THIAGO BERNARDINO DE ALMEIDA

BOAR WELFARE INFLUENCE THE QUALITY OF THE GAMETES

PIRASSUNUNGA

2021

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Thesis submitted to the Postgraduate Program in Experimental Epidemiology Applied to Zoonosis of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

Department:

Preventive Veterinary Medicine and Animal Health

Area:

Experimental Epidemiology Applied to Zoonosis

Advisor:

Prof. Adroaldo José Zanella, Ph.D.

PIRASSUNUNGA

2021

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T. 4077
FMVZ

Almeida, Thiago Bernardino de
Boar welfare influence in the quality of the gametes / Thiago Bernardino de Almeida. – 2021.
109 f. : il.

Título traduzido: Influência do bem-estar do macho suíno na qualidade dos gametas.

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Medicina Veterinária Preventiva e Saúde Animal, Pirassununga, 2021.

Programa de Pós-Graduação: Epidemiologia Experimental Aplicada às Zoonoses.
Área de concentração: Epidemiologia Experimental Aplicada às Zoonoses.
Orientador: Prof. Dr. Adroaldo José Zanella.

1. Alojamento de cachaço. 2. Comportamento do cachaço. 3. Cortisol salivar. 4. Enriquecimento ambiental. 5. Suínos reprodutores. I. Título.

Ficha catalográfica elaborada pela bibliotecária Maria Aparecida Laet, CRB 5673-8, da FMVZ/USP.

São Paulo, 19 de maio de 2021
CEUA N 3612010616
(ID 007835)

IImo(a). Sr(a).
Responsável: Adroaldo José Zanella
Área: Epidemiologia Experimental Aplicada As Zoonoses

Título da proposta: "O BEM-ESTAR DO MACHO SUÍNO INTERFERE NOS INDICADORES DE SAÚDE, DESEMPENHO E BEM-ESTAR DOS LEITÕES".

CERTIFICADO (Emenda versão de 19/maio/2021)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 19/maio/2021) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "O trabalho de doutorado do Thiago Bernardino de Almeida incluiu 26 machos suínos alojados na Granja TOPGEN, cujo protocolo original CEUA, contempla os animais somados a 27 animais de uma população de 30 animais adquiridos para a continuidade dos trabalhos. Três dos animals não se mostraram aptos para coleta de sêmen e não foram incluídos no estudo. Ou seja no trabalho de doutorado do Thiago Bernardino de Almeida foram estudados 53 machos suínos adultos. O projeto revisado que foi anexado ao protocolo original oferece detalhes da abordagem experimental conduzida com os animais. Para fins de evitar qualquer problema vamos solicitar a inclusão de 30 novos animais, mesmo considerando que três dos machos não foram utilizados no estudo. Nesta emenda vamos informar que a totalidade de animais que o Thiago Bernardino trabalhou foram 55 animais. 26 animals na Granja Topgen. 30 animais alojados no Campus Fernando Costa, dos quais somente 27 foram estudados.".

Animais a serem adicionados							
Origem:	Animais de proprietários						
Espécie:	Suínos	sexo:	Machos	idade:	1 a 7 anos	N:	26
Linhagem:	Large white e landrace			Peso:	150 a 300 g		
Animais a serem adicionados							
Origem:	Faculdade de Medicina Veterinária e Zootecnia da USP						
Espécie:	Suínos	sexo:	Machos	idade:	8 a 18 meses	N:	30
Linhagem:	Large white e landrace			Peso:	100 a 250 g		

Comentário da CEUA: Aprovado

Prof. Dr. Marcelo Bahia Labruna
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EVALUATION FORM

Author: ALMEIDA, Thiago Bernardino

Title: Boar welfare influence the quality of the gametes

Thesis submitted to the Postgraduate Program in Experimental Epidemiology Applied to Zoonosis of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

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Professor. _____

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ACKNOWLEDGEMENTS

Inicialmente eu gostaria de agradecer à Deus e à minha família. Meus pais, Afonso e Zulmira, meus amados irmãos, Juninho e Greice, meus queridos pets, Boris, Beni e Toni e também à minha amada companheira Anna. Sem vocês comigo, a vida seria cinza, sem alegria e sem amor. Obrigado por serem parte da minha vida.

Agradeço ao meu orientador e grande amigo, Adroaldo. Obrigado pela confiança, parceria, conversas, companheirismo, muitas conversas, muitos cafés e pelos trabalhos que desenvolvemos juntos nesses últimos 7 anos. Me orgulho muito de tudo que fizemos e do modo que fizemos. Aproveito para agradecer todos os amigos e colegas que fizeram parte do CECSBE. Arthur, Beatrice, Bruna, Denis, Evandro, Fred, Jivago, Laura, Leandro (especialmente pela parceria nos trabalhos desta tese), Luana, Marcia, Marisol, Patricia, Shara. Todos os alunos de graduação, estagiários que interagiram, direta ou indiretamente comigo, aos que avaliaram os vídeos, aos que auxiliaram na colheita de dados, meu muito obrigado. Todas as conversas, risadas, tudo contribuiu para o que sou hoje e por isso sou muito grato. Aos colaboradores do VPS, em especial ao Danival, João Augusto e ao José Roberto (Ni). Vocês são pessoas maravilhosas, sempre dispostos a ajudar.

Agradeço também à Universidade de São Paulo. Conheci grandes pesquisadores e fiz ótimos amigos. Esse ambiente aqui no campus de Pirassununga é muito aconchegante e vivi muitos momentos felizes nesse lugar. Agradeço também ao meu departamento, o VPS. Sempre muito sensatos, compreensíveis e, de fato, preocupados com as pessoas. Em diversos momentos me senti privilegiado de fazer parte deste departamento. Agradeço também à Prefeitura do Campus Fernando Costa. Aos colaboradores da suinocultura do campus e também aos colaboradores do abatedouro. Agradeço também ao Laboratório de Pesquisa em Suínos e todos os colaboradores, em

especial ao Professor Cesar, pela disponibilidade do espaço físico para a realização do segundo experimento.

Agradeço também ao amigo Leonardo Batissaco, a Gabriela Marques e também à Professora Carla Celeghini. A parceria de vocês foi fundamental para os resultados de termografia e ultrassonografia deste trabalho. Agradeço também à Patricia Carvalho e ao Professor Rubens Arruda pela valiosa colaboração nos dados do CASA e de morfologia espermática. Muito obrigado à Giuliana Ferronato e ao Professor Juliano Silveira. Os esforços de vocês foram essenciais para que eu concluísse o meu capítulo com os dados moleculares.

I would like to thanks all of my US friends, Andressa, Bek, Cassie, Chris, Eldin, Jeff, Jessica, Katie, Khristen, Kylie, Liz, Marcel, Nickole, Patrick, Ruth, Shwetank, Scott, Tracy, Uriel, Victoria, and Yasmin. You made my time in the US much cozier and comfortable. Thank you so much for all shared love.

Agradeço também à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e à CAPES (Processo número 2017/05604-2 e BEPE 2019/11026-7) pelos recursos fornecidos para a condução deste trabalho. Obrigado Certified Humane por doar os recursos para aquisição dos animais e das reformas físicas do LPS. Muito obrigado à TopGen pelo acesso aos animais no experimento com LPS.

É muito complexo (para não dizer impossível) traduzir os sentimentos que sinto nesse momento do término do doutorado. É uma mistura de emoções que, no final, as que mais se destacam envolvem aquela sensação de dever cumprido. Ainda mais nos tempos que vivemos, da negação da ciência. Continuar nessa luta, é um ato de revolução. Por isso, dedico esse trabalho a todos os profissionais que se dedicam à pesquisa, que doam seus esforços para tornar o mundo cada dia melhor. Dedico também ao Ciro, que, onde você estiver, o seu lugar no meu coração está reservado.

“Educação não transforma o mundo.

