UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

MAELINE SANTOS MORAIS CARVALHO

EFEITO DO MÉTODO DE MONITORAMENTO E DO ENRIQUECIMENTO AMBIENTAL SOBRE AS RESPOSTAS AUTONÔMICAS E CARDIOVASCULARES DE RATOS SUBMETIDOS AO ESTRESSE CRÔNICO

EFFECT OF MONITORING METHOD AND ENVIRONMENTAL ENRICHMENT ON AUTONOMIC AND CARDIOVASCULAR RESPONSES IN RATS SUBJECTED TO CHRONIC STRESS

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Odontologia, na Área de Fisiologia Oral.

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Orientadora: Profª Dra. Fernanda Klein Marcondes

Este exemplar corresponde à versão final da tese defendida pela aluna Maeline Santos Morais Carvalho, e orientada pela Profª. Dra. Fernanda Klein Marcondes.

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RESUMO

Modelos animais são essenciais para compreender os mecanismos que estão envolvidos no desenvolvimento de doenças emocionais, metabólicas e cardiovasculares, bem como sua relação com o estresse. Porém, considerando que métodos de monitoramento dos parâmetros cardiovasculares, que requerem manejo, aquecimento e restrição, podem influenciar negativamente a precisão e reprodutibilidade dos dados, um dos objetivos deste estudo foi avaliar a hipótese de que animais expostos ao modelo de estresse crônico moderado e imprevisível (ECMI), monitorados por pletismografia, poderiam apresentar medidas de pressão arterial sistólica (PAS) e de frequência cardíaca (FC) diferente daqueles monitorados por telemetria. Adicionalmente, considerando a importância de compreender se intervenções ambientais, como o enriquecimento ambiental (EA), poderiam amenizar os efeitos negativos do estresse crônico, foi também estudado o efeito do EA sobre as respostas cardiovasculares e autonômicas de ratos expostos ao ECMI. Foram utilizados ratos machos, Sprague- Dawley, livre de patógenos específicos, com 2 meses de idade e peso entre 250 e 300 gramas, e os resultados foram organizados em 2 artigos. No artigo 1, trinta e oito animais foram utilizados e divididos em quatro grupos experimentais: controle-telemetria, ECMI-telemetria, controle-pletismografia e ECMI-pletismografia. Os animais foram monitorados por telemetria ou pletismografia durante as 6 semanas do protocolo experimental, e submetidos ao ECMI durante as semanas 2, 3 e 4. Quinze dias após aplicação do ECMI os animais foram eutanasiados para determinação dos níveis de corticosterona plasmática. Animais submetidos ao ECMI apresentaram níveis elevados de corticosterona plasmática em comparação ao seu respectivo grupo controle, porém, não houve efeito do método sobre os níveis de corticosterona em animais submetidos ou não ao ECMI. A média da PAS e da FC dos animais controle ou estressados, monitorados por pletismografia, foi significativamente maior em determinadas semanas do protocolo experimental em comparação aos monitorados por telemetria. No artigo 2, outros 21 animais com implantes radiotelemétricos inseridos em aorta descendente foram distribuídos nos grupos: controle, ECMI, EA e EA + ECMI. O EA foi realizado durante sete semanas, 5 vezes por semana, 2 horas por dia, enquanto o ECMI foi aplicado nas semanas 3, 4 e 5. Após quinze dias de aplicação do ECMI, os animais foram submetidos à eutanásia e avaliados quanto à concentração plasmática de corticosterona, à modulação autonômica cardíaca, os parâmetros cardiovasculares e à atividade locomotora. O ECMI aumentou a concentração de corticosterona plasmática, a variabilidade da pressão arterial sistólica (VPAS) e diminuiu a variabilidade da frequência cardíaca (VFC). Além

disso, aumentou a pressão arterial média (PAM), no período escuro, a FC e a atividade locomotora no período claro. Por outro lado, o EA impediu o aumento da concentração de corticosterona plasmática, da FC e da VPAS, aumentou a VFC, a modulação parassimpática e reduziu a modulação simpática. Os resultados obtidos neste estudo sugerem que, no contexto do estresse crônico, as metodologias, mesmo que minimamente invasivas e que permitem o condicionamento dos animais, interferem sobremaneira nos resultados de avaliação da pressão arterial. Além disso, mostram que o enriquecimento ambiental, usado como uma estratégia não farmacológica reduziu os efeitos cardiovasculares induzidos pelo estresse crônico.

Palavras-chave: Estresse. Sistema nervoso autônomo. Telemetria. Pletismografia. Enriquecimento ambiental (Cultura animal). Animais - Proteção. I.

ABSTRACT

Animal models are essential to understanding the mechanics involved in the development of emotional, metabolic, and cardiovascular diseases, as well as their relationship with stress. However, considering that methods of monitoring cardiovascular parameters, which require handling, heating and restriction, can negatively influence the accuracy and reproducibility of the data, one of the objects of study was to evaluate the hypothesis that animals subjected to the unpredictable mild chronic stress (CMS) model, monitored by tail plethysmography, could present systolic blood pressure (SBP) and heart rate (HR) measurements different from those monitored by telemetry. Additionally, considering the importance of understanding whether environmental interventions, such as environmental enrichment (EE), could mitigate the negative effects of chronic stress, the effect of EE on the cardiovascular and autonomic responses of rats exposed to CMS was also studied. Rats male, Sprague-Dawley, specific pathogens free with 2 moths and weight between 250 and 300 g were used. In article 1, thirty-eight animals were used and divided into four experimental groups: control-telemetry, CMS-telemetry, control-plethysmography and CMS-plethysmography. The animals were monitored during 6 weeks of the experimental protocol, and submitted to the CMS in weeks 2, 3 and 4. Fifteen days after CMS, the animals were euthanized for determination of plasma corticosterone concentration. Animals submitted to CMS presented higher plasma corticosterone levels in comparison with respective control group, however, there was no effect of the method on corticosterone levels in animals subjected or not to CMS. The mean SBP and HR of control or stressed animals, monitored by plethysmography, was significantly higher in certain weeks of the experimental protocol compared to those monitored by telemetry. In article 2, another twenty-one animals with radiotelemetric implants inserted in abdominal aorta were distributed in the groups: control, CMS, EE and EE + CMS. EE was performed for seven weeks, 5 times a week, 2 hours a day, while CMS was performed at 3, 4 and 5 weeks. After 15 days of CMS application, the animals were euthanized and evaluated for plasma corticosterone concentration, cardiac autonomic modulation, cardiovascular parameters and locomotor activity. The CMS increased the concentration of plasma corticosterone, the systolic arterial pressure variability (SAPV), and decreased the heart rate variability (HRV). In addition, it increased mean blood pressure (MAP), in dark period, HR, and locomotor activity in light period. On the other hand, EE prevented the increase in plasma corticosterone, HR, and SAPV, increased HRV, parasympathetic modulation, and decreased sympathetic modulation. The results obtained in this study suggest that in the context of

chronic stress, methodologies, even if minimally invasive and allowing the conditioning of animals greatly interfere in the results of blood pressure assessment. Furthermore, they show that environmental enrichment, used as a non-pharmacological strategy reduced the cardiovascular effects induced by chronic stress.

Keywords: Stress. Autonomic nervous system. Telemetry. Plethysmography. Environmental enrichment (Animal culture). Animal welfare.

SUMÁRIO

1 INTRODUÇÃO

O estresse é um dos principais fatores de risco para o desenvolvimento de doenças cardiovasculares, emocionais e metabólicas (Purdy, 2013; Stayjee et al., 2020), comprometendo o estado de saúde físico e mental do indivíduo (Ursin e Eriksen, 2004; Charmandari et al., 2005; Nicolaides et al., 2015). Diante das altas taxas de mortalidade e morbidade das doenças cardiovasculares e sua associação com o estresse crônico, tornou-se necessário conhecer os mecanismos fisiopatológicos envolvidos no desenvolvimento dessas doenças para que novas estratégias profiláticas e terapêuticas sejam desenvolvidas.

A utilização de modelos animais representa uma importante ferramenta experimental para elucidar os mecanismos fisiopatológicos envolvidos no desenvolvimento de doenças cardiovasculares, complementando estudos clínicos e epidemiológicos. Neste contexto, o protocolo de estresse crônico moderado e imprevisível (ECMI) tem sido utilizado para o estudo dos efeitos cardiovasculares do estresse. Esse modelo consiste na aplicação alternada e prolongada de diferentes estímulos estressores, durante 3 semanas consecutivas, o que dificulta a adaptação do animal (Moreau, 1997; Neves et al., 2012). Aumento na pressão arterial sistólica (PAS), diastólica (PAD) e frequência cardíaca (FC), concentrações sanguíneas elevadas de corticosterona, catecolaminas e angiotensina II, hipertrofia da aorta e diminuição do lúmen dos vasos foram alguns dos efeitos nocivos do ECMI evidenciados por nosso grupo de pesquisa (Neves et al., 2009, 2012; Marcondes et al., 2011; Firoozmand et al., 2018; Costa et al., 2020, 2021).

Apesar das significativas mudanças neuroendócrinas, morfológicas e funcionais no sistema circulatório induzidas pelo ECMI mencionadas acima, os resultados epidemiológicos que indicam uma correlação entre o estresse crônico e hipertensão arterial ainda são difíceis de replicar em pesquisas pré-clínicas (Markovitz et al., 2004; Liu et al., 2022). Neste sentido, alguns estudos em modelos animais têm mostrado alterações na pressão arterial (PA) e na FC decorrentes da exposição ao estresse, no entanto, outros estudos não encontraram nenhuma mudança nesses índices, ou eles foram mais discretos (Nalivaiko, 2011; Crestani, 2016). Assim, embora mudanças na PA e na FC sejam frequentemente observadas em resposta ao estresse, sabe-se que elas podem variar de acordo com as características e tipo dos estímulos estressores aplicados (duração, frequência, intensidade, previsibilidade ou imprevisibilidade, homotípico, heterotópico), (Koolhaas et al., 2011; Crestani, 2016), idade, sexo e linhagem do animal (Duarte et al., 2015; Cruz et al., 2016; Crestani, 2016; Vieira et al., 2018). Outro fator relevante a ser considerado é a influência do método de monitoramento utilizado para avaliar as respostas cardiovasculares em modelos de

estresse. De acordo com a revisão de Nalivaiko (2011) e de Crestani (2016), a maioria dos estudos que apontaram mudanças significativas na PA e na FC após exposição a estressores crônicos utilizou a pletismografia de cauda para avaliar esses parâmetros, e quando foram utilizados métodos invasivos para validar os achados identificados por pletismografia de cauda, o aumento da PA e da FC foi menor.

Assim, considerando esses fatores, um dos objetivos desta tese foi avaliar a hipótese de que, animais com mesma linhagem, sexo, idade, expostos ao modelo ECMI, monitorados por pletismografia, poderiam apresentar medidas de pressão arterial sistólica (PAS) e de frequência cardíaca (FC) diferente daqueles monitorados por telemetria.

Considerando os prejuízos ao sistema cardiovascular decorrente de estímulos estressores e o impacto negativo das doenças cardiovasculares sobre a saúde física, emocional e financeira da sociedade (Abegunde et al., 2007; Mozzaffarian et al., 2016), tem cada vez mais sido reconhecido a importância de múltiplas intervencões na saúde e bem-estar do indivíduo. A prática regular de atividade física e o engajamento em atividades social e mentalmente estimulantes têm sido sugeridas para prevenir ou melhorar a qualidade de vida de indivíduos com desordes cardiovasculares, cognitivas ou emocionais (Carlson et al., 2012; Lin et al., 2014; Fissler et al., 2018; Dauwan et al., 2021; Scarfò et al., 2023). Para mimetizar essas intervenções, em estudos pré-clínicos, o enriquecimento ambiental (EA) tem emergido como uma modelo experimental que potencializa a interação social e promove o desenvolvimento sensorial e motor (Segovia et al., 2009). A exposição do animal a um ambiente contendo uma variedade de objetos como roda de exercício, túneis, objetos com diferentes texturas e outros animais (Fernandez - Turuel et al., 2002; Fox et al., 2006; Zanca et al., 2015; Costa et al., 2020, 2021), estimula o comportamento natural da espécie e tem sido utilizado como uma importante estratégia na modulação da reação ao estresse (Costa et al., 2020, 2021), além de prevenir doenças como a hipertensão arterial (Garbin et al., 2012; Sousa et al., 2019). Melhora no aprendizado, memória, redução de comportamentos análogos a depressão humana (Costa et al., 2020, 2021) e ansiedade (dados não publicados), melhor recuperação motora após eventos cerebrovasculares como acidente vascular encefálico (AVC), são alguns dos benefícios do EA (Livingston – Thomas et al., 2016). Adicionalmente, estudos têm sugerido que o EA pode modular o eixo hipotálamo-hipófise- adrenal (HHA) e a atividade simpática, reduzindo respostas hormonais ao estresse crônico (Costa et al., 2020, 2021).

Diante disso, sabe-se da importante influência do sistema nervoso autônomo (SNA) em regular os processos fisiológicos e controlar a PA e FC (Malpas, 2010), e estudos

têm apontado que disfunções nesse sistema pode contribuir para o desenvolvimento de hipertensão arterial, doença coronariana, arritmias e parada cardíaca súbita (Grippo et al., 2009; Thayer et al., 2010; Wulsin et al., 2015; Mucci et al., 2016; Buckley e Shivkumar 2016, Sara et al., 2018; Chang Liu et al., 2021). Neste contexto, uma das formas utilizada para avaliar o SNA é a partir do estudo da variabilidade da frequência cardíaca (VFC), e variabilidade da pressão arterial (VPA). De maneira geral, a VPA tem sido considerada um marcador fisiológico do controle do SNA e seu aumento tem sido associado ao aumento do risco cardiovascular e danos em órgãos alvos, enquanto que a redução da VFC está associada à hipertensão e ao risco elevado de morte súbita em indivíduos com insuficiência cardíaca crônica (Malpas, 2010; Prinsloo et al., 2014; Grassi et al., 2015; Irigoen et al., 2016).

Em humanos e animais é possível determinar a VFC e VPA no domínio do tempo e da frequência, utilizando, por exemplo, a análise espectral autoregressiva (Farah et al., 2004; Joaquim et al., 2004). A análise espectral autoregressiva é um modelo matemático que permite a análise da atividade do SNA de maneira não invasiva, a partir de valores sucessivos de FC, intervalo de pulso (IP) e PA, bem como oscilações rítmicas em diferentes frequências, que podem refletir a modulação parassimpática ou simpática sobre o sistema cardiovascular. Dessa forma, os componentes de alta frequência (Hight Frequency - HF - 0,75 a 3,00 Hz), representam a modulação respiratória e são considerados um indicador da atuação do nervo vago sobre o coração. Já os de baixa frequência (Low- Frequency - LF - 0,20 - 0,75 Hz), representam a modulação vagal e simpática sobre o coração, com predominância do simpático (De Angelis et al., 2004; Silva, 2015; Irigoen et al., 2016; Rabello Casali et al., 2016).

