anti-inflammatory processes (Simonneaux, 2011). During development, melatonin is inherently involved in neuronal proliferation, myelination, differentiation and migration, dendritic formation and neuronal plasticity (Fu et al., 2011; Villapol et al., 2011). Therefore, since neonatal reflexes may be considered as an index of brain maturation, the present findings suggest that maternal melatonin deprivation during perinatal period affects fetal brain development mechanisms involved with the correct programming of the brain, with long-term consequences.

Concerning body weight, the lack of maternal melatonin did not

influence the preweaning weight of the offspring. However, it led to overweight in the adolescent and adult offspring. The daily rhythmic melatonin replacement therapy to dams completely reverses this effect in both sexes, indicating a transgenerational anti-obesogenic and weight-reducing effect of melatonin (Cipolla-Neto et al., 2014). Our data corroborate previous studies stating that the adult offspring of melatonin-deficient dams display glucose intolerance, insulin resistance and serious impairment in the glucose-induced insulin secretion by isolated pancreatic islets. These programming effects disappear with the appropriate schedule of melatonin replacement therapy to the mothers during gestation (Ferreira et al., 2012).

When considering these findings, it is important to keep in mind that our model of gestational hypomelatoninemia does not allow to distinguish the direct effects of melatonin restriction on pup development from possible indirect effects of an impaired maternal behavior/physiology. Previous studies suggest that alterations in the maternal behavior could act as a disruptive factor for optimal offspring development and could cause short and long term consequences, impacting the neuroendocrine and behavioral responses, as well as the physical growth and development of the offspring (Champagne et al., 2003; Liu et al., 2000; Liu et al., 1997; Toki et al., 2007). Further studies may be conducted to evaluate maternal behavior in the hypomelatoninemia model used in this work.

In addition to the importance of melatonin to embryo development, the absence or the administration of melatonin to pregnant animals seem to be able to differentially influence male and female offspring development, in accordance to the present data (López et al., 1995). Similar findings have been reported in intrauterine programming of metabolic function for high-protein diet (Thone-Reineke et al., 2006), obesity (Lecoutre et al., 2016) and glucocorticoid exposure (O'Regan et al., 2004).

We have demonstrated as well, that maternal melatonin deprivation leads to impairment in the spatial reference memory of the adult male offspring. PINX-F1 rats presented longer latency during acquisition, but they improved their spatial memory across the training. In the probe test, all rats showed a similar latency to reach the target annulus than during the previous training session, thus indicating that PINX-F1 rats did acquire relevant information about the reference memory task. This pattern of results is typical of animals with hippocampal damage (Xavier and Costa, 2009) and seems to be related to the behavioral strategy used by the animals to perform this task: rats with hippocampal damage lose their ability to use cognitive maps but keep guidance and orientation strategies. Besides the experimental approach, our data are in accordance with previous evidence that showed that LL-exposed dams that presented a blockage of melatonin production, among other consequences, leads to a significant deficit of reference spatial memory in the adult offspring (Vilches et al., 2014).

The lack of maternal melatonin not only impaired spatial reference memory but also the working memory. The PINX-F1 rats exhibit poor performance regardless of being tested with an ITI of 10 min or even when the ITI was virtually 0 min. This observation suggests that PINX-F1 rats have some disability to change their strategies to find a new platform position, indicating lack of flexibility in the use of previously acquired information (Xavier and Costa, 2009).

The hippocampus is a critical structure that is responsible for spatial learning and memory, processes that are dependent on neural plasticity and changes in the connectivity between neurons. These spatial memory deficits could also be related to a change in neurogenic processes. Restricting the development of new neurons has an adverse impact on the performance of various forms of learning and hippocampal-dependent memory (for review Yau et al., 2015; Gonçalves et al., 2016).

It should be stressed, that the proper rhythmic melatonin replacement therapy to the mother during gestation and lactation was able to prevent the neurodevelopmental effects resulting in cognitive deficits in the adult male offspring.

In the present study, we demonstrated that maternal melatonin replacement significantly increased the number of Ki67⁺ cells in the hippocampus DG in adult rats. This observation is consistent with previous reports that showed that melatonin plays a role in proliferation of new neurons in the adult brain (Kim et al., 2004; Ramirez-Rodriguez et al., 2009; Chu et al., 2016; Yu et al., 2017). This enhancement of proliferation might affect the neural progenitor pool and influences subsequent neurogenesis, further leading to the beneficial effects on hippocampus-related spatial memory.

Despite the observed beneficial effects, melatonin did not increase the number of young neurons in our model. Treatment with melatonin in adult animals increases the number of DCX-positive immature neurons (Ramirez-Rodriguez et al., 2011), but maternal melatonin replacement seems to be incapable to enhance the number DCX-positive cells above the ordinary level. PINX-F1 showed a trend to decrease the number of proliferative cells and did not show any difference in the number of immature neurons (DCX-IR cells) in the adult male offspring. This observation is consistent with previous reports that showed that different aspects of adult hippocampal neurogenesis (i.e., proliferation, differentiation, and survival) are differentially affected by various molecular cues.

Classically, interventions that enhance neurogenesis are linked to improved spatial cognition (Yau et al., 2015). Yet in our current study, MMD did not affect hippocampal proliferation and differentiation while spatial memory performance declined. One explanation might be that MMD decreases the number of survived newborn neurons that would be incapable to integrate into existing hippocampal circuits and to contribute to spatial memory performance. We can also speculate that lack of maternal melatonin could influence other stages of adult hippocampal neurogenesis including synaptogenesis and recruitment of new neurons. Therefore, we consider our results as hints on how circadian maternal melatonin might affect structural and functional brain plasticity on a cellular level.

In summary, we provide the first evidence that maternal melatonin deprivation during gestation delays the appearance of some physical features and neurological reflexes development and confirmed that the absence of maternal melatonin has long-term effects on neurogenesis and cognitive function as indicated by impairing learning and memory processes and that several of these effects are sex-dependent. Although there are limitations in translation of the present data to humans, our findings suggest that pregnant and lactation women should avoid any environmental condition that leads to a reduction in melatonin production such as exposure to light at night and nocturnal shift work.

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Authorship contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Motta-Teixeira: conception and design, performance of experiments, data analysis and interpretation, and final manuscript writing; Machado-Nils, Battagello, Diniz; performance of experiments, data analysis and interpretation, and manuscript writing; Andrade-Silva, Silva-Jr, Matos, performed experiments, data analysis and interpretation; Korosi, Bittencourt, Xavier, Lucassen, Amaral, Reiter, data analysis and interpretation, and manuscript writing; Cipolla-Neto

conception and design, data analysis and interpretation and final writing manuscript.

Conflict of interest statement

No conflict of interest to declare.

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