Educação muda as pessoas. Pessoas

transformam o mundo”

Paulo Freire

RESUMO

Almeida, T. B. **Influência do bem-estar do macho suíno na qualidade dos gametas.** 2021. 109 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Em granjas comerciais de suínos, os cachaços são frequentemente expostos a situações estressantes, tais como alojamento individual, temperatura ambiente inadequada, restrição alimentar, falta de interação social, doenças, entre outros desafios. As consequências de tais situações estressantes para o bem-estar dos machos reprodutores e os possíveis impactos na qualidade do sêmen e nos resultados de desenvolvimento de sua prole são amplamente desconhecidas. Pesquisas recentes, em roedores, indicaram que efeitos epigenéticos no sêmen são responsáveis por alterações no metabolismo e comportamento da prole, inclusive para as gerações subsequentes, modulando assim a resiliência dos animais e, consequentemente, seu bem-estar. Após a caracterização do impacto do ambiente nos indicadores fisiológicos e comportamentais de bem-estar, investigamos a população de micro RNAs não codificantes no sêmen que é o nosso alvo de estudo pois representa os mecanismos potenciais para explicar os resultados do desenvolvimento tais como sobrevivência, crescimento, modulação comportamental e fisiológica, relatados anteriormente no nosso grupo de pesquisa, na prole. Essas pesquisas foram realizadas em dois experimentos separados, mas complementares. Nosso objetivo foi medir o impacto de um desafio de doença (n = 13) ou solução salina (n = 13) em 26 cachaços alojados em cela que foram escovados (n = 13) ou não escovados (n = 13), como um protocolo para promover o enriquecimento ambiental, e as consequências do protocolo experimental para o seu comportamento e características do sêmen, e o impacto de diferentes condições de alojamento, celas (n = 9), baias (n = 9) e baias com enriquecimento ambiental (n = 9), em parâmetros comportamentais, fisiologia do estresse (n=27) e indicadores moleculares seminais (n=18) de machos reprodutores jovens. Nós identificamos que o desafio de doença alterou a temperatura retal e a concentração de cortisol salivar de cachaços alojados em celas e que a escovação modulou seu comportamento e sua resposta de cortisol durante o desafio. Em nosso estudo, o desafio com LPS não alterou as características do sêmen. No segundo estudo, identificamos que os machos alojados em celas manifestaram mais

comportamento anormal quando comparados com os machos alojados em baias ou baias enriquecidas. Os machos mantidos em baias enriquecidas mostraram uma proporção significativamente maior entre os níveis de cortisol AM e PM, que são indicadores do funcionamento apropriado do eixo HPA, quando comparados aos machos alojados em celas e os machos mantidos em baias não enriquecidas. Além disso, os machos alojados em celas tinham indicadores robustos de saúde testicular comprometida, temperatura escrotal superficial mais elevada, incluindo alterações na motilidade e aglutinação dos espermatozoides. O estudo da população de microRNAs demonstrou diferenças significativas em 12 dos 266 microRNAs estudados. Os microRNAs que apresentaram diferença na abundância, em machos mantidos em baias com enriquecimento ambiental, podem explicar os melhores índices de qualidade seminal e o maior número de leitões nascidos vivos, quando comparados com a produtividade e com a progênie dos outros tratamentos. Este trabalho é pioneiro na investigação do impacto das condições de alojamento de cachaços no seu bem-estar e desempenho. Os cachaços mantidos em celas tinham a fisiologia e a saúde dos testículos comprometidas, por isso, os cachaços de reprodução devem ser mantidos em baias.
Palavras-chave: alojamento de cachaço; bem-estar animal; comportamento do cachaço; cortisol salivar; enriquecimento ambiental; suínos reprodutores.

ABSTRACT

Almeida, T. B. **Boar welfare influence the quality of the gametes.** 2021. 109 p.

Thesis (PhD in Sciences) – School of Veterinary Medicine and Animal Science,

University of São Paulo, São Paulo, 2021.

In commercial pig breeding farms, boars are often exposed to stressful situations, such as individual housing, inadequate ambient temperature, food restriction, lack of social interaction, illness, among other challenges. The consequences stressful situations for the welfare of the breeding boars and possible impacts in semen quality and developmental outcomes of their offspring are largely unknown. Recent research, in rodents, indicated that epigenetic effects in semen are responsible for changes in the metabolism and behavior of the offspring, including for subsequent generations, thus modulating the resilience of the animals and, consequently, their welfare. Small non-coding RNAs population in semen is our study targets because it represents the potential mechanisms to explain developmental outcomes represented as survival, growth, behavioral and physiological modulation, previously reported, from our own research group, in the offspring. This research were carried out in two separate, but complementary, experiments. Our aim was to measure 1) the impact of an endotoxin challenge (n=13) or saline (n=13) in 26 crated boars that were brushed (n=13) or not brushed (n= 13), as a protocol to promote environmental enrichment, and the consequences of the experimental protocol to their behavior and semen characteristics, and the impact of different housing conditions, conventional crates (n=9), pens (n=9) and environmental enriched pen (n=9), on behavioral parameters, stress physiology (n=27) and molecular seminal indicators (n=18) of young breeding boars. We identified that the endotoxin challenge changed the rectal temperature and the salivary cortisol concentration of crated boars and that brushing modulated their behavior and their cortisol response during challenge. In our study, LPS challenge did not change semen features. In the second study, we identified that boars housed in crates performed more abnormal behavior when compared with boars housed in pens or enriched pens. Boar kept in enriched pens showed a significant higher ratio between AM and PM cortisol levels, which are indicators of appropriated HPA axis functioning, than crated boars and boars kept in non-enriched pens. Moreover, boars housed in crates had robust indicators of compromised testis health, higher superficial scrotum temperature, including

alterations in sperm motility and sperm agglutination. The study of microRNAs showed significant differences in the abundance of 12 microRNAs out of 266 studied. The microRNAs which showed differences in the abundance on the ejaculate of boars kept in enriched pens could explain the superior markers in semen quality indicators and larger number of piglets born alive, observed in these boars, when compared with boars kept in crates or non-enriched pens. This work is pioneer in investigate the impact of housing conditions of boars in their welfare and performance. Boars kept in crates had testis physiology and health compromised, and for this reason, breeding boars should be kept in pens.

Keywords: Animal welfare; boar behavior; boar housing; breeding pigs; environmental enrichment; salivary cortisol.

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SUMMARY

1. INTRODUCTION

Social stress in boars has been poorly studied, with a limited number of publications on the subject. Boars are usually kept in individual pens or even in crates, similar to the gestation crates for sows, but with larger dimensions (FLOWERS, 2015), both in commercial farms or semen collection centers. The inadequate social environment is an important stressor for pigs, since this species has a complex social structure (MARINO; COLVIN, 2015). The restrictions of movement and reduction of social interactions are harmful to sows, altering opioid receptors in the brain (ZANELLA, 1996), altering the quality of bone mineralization (MARCHANT; BROOM, 1996), increasing the performance of abnormal behavior (BROOM; MENDL; ZANELLA, 1995), and these are indicators of reduced welfare. Social stress is also an important contributor to poor welfare outcomes in pigs. Socially isolated and prematurely weaned pigs showed differences in the central nervous system compromising the modulation of stress responses (POLETTO et al., 2006).

When boars are housed in crates, they are exposed to movement restriction and they manifest abnormal behaviors, similarly to what has been reported for crated sows (TERLOUW; LAWRENCE; ILLIUS, 1991). Moreover, when boars are housed in pens, which is a preferred environment to the conventional crates, it usually has solid walls, without visual contact with conspecifics. It was already shown that social isolation may be negative and a very severe and lasting experience, which can affect the sexual performance of the animal (HEMSWORTH; BEILHARZ; GALLOWAY, 1977). In most pig farms, the housing of boars does not have any kind of enrichment, making the environment very barren which could increase the performance of abnormal behaviors, and may compromise their welfare (FLOWERS, 2015). In addition, the nutrition given

to boars is not always adequate, as they often receive the same diet of pregnant sows, in a slightly larger amount (AMARAL et al., 2006). The nutritional requirements of boars are different from sows (NRC, 2012), and therefore must be developed as accurately as possible to achieve the levels required for optimum productivity.