Diante dos benefícios do EA em modular as respostas ao estresse crônico, e a carência de estudos que avaliaram os efeitos do EA sobre as respostas cardiovasculares e autonômicas cardíacas em ratos submetidos a estressores crônicos variados, buscou-se também nesta tese, investigar se o EA, utilizado como uma estratégia não farmacológica poderia prevenir ou reduzir as mudanças cardiovasculares e autonômicas cardíacas induzidas pelo ECMI.

As respostas para essas hipóteses, assim como a fundamentação teórica dos estudos, o delineamento experimental, os resultados e discussões estão apresentadas nos artigos 1 e 2 de acordo com as normas estabelecidas pela deliberação 001/2015 da CCPG da Universidade Estadual de Campinas (UNICAMP).

2 ARTIGOS

2.1 Use of Telemetry as a Refinement Tool for the Evaluation of Cardiovascular Responses in Rats Submitted to Chronic Stress

Artigo submetido ao periódico Laboratory Animals em 19/01/2024.

Maeline S Morais-Carvalho; Rafaela Costa; Gizele B Barankevicz; Carlos T dos S Dias; Dulce E Casarini; Fernanda K Marcondes.

Comprovante de submissão eletrônica do artigo está disponível no Anexo 2.

Use of Telemetry as a Refinement Tool for the Evaluation of Cardiovascular Responses in Rats Submitted to Chronic Stress

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Abstract

Animal models play an essential role in research on cardiovascular diseases, however the stress caused by laboratory procedures represents a significant challenge in animal research. Therefore, the purpose of this work was to evaluate the temporal evolution of cardiovascular parameters, monitored by telemetry or plethysmography, in 2-month-old male Sprague-Dawley rats submitted to chronic mild unpredictable stress (CMS) protocol. In experiment 1, the animals (control and CMS - telemetry) were submitted to surgery to insert a transducer, followed by monitoring of blood pressure and heart rate for 6 weeks. In experiment 2, other animals were divided into 2 groups (Control and CMS - Plethysmography) and after being submitted to the habituation protocol, weekly recording of blood pressure and heart rate was performed by plethysmography. In both experiments, the CMS protocol was applied in weeks 2, 3, and 4, with cardiovascular measurements before, during, and after the application of stress. In both experiments, the CMS protocol caused significant increases of plasma corticosterone, compared to the respective control groups. Animals displayed blood pressure and heart rate behaviors that differed according to the measurement method employed, in certain weeks during the experimental period. Prolonged effect to CMS, and significantly higher average of blood pressure and heart rate were observed in animals monitored by plethysmography. The use of telemetry as method of monitoring cardiovascular parameters prevented changes in the animals' routine, which contributed to greater welfare, less distress and discomfort, and physiological responses free of the experimental stress triggered by the method.

Keywords: Stress, telemetry, plethysmography, welfare, blood pressure.

Introduction

Stress is a risk factor for the development of cardiovascular diseases,[1] independent of traditional risk factors.[2,3] Given the high rates of morbidity and mortality associated with these diseases, together with the costs of treatment,[4,5] it is essential to identify risk factors. These include arterial hypertension,[6] which has a complex multifactorial etiology[7] and is increasingly common as a result of sedentary lifestyles, obesity, and stress.[8]

With animal experimentation it became possible to discover and test novel preventive and curative therapies for a series of diseases by understanding the mechanisms and factors involved that can contribute to their development. Nonetheless, experimental stress is one of the main challenges faced by researchers in animal research. Many routine procedures performed in the laboratory environment (handling, physical restraint, dosing and blood sampling) negatively impacts animal welfare, and consequently, lead to cardiovascular, hormonal changes[9–11] that compromise the interpretation and reliability of the data obtained. Other factors that can interfere and lead to different cardiovascular responses are the animal's age, strains, sex, applied stress protocol and types of stressor stimuli (homotypic or heterotypic).[12–14]

In this way, the use of technology-based approaches may reduce the stress caused to the animal during the procedures performed and avoid changes in its routine, contributing to animal welfare, better reliability and accuracy of the results obtained.[15] Furthermore, it upholds the principles of the 3Rs in experimentation, as proposed by Russell and Burch in 1959.[16] These principles involve Reduction, Refinement and Replacement of procedures used in the animal experimentation, aiming the use of a smaller number of animals, as well as experimental procedures that minimize pain, suffering and more accurate equipment for data collection.[16]

In this context, telemetry and plethysmography are two methods in the literature that enable the monitoring of cardiovascular parameters *in vivo*. And, although it has its advantages, disadvantages and recommendations for use previously described by Kurtz in 2005,[17] it still requires greater knowledge regarding its use in studies on the effects of chronic stress on the development of arterial hypertension.

In this sense, due to the factors mentioned above, sought with this study, to follow the evolution of blood pressure and heart rate in Sprague-Dawley rats, with the same weight, age and sex, over six weeks, before, during and after the application of the chronic mild

unpredictable stress (CMS) protocol, monitored using telemetry or plethysmograph, and to assess whether the monitoring method could interfere with the physiological responses triggered or not by chronic stress.

Animals, material and methods

Animals and experimental design. Thirty-eight male Sprague-Dawley (NTacUnib:SD), specific pathogen free rats,[18] aged 2 months and weighing between 250 and 300 g at the start of the experiment, were provided by the Multidisciplinary Biological Research Center (CEMIB) of the State University of Campinas. During the experimental period, the animals were housed in individual cages containing autoclaved sawdust, in an air conditioned room (22 ± 2 ºC), with a 12 h/12 h light/dark cycle (lights switched on at 06:00 am) and *ad libitum* feed. The animals were handled during changing and cleaning the cages. All the procedures were approved by the Animal Use Ethics Committee of the State University of Campinas (CEUA processes #4219-1 and #900-1) and complied with the requirements of the National Council for Control of Animal Experimentation (CONCEA) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The experiments had durations of 6 weeks (Figure 1).

In Experiment 1, 16 rats aged 2 months were submitted to surgery for insertion of a transducer (Data Sciences International, St. Paul, MN Model TA11PA-C40),[19] for subsequent recording of systolic and diastolic blood pressure and heart rate and corticosterone concentrations. Following recovery from the surgery, after 15 days, with reestablishment of normal weight,[20] blood pressure and activity,[21] and heart rate,[22] the animals were randomly divided into two groups: control and CMS and were continuously monitored for 6 weeks. During weeks 2, 3, and 4, the rats in the stress group were submitted to CMS,[23] with adaptations (Table 1). Fifteen days after the end of the CMS protocol, the animals were killed by decapitation (Figure 1). Of the sixteen animals, 6 (telemetry control group) were kept under similar experimental conditions for 6 weeks, but without CMS application, for the determination of corticosterone concentrations, systolic and diastolic blood pressure and heart rates (Figure 1).

In Experiment 2, 22 rats were used. Eleven animals were submitted to weekly measurements of blood pressure and heart rate by tail plethysmography.[24] During weeks 2, 3, and 4, the animals were submitted to CMS (Table 1). Fifteen days after the end of the CMS protocol, the animals were killed by decapitation (Figure 1). The other 11 animals (control group) were kept for 6 weeks under the same experimental conditions, without CMS

application, and were used for determination of corticosterone levels and hemodynamic parameters. The control animals were submitted to plethysmography during the same periods as the animals submitted to CMS (Figure 1).

The temporal evolution of blood pressure and heart rate was monitored for each animal, considering the various factors, described above, that could influence identification of the hemodynamic alterations due to stress induced by the CMS protocol. In both experiments, baseline values were obtained by measurement of the cardiovascular parameters one week before the start of the CMS protocol (week 1). Hence, the blood pressure and heart rate of the animals were monitored before, during, and after application of the stress stimuli.

The sample size was estimated based on previously published studies,[23] the number of experimental groups (4), considering α=0.05 and statistical power of the test of 95%. All animals were male to control the effects of the reproductive cycle, which would be additional factors in addition to the monitoring method and stress. Furthermore, the selected strains and age allowed better comparison with studies published by our group and others.[25]

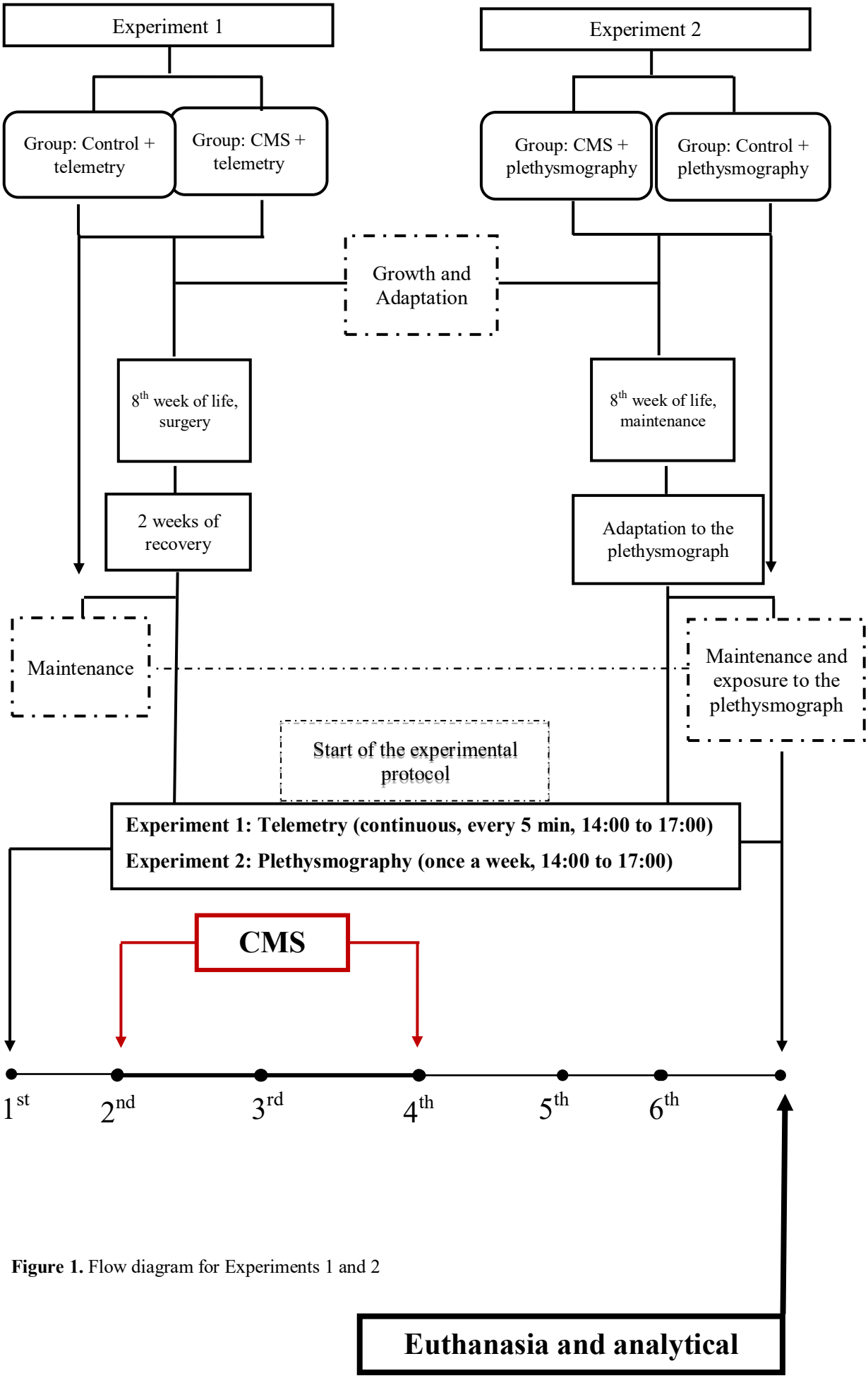

Figure 1. Flow diagram for Experiments 1 and 2

Chronic mild unpredictable stress (CMS). The CMS protocol (Table 1) consisted of applying different stress stimuli over 7 days, repeating the procedures for 3 consecutive weeks,[23] with adaptations.

Table 1. Chronic mild unpredictable stress (CMS) protocol

	Morning	Afternoon
Monday	**8:00 – 9:00** Immobilization.	**13:00 – 14:00** Immobilization. **18:00** Continuous illumination during the night.
Tuesday	**8:00 – 9:00** Immobilization.	**14:00 – 15:00** Immobilization followed by deprivation of water and food for 17 h.
Wednesday	**8:00 – 10:00** Restricted access to food for 2 h.	**14: 00 – 15:00** Immobilization followed by accommodation in cages containing wet wood shavings.
Thursday	**8:00 – 9:00** Immobilization.	**13:00 – 14:00** Immobilization followed by deprivation of water for 18 h.
Friday	**8:00 – 10:00** Exposure to empty water bottles. **11:00 – 12:00** Immobilization	**18:00** Inverted light/dark cycle (until 8:00 on the following Monday).

Determination of blood pressure and heart rate by telemetry. Fifteen days after recovery from the surgery, the animals were submitted to the procedure for recording blood pressure and heart rate. On the Monday of week 1, the telemetry was activated and the signals from the transmitters implanted in the animals were captured by the PhysiolTel RPC-1 receivers (Data Sciences International, St. Paul, MN, USA) positioned below the cages. The signals were processed using a Data Exchange Matrix and were sent to the computer, where data acquisition was performed using Dataquest ART 4.3 software. The data were obtained continuously, with the systolic blood pressure and heart rate of each animal being recorded every 5 min,[26] at a sampling rate of 500 Hz,[21,22] on Fridays between 14:00 and 17:00. The data were stored and extracted for analysis using the proprietary software, following the instructions of the manufacturer (Data Sciences International, St. Paul, MN, USA).