Semen quality is known to be affected by environmental temperature and by the presence of straw (CORCUERA et al., 2002). In this work, the researchers found that motility and acrosome integrity were better in the semen of boars housed in temperature-controlled systems. Similar results were found assessing motility and acrosome integrity, which showed better indicators for animals housed with straw bedding during the summer, than in animals kept without straw bedding (CORCUERA et al., 2002). Moreover, Flowers (2015) reported other features that could impact boar performance, such as housing conditions, nutrition, photoperiod and temperature. Age and season can also contribute to the variation of reproductive tract function, such as volume, sperm concentration, motility, pH, morphology, and protein content of seminal plasma (FRASER et al., 2016).

Our team has been involved in studies evaluating the maternal effect, in the prenatal period, in the offspring (COULON et al., 2013; HENRIQUE, 2015; PETIT et al., 2015; BERNARDINO et al., 2016), however, little has been investigated addressing the possible paternal effect on offspring (BALE, 2015). Some studies indicated that epigenetic changes in male germ cells may be responsible for behavioral changes in offspring (BRAUN; CHAMPAGNE, 2014; TRIANTAPHYLLOPOULOS; IKONOMOPOULOS; BANNISTER, 2016). According to these same authors, the inherited epigenetic variation is, possibly, responsible for altering the behavior, metabolism and neurobiology of subsequent generations. This is the mechanism by which the environment interferes in the animal's genome, through changes in gene

expression, possibly affecting the phenotype, without altering the DNA sequence (NESTLER, 2016). The changes reported previously may have a transgenerational and long-lasting effect, involving the deletion and replacement of epigenetic marks (BERGER et al., 2009).

Epigenetic changes are found in germ cells, which can be maintained for generations even if that organism is not exposed to the initial stimulus (FRANKLIN et al., 2010). These changes can be caused by exposure to stress or even by the effect of the environment that the animal is inserted in, causing an individual change in the germ cells, which allows the transmission to offspring, modulating, for example, the vulnerability of the offspring to stress (NESTLER, 2016). Example of such effect was identified by Dietz et al. (2011b), when adult male mice were subjected to a challenge called defeat chronic social stress, they generated an offspring more vulnerable to various stressful stimuli. It is likely that semen acts as a transmission route to modulate the susceptibility of stress from one generation to the next, contributing to the modulation of the phenotype of the progeny and future descendants (FRANKLIN et al., 2010; BOHACEK; MANSUY, 2015). Another example is the one studied by Weaver et al. (2004), where maternal care, characterized by licking and grooming, altered the epigenetics of the glucocorticoid promoter gene in offspring of female rats.

It is known that some environmental changes alter the epigenome, measured by DNA methylation, of semen, and these changes can be identified by up to three generations in *C elegans* (DAXINGER; WHITELAW, 2010). The methylation of paternal DNA is essential for initial development (GU et al., 2010), so it is an important of concerns about such changes. In addition, males are susceptible to changes in their germ cells not only during puberty but also in adulthood (RODGERS et al., 2013a). Some DNA-specific regions, such as the CpG islands, may be potential targets for

environmental-mediated epigenetic change (SINCLAIR et al., 2016). In pigs it has already been shown that methylation of paternal and maternal DNA is preserved after fertilization (JEONG et al., 2007). However, it should be noted that not all epigenetic labels remain after fertilization, presenting different patterns of methylation when compared to germ cells and embryos (TRIANTAPHYLLOPOULOS; IKONOMOPOULOS; BANNISTER, 2016).

Micro RNAs are defined as a single stranding non-coding RNA, which regulate gene expression at the post-transcriptional level, and are highly conserved among different species (WU et al., 2013; ALVES et al., 2021). A population of microRNAs is involved in transgenerational transmission (BELLEANNÉE, 2015), and may be involved in gene silencing by up to five generations in the paternal line in nematodes (FIRE et al., 1998). Thus, the population of microRNAs, together with other epigenetic changes are probably crucial for the transmission of paternal stress to offspring (RODGERS; BALE, 2015). Another study has shown that when a certain population of micro RNAs from stressed animals are inserted into an oocyte, they can alter the phenotype of offspring as well as in natural fertilization (GAPP et al., 2014). Epigenetics modulation is crucial for survival, since cloned animals often present pathologies and inadequate nuclear reprogramming, thus relating disease and epigenetics (TRIANTAPHYLLOPOULOS; IKONOMOPOULOS; BANNISTER, 2016). The population of seminal micro RNAs has potential to be altered in intracellular communication even in a mature and inert spermatozoon.

Environmental enrichment protocols have demonstrated to be a powerful strategy to mitigate the negative consequences of stressors in pigs (TATEMOTO et al., 2020). Natural behaviors of boars include rooting, manipulating substrate, wallowing and they respond very positively to tactile stimulation, in certain body regions.

Our goal was to evaluate boar welfare and how the surrounding environment can modulate its state, measured using physiological and behavioral indicators, in addition to reproductive tract functions, such as sperm motility and morphology, and microRNA population. With a detailed evaluation of germ cell epigenetic changes, it is possible to better understand the effect and the possible mechanisms by which the welfare of the paternal lineage can modulate its welfare and homeostasis and their offspring physiology and behavior.

The aim of this study was to investigate the consequences of different housing conditions for breeding boars for their welfare, physiology, performance, and molecular impact on germ cells epigenetics, assessing microRNA population in their semen.

Our hypothesis is that boars kept in environments that allows them to perform more biologically relevant behavior with environmental enrichment will have better welfare and performance outcomes. We expected that animals kept in a barren and stressful environment, such as crates, will show indicators of poor welfare and lower performance.

In order to test our hypothesis, we carried out two distinct trials. In the first experiment, carried out on a commercial farm, reported in chapter 1, we evaluated the impact of an environmental enrichment protocol, brushing crated boars that were challenged or not challenged with endotoxin, to stimulate a disease process. We measured behavior, salivary cortisol, rectal temperature and semen quality. In the second trial, carried out at the University of São Paulo, Fernando Costa Campus, in Pirassununga, we studied animals, from the same genetic background in a more controlled environment. The results are reported in chapters 2, 3, and 4. We housed boars in crates, similar to the experiment 1 and had two additional treatments. We provided a more complex environmental enrichment, with tactile stimulus, brushing,

water baths, and with rooting material (hay), and a control barren pen, with the same measures as the enriched treatment. We collected more information from the animals, such as testicular thermograph images, ultrasound, and we used the infrastructure to collect more objective information, such as Computer Assisted Sperm Analyses (CASA), sperm morphology measures, and to carry out the molecular work.

2. Chapter 1 – The impact of an LPS challenge in crated boars

2.1.INTRODUCTION

The most genetic and monetary valuables animals in the pig production chain are housed in breeding pig farms. The industry dedicates special attention to those animals, regarding their health and performance. However, low attention has been given to welfare, particularly of boars. In many countries and the majority of the states the United States, boars are housed in crates (FLOWERS, 2015).