Determination of blood pressure and heart rate by plethysmography. Two weeks before the start of the experimental protocol, the animals were habituated once weekly to the equipment (BP-2000 Blood Pressure Analysis System, Visitech Systems) and to the procedure for recording blood pressure and heart rate. After the habituation sessions, the blood pressures and heart rates were measured by connecting a plethysmograph to the tails of the animals,[24] in a soundproof room, on Fridays at 14:00 (weeks 1 to 6). Before starting the procedure, the rats were removed from animal house and kept for 30 min in the experiment laboratory with the same environmental conditions (temperature, light, acoustic insulation), to adapt to the environment. The procedure was performed by placing the rat in a suitably sized rectangular metal tube attached to a platform heated at 36 ºC. The tail was attached to a pulse transducer and a rubber cuff connected to a sphygmomanometer. After 5 min, the equipment was activated, the cuff was inflated and deflated automatically, and the pulse transducer captured the variations of light transmitted through the tail. The systolic blood pressure and heart rate values were recorded on the computer using the equipment software (BP-2000 Analysis Software, Visitech Systems). The systolic pressure was obtained by monitoring vessel dilation as the cuff was inflated and deflated. The blood pressure and heart rate values were obtained as the average of ten measurements.

Corticosterone determination. After fifteen days following the conclusion of the CMS protocol, the animals were killed by decapitation [27] without any prior anesthesia, since anesthesia can increase corticosterone levels.[28,29] Three mL of blood from each animal were collected in a Falcon tube containing anticoagulant, the tube was centrifuged (1000 g for 20 min at 4 ºC), the supernatant was transferred to microtubes and were stored at -80 ºC for further assay.

The plasma corticosterone concentration was determined by colorimetric enzymatic assay, using a commercial kit (Enzo Life Sciences, Inc., Ann Arbor, MI, USA). The detection limit was 0.027 ng/mL and the intra- and inter-assay coefficients of variation were 7.7 and 9.7%, respectively.

Statistical analysis. In experiments 1 and 2, exploratory analysis was performed and assumptions were verified, such as normality of error, homogeneity of treatment variances and existence of discrepant points. Data were analyzed by Three-way analysis of variance (ANOVA), followed by Tukey's post hoc test, where appropriate, considering the factors: stress, monitoring method and weeks, employing GraphPad Prism v. 8.0.1software.

Statistical analysis of the corticosterone concentration employed two-way ANOVA followed by Tukey's test for multiple comparisons of means. Evaluation was made of the effects of the main factors, monitoring method, and application of stress, as well as their interactions. Two levels were considered for the effects of the method (telemetry and plethysmography) and stress (control and stress groups). A significance level of $p < 0.05$ was adopted. The results were presented as means ± standard errors.

Results

The CMS caused a significant increase in plasma corticosterone levels, compared to the control animals, in the groups submitted to both methods of blood pressure assessment ($F(1.36) = 71.79$ $p < 0.0001$, Figure 2). There was no effect of the monitoring method (telemetry or plethysmography) on the plasma corticosterone concentrations of the animals submitted to the stress or the controls ($p = 0.8029$, Figure 2).

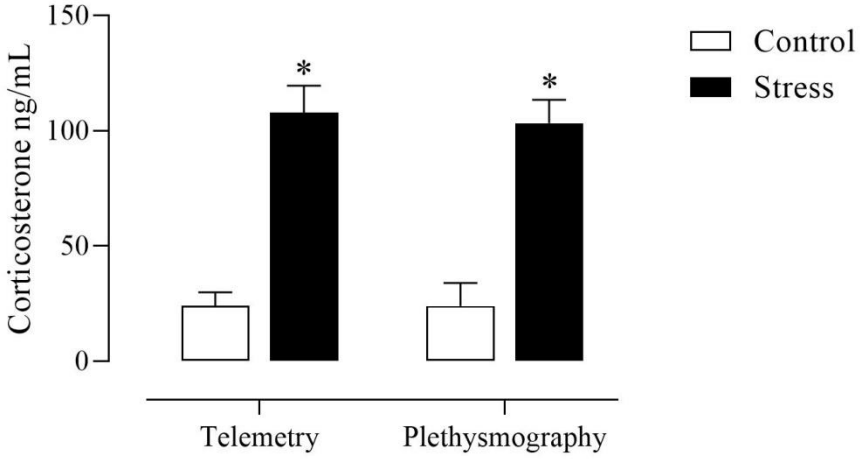

Figure 2. Plasma corticosterone concentrations in the CMS and control group rats monitored using plethysmography or telemetry and killed 15 days after the end of CMS application. The results are presented as means ± standard errors. * Significant difference relative to the control group (n = 10 per group, two-way ANOVA followed by Tukey's test, $p < 0.05$).

Three-way ANOVA performed for systolic blood pressure showed a significant interaction among week/stress effects (F (5.207) = 4.028 p = 0.0016, Figure 3), stress/monitoring method (F (1.207) = 24.24 p = < 0.001, Figure 3), without interaction week/monitoring method (F (5.207) = 0.2913 p = 0.9174, Figure 3). The control-plethysmography group presented higher systolic blood pressure values, compared to the control-telemetry during the entire experimental protocol ($p < 0.05$, Figure 3).

The systolic blood pressure of the CMS-plethysmography group was significantly higher at week 5 (p = 0.0006, Figure 3), compared to the CMS-telemetry group, with no significant difference at weeks 1, 2, 3, 4, and 6 ($p > 0.05$, Figure 3). The stressed animals monitored by telemetry presented an increase in systolic blood pressure during the three weeks of application of the CMS protocol (weeks: 2, 3, 4) when compared to the respective control group, also evaluated by telemetry ($p < 0.05$, Figure 3). There was no difference in systolic blood pressure values among the control and stress groups monitored by plethysmography ($p > 0.05$, Figure 3).

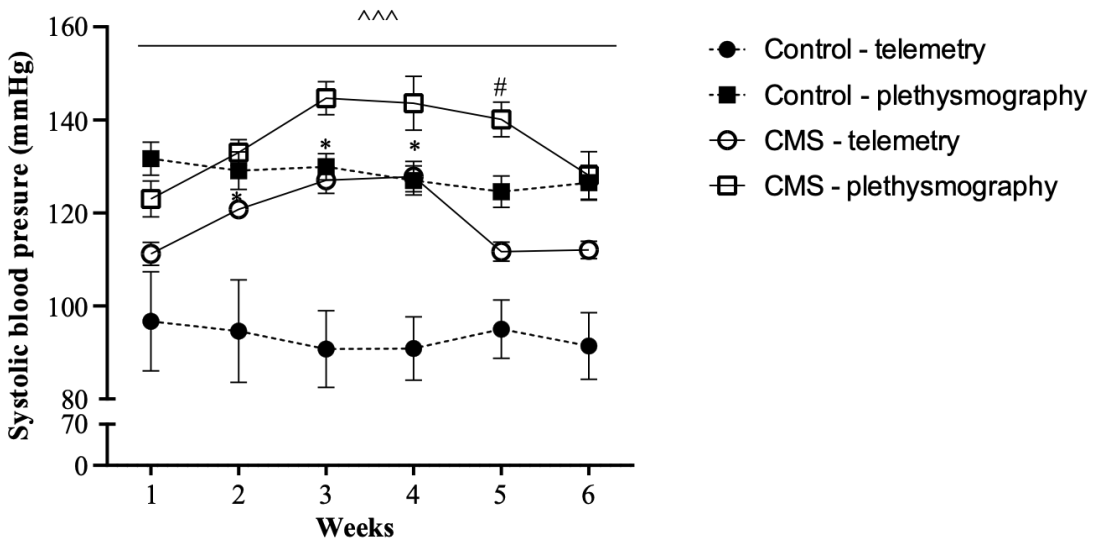

Figure 3. Comparison of acquired systolic blood pressure (SBP - mmHg) recordings nonsimultaneously in the CMS and control group rats monitored using plethysmography or telemetry, during 6 consecutive weeks, and killed 15 days after the end of CMS application. *p< 0,05 telemetry records in rats submitted the CMS v.s. their respective control group. #p< 0,05 telemetry records in rats submitted the CMS v.s. the plethysmografy records in rats submitted the CMS. ^^^p< 0,05 telemetry records control v.s. the plethysmografy records control group. The results are presented as means ± standard errors, (n = 6-11 per group, three-way ANOVA followed by Tukey's test where apropriate, $p < 0.05$).

There was a significant interaction between the effects of stress/week/monitoring methods (F (5.208) = 2.642, p = 0.0243, Figure 4) on heart rate. Animals in the control-plethysmography group had significantly higher heart rate values than the control-telemetry group during the entire experimental protocol (p= 0.0001, Figure 4). The heart rate of the CMS-plethysmography group was significantly higher in weeks 1, 2, 5, 6 of the experimental protocol in relation to the CMS-telemetry group (p = < 0.0001, Figure 4), with no difference in weeks, 3 (p = 0.9999, Figure 4) and 4 (p = 0.7267, Figure 4). Stressed animals monitored by telemetry showed an increase in heart rate during two weeks of application of the CMS protocol (weeks: 3 and 4) when compared to the respective control group monitored by telemetry (p = 0.0001, Figure 4). There was no different among CMS and control groups monitored by plethysmography during experimental protocol (p > 0.05, Figure 4).

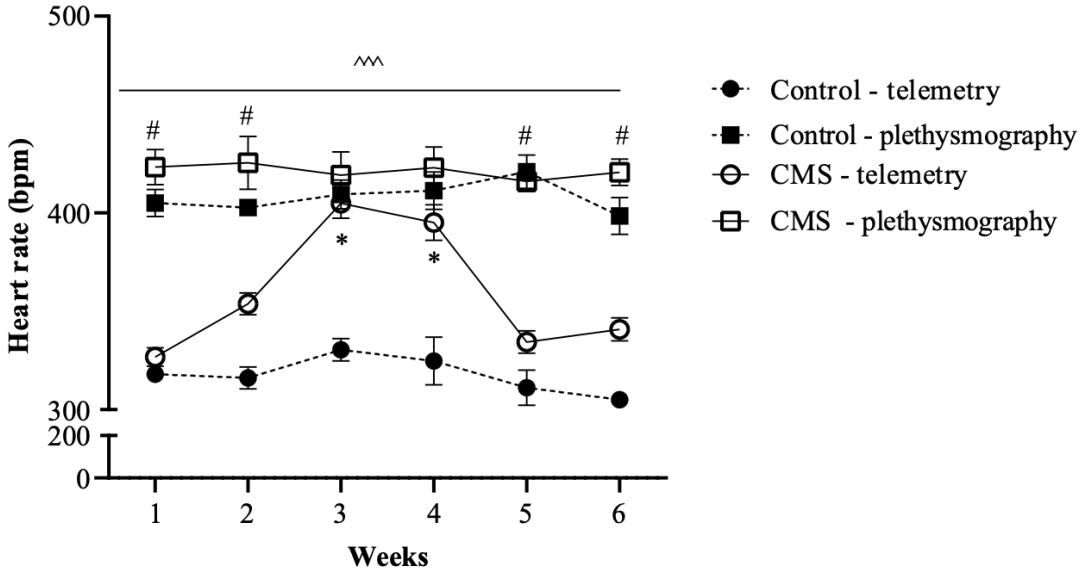

Figure 4. Comparison of acquired heart rate (HR - bpm) recordings nonsimultaneously in the CMS and control group rats monitored using plethysmography or telemetry, during 6 consecutive weeks, and killed 15 days after the end of CMS application. *p< 0,05 telemetry records in rats submitted to CMS v.s. their respective control group. #p< 0,05 telemetry records in rats submitted the CMS v.s. the plethysmografy records in rats submitted the CMS. ^^^p< 0,05 telemetry records control v.s. the plethysmografy records control group. The results are presented as means ± standard errors, (n = 6-11 per group, three-way ANOVA followed by Tukey's test, p < 0.05).

Discussion

The main findings of the present study were that (1) exposed animals to the CMS monitored by plethysmography exhibit prolonged systolic blood pressure and heart rate responses compared to animals monitored by telemetry, (2) the control and CMS groups

monitored by plethysmography showed the same physiological responses and (3) the animals in the control group monitored by telemetry showed physiological responses significantly lower than those observed in the control group monitored by plethysmography since the beginning of the experiment.

Changes in animal welfare or stress are identified through elevated levels of corticosterona,[23,30,31] catecholamines[30] and increase in blood pressure, heart rate and body temperature.[32,33] Monitoring methods that involve handling, warming and restraint have a negative effect these parameters and may compromise the interpretation of results.[14,22,24,34,35]

Our results show that similar physiological responses were triggered in control or stressed animals monitored by plethysmography. No statistical difference was observed in systolic blood pressure and heart rate, including in the weeks of application of the CMS protocol (weeks: 2, 3 and 4), between control and CMS groups monitored by plethysmography. In addition, the control group monitored by telemetry showed significantly lower values of systolic blood pressure and heart rate since the beginning of the experiment. It would be reasonable to conclude, based on these results, that although the habituation protocol was applied, it was not sufficient to make the animals habituated to the procedure and prevent physiological responses to experimental stress from being triggered.

Restraint, warming and handling are some of the different factors that, associated or not, could explain the increase in blood pressure and heart rate in animals monitored by plethysmography compared to those monitored by telemetry. Although common and often necessary, physical restriction promotes cardiovascular and hormonal changes[9] and symptoms analogous to human depression, anedonia.[11] In this regard, various habituation protocols have been described to minimize stress during the recording of cardiovascular parameters by plethysmography. However, studies have shown no reduction in blood pressure or heart rate as a result of repeated exposure to the technique in mice[33] and rats.[36]

Moreover, alterations in body temperature due to confinement in restraining cages[37] or intentional warming platforms, as recommended by the equipment manufacturer, were found to increase systolic blood pressure values in spontaneously hypertensive rats compared to other techniques without warming.[35]

Another important finding is the prolonged effect of CMS observed in animals monitored by plethysmography. In contrast, animals monitored by telemetry exhibited normal values right after the end of the application of stressor stimuli. Similar prolonged effects to stress were observed in spontaneously hypertensive rats monitored by telemetry when compared to normotensive rats in a study where animals were exposed to air-jet stress

followed by a further 10 consecutive days of restraining.[13] In this sense, the reduction in systolic blood pressure and heart rate shortly after the end of stressful stimuli in animals monitored by telemetry may be associated with the lack of previous experience with the discomforts generated by the procedure. Although the chronic stress protocol did not induce arterial hypertension, and no statistical difference was observed in systolic blood pressure in the weeks of applying the stress protocol, a significant higher average was noted in animals monitored by plethysmography compared to those measured by telemetry. In contrast to these findings, similar readings they were obtained in mice not disturbed, when monitored by telemetry and tail-cuff.[33] These findings support the hypothesis of a more robust reaction of stressed animals to the monitoring method and align with studies suggesting that chronic stress exposure can result in a more pronounced physiological response when subjected to acute stress stimuli.[38]

Simple refinement strategies have positive impact on welfare and have been used to minimize the stressful effects that common procedure used in the laboratory environment can cause to animals during research. In addition to reducing behaviors related to fear and anxiety[39,40] changes in handling decreased elevated levels of corticosterone and blood glucose in mice.[41] In this sense, the use of telemetry, in addition to reducing the number of animals,[32] enables continuous monitoring and the acquisition of more accurate data.[42] It can serve as a refinement tool as it allows for monitoring the animal in its natural environment, minimizing any suffering or distress caused by the procedure. On the other hand, while plethysmography is a non-invasive and pain-free technique, measurements are typically conducted during the day, disrupting rodent sleep cycles.[17] Additionally, the requirement for handling, warming, and restraint has a negative impact on animal welfare, as discussed earlier and demonstrated in our results.

Despite the surgery performed for the implantation of the transducers in the animals' aorta, no impact on the animals' welfare was evidenced after the recovery period (animals ate, moved normally and did not show any signs of pain or discomfort). As described in methodology, the animals were carefully medicated and evaluated by pain face scale during the recovery period. In addition, weight gain was monitored because a sustained reduction in weight could indicate pain and suffering. In contrast, many animals ate and moved around in their cages shortly after the end of surgery.

Based on our experiences, the surgery time, specifically the aortic clamping time, has a significant impact on the animal's recovery time. A faster the surgery results in less

impact on the animal's welfare and, consequently, a shorter recovery time. However, further studies will be needed to confirm the perceptions identified in the laboratory.

Considering that corticosterone is an important biomarker in stress studies, in the present work, both monitoring methods revealed elevated plasma corticosterone levels 15 days after the conclusion of the stressful stimuli, indicating the effectiveness of the CMS protocol and the absence of adaptation to the stress stimuli. Nevertheless, it should be noted that a limitation of the present study was that corticosterone levels were not assessed during or shortly after the monitoring of blood pressure and heart rate in the experiments.

Our study presents new and significant discoveries about the behavior of blood pressure and heart rate in response to chronic stress, monitored by telemetry or plethysmography. Through this longitudinal monitoring, we were able to identify both distinct and similar physiological responses to chronic stress, as well as responses triggered by experimental stress. Furthermore, it demonstrated that the CMS protocol did not induce hypertension in animals. These findings strongly support the hypothesis that hypertensive effect identified by plethysmography may be attributed to the heightened stress experienced by animals during the procedure.[14]

In conclusion, utilizing methods that minimize the number of laboratory procedures and avoid disruptions to the normal activities of laboratory animals, enables the acquisition of data devoid of physiological responses induced by experimental stress. This approach can contribute to enhanced welfare in research animals. Thus, in particular, facilitated the tracking of physiological changes induced by the stress protocol without associating stressor stimuli with the monitoring method. Moreover, it demonstrated to be an excellent tool for refinement in experimental procedures.

Conclusion

The application of plethysmography and telemetry for measuring cardiovascular parameters in rats subjected to the CMS protocol revealed differences between the techniques in terms of the temporal patterns of decreased systolic blood pressure. Additionally, the similarity in cardiovascular responses between the control and stress group suggests aversive reactions to the monitoring method. In studies of this nature, the use of telemetry can enhance the precision of results obtained for temporal variations in these parameters.

Declaration of Conflicting Interests

The authors declare that there are no conflicts of interest.

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Data Availability Statement

All data will be available under request to correspondent author. Email: ferklein@unicamp.br.

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Supplementary information

Telemetry

Surgical procedure. For insertion of the transducer to record the cardiovascular parameters by telemetry, the animals were anesthetized using 5% isoflurane in 1 L of oxygen, in an anesthesia chamber (13 cm width x 25 cm length x 12.5 cm height).[1,2] During the surgery, anesthesia was maintained using 2.5-3.0% isoflurane,[2,3] with the animals manipulated in a dorsal decubitus position on the surgical table during approximately 40 min.

After induction of anesthesia, trichotomy was performed of approximately 5 cm of the abdominal region. Pre-asepsis was performed with 10% iodized alcohol and, using an aseptic technique, a 5 cm laparotomy was performed on the abdominal midline. The peritoneal cavity was exposed and the intestine was gently moved within the abdominal cavity, using gauze soaked in sterile physiological solution, enabling access to the abdominal aorta.[4] After locating the abdominal aorta, a suture line was introduced in the ascending region of the aorta and a clamp was positioned in the descending region to interrupt the local blood flow.[1,3,5]

Using an incision in the abdominal aorta, the catheter of the TA11PA-C40 measurement transducer (Data Sciences International, St. Paul, MN, USA) was inserted into the lumen of the vessel and fixed into position with approximately 1-2 drops of tissue glue (Vetbond, 3M, St. Paul, MN, USA).[4] Once successful fixation of the catheter had been confirmed by observing an absence of hemorrhage, the transducer was attached to the internal layer of the abdomen and the skin was sutured with Suturin thread, followed by disinfection with 10% iodine. After fixing the catheter, the animals were medicated subcutaneously with enrofloxacin (antibiotic, 5 mg/kg) and ketoprofen (anti-inflammatory, 5 mg/kg), and remained in the surgery room until return from anesthesia. A few seconds before application of the drugs, the isoflurane was discontinued and the animal remained with inhalation of oxygen. The animals were checked daily by the researcher during one week after the surgical procedure, for possible signs of pain, infection, or stress caused by the surgical procedure.[6] For the relief and control of pain, a second dose of anti-inflammatory was administered 12 h after the end of the surgical procedure.[3] Two weeks were allowed for weight recovery and restoration of circadian rhythms, blood pressure, and heart rate. The experimental groups (CMS + Telemetry and Control + Telemetry) included animals that, after the recovery period and beginning of recordings, showed no losses in diet, activity and blood pressure and heart rate values.

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2.2 The Effects of Environmental Enrichment on Cardiovascular Responses to Chronic Stress

O artigo será submetido após sugestões da banca examinadora.

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The Effects of Environmental Enrichment on Cardiovascular Responses to Chronic Stress

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Abstract

Stress can cause changes in cardiac structure and/or function that can contribute to the development of heart disease. On the other hand, several studies have shown the positive effects of environmental enrichment (EE) on stress responses. In this sense, the objective of the present study was to investigate the effects of EE on cardiovascular parameters and autonomic cardiac activity in rats subjected to moderate and unpredictable chronic stress (CMS). Male Sprague-Dawley rats with radiotelemetry catheters inserted into the descending aorta were randomized into Control, Stress (CMS), Environmental Enrichment (EE) and Environmental Enrichment + Stress (CMS + EE). EE was applied for 7 weeks, 5 days per week, 2 hours per day, while CMS was applied for 3 consecutive weeks. Fifteen days after application of CMS, the animals were decapitated and plasma corticosterone levels were determined. CMS increased plasma corticosterone concentration, systolic arterial pressure variability (SAPV) and decreased heart rate variability (HRV). Futhermore, CMS increased mean arterial pressure (MAP) in dark period, heart rate and locomotor activity in light period. EE decreased corticosterone secretion, prevented an increase in heart rate and SAPV, increased HRV and parasympathetic tone, and reduced sympathetic tone. The results indicated that EE appears to prevent CMS-induced hormonal and cardiac autonomic dysregulation.

Keywords: Stress. Autonomic nervous system. Environmental enrichment. Hypertension. Cardiovascular.

Introduction

The increase in neuroendocrine activity characterized by the hypothalamic-pituitary-adrenocortical (HPA) and sympatho-adrenomedullary axes constitute the set of important adaptive responses generated in the face of stressful stimuli (McCarty, 2016; Crestani, 2016). However, prolonged or intense exposure to stressor stimuli might induce metabolic, cardiovascular and emotional disorders (Grippo and Johnson, 2009; Steptoe and Kivimäki, 2012; Golbini et al., 2015; Sun et al., 2021). Considered the main cause of death worldwide, cardiovascular diseases have become an important health problem given the high costs involved in their treatment and the possible financial and quality of life losses (Abegunde et al., 2007; World Health Organization, 2020; van der Velden et al., 2022).

High blood pressure is one of the factors that contributes to the high number of cases of cardiovascular diseases, and is closely related to stress, sedentary lifestyle and obesity (Miura et al., 2013; Pearson et al., 2019). According to data from the Brazilian cardiologist institute, in 2023 the number of hypertensive patients broke a record. These results are not only alarming due to the increase in the number of cases, but also due to the increase in cases among young people aged 18 to 24 (Migowski and Tavares, 2024).

One of the causes behind cardiovascular disease in humans is autonomic cardiovascular imbalance, which is characterized by high sympathetic modulation, decreased parasympathetic modulation, or both (Thayer et al., 2010). In experimental and clinical studies the reduction in heart rate variability (HRV) has been associated with stressful situations, high blood pressure, depression and cardiovascular diseases (Grippo et al., 2003; Costoli et al., 2004; Thayer et al., 2010; Carnevali et al., 2014; Morais-Silva et al., 2019; Park et al., 2017). Meanwhile, increased blood pressure variability (SAPV) has been linked to organ damage (Parati et al., 1987, 2012; Hansen et al., 2010).The use of telemetry, in addition to allowing the monitoring of cardiovascular parameters in animals without adding stressful factors such as (handling, heating, restriction), also allows research into the autonomic functioning of the heart through autoregressive spectral analysis (Farah et al., 2004; Joaquim et al., 2004).

The chronic mild stress (CMS) model has been used by our research group and other authors to analyze the effects of chronic stress on cardiovascular, behavioral and metabolic disorders. Prolonged and unpredictable exposure to a variety of stressful stimuli prevents animals from habituating and simulates, in a more realistic way, the stressors of daily life (Willner, 2005; Neves et al., 2009, 2012; Firoozmand et al., 2018; Costa et al., 2020,

2021). Increases in systolic and diastolic blood pressure and heart rate, reduction in HRV, increase in sympathetic tone, endothelial dysfunction associated with reduced bioavailability of nitric oxide, hyperactivity of the renin angiotensin system (RAAS), and increased deposition of perivascular collagen are some of the changes identified in the cardiovascular system induced by CMS (Grippo et al., 2003; Neves et al., 2009, 2012; Marcondes et al., 2011; Firoozmand et al., 2018).

Environmental enrichment (EE) has emerged an important strategy for mitigating stress-mediated responses (Fern´andez-Terue et al., 2002; Wright and Conrad, 2008; Shilpa et al., 2017). Exposing lab animals to a range of stimuli (sensory, motor, social and physical), whithin an environment containing objects of different textures, climbing structures, and opportunities for voluntary physical activity, has demonstrated positive effects on cognitive, behavioral and neuroendocrine response to chronic stressors (Rosenzweig and Bennett 1996; Simpson and Kelly 2011; Peña and Prunel 2006; Costa et al., 2021). However, there is limited research exploring the effects of EE on cardiovascular responses to chronic stressors. It is noteworthy that in these studies (Norman et al., 2018), others types of stressor were applied (e.g social stress). Therefore, the objective of our study was to investigate the effects of EE on cardiovascular parameters and cardiac autonomic activity in rats subjected to CMS.

Materials and Methods

Animals and experimental design

Twenty one male Sprague-Dawley SPF (specific pathogen free) (Costa et al., 2012) rats, aged 2 months and weighing between 250 and 300 g at the start of the experiment, were provided by the Multidisciplinary Biological Research Center (CEMIB) of the Universidade Estadual de Campinas. Three weeks before the start of the experimental protocol, the animals were housed individually in standard polycarbonate cages, measuring (65 x 25 x 15 cm), containing only autoclaved sawdust. The standard cages were kept in a ventilated rack (Alesco® - Individual Ventiled Caging Systems) in a room with temperature and humidity controlled (22 ± 2 °C), (50-70%), and a 12:12 light and dark period. The animals received filtered water and food ad libitum and were randomized into control (n=5), chronic mild unpredictable stress (n=7) (CMS), environmental enrichment (n=4) (EE), and CMS + EE (n=5). When they reached 2 months of age, the animals underwent surgery to implant the blood pressure measurement transducer in the abdominal aorta. Fifteen days after surgery, recording of blood pressure and heart rate and the EE protocol began, during the 7 weeks of the experimental protocol. The CMS was applied for 21 days in weeks 3, 4, 5. Two

weeks after the stress protocol, the animals were decapitated and blood samples were collected for later analysis (Figure 1). All the procedures were approved by the Animal Use Ethics Committee of the Universidade Estadual de Campinas (CEUA processes 5195-1/2019) and complied with the requirements of the National Council for Control of Animal Experimentation (CONCEA) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

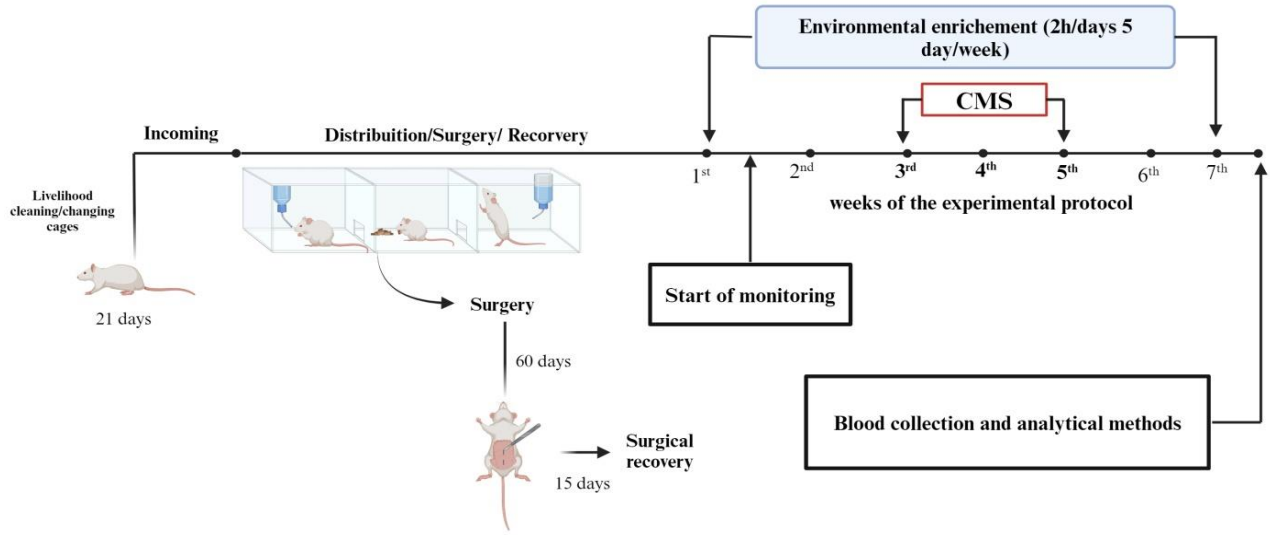

Figure 1. Experimental design. The animals arrived at 21 days old. One week prior to the surgery, the animals were divided into experimental groups: control, CMS, EE, and CMS + EE. At 2 months of age, the animals underwent surgery to implant the blood pressure measurement transducer in the abdominal aorta. After 15 days of recovery, the experimental protocol began, which lasted 7 weeks. The environmental enrichment protocol was performed for 7 weeks, 5 days per week, 2 h per day (4 to 6 p.m.). A chronic stress protocol was applied for 21 days in weeks 3, 4, 5. Continuous monitoring of blood pressure and heart rate began in week 1 and until week 7 of the experimental protocol. Fifteen after the end of the application of the CMS protocol, the animals were euthanized for decapitation. CMS: chronic mild unpredictable stress; EE: environmental enrichment. Created with Biorender.com.