Since the breeding farms kept valuable animals, the protocols regarding health and biosecurity are well stablished. However, even in breeding farms, *Escherichia coli* has high prevalence in pig herds. *Escherichia coli* is a gram-negative bacteria, which can affect sperm cells, and impact litter size of contaminated semen samples (MAROTO MARTÍN et al., 2010). Lipopolysaccharide (LPS) is the major part of external membrane of Gram-negative bacteria and stimulates an intense immune response (OSBORN et al., 1964), and it has been used as a disease model in vertebrates (LLAMAS MOYA et al., 2006; LIU et al., 2019). It has been demonstrated that LPS in boar semen can increase oxidative stress, mitochondrial peroxidation, and decrease

motility of sperm cells (HE et al., 2017). In single housed young female pigs, an inoculation of LPS can modulate the brain noradrenaline, as well as cytokines in the brain (NORDGREEN et al., 2018). It is important to state that there is a gender difference in the response of pigs to LPS, since adrenal response in males and females are distinct (LLAMAS MOYA et al., 2006).

Indicators based on boar behavior and welfare are limited. Previous research addressed resting behavior (PETAK et al., 2010; TELES et al., 2017), sexual behavior during natural mating (HODEL; NATHUES; GRAHOFER, 2021), and effects of group housing in boars (CORDOBA-DOMINGUEZ et al., 1991). However, compared to the extensive literature published in pregnant sows, piglets, and finishers, boar behavior and welfare have been neglected and poorly investigated and reported in literature over the years.

Environmental enrichment can mitigate the negative impact of stressors in domestic pigs (TATEMOTO et al., 2019a). Tactile stimulation are powerful mediators of positive states in several species, including boars. Brushing is widely used in dairy cattle and there are reports in the literature of increased milk production (SCHUKKEN; YOUNG, 2009) and also as environmental enrichment (WILSON et al., 2002), since mechanical brushes can reduce stress or frustration when animals are housed in poor environments (DEVRIES et al., 2007). In addition, it was reported that the environmental enrichment was beneficial not only for the dam, but also for the offspring (TATEMOTO et al., 2019a, 2020). We did not find publications on boar environmental enrichment, despite the fact that the paternal lifestyle can impact offspring behavior (DIETZ et al., 2011a; RODGERS et al., 2013b; BOHACEK et al., 2015; RANDO; SIMMONS, 2015; SHORT et al., 2016; CHAN; NUGENT; BALE, 2018).

Considering the above mentioned information, we investigated the impact of a LPS challenge in crated breeding boars, kept in a commercial facility in the south of Brazil. Our aim was to determine semen features, rectal temperature, behavior, and salivary cortisol concentration of crated boars challenged with LPS with or without brushing.

2.2.MATERIALS AND METHODS

This study was approved by the Committee on Ethics in Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science (FMVZ), under protocol no. 3612010616.

2.2.1. Animals

Twenty-six adult boars, aged between 8 and 31 months, kept on a breeding farm, were studied. They were housed in stalls measuring 245 x 73 centimeters. Concentrated diet was provided in two moments, at 0700h and 1300h, with the same diet offered for all animals. The diet consisted in corn, soybean meal and mineral premix, specific for breeding boars. The boars, landrace (n=13) and large white (n=13) were allocated by age and breed into four treatments, a) one time inoculation of LPS (2 µg/kg) with brushing (8 animals); b) one time inoculation of LPS (2 µg/kg) without brushing (5 animals); c) inoculation of 0.9% saline solution with brushing (5 animals); and d) inoculation of 0.9% saline solution without brushing (8 animals).

Table 1 contains the detailed description of the groups. The animals were distributed according to their age, in ascending chronological order. Inoculation of

saline solution was performed so that the animals of all treatments were submitted to the same handling protocol.

Table 1 - Individual information about the studied boars. Data about the breed of the studied boars (LR = landrace; LW = large white), birth date, weight and treatment group.

Treatment	Animal	Birth date	Weight (kg)
LPS brushed	LW 125	October, 2015	266.5
	LW 604	January, 2016	255.4
	LR 992	September, 2016	209.1
	LR 1447	November, 2016	237.4
	LW 1039	December, 2016	201.9
	LW 1073	December, 2016	228.8
	LR 1064	May, 2017	188.6
	LW 438	September, 2017	204.6
LPS control	LR 779	December, 2015	246
	LW 258	August, 2016	236.3
	LR 900	September, 2016	262.2
	LR 1494	November, 2016	220.7
	LW 1440	February, 2017	197.7
Saline brushed	LR 463	June, 2016	260
	LW 292	August, 2016	213.3
	LR 1296	November, 2016	218.8
	LR 218	January, 2017	211.3
	LW 1419	February, 2017	206.2
Saline control	LW 543	January, 2016	251.3
	LW 253	August, 2016	249.5
	LR 343	September, 2016	271.3
	LW 477	September, 2016	232.2
	LR 1171	November, 2016	260.7
	LW 1176	January, 2017	210.9
	LR 529	March, 2017	196.3
	LW 337	May, 2017	188

2.2.2. Experimental treatments

According to the adopted protocol, four distinct groups were formed. One group that we inoculated LPS and were not brushed (LPS without brushing), inoculated with LPS and brushed (LPS with brushing), the group inoculated with 0.9% saline and were

not brushed (saline without brushing) and the group inoculated with 0.9% saline and have been brushed (saline with brushing).

2.2.3. Tactile enrichment – brushing

The day before that the animals were inoculated with LPS, half of them were assigned to brushing, in order to enrich the environment. The animals were manually brushed, using a 48 centimeters broom with nylon bristles for two uninterrupted minutes, every day after inoculation (D32) until the end of the experiment (D86). Brushing was performed in the late afternoon, starting at 5 pm.

2.2.4. LPS and saline challenge

On day 32 (D32) after the beginning of the evaluations, the animals were inoculated with LPS (2µg / kg, Sigma Aldrich, 0111:B4) or 0.9% saline. The procedure occurred after the animals were restrained using the "snaring method" with inoculation in the lateral auricular vein. All inoculation volumes were standardized to 1 milliliter, using a 1 ml syringe with a 13 x 0.45 mm needle. The inoculation procedure for all animals lasted 72 minutes. After inoculation, collections were performed on days D42, D56, D71, and D86. On the day of inoculation, saliva was also collected from the animals. There were six collections per animal during the first 12 hours after inoculation, totaling 156 saliva samples, for cortisol measurement.

After inoculation, all animals were followed up clinically, with measurement of rectal temperature and respiratory rate. If the measured rectal temperature exceeded 40 ºC, the animal received a water shower at room temperature. If it was necessary, we repeated the shower. In the approved protocol, if the temperature did not drop in

response to the water shower, we would treat them with dipyrone, but that was not necessary for any boar. 10 measurements of rectal temperature were performed in an interval of 24 hours. Immediately after inoculation, the animals were followed up, hourly, for six hours. In the next four measurements, the interval was two hours and the last measure was performed 24 hours after inoculation.

2.2.5. Behavior

The boar's behavior was measured for 2 consecutive days Saturdays and Sundays, which were days with limited management activities at the breeding farm, and the semen sampling was performed the day after the behavior collection, on Mondays. All animals were observed, directly, for 2 uninterrupted minutes, four times in a sequence, in three different moments throughout the day, using focal sampling and continuous observation (MARTIN; BATESON, 2007). The first observation was performed between 8:40h to 9:40h (C1, C2, C3 e C4); the second observation was performed between 10:40h to 11:40h (C5, C6, C7 e C8); the third observation was performed between 17:30h to 18:30h (C9, C10, C11 e C12). Thus, each animal was evaluated for 24 minutes in each observation day, totaling 228 minutes per animal (C0 e C1, C14 e C15, C40 e C41, C54 e C55, C70 e C71, C85 e C86). On the same days of the behavioral observation, saliva samples were collected, following the methodology of Siegford et al. (2008), at 7:00 am and at 7:00 pm, in order to measure the salivary cortisol concentration. Table 2 shows the definition of the observed behaviors. The behavior "inactivity" was calculated based on the activities performed during the observation period, excluding "sleeping" behavior. As an example, if the boar spent 80 seconds performing sham-chewing, it performed the "inactivity" behavior for 40 seconds.