Stress protocol

The CMS protocol was applied for 21 days, during weeks 3, 4 and 5 of the experimental protocol (Table 1), (Moreau et al., 1997; Neves et al., 2009).

Table 1. Chronic mild unpredictable stress procedure.

	Morning	**Afternoon**
Monday	8 a.m.: 1 h immobilization	1 p.m.: 1 h immobilization 6 p.m.: overnight illumination
Tuesday	8 a.m.: 1 h immobilization	2 p.m.: 1 h immobilization 6 p.m.: overnight - water and food deprivation
Wednesday	8 a.m.: access to restricted food for 2 h	2 p.m.: 1 h immobilization 6 p.m.: overnight - wet bedding
Thursday	8 a.m.: 1 h immobilization	1 p.m.: 1 h immobilization 6 p.m.: overnight - water deprivation
Friday	8 a.m.: exposure to empty water bottle for 2 h 11 a.m.: 1 h immobilization	6 p.m.: reversed light/dark cycle throughout the weekend.

Environmental enrichment (EE)

The EE protocol was conducted as previously described by Costa et al. (2021). Briefly, during the 7 weeks of the experimental protocol, 2 hours (4 to 6 p.m) per day, 5 days per week, animals were removed from their standard cages by the same researcher, and placed in groups of 6 animals from the same experimental group (EE or CMS +EE) in a cage containing three floors, ramps, wheels, and objects with different shapes and textures, as well as food and water ad libitum. All objects were changed weekly to maintain newness. The room used to apply the EE model was maintained at a temperature and humidity similar to the standard room. (Figure 2).

Figure 2: Environmental enrichment cage.

Cardiovascular parameters and locomotor activity

Cardiovascular parameters and locomotor activity were monitored via telemetry. Upon reaching 2 months of age, the animals were anesthetized using 5% isoflurane in 1 L of oxygen, within an anesthesia chamber measuring 13 cm in width, 25 cm in length, and 12.5 cm in height. Catheters (model TA11PA-C40, Data Sciences International, St. Paul, MN, USA) were inserted into the lumen of the descending aorta, following previously described methods (Steiner et al., 2017). Fifteen days after recovering from the surgery, the animals underwent blood pressure and heart rate recording procedures. Telemetry was activated on Monday of week 1, and the signals from the transmitters implanted in the animals were captured by the PhysiolTel RPC-1 receivers (Data Sciences International, St. Paul, MN, USA) positioned beneath the cages. The signals were processed using a Data Exchange Matrix and transmitted to the computer, where data acquisition was conducted using Dataquest ART 4.3 software. Data were continuously obtained, with the systolic blood pressure, diastolic blood pressure, heart rate, and locomotor activity of each animal recorded every 5 min (Steiner et al., 2017), at a sampling rate of 500 Hz (Whitesall et al., 2004; Greene et al., 2007).

Recordings were made on Fridays between 2 and 5 pm during the light period and 2 and 5 am during the dark period. The data were stored and extracted for analysis using the proprietary software, following the manufacturer's instructions (Data Sciences International, St. Paul, MN, USA). To stablish baseline values, recorded data from the second week of the experimental protocol were considered, taking into account the potential stressful effects of EE on cardiovascular parameters (Lyons et al., 2009; Crofton et al., 2015).

Variance and spectral analysis

To assess cardiovascular autonomic control, power spectral analysis was applied to series of tachograms and systograms derived from systolic arterial pressure (SAP) and pulse interval (PI). Frequency domain analysis of heart rate variability (HRV) and arterial pressure variability (APV) was conducted using an autoregressive algorithm (Malliani et al., 1991) on stationary sequences comprising 200 beats, verified by the stationary test (Porta et al., 2004). The low-frequency (LF, 0.2–0.75 Hz) and high-frequency (HF, 0.75–3.0 Hz) spectral components of PI and SBP were quantified in both absolute terms (ms^2 and $mmHg^2$), respectively) and normalized units (NU). These normalized units were derived by calculating the LF and HF powers and correlating them with the total power after excluding the very low frequency component (frequencies < 0.2 Hz) (Montano et al., 2009). This approach facilitates the estimation of the center frequency and power of each relevant oscillatory component, indicating the involvement of central control over sympathetic/parasympathetic systems in cardiovascular responses (Malliani et al., 1991; Fazan et al., 2005; Montano et al., 2009; Tobaldini et al., 2009; Quagliotto et al., 2012). The LF/HF ratio indicates the sympathovagal balance (Montano, 2009).

Determination of plasma corticosterone

The plasma corticosterone concentration was determined by colorimetric enzymatic assay, using a commercial kit (Enzo Life Sciences, Inc., Ann Arbor, MI, USA). The detection limit was 0.027 ng/mL and the intra- and inter-assay coefficients of variation were 7.7 and 9.7%, respectively.

Statistical analysis

The Shapiro-Wilk normality test was conducted on all of the analyzed data. Subsequent to verifying the normality of the data, statistical analyses were conducted using the two-way (ANOVA) test, followed by Tukey's post hoc test. The stress and enrichment factors, as well as their interaction, were examined. When $p < 0.05$ was considered statistically significant, the values were presented as mean ± standard error of the mean (SEM).

Results

The effects of enrichment and stress on plasma corticosterone concentrations interacted significantly ($F(1.20) = 6.160$, $p = 0.0221$, Figure 3). The stressed group (CMS) exhibited higher plasma levels of corticosterone compared to the control ($p= 0.0029$), EE ($p= 0.0045$) and CMS + EE ($p=0.0168$) groups, while there was no difference between the control, EE and CMS + EE groups ($p > 0.05$, Figure 3).

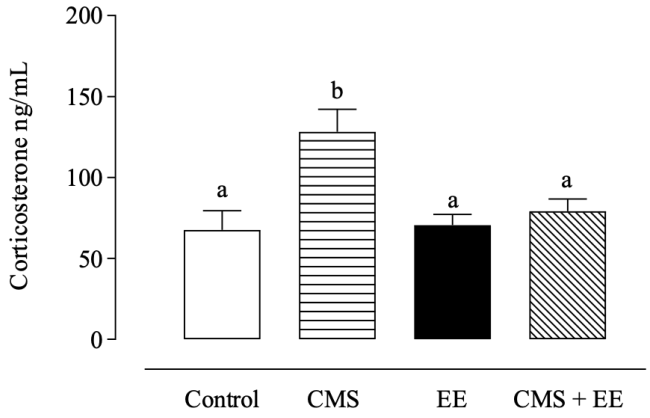

Figure 3. The impact of environmental enrichment on plasma corticosterone concentrations in animals subjected or not to the moderate and unpredictable chronic stress model. Values are shown as mean ± standard error (n = 6 per group). Significantly diverse groups are indicated by different letters. (two-way ANOVA followed by Tukey; $p < 0.05$).

Regarding cardiovascular parameters, a significant interaction between stress and enrichment effects ($F(1.16) = 7.984$, $p=0.0122$, Figure 4A) was observed on heart rate during the light period. The CMS group exhibited elevated heart rate during light period compared to the Control, EE and CMS + EE groups ($p< 0.05$, Figure 4A), with no difference between the Control, EE and CMS + EE groups ($p> 0.05$, Figure 4A). In the dark period, there was a significant main effect of enrichment ($F(1.17) = 29.93$, $p<0.0001$, Figure 4B) without a stress effect ($F(1.17) = 0.7799$, $p=0.3895$, Figure 4B) or interaction $F(1.17) = 1.884$, $p=0.1878$,

Figure 4B). The results indicate a decrease in the heart rate in animals exposed to enrichment (EE and CMS + EE) compared to the control and CMS groups ($p< 0.05$, Figure 4B). A significant effect of stress was observed on mean arterial pressure (MAP) during the dark period $F (1.17) = 5.334$, $p=0.0337$, Figure 5B) with no effect of environmental enrichment ($F (1.17) = 0.001791$, $p=0.9667$, Figure 5B) or interaction ($F (1.17) = 0.5455$, $p=0.4702$, Figure 5B). In the CMS and CMS + EE groups, MAP was higher compared to the Control and EE groups ($p< 0.05$, Figure 5B). Additionally, there was no difference between the Control and EE groups during the dark period. There was no effect of stress or environmental enrichment on MAP analyzed during the light period for any of the groups ($p>0.05$, Figure 5A).

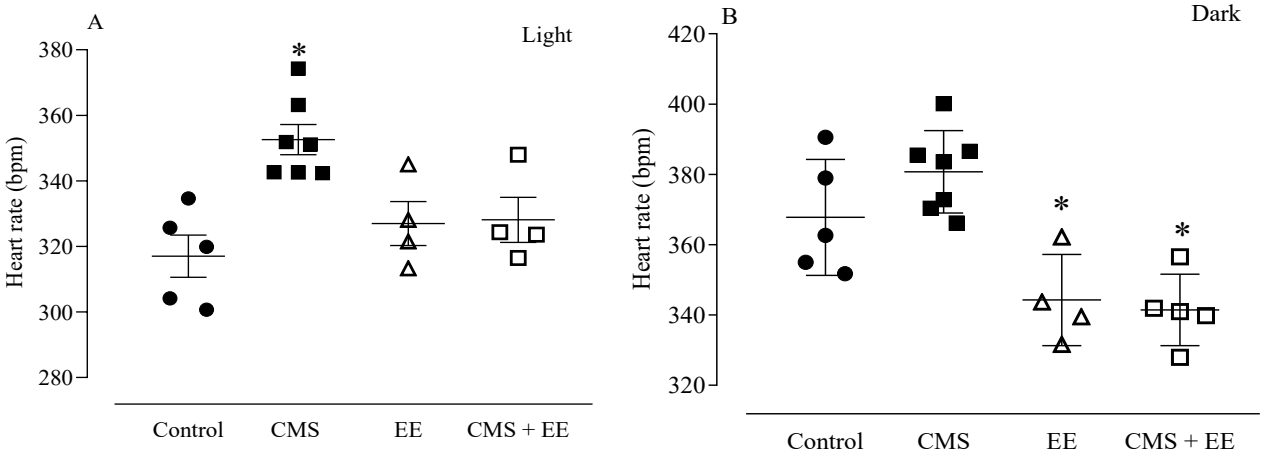

Figure 4. Heart rate (HR - bpm) measurements in stressed or control rats, with or without environmental enrichment during the light and dark periods. Values are presented as mean ± standard error. Significance was determined by two-way ANOVA post-hoc test/ Tukey, when appropriate. Cardiovascular parameters were recorded continuously for 24 hours during 6 weeks, considering: 2 am - 5 am for the dark period and 2 pm - 5 pm (light period) of each week. For analysis, the average of all weeks was considered (1 to 6) of the experimental protocol. Light: *$p < 0.05$ vs. Control, EE and CMS + EE Groups. Dark: *$p < 0.05$ vs. Control and CMS Groups. CMS: unpredictable mild chronic stress (n=7); EE: environmental enrichment (n=4); Control (n=5); CMS + EE (n=4-5).

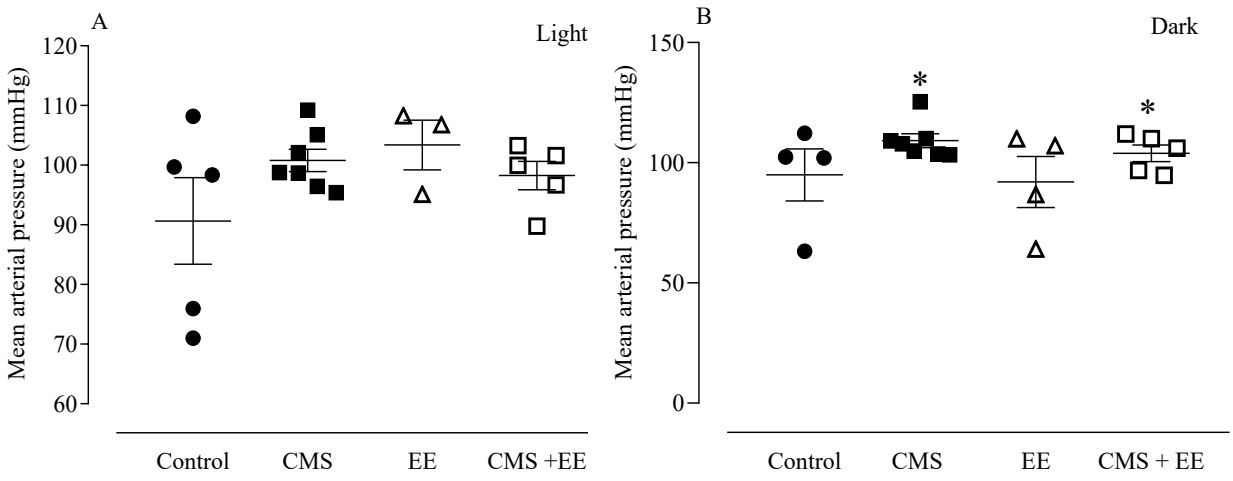

Figure 5. Mean arterial pressure (MAP) stressed or control rats, with or without environmental enrichment during the light and dark periods. Values are presented as mean ± standard error. Significance was determined by two-way ANOVA/Tukey post-hoc test, when appropriate. Cardiovascular parameters were recorded continuously for 24 hours for 6 weeks, considering: 2 am - 5 am for the dark period and 2 pm - 5 pm light period) of each week. For analysis, the average of all weeks (1 to 6) of the experimental protocol was considered.Dark: *p< 0.05 vs. Control and EE groups. CMS: unpredictable mild chronic stress (n=7); EE: environmental enrichment (n=3-4); Control (n=4-5); CMS + EE (n=5).

Stress had significant effect (F (1.17) = 4.957, p=0.0398, Figure 6A), with no effect of environmental enrichment (F (1.17) = 0.3213, p=0.5782, Figure 6A) or interaction (F (1.17) = 1.524, p=0.2338, Figure 6A) on animals' locomotor activity during the light period. Results show a considerable increase in locomotor activity in the CMS and CMS+ EE groups during the light period compared to the Control and EE groups (p< 0.05, Figure 6A). In the dark period, there was no effect of stress (F (1.16) = 1.594, p=0.2249, Figure 6B), or interaction F (1.16) = 1.874, p=0.1900, Figure 6B), but a significant environmental enrichment effect F (1.16) = 28.71, p<0.0001, Figure 6B). Compared to the control and CMS groups, the EE and CMS+EE animals had a reduced locomotor activity during the dark period.

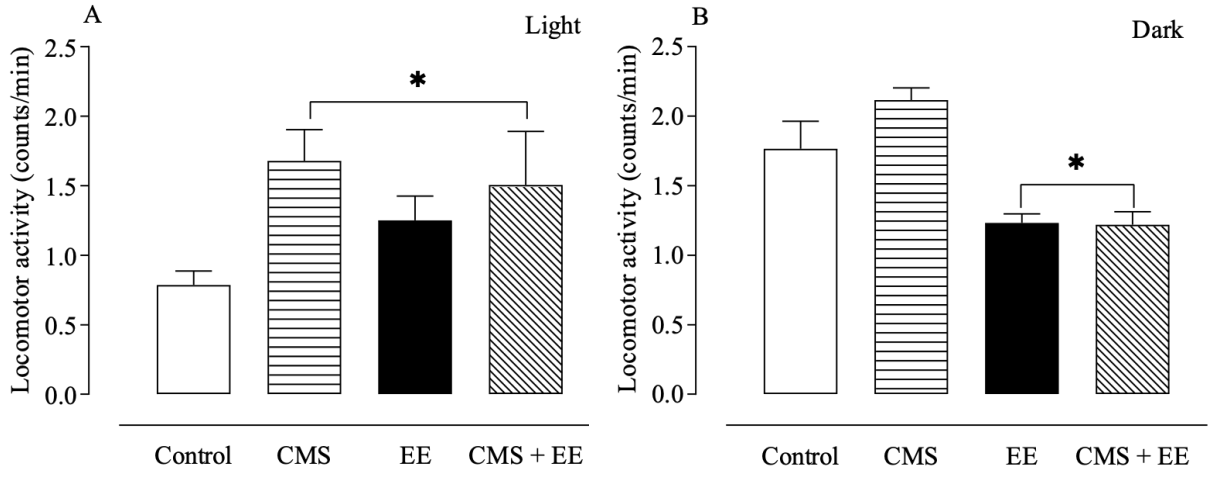

Figure 6. Locomotor activity stressed or control rats, with or without environmental enrichment during the light and dark periods. Values are presented as mean ± standard error. Significance was determined by two-way ANOVA/Tukey post-hoc test where appropriate. Behavioral parameters were recorded continuously for 24 hours for 6 weeks, for these analyzes the time interval was considered: 2am – 5am for the **dark period** and 2pm – 5pm **light period**) of each week. For analysis, the average of all weeks (1 to 6) of the experimental protocol was considered.Light: *p < 0.05 vs. Control and EE groups. Dark: *p< 0.05 vs. Control and CMS groups. CMS: chronic mild unpredictable stress (n=7); EE: environmental enrichment (n=3-4); Control (n=5); CMS + EE (n=5).

Heart rate (HR) variability evaluated in time (variance) and frequency domains using autoregressive spectral analysis showed a significant main effect of environmental enrichment (F (1.15) = 8.860, p=0.0094, Figure 7A) and stress (F (1.15) = 5.639, p=0.0313, Figure 7A) without interaction (F (1.15) = 0.1581, p=0.6965, Figure 7A) during the light period. The CMS and CMS + EE groups exhibited a reduction in heart rate variability compared with Control and EE groups. Additionally, in the EE and CMS + EE groups, these values were significantly higher in comparison to the control and CMS groups, respectively. (p < 0.05; Figure 7A).

In the dark period, there was a notable impact of environmental enrichment (F (1.15) = 4.561, =0.0496, Figure 7B) on heart rate variability, without a stress effect (F (1.15) = 2.388 P=0.143, Figure 7B) or interaction (F (1.15) = 1.441, p=0.2486, Figure 7B). The EE and CMS + EE presented an increase in heart rate variability compared to the Control and CMS groups, while no difference was between Control and CMS groups (p < 0.05, Figure 7B). For low-frequency (LF) oscillations, during the light period, there were no significant effects of stress (F (1.16) = 1.609 p=0.2227, Figure 7C), environmental enrichment (F (1.16) = 0.2187, p=0.6464, Figure 7C) or interaction (F (1.16) = 2.674, p=0.1215, Figure 7C) for either group. In contrast, in the dark period, there was a significant effect of environmental

enrichment (F (1.15) = 6.624, p=0.0212, Figure 7D), but no effect of stress (F (1.15) = 0.007675, p =0.9313, Figure 7D) or interaction (F (1.15) = 0.8710, p=0.3654, Figure 7D). In the dark period, animals from the EE and CMS + EE groups exhibited a decrease in LF oscillations compared to animals in the Control and CMS groups during the same period. There were no significant main effects of stress (F(1.16) = 2.672, p = 0.1216, Figure 7E), environmental enrichment (F(1.16) = 0.6039, p = 0.4484, Figure 7E), or interaction (F (1.16) = 4.202, p=0.0571, Figure 7E) in HF oscillations during the light period. In the dark period, the results show a significant effect of environmental enrichment F (1.15) = 6.450, p=0.0227, Figure 7F), without any effect of stress (F (1.15) = 0.4206, p= 0.5265, Figure 7F) or interaction F (1.15) = 0.8471, p=0.3719, Figure 7F). In the EE and CMS + EE groups, there was an increase in HF oscillations in comparison to the control and CMS groups. (p< 0.05, Figure 7F), while the CMS group showed no changes compared to the control group (p> 0.05< Figure 7F). Regarding sympathovagal balance assessed by the LF/HF ratio, there was no main effect of stress (F (1.16) = 0.8135 p=0.3805, Figure 7G), environmental enrichment (F (1.16) = 0.1344, p=0.7187 Figure 7G) or interaction (F (1.16) = 1.661, p=0.2157, Figure 7G) in the light period. Two-way ANOVA revealed no significant effects of stress (F(1.14) = 0.08055, p = 0.7807, Figure 7H) or interaction (F(1.14) = 0.1093, p = 0.7458, Figure 7H) during the dark period, but rather an enrichment effect (F(1.14) = 5.827, p = 0.0301, Figure 7H).

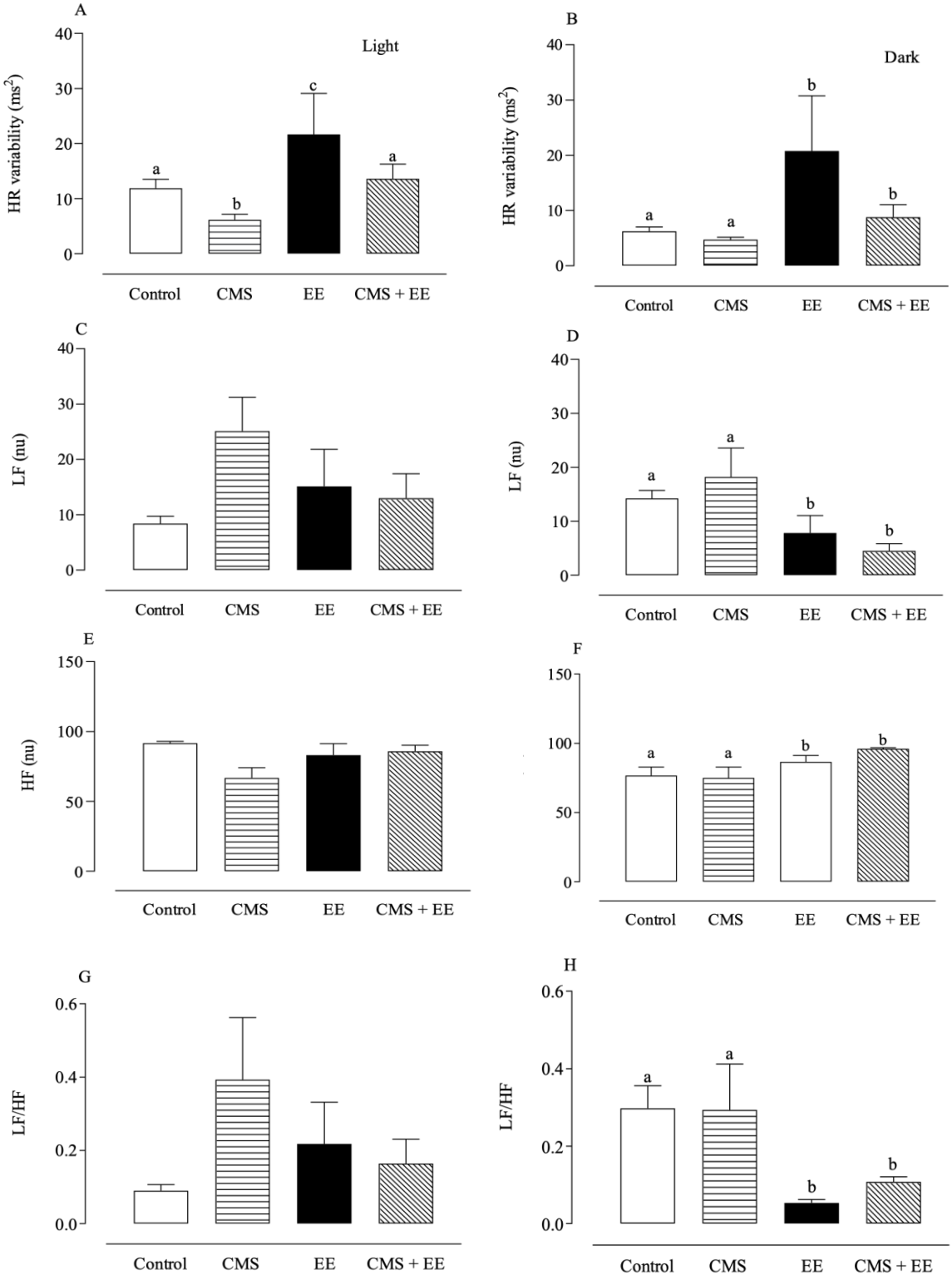

Figure 7. Heart rate variability (HRV) in the time and frequency domains stressed or control rats, with or without environmental enrichment during the light (A, C, E and G), and dark periods (B, D, F and H).(LF: low frequency oscillations (0.2–0.75 Hz) (C and D)), (HF: high frequency oscillations (0.75–3.0 Hz) (E and F)) and (LF/HF ratio (G and H)). Values are presented as mean ±standard error. (n = 4-6 per group) (two-way ANOVA/Tukey post-hoc test ($p < 0.05$)). CMS: chronic mild unpredictable stress (n=6-7); EE: environmental enrichment (n=3-4); Control (n=4); CMS + EE (n= 5).

Considering the systolic arterial pressure (SAP) variability, the two-way ANOVA showed a significant interaction between the effects of stress/enrichment in both the light ($F(1.14) = 6.927$, $p=0.0197$, Figure 8A) and dark periods ($F(1.13) = 11.96$, $p=0.0042$, Figure 8B). According to post-hoc analyses, animals under chronic stress (CMS) exhibited higher variability in their heart rates than those in the Control, EE, and CMS + EE groups. ($p < 0.05$, Figure 8B), while there was no difference between the Control, EE and CMS + EE. EE groups in both periods ($p > 0.05$, Figure 8A and 8B). Likewise, in the analyzes performed to evaluate the power of LF oscillations, two-way ANOVA test revealed a significant interaction between the stress/enrichment effects in the light ($F(1.15) = 12.12$, $p=0.0033$, Figure 8C) and dark periods ($F(1.13) = 10.34$, $p=0.0068$, Figure 8D). There was a significant increase in LF in animals subjected to CMS during the light and dark period ($p < 0.05$, Figure 8C and 8D) and no changes in Control, EE and CMS + EE evaluated in both periods ($p > 0.05$, Figure 8C and 8D). Stress and enrichment effects interacted significantly during the dark period for HF oscillations ($F(1.11) = 11.84$, $p=0.0055$, Figure 8F). The CMS group showed a clear increase in HF in comparison to the Control, EE and CMS+EE group. In contrast, the CMS + EE group showed no changes in HF compared to the Control and EE group ($p > 0.05$, Figure 8F). Stress or enrichment had no discernible influence on the HF oscillations during the light period ($p>0.05$, Figure 8E).