Table 2 – Definition of the behaviors used in the ethogram for behavioral evaluation of the boars

Behavior	Definition
Sleep	Eyes closed, activity absent
Lying ventrally	Lying with the belly facing the ground with all limbs under the body.
Lying laterally	Lying sideways, with all the limbs extended laterally
Sitting	The animal has the forelimbs on the floor and the hind limbs flexed
Standing	The whole body supported by the four limbs
Sham-chewing	Continuous chewing without the presence of any visible food in the oral cavity
Bar biting	The animal bite any part of the crate or the wire
Licking floor	The tongue touches the ground, followed by head movements
Head weaving	The animal moves their head laterally or vertically, repeated times
Licking empty trough	The tongue touches the trough with no food, without drinking water
Eliminate (E)	The action of urinate or defecate
Social behavior (E)	The boar interacts friendly with the boar housed beside it
Aggressive behavior (E)	Any attempt of bite, facing or physical interaction
Vocalization (E)	Any sound emitted by the animal
Inactive	Calculated by the difference between the total observation time and the activities performed. The “sleep” time was not taken in the account

Measured behaviors for boars housed in crates or pens. The letter (E) indicates that this behavior was measured as event instead duration
Adapted from Zonderland et al. (2004); Bernardino (2016); Tatemoto et al (2019b)

2.2.6. Semen sampling

In parallel to the reported experiment, the semen of the boars were used to inseminate sows from three different farms. Because of that, the semen collection routine was intense, with, at least, three semen collections per week, alternating among

the boars. All seminal features, including volume, concentration, and subjective motility, were registered and analyzed by the same farm collaborator, in all weeks.

Semen was collected by the gloved hand method from all boars every two weeks. This method provided adequate tactile stimulus to active optimum sperm features and is indicated for routine collection in boars (KING; MACPHERSON, 1973). Before moving the animal to the collection room, the boar was placed in a specific crate, and had the preputial area cleaned, with dry paper and the hairs on the prepuce were trimmed if necessary. The preputial diverticulum was emptied by manual pressure. After that, the boar was guided to a pen, containing a proper mannequin and after jumping in the mannequin, the collection was started. The initial jets of the ejaculate were discarded, and the semen was directed to a specific cup, using a circular paper filter (DeLaval, 240mm, 20g) at the top to separate the gelatinous fraction from the ejaculate.

Two semen collections were performed previously to the LPS or saline inoculation (D1 and D14) and semen collection was carried out every two weeks, until the end of the experimental period (D86).

2.2.7. Semen analysis

Sperm motility, sperm concentration, and volume were immediately evaluated. All analyzes were performed in a laboratory, attached to the collection room, with controlled internal temperature. To perform sperm motility, 10 µl of semen was inserted into a 26x76 mm glass slide with a 22x22 mm cover slip, both heated on a heating platform. Afterwards, the slide was inserted into an optical microscope, using the 40-fold magnification objective. Motility analysis was estimated qualitatively, under the percentage of mobile sperm cells, in at least three fields. The percentage was

determined by the evaluator and only the cells that presented progressive and rectilinear movement were classified with maximum vigor score (CELEGHINI et al., 2019).

All analyzes were performed by the same employee who performs this activity for the farm. The protocol for measuring sperm concentration was developed by a company that provides technical assistance in the breeding for the farm. The process consists of using a spectrophotometer to measure the absorbance of the solution. Initially, 3 milliliters of 0.9% saline solution were deposited in a plastic cuvette and this was inserted into the spectrophotometer (Quimis Q898DRM®) for blanking. Thereafter, 30µl of semen was inserted into the saline solution, homogenized and read again in the spectrophotometer. The absorbance value was used to estimate the concentration of cells per mm^3, according to a table pre-established by the company. After such results, two variables were obtained. The total number of sperm per ejaculate (multiplying the volume and concentration), and the number of possible doses to be prepared (volume x concentration x motility / 2.5, with each dose containing 2.5 million sperm).

2.2.8. Statistical analyses

All the data were analyzed with the package Statistical Analysis System 9.4 (SAS Inst., Inc., Cary, NC). Initially, the data were checked for the presence of discrepant information (outliers) and we verified the residual normality, through the Shapiro-Wilk test. When the normality assumption was not met, the transformation by logarithmic, the square root or ac sine was used. After those approaches, the data were analyzed by ANOVA with PROC GLIMMIX of SAS using a treatment as principal effect over the time, and a block as random effect. The block was the semen quality of the animals, distributing them equally in the treatments. Also, the command REPEATED was added

to the program, in order to analyze the time effect. Thus, we had the effect of treatment, the effect of time, and the interaction between these two factors.

Moreover, for all analyses, among the 15 different covariance structures were tested and the one that best fits the statistical model was chosen, based on the lower value of the Akaike Index correction criterion (AICC) (WANG; GOONEWARDENE, 2004). When necessary, for a pos-hoc test, we used the Tukey-Kramer test.

The data collected prior to the treatments was used for the initial analysis. However, for the presented results, we used the data only after the allocation of the animals in the treatments. The means and the standard error mean (SEM) were obtained from raw data. For all statistical analyses performed, a 5% level of significance was adopted.

2.3.RESULTS

The data from behavioral evaluations are summarized in table 3 and table 4. For the behavior head weaving, we identified a time effect. The Tukey-Kramer test showed that the boars manifested this behavior longer at the day 51 (P = 0.0037). The tendency found for lying laterally, showed that the animals from saline with brushing lay laterally longer than the other treatments (P = 0.0688). Additionally, the behavior lying ventrally was different among treatments. Moreover, regarding the lying laterally behavior, the Tukey-Kramer test results are presented in table 4. The behavior lying ventrally was similar. The non-brushed animals lay ventrally longer than the brushed animals.

Table 3 – Principal effects of treatment, time, and their interaction of evaluated behavior from crated boars, challenged with LPS or saline, brushed or non-brushed

Activity	Treatment	Time	Interaction
Kneeling	0.39	0.38	0.89
Head weaving	0.33	0.03	0.58
Drinking	0.36	0.04	0.16
Scratching	0.55	0.02	0.02
Lying laterally	0.07	<0.01	0.11
Lying ventrally	0.01	0.71	0.83
Standing	0.64	<0.01	0.87
Licking empty trough	0.31	0.04	0.05
Licking floor	0.20	0.01	0.54
Sham chewing	0.88	<0.01	0.05
Bar biting	0.05	0.02	0.23
Inactivity	0.67	<0.01	0.78
Grinding teeth	0.18	0.10	0.63
Siting	0.97	0.63	0.28
Social	0.35	<0.01	0.26
Sleep	0.46	<0.01	0.59
Eliminate	0.35	0.03	0.20
Vocalization	0.74	<0.01	0.23

Table 4 - The Tukey-Kramer test result for lying laterally behavior of crated boars

Treatment	Mean (in seconds)	Tukey-Kramer result
LPS with brushing	43.1738	A
LPS without brushing	30.6129	B
Saline with brushing	49.0342	A
Saline without brushing	32.0834	B

Regarding the behavior scratching, we found an interaction between time and treatment. After the Tukey-Kramer test, we saw that there were fewer differences between some treatments in 2 different times.

At the D54, the treatment saline with brushing and saline without brushing were different. In this same day, the treatment saline with brushing differed from LPS with brushing. In the both previous differences, the saline with brushing animals showed a longer duration for the behavior scratching. Those above mentioned interactions were the only ones found after the post-hoc test.

For the sham-chewing behavior, we found an interaction between treatment and time ($p = 0.047$). The post hoc test showed that this interaction was only in the day 37, comparing the treatments saline with brushing and saline without brushing, ($p = 0.0229$). We did not find any difference among the other treatments.

For the bar biting behavior, we also identified a tendency ($p = 0.051$). For this behavior, the post-hoc test showed that the animals from saline without brushing performed this behavior the longest (9.2624 seconds). This is the only dissonant treatment impact in behavior among treatments. (LPS without brushing = 1.7825 seconds; LPS with brushing = 2.3194 seconds; Saline with brushing = 3.8848 seconds).

We did not identify any difference in semen features. A time effect was observed for the following variable: doses, total of sperm cells, temperature, and volume. However, according to our experimental design, we were not interested in time effect without interaction with treatment and these results will not be discussed. Table 5 content is the p-value for all variables related to the semen parameters.

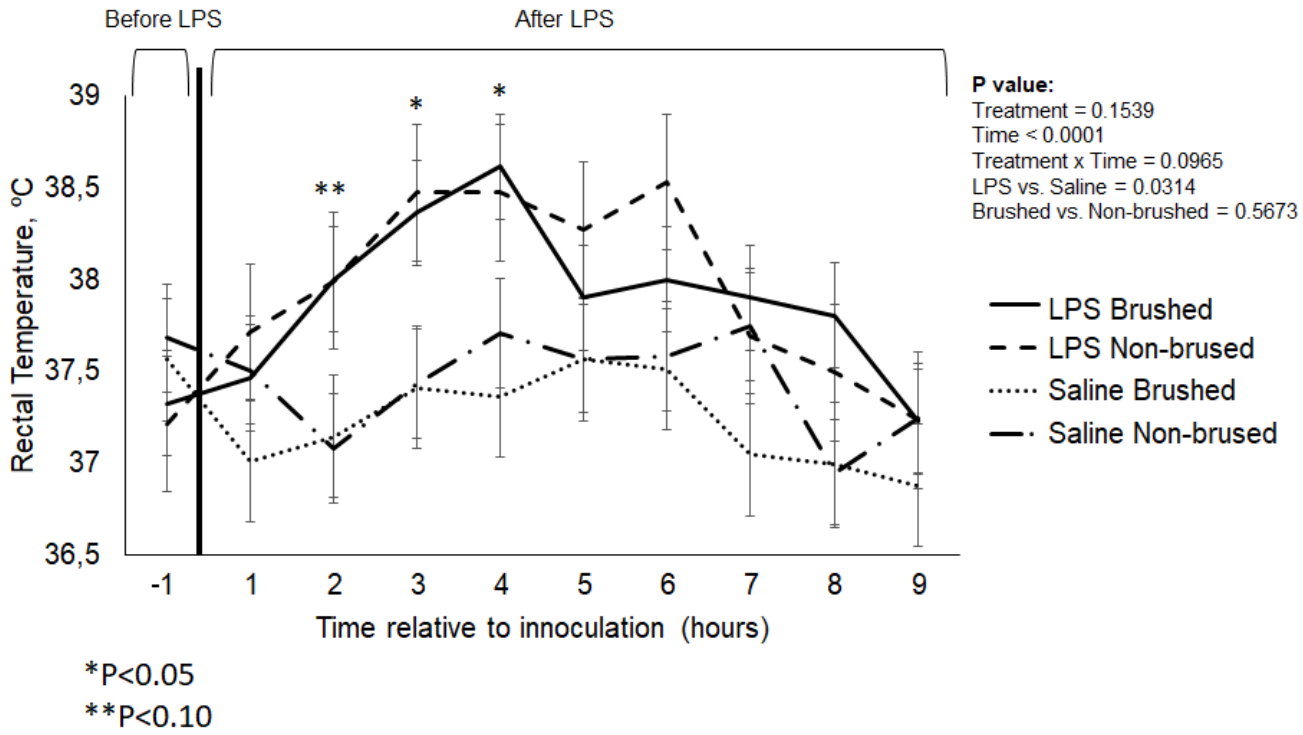
Table 5 - The p value of all the semen features studied

Variable	TREATMENT	TIME	INTERACTION

Concentration	0.477	0.6999	0.312
Doses	0.9945	0.0041	0.459
Motility	0.4069	0.8214	0.8554
Total of sperm cells	0.9839	0.0035	0.413
Temperature	0.0765	<0.0001	0.9179
Volume	0.5573	0.002	0.7779

We identified a difference in rectal temperature among boars challenged with LPS or saline. Boars which received LPS showed higher rectal temperature 2, 3, and 4 hours after the inoculation. This difference was not identified prior or 5 hours after the inoculation (figure 1).

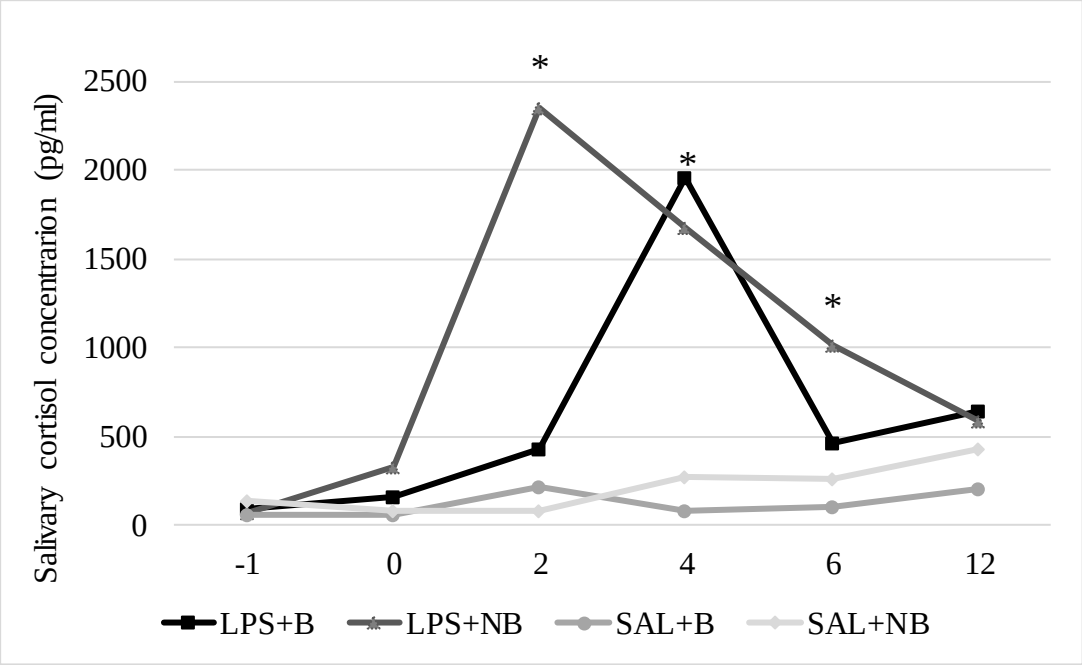
Figure 1 – Rectal temperature (ºC) of boars challenged with LPS or saline, brushed or non-brushed during 10 hours



*P<0.05
**P<0.10

The saliva samples from the day of the LPS challenge showed an increase in cortisol concentration for LPS challenged boar. The graph 2 shows that the difference among treatment was found, two, four, and six hours after inoculation. Twelve hours after LPS inoculation, we did not find any differences among treatments in salivary cortisol concentration. The Tukey-Kramer test showed that after 6 hours of inoculation, only boars from LPS without brushing had higher salivary cortisol concentration.

Figure 2 – Salivary cortisol concentration of boars challenged with LPS or saline, brushed or non-brushed during 13 hours interval



The stars (*) indicate a P value lower than 0.05. X axis is represented in hours after inoculation.

2.4.DISCUSSION

We did not identify many differences on behavioral data, when contrasting the treatments in our study. We assumed that because all animals were housed in crates, the most common scenario in Brazil and global commercial farms. They are housed in

crates for their entire life, without any previous experience of environmental enrichment, which certainly influenced the impact of brushing on the behavior results. It is important to mention that brushing addressed limited aspects of the animal's biological needs. In addition, we understood that it was necessary to study boars in different housing conditions.