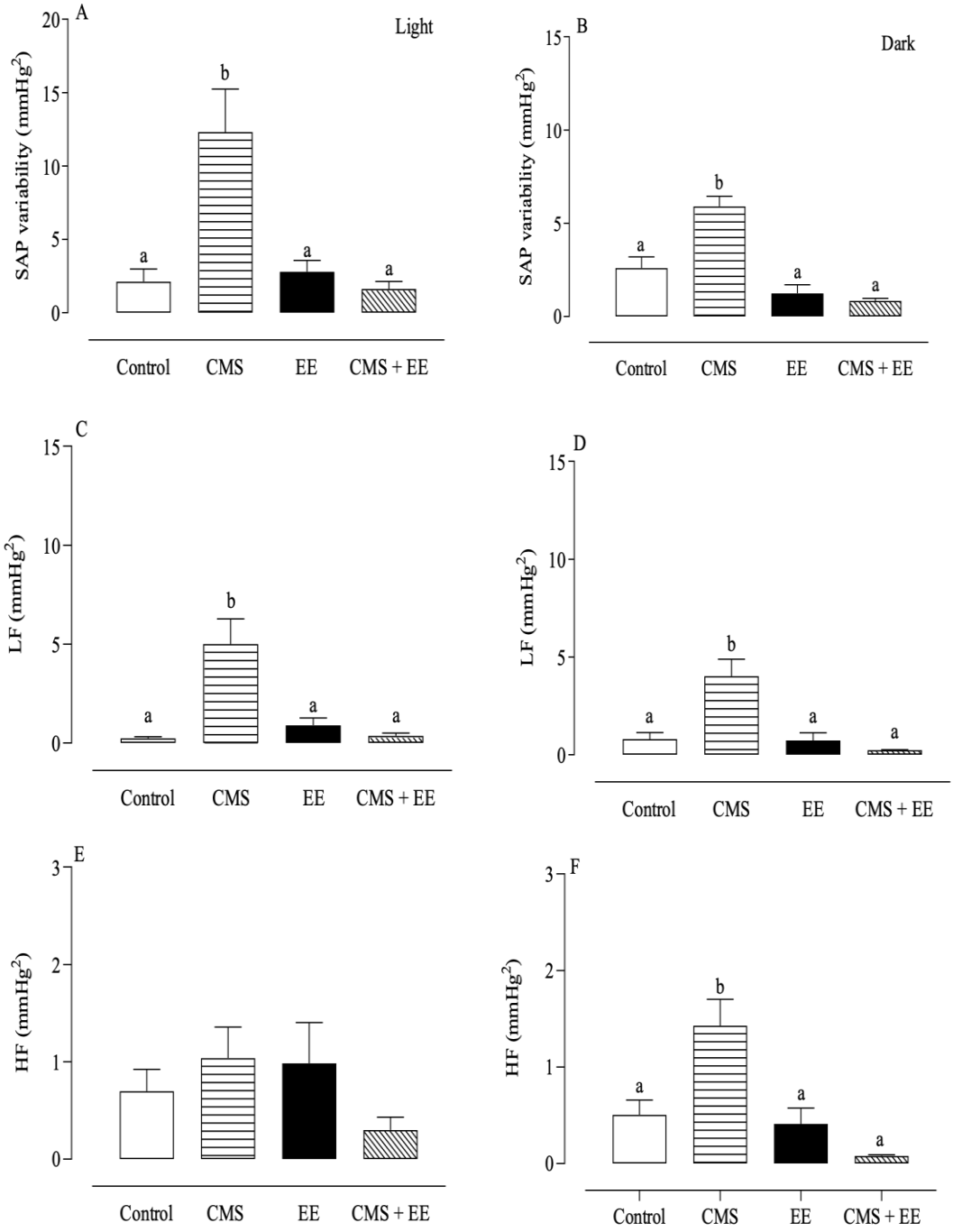

Figure 8. Systolic arterial variability (SAPV) in time and frequency domains in stressed or control rats, with or without environmental enrichment during the light (A, C and E) and dark period (B, D and F). (LF: low frequency oscillations (0.2–0.75 Hz) (C and D)), (HF: high frequency oscillations (0.75–3.0 Hz) (E and F)). Values are presented as mean ±standard error. Significantly diverse groups are indicated by different letters (two-way ANOVA/Tukey post-hoc test ($p < 0.05$)). CMS: chronic mild unpredictable stress (n=4-7); EE: environmental enrichment (n=3-4); Control (n=3-4); CMS + EE (n=4-5).

Discussion

In the present study, we explored the impact of environmental enrichment (EE) on hormonal responses and cardiac autonomic activity in rats exposed to chronic mild unpredictable stress (CMS). Our findings revealed several beneficial effects of EE: (1) prevention of the increase in heart rate during the light period, (2) reduction of locomotor activity and heart rate during the dark period, (3) increase in heart rate variability, indicating enhanced cardiac autonomic regulation, (4) promotion of parasympathetic tone and reduction of sympathetic tone, (5) prevention of the increase in systolic arterial pressure variability and (6) prevention of the rise in plasma corticosterone levels. Taken together, these results suggest that just 2 hours of environmental enrichment could modulate the activity of the hypothalamic pituitary adrenal (HPA) axis and sympathetic nervous system in response to stressful situations.

Elevated levels of corticosterone and catecholamines are commonly observed in aversive situations (McCarty, 2016), reflecting the activation of the hypothalamus-pituitary-adrenal axis and autonomic nervous system (ANS). This physiological response aims to maintain homeostasis and ensure survival (Sterling, 2012; Crestani, 2016). However, prolonged exposure to these stress responses, coupled with the organism's maladaptation to stressful stimuli, can contribute to the development of various diseases (Danese and McEwen, 2012; Herman, 2013; McEwen and Seeman, 1998).

In the present study, high levels of corticosterone were observed in animals exposed to CMS, even 15 days after the end of the application of stressful stimuli. Although we did not assess plasma catecholamine levels in this study, previous research conducted by our group using the same experimental protocol has demonstrated an increase in both plasma corticosterone and catecholamines (Neves et al., 2009, 2012; Firoozmand et al., 2018; Costa et al., 2020, 2021). These findings collectively suggest a lack of adaptation to stressors and neuroendocrine dysregulation (Costa et al., 2021).

On the contrary, studies have proposed that environmental interventions, such as EE, could improve negative feedback sensitivity and promote greater efficiency and adaptability of the HPA axis (Mora et al., 2007; Segovia et al. 2009, Costa et al., 2021). This hypothesis is supported by research demonstrating a reduction in stress-induced catecholamine and corticosterone release after environmental enrichment (Costa et al., 2021) and a quicker return of coticosterone to baseline levels (Konkle et al., 2010). Consistent with these findings, our study revealed that environmental enrichment prevented the increase in plasma corticosterone concentrations in animals subjected to CMS.

In addition to the aforementioned changes, accumulating evidence implicates stress in the development of various diseases, including hypertension, coronary heart disease, arrhythmias and sudden cardiac death (Mucci et al., 2016; Buckley et al., 2016 Sara et al., 2018; Chang Liu et al., 2021). Autonomic imbalance has emerged as a key mechanism underlying in cardiac, emotional and metabolic disorders (Farah et al., 2006; Grippo et al., 2009). Over recent decades, mounting evidence suggests that high blood pressure variability (BPV) is linked to cardiac and vascular injuries and increased mortality from cardiovascular diseases, while reduced heart rate variability is associated with hypertension and an elevated risk of sudden death in individuals with chronic heart failure (Malpas, 2010; Prinsloo et al., 2014; Grassi et al., 2015).

It is crucial to recognize that changes in heart rate variability or blood pressure may precede or coincide with alterations in these parameters (Galinier et al., 2000; La Rovere et al., 2003). In our study, CMS led to an increase in mean arterial pressure values during the animals' active period. Interesting, this rise was not accompanied by an increase in heart rate and locomotor activity. These findings underscore alterations in the day/night pattern and demonstrate that the changes observed in blood pressure extend beyond the increase in locomotor activity and heart rate.

In a previous investigation conducted by our research group, CMS produced morphological and functional alterations in the aorta of rats, which were associated with a reduction of nitric oxide (Neves et al., 2009). Furthermore, CMS reduced blood vessel caliber by increasing perivascular collagen (Firoozmand et al., 2018). These observations were linked to an increase in circulating corticosterone, cathecholamines and angiotensin II, suggesting the involvement of the renin angiotensin system (RAS) in the changes induced by CMS (Marcondes et al., 2011; Firoozmand et al., 2018; Bangsumruaj et al., 2022).

In our current study, we found that mild nocturnal hypertension was associated with an increase in BPV and sympathetic modulation during both analyzed periods. Interestingly, we also observed an increase in parasympathetic modulation in stressed animals during the dark period. These results suggest that compensatory mechanisms were activated to regulate stress-induced changes. On the other hand, while EE did not prevent or reduce MAP values during the dark period, animals exposed to environmental enrichment did not exhibit changes in BPV in either period. These findings may be attributed to the absence of alterations in sympathetic and parasympathetic tone. Our data provide new evidence suggesting that EE could modulate cardiac autonomic activity and prevent the development of cardiovascular disorders such as those mentioned above.

Considering the effects of stress on HR, the data from the present study indicate that, unlike MAP, HR increased during the light period with no changes observed during the dark period. It is noteworthy that the locomotor activity of stressed animals was heightened during the light period but remained unchanged during the dark period. It is important to note that the light period typically corresponds to a time of decreased animal activity. However, despite the elevated activity levels observed in animals subjected to stress and EE were elevated during the light period, this elevation was not sufficient to induce an increase in HR. Therefore, the alterations in HR observed in this study may linked to fear, anxiety, and the influence of catecholamines on cardiac β-adrenergic receptors in the sinoatrial node (Kannel et al., 1987; Malpas, 2010; Grassi et al., 2015), which can also be triggered by stress (Firoozmand et al., 2018; Costa et al., 2020, 2021). Alongside the changes in HR, stress also reduced HRV during the light period, but had no discernible effect during the dark period. As evidenced in our findings, previous studies have reported a reduction in HR variability in response to stress (Grippo et al., 2003; Normann et al., 2018).

In contrary to chronic stress, EE mitigated the adverse impacts of stress and enhanced variability in both periods. This augmentation seems to correlate with a decrease in sympathetic modulation and an increase in parasympathetic modulation during the dark period. The positive influences of EE on cardiovascular reactions, as noted in this study, may be associated with an improved capacity to confront new challenges, decreased secretion of corticosterone and catecholamine, and regulation of RAAS. Nonetheless, further research will be necessary to validate the influence of EE on RAAS regulation.

In addition to the potential mechanisms mentioned earlier, the voluntary physical activity facilitated by EE might have contributed to the regulation of autonomic cardiac activity. There is evidence of an increase in heart vagal tone in healthy individuals or those engaging in physical activity (Prisloo et al., 2014). This increase has also been observed in animal models linked to overall cardiovascular health improvements, including reduced body fat, decreased inflammation cytokines, and less arterial plaque accumulation (Wang et al., 2010). Studies suggest that engagement in diverse sensory, motor, cognitive and social activities yields positive behavioral, cognitive, and neurobiological effects, which may enhance stress responses in humans and animal models (Norman et al., 2018). Additionally, involvement in mentally stimulating activities appears to correlate with heightened baseline HRV and altered HRV reactivity to stress (Lin et al., 2014).

Therefore, it is important to highlight that the benefits of EE, as observed in the current study and in previous research, stem from a variety of complex mechanisms activated

by the diverse stimuli provided. Exposure to novelty, which may initially be mildly stressful (Lyons et al., 2009; Crofton et al., 2015), could enhance animals' capactity to cope with stressful situations, fostering resilience (Saavedra et al., 2011; Costa et al., 2021).

In conclusion, this study adds to prior research highlighting the functional and structural harm to the cardiovascular system caused by chronic stress and introduces significant new insights into the advantageous effects of environmental enrichment. We observed an augmentation in heart rate variability alongside no alterations systolic blood pressure variability. These changes might be mediated by an elevation in parasympathetic activity and a decreased in sympathetic activity. Our findings underscore the benefits of non-pharmacological interventions in mitigating and preventing stress-induced cardiovascular and/or hormonal disorders.

Conclusion

This study demonstrated that environmental enrichment can protect against hormonal and cardiovascular problems related to chronic stress. The improvement in the ability to face new challenges and the modulation of stress-induced neuroendocrine responses appear to be related to the sensory, motor, behavioral and cognitive stimuli elevated by environmental enrichment.

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3 DISCUSSÃO

O presente estudo avaliou as respostas hormonais, cardiovasculares e autonômicas cardíacas em ratos adultos jovens Sprague-Dawley submetidos ao ECMI, bem como os efeitos do método de monitoramento e do enriquecimento ambiental (EA), sobre as mesmas. Para o estudo dos efeitos do ECMI e do método de monitoramento sobre a PAS e FC, os animais controle ou submetidos ao ECMI foram monitorados por telemetria ou pletismografia de cauda antes, durante e até 15 dias após a aplicação do protocolo ECMI. Os animais monitorados por telemetria e submetidos ao ECMI mostraram aumento na PAS durante as três semanas de aplicação dos estímulos estressores. Já o aumento da FC ocorreu nas duas últimas semanas do protocolo ECMI, semanas 3 e 4. Ao contrário desses resultados, os animais monitorados por pletismografia de cauda, submetidos ao ECMI, não apresentaram diferenças significativas nos valores de PAS e FC em comparação ao grupo controle monitorado por pletismografia de cauda, mesmo nas semanas de aplicação do protocolo ECMI.

Além de analisar os resultados de cada grupo usando a mesma abordagem de monitoramento para avaliar os efeitos do ECMI sobre a PAS e FC, também investigamos se havia diferenças entre os grupos que tinham as mesmas condições experimentais, porém foram monitorados por métodos distintos. Os resultados mostraram que, durante todo o protocolo experimental, o grupo controle monitorado por pletismografia de cauda apresentou valores de PAS e FC significativamente maiores do que o grupo controle monitorado por telemetria. Além disso, nos grupos submetidos ao estresse, os animais monitorados por pletismografia de cauda mostraram efeito prolongado do ECMI sobre a PAS e FC. Enquanto os animais monitorados por telemetria apresentaram retorno da PAS logo após o fim da aplicação dos estímulos estressores, nos animais monitorados por pletismografia de cauda este retorno só ocorreu 15 dias após cessar o estresse. Por outro lado, não houve mudanças na FC dos animais monitorados por pletismografia de cauda durante todo o protocolo experimental.

Diante disso, apesar dos modelos animais representarem uma importante ferramenta para a compreensão dos mecanismos envolvidos no desenvolvimento de doenças cardiovasculares bem como sua associação com o estresse. O estresse experimental é um dos principais desafios em estudos que utilizam modelos animais. Neste sentido, diversos estudos têm investigado o impacto de diferentes estratégias utilizadas para minimizar os efeitos estressores que procedimentos comuns realizados no ambiente laboratorial poderia acarretar aos animais. Melhora no bem-estar, redução nos níveis de corticosterona, glicose e de

comportamentos semelhantes ao medo e ansiedade são alguns dos benefícios encontrados em estudos que utilizaram maneiras diferentes de manipular o animal durante experimentos (Gouveia e Hurst, 2013; Nakamura e Suzuki, 2018; Davies et al., 2022).

Além da manipulação, outros fatores que podem ter contribuído para as divergências observadas em nosso estudo, podemos destacar o estresse térmico e de contenção que são necessários para o registro dos parâmetros cardiovasculares por pletismografia de cauda. Neste sentido, embora haja significativos esforços dos pesquisadores para treinar e aclimatar os animais ao procedimento, ainda há dúvidas que os animais possam habituar-se as medidas repetidas por pletismografia de cauda (Sikora et al., 2016; Wilde et al., 2017). No estudo realizado por Wilde et al. (2017), a pressão arterial sistólica e a frequência cardíaca dos animais monitorados por plestimografia de cauda durante 3 semanas não sofreu qualquer alteração durante os testes de habituação. Assim, as diferenças identificadas na PAS e na FC dos animais monitorados por pletismografia de cauda podem estar relacionadas ao estresse experimental gerado pelos procedimentos realizados no ambiente laboratorial. Portanto, o uso de métodos de monitoramento que requerem manipulação, aquecimento e restrição podem resultar em respostas hemodinâmicas mais acentuadas e comprometer a precisão e reprodutibilidade dos resultados. Por outro lado, o monitoramento contínuo e livre dos parâmetros cardiovasculares por telemetria parece não adicionar os negativos efeitos do estresse experimental sobre esses parâmetros, principalmente em estudos com animais submetidos ao estresse.