Boars from the non-brushed treatments, both from saline and LPS inoculation, did lay ventrally longer than the animals brushed (see table 4). These results are intriguing because it shows that the animals from brushed treatments changed their resting behavior. Even though boars were housed in crates, a well-known source of stress for pigs, the brushing approach, to enrich their environment, appears to be effective in modulating lying behavior. Resting is the most representative behavior in finishing pigs (EKKEL et al., 2003), which may differ for boars, also because of the difference in the social structure of breeding animals.

Analyzing the other behaviors and the semen features, we just identified a time effect. According to our experimental design, the time effect without interaction with treatment was not part of our study. Therefore, we will not discuss the time effect. Additionally, the semen data was all analyzed through subjective methodologies, since we did not have access to objective methods at the farm. Object methodologies, such as Computer Assisted Sperm Analyses (CASA) would contribute to more detailed information about the motility features. As already mentioned, the boars that we sampled had a great variation in their ages. This fact itself could play a big role in the morphology and sperm quality results.

Body temperature and cortisol responses were affected by the previous experience with brushing. LPS inoculation caused an increase in the rectal temperature of the boars. The animals inoculated with LPS showed higher rectal temperature after 2, 3, and 4

hours after the inoculation. We did not identify differences in the rectal temperature between LPS treatment and non-LPS treated boars after 5 hours of inoculation. LPS is known to cause sickness behavior, such as hyperthermia, prostration, emesis, among others. This result is important because, based on it, we can guarantee that the LPS caused a stressful event, which was resolved a few hours later. This is part of our hypothesis.

As observed in Figure 1, the boars from the LPS group, brushed or non-brushed, seems to have different behavior after 4 hours of inoculation. Despite the absence of statistical analyses, there is a dramatic decrease in rectal temperature from LPS brushed boars 5 hours after the inoculation. However, for LPS non-brushed boars, the rectal temperature decreased only after 7 hours post inoculation. We believe that in a larger population, these results could be different, indicating that the applied enrichment could mitigate the impact of a LPS challenge in boars. Salivary cortisol concentration was higher for LPS challenged boars 2, 4 and 6 hours after inoculation. The salivary cortisol concentration results were very similar to the rectal temperature. The LPS brushed boars showed a delay to raise up the salivary cortisol concentration compared with LPS non-brushed boars. Moreover, the LPS non-brushed boars delayed the decrease in the salivary cortisol concentration, corroborated by the difference only for LPS non-brushed boars after 6 hours of inoculation. It is possible that the positive human-animal interaction, caused by brushing, mitigated the anticipatory responses of the boars to the treatment.

Another interesting result that can be added to the hypothesis that the quality of the interactions with humans improved with the brushing protocol is the resting behavior of brushed boars (see table 4). Brushing boars can modulate laterally lying behavior, even in a stressful individual confined housing.

To the best of our knowledge, this is the first report that a LPS challenge in boars can modulate rectal temperature and salivary cortisol concentration.

2.5.CONCLUSION

Our results showed that the treatments were able to modify the behavior of the boars, particularly lying behavior. The resting behaviors, represented by lying laterally and ventrally, were different among treatments. In both behaviors, after the Tukey-Kramer tests, the results were the same. The animals from the brushed treatments (independent of the LPS or saline inoculation) did lay down longer than no brushed boars.

LPS challenge can change the rectal temperature of crated boars, when inoculated with the dose of 2µg / kg, as well as increase salivary cortisol concentration. Combining the results from rectal temperature and salivary cortisol concentration, to provide an environmental enrichment such as brushing and can, somehow, modulate the physiology response to a disease challenge, hereby represented by LPS inoculation. We anticipate that an improvement in the relationship with humans is a result of the positive experience represented by that brushing protocol and could explain, partially, the differences. Comparing the animals from the LPS group, brushed boars showed rectal temperature and salivary cortisol concentration results that indicate they responded in a less intense way, which could mitigate the negative impact of an endotoxin challenge.

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3. Chapter 2 – Behavior and physiology of boars housed in three different environments

3.1.INTRODUCTION

Boars housed in commercial conditions are subjected to low-stimulus environments, which may impact the expression of normal species specific behaviors and compromise its health (TELES et al., 2017). The behavior of boars has not been reported in literature as detailed as the behavior of sows, piglets, or finishers. It is known that the temperature, photoperiod, and nutrition can impact the reproductive performance of boars, however, there is limited information about semen collection frequency and housing condition in the previous studies (FLOWERS, 2015). Semen from boars housed in a facility with a controlled environment temperature showed higher motility and higher normal acrosomes, when contrasted with boars without proper temperature control in their environment (CORCUERA et al., 2002). In addition, in this same research, the authors provided straw bedding, which was beneficial in increasing the same parameters in the winter months. To our knowledge, there is no information in scientific literature about the influence of housing conditions in the welfare and physiology of boars, while this subject has been extensively reported in pregnant sows (SCHWARZ et al., 2021).

Crates are a well reported source of chronic stress for adult sows, increasing cortisol concentrations (HEMSWORTH, 2018). Crates can compromise the ability of the animals to seek social contact, to explore, and to show natural behavior such as rooting, separating clean and dirty areas, exploratory behaviors, among others (BROOM, 1986). Moreover, crates with a concrete floor provide an inadequate environment, since boars can only interact with food, metal bars, and with their own

feces (PETAK et al., 2010). In semi-natural conditions, boars can spend 27% of their day time grazing (STOLBA; WOOD-GUSH, 1989), indicating a relevant behavior for adult males. A valuable alternative that should be provided in this scenario is environmental enrichment. However, the scientific community has not dedicated attention to this issue, especially for breeding boars (TELES et al., 2017).

Another possibility to improve the social environment of breeding boars is keeping the animals in groups. Kunavongkrit et al., (2005) reported that it is difficult to house boars in groups, unless electronic feeding is provided. Another issue is regarding sodomy and aggressive behaviors, which could compromise performance and welfare of the animals (KUNAVONGKRIT et al., 2005). A previous study evaluated group housed boars, with 4-5 animals per pen (CORDOBA-DOMINGUEZ et al., 1991). The boars were reared together from an early age, and the authors reported few undesirable behaviors within this management scenario. Previous research showed that group housing for boars is a good tool to improve social interactions and welfare (HEMSWORTH; BEILHARZ, 1979; CORDOBA-DOMINGUEZ et al., 1991). It has been hypothesized that limited opportunities for social behavior such as snout-snout contact with another boar would be a good alternative to improve welfare (CORCUERA et al., 2002), recommending that solid walls should be avoided.

Salivary cortisol sampling can provide key information about the hypothalamic-pituitary-adrenal axis activity in pigs as it represents the free, unbound fraction, hence biologically active, glucocorticoid, provided that samples are taken in the morning and afternoon (ZANELLA et al., 1998). High cortisol levels may compromise reproductive efficiency and fertility in gilts (TSUMA et al., 1996). However, to the best of our knowledge, little information is available about salivary cortisol levels in boars.

Teles et al., (2017) reported that crated boars with a coffee husk bed changed their resting behavior and showed higher salivary cortisol concentration than boars kept without coffee husk bedding material. Likewise, using the same bedding material for boars housed in pens showed a reduction in subjective motility (TELES et al., 2016).

The aim of the present study was to evaluate the impact of three different housing conditions for boars on their behavior and salivary cortisol concentration.

3.2.MATERIALS AND METHODS

This study was approved by the Committee on Ethics in Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science (FMVZ), University of São Paulo (USP) under protocol no. 3612010616.