Portanto, é importante entender as limitações inerentes às técnicas de registro de PA e FC e garantir que a metodologia de pesquisa seja adequada ao objetivo estudado. Deste modo, destacamos que, embora a telemetria seja uma metodologia invasiva que inicialmente exige gastos substanciais para a aquisição do equipamento, bem como para a manutenção dos transmissores de rádio implantáveis, ela permite o monitoramento contínuo (24 horas) sem que seja necessária a transferência do animal para ambientes experimentais, assim como interromper seu ciclo de sono, aquecer e/ou restringir seus movimentos (Kurtz et al., 2005), sendo assim, capaz de minimizar os desconfortos gerados para o registro dos parâmetros cardiovasculares, um dos princípios dos 3Rs descritos por Russel e Burch em 1959.

Além de evitar a manipulação, aquecimento e restrição, procedimentos que podem afetar o bem-estar animal e contribuir para as divergências encontradas em nosso estudo, o monitoramento contínuo por telemetria nos permite investigar de maneira não invasiva a função autonômica cardíaca a partir da análise da variabilidade da frequência cardíaca (VFC) e da pressão arterial (VPA). Assim, considerando que o estresse crônico pode levar a

alterações anatômicas e/ou funcionais do sistema cardiovascular, assim como, disfunção do SNA caracterizado pela diminuição da variabilidade da frequência cardíaca e aumento da modulação cardíaca simpática (Gripo et al., 2003; Neves et al., 2009, 2012; Marcondes et al., 2011; Firoozmand et al., 2018; Bangsumruaj et al., 2022). Buscou-se no segundo artigo que compõem esta tese investigar os efeitos do EA sobre as respostas hormonais e atividade autonômica cardíaca de ratos submetidos ao estresse crônico monitorado por telemetria. O EA tem sido considerado um modelo experimental capaz de modular o eixo hipotálamo-hipófise-adrenal (HHA) e a atividade simpática, reduzindo as respostas hormonais ao estresse crônico (Costa et al., 2020, 2021), cancelando o comprometimento da aprendizagem, memória induzida por estressores crônicos (Costa et al., 2021), e comportamentos análogos a depressão humana e ansiedade (Sampedro-Piqueiro et al., 2013; Seong et al., 2018; Keloglan Musuroglu et al., 2022).

Nossos resultados mostraram que o EA aumentou a variabilidade da frequência cardíaca, a atividade parassimpática e mitigou a atividade simpática dos animais expostos ou não ao estresse. O EA também impediu o aumento da variabilidade da pressão arterial e da frequência cardíaca e dos níveis plasmáticos de corticosterona induzidos pelo estresse crônico. Portanto, os dados obtidos no presente estudo sugerem que 2 horas diárias de EA podem minimizar os efeitos negativos do estresse a partir da modulação do eixo (HHA) e da atividade autonômica cardíaca.

O conjunto de estímulos, sensorial, motor e social, proporcionados pelo EA sugerem ser importantes na saúde cardiovascular, metabólica, emocional e cognitiva (Queen et al., 2020). No entanto, não é evidentemente claro se os estímulos aplicados individualmente teriam os mesmos efeitos do EA. Neste sentido, apesar de no estudo realizado por Grippo et al. (2014), os autores terem demostrado que o EA e o exercício físico isolado foram igualmente eficazes para atenuar comportamentos análogos à depressão humana, no mesmo estudo, o exercício físico isolado não teve o mesmo efeito sobre o comportamento relacionado à ansiedade, em animais exposto ao isolamento social. Interessantemente, Norman et al. (2018) também mostrou que o EA e o contato social de ratazanas da pradaria com machos previamente conhecidos reduziu a FC e aumentou a variabilidade da FC em comparação aos animais que continuaram em isolamento social. Assim, embora esses estudos tragam importantes evidências sobre o impacto positivo do EA sobre as respostas comportamentais e autonômicas cardíacas, eles foram feitos utilizando uma linhagem de ratos específicas, e foram consideradas apenas um sexo, feminino, e um tipo específico de estímulo estressor, o isolamento social. Portanto, para melhor entender se o conjunto de estímulos que reduzem os

efeitos negativos induzidos pelo estresse supera ou é igual aos estímulos aplicados separadamente, pretendemos em futuros estudos investigar os efeitos do EA sobre as respostas metabólicas, comportamentais e cardiovasculares de ratos machos submetidos ao modelo de estresse crônico variado.

Com base nos resultados apresentados nos dois estudos que compuseram esta tese, enfatizamos sobre a importância do uso de modelos animais e da utilização de métodos que minimizem os desconfortos gerados no ambiente laboratorial para compreender os eventos envolvidos no desenvolvimento de doenças cardiovasculares , assim como, o uso do EA como medida terapêutica ou profilática para redução dos efeitos deletérios do estresse crônico.

4 CONCLUSÃO

Os resultados obtidos nesta tese mostram que a avaliação dos parâmetros cardiovasculares por pletismografica de cauda, em ratos submetidos ao estresse crônico, pode gerar reações fisiológicas relacionadas ao estresse experimental o que poderia comprometer a interpretação dos resultados obtidos, e evidenciam efeitos protetores do enriquecimento ambiental contra as desordens cardiovasculares e hormonais induzidas pelo estresse crônico.

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ANEXOS

ANEXO 1 – Certificação dos Comitês de Ética

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que a proposta intitulada **ELEVAÇÃO DA PRESSÃO ARTERIAL INDUZIDA POR ESTRESSE CRÔNICO EM RATOS: PARTICIPAÇÃO DO SISTEMA RENINA ANGIOTENSINA**, registrada com o nº **4219-1**, sob a responsabilidade de **Profa. Dra. Fernanda Klein Marcondes e Maeline Santos Morais Carvalho**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **20 de maio de 2016**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**05/06/2016-05/02/2018**
Vigência da autorização para manipulação animal:	**05/06/2016-05/02/2018**
Espécie / linhagem/ raça:	**Rato heterogênico / NTacUnib:SD (Sprague Dawley)**
No. de animais:	**72**
Peso / Idade:	**02 meses / 300g**
Sexo:	**machos**
Origem:	**CEMIB/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

Campinas, 20 de maio de 2016.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que a proposta intitulada **EFEITO DO ENRIQUECIMENTO AMBIENTAL SOBRE A PRESSÃO ARTERIAL E FREQUÊNCIA CARDÍACA DE RATOS SUBMETIDOS A ESTRESSE CRÔNICO**, registrada com o nº **5195-1/2019**, sob a responsabilidade de **Prof. Dr. Fernanda Klein Marcondes e Maeline Santos Morais Carvalho**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA)**, **tendo sido aprovada pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP**, em reunião de **11/04/2019**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	01/08/2019 a 22/02/2022
Vigência da autorização para manipulação animal:	11/04/2019 a 22/02/2022
Espécie / linhagem/ raça:	Rato heterogênico / NTacUnib:SD (Sprague Dawley)
No. de animais:	12
Idade/Peso:	21.00 Dias / 250.00 Gramas
Sexo:	12 Machos
Espécie / linhagem/ raça:	Rato heterogênico / NTacUnib:SD (Sprague Dawley)
No. de animais:	12
Idade/Peso:	21.00 Dias / 250.00 Gramas
Sexo:	12 Machos
Espécie / linhagem/ raça:	Rato heterogênico / NTacUnib:SD (Sprague Dawley)
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Espécie / linhagem/ raça:	Rato heterogênico / NTacUnib:SD (Sprague Dawley)
No. de animais:	12
Idade/Peso:	21.00 Dias / 250.00 Gramas
Sexo:	12 Machos
Origem:	CEMIB
Biotério onde serão mantidos os animais:	Biotério da Faculdade de Odontologia de Piracicaba, FOP/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização a junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Universidade Estadual de Campinas
Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **900-1**, sobre "**RESPOSTAS METABÓLICAS, CARDIOVASCULARES E COMPORTAMENTAIS INDUZIDAS POR DIETA HIPERCALÓRICA E ESTRESSE CRÔNICO, EM RATOS**" sob a responsabilidade de **Profa. Dra. Fernanda Klein Marcondes** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 13 de setembro de 2005.

CERTIFICATE

We certify that the protocol nº **900-1**, entitled "**METABOLIC, CARDIOVASCULAR AND BEHAVIORAL RESPONSES INDUCED BY HIPERCALORIC DIET AND CHRONIC STRESS IN RATS**", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on September 13, 2005.

Campinas, 13 de setembro de 2005.

Profa. Dra. Ana Maria A. Guaraldo
Presidente - CEEA/IB/UNICAMP

Fátima Alonso
Secretária - CEEA/IB/UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA
CIDADE UNIVERSITÁRIA ZEFERINO VAZ
CEP 13.081-970 - CAMPINAS - SP - BRASIL

TELEFONE 55 19 3788-6333
FAX 55 19 3289-3124

ANEXO 2 – Comprovante de submissão eletrônica ao periodico Laboratory Animals

A manuscript titled Use of Telemetry as a Refinement Tool for the Evaluation of Cardiovascular Responses in Rats Submitted to Chronic Stress (LA-24-025) has been submitted by Professor Fernanda Marcondes to Laboratory Animals.

You are listed as a co-author for this manuscript. The online peer-review system, ScholarOne Manuscripts, automatically creates a user account for you and you may receive e-mails and communications from the journal editorial office staff regarding your manuscript based on the authorization you provided to the co-author who created the account.

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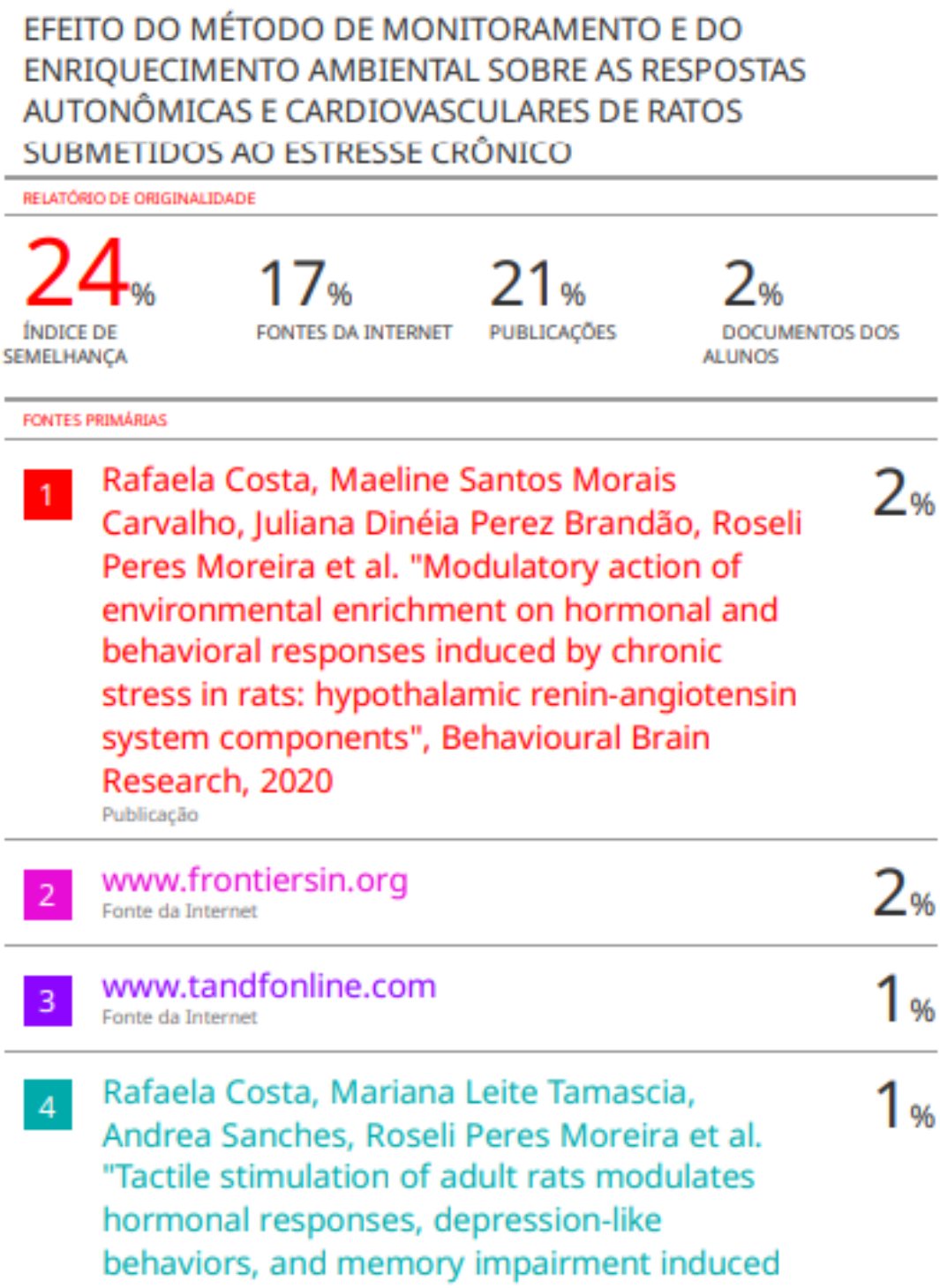
ANEXO 3– Verificação de originalidade e prevenção de plágio.

Este relatório foi gerado por meio da plataforma *Turnitin*. De acordo com norma da Cordenadoria de Pós-graduação (CPG) da Universidade Estadual de Campinas a similaridade aceita é de até 24% de similaridade.



EFEITO DO MÉTODO DE MONITORAMENTO E DO ENRIQUECIMENTO AMBIENTAL SOBRE AS RESPOSTAS AUTONÔMICAS E CARDIOVASCULARES DE RATOS SUBMETIDOS AO ESTRESSE CRÔNICO

RELATÓRIO DE ORIGINALIDADE

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ÍNDICE DE SEMELHANÇA	FONTES DA INTERNET	PUBLICAÇÕES	DOCUMENTOS DOS ALUNOS

FONTES PRIMÁRIAS

1	Rafaela Costa, Maeline Santos Morais Carvalho, Juliana Dinéia Perez Brandão, Roseli Peres Moreira et al. "Modulatory action of environmental enrichment on hormonal and behavioral responses induced by chronic stress in rats: hypothalamic renin-angiotensin system components", Behavioural Brain Research, 2020 Publicação	2%
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