3.2.1. Animals

For this study, 27 boars (F1 large white x landrace) 10 months old, were fed twice daily, 0700am and 1300pm, with 2.8kg of concentrate per day, and had *ad libitum* access to a nipple drinker. The diet consisted in corn, soybean meal and mineral premix (3300 kcal per kg, calculated value). Prior to treatment allocation, all animals were housed in individual pens, measuring 3.85 meters x 1.2 meters. Afterwards, animals were allocated in one of the assigned three housing conditions, blocked by their seminal quality, measured using Computer Assisted Sperm Analysis (CASA) and sperm morphology.

The three housing conditions consisted in a) boars kept in the same pens (n = 9), b) identical pens with an enrichment protocol (n = 9), and c) gestation crates (n = 9). For the enriched pens, environmental enrichment was offered twice daily, one hour after

feeding represented by brushing the animals for two minutes using a broom, showering the animals with water for 30 seconds, and hay was provided as rooting material.

3.2.2. Behavior

Boar behavior was recorded with cameras (Intelbras VHD 1220 B – G4), in full high-definition quality, and stored in a hard drive disk until further analyses. We analyzed six minutes each hour, from 7:00 h until 17:00 h. The behavior was analyzed by six different trained observers, at week 0 (prior to the treatment allocation), week 4 (30 days after treatment assignment), and week 8 (60 days after treatment assignment). The behavioral observation was performed by a combination of scan sampling, followed by a focal animal and continuous recording (MARTIN; BATESON, 2007), with each animal observed for one hundred and twenty uninterrupted seconds. Each boar was observed three times in each hour of the day, totaling 54 minutes per day of observation. Six trained observers had access to the videos and, in a randomized way, gathered data from the studied animals. All collection periods consisted in two consecutive days to avoid interference of possible events in the behavioral data (e.g. on days D-1, D0, D29, D30, D59, and D60). An ethogram was developed, based on the boar ethogram validated by Zonderland et al. (2004), Bernardino (2016), and Tatemoto et al. (2019c).

Table 1 has the behavioral variables used in the behavioral observation protocol. We obtained the variable abnormal behavior by the sum of the following behaviors: head weaving, biting bars, licking floor, sham-chewing, drinking manipulation, and licking empty trough.

3.2.3. Salivary sampling

We followed the methodology of Siegford et al. (2008), which consisted in presenting hydrophilic cotton, two roller-shaped units, tied to a dental floss with long tips and offered to the animals. The boar chewed the cotton until it was saturated with saliva. The first sample was discarded; we repeated the protocol to collect only recently produced saliva. After the second sample was collected, it was placed in a 15 ml conic tube, identified with the animal's number and time of collection. Subsequently, the tube was packed in an ice cube box until the end of the collection, and then frozen at -20 °C until processing. The thawing was done in a container containing ice. After the complete thawing of the sample, the sample was centrifuged for 2 minutes at 1000 x g (Celm Combate), and then the supernatant was aliquoted into 1.5 ml micro tubes and again frozen at -20°C until analysis. This process assists in the removal of mucins and other components that may interfere with the analysis protocol. We used a cortisol enzyme immunoassay, without extraction, to measure the cortisol concentration (PALME; MÖSTL, 1997). All samples were analyzed in duplicates.

Saliva was collected before feeding time (6:30h-7:30h) and in the afternoon (18:00-19:00) during the following periods of behavioral observations. Samples from two consecutive days were pooled for AM and PM collection times. Salivary cortisol was analyzed using an EIA (PALME; MÖSTL, 1997). In addition, we calculated the AM/PM ratio in salivary cortisol samples. To determine the AM/PM ratio, we used the salivary cortisol concentration from the morning and divided it for the cortisol concentration found in the afternoon. This methodology was used in humans for distinct approaches (HAN et al., 2019).

3.2.4. Statistical analyses

All the data were analyzed with the package Statistical Analysis System 9.4 (SAS Inst., Inc., Cary, NC). Initially, the data were checked for the presence of discrepant information (outliers) and we verified the residual normality, through the Shapiro-Wilk test. After those approaches, the data were analyzed by ANOVA with PROC GLIMMIX of SAS using a treatment as principal effect over the time and a block (seminal quality) as a random effect. In addition, the command REPEATED was added to the model, to analyze the time effect. Thus, we had the effect of treatment, the effect of time, and the interaction between these two factors.

For all analyses, among the 15 different covariance structures were tested and the one that best fits the statistical model was chosen, based on the lower value of the Akaike Index correction criterion (AICC) (WANG; GOONEWARDENE, 2004). When necessary, for a post-hoc test, the Tukey's test was used.

We used this approach for the behavior variables and AM/PM ratio. For the variable salivary cortisol concentration, we added the time of collection in the model (morning and afternoon). Thus, for this variable, the boar was considered as plot, the week as sub-plot, and the time of collection was classified as a sub-sub plot.

The means and the standard error mean (SEM) were obtained from raw data. For all statistical analyses performed, a 5% level of significance was adopted.

3.3. RESULTS

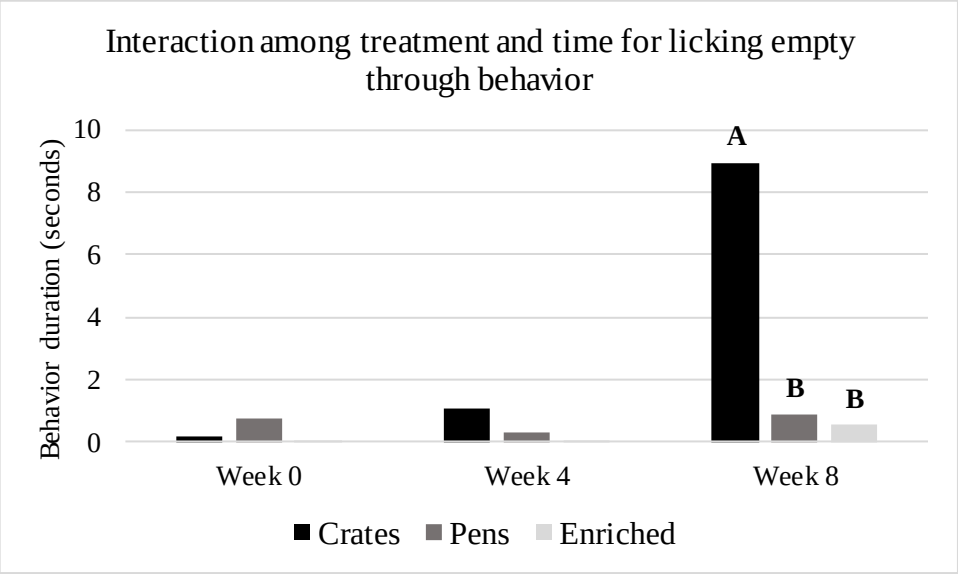
We found several differences among treatments on behavior, which are summarized in table 1. Most differences found were caused by time, however, there were several interactions among treatment and time.

Table 1 – Mean duration and probability of the analyzed behaviors of boars housed in three different conditions.

	Mean duration (seconds)			**Probability**		
Behavior	Pens	Crates	Enriched pens	Treatment	Week	Interaction
Lying ventrally	34.53	32.00	36.81	0.6590	0.0059	0.1823
Lying laterally	37.32	28.65	31.95	0.2933	< 0.0001	0.2664
Sitting	4.15	8.50	4.81	0.6850	0.9152	0.2746
Standing	43.18	49.16	45.73	0.5971	< 0.0001	0.0765
Biting bars	0.03	0.02	0.03	0.4414	0.0195	0.9258
Licking floor	0.36	0.02	0.24	0.9373	0.0314	0.2781
Sham-chewing	0.05	0.11	0.04	0.3527	0.1376	0.7774
Licking empty through	0.97	1.01	0.71	< 0.0001	< 0.0001	< 0.0001
Eating	4.14	3.59	4.08	0.4533	0.2507	0.5221
Resting	0.06	0.13	0.01	0.0939	< 0.0001	0.0030
Abnormal behavior	2.47	5.23	2.93	0.0500	0.0028	0.7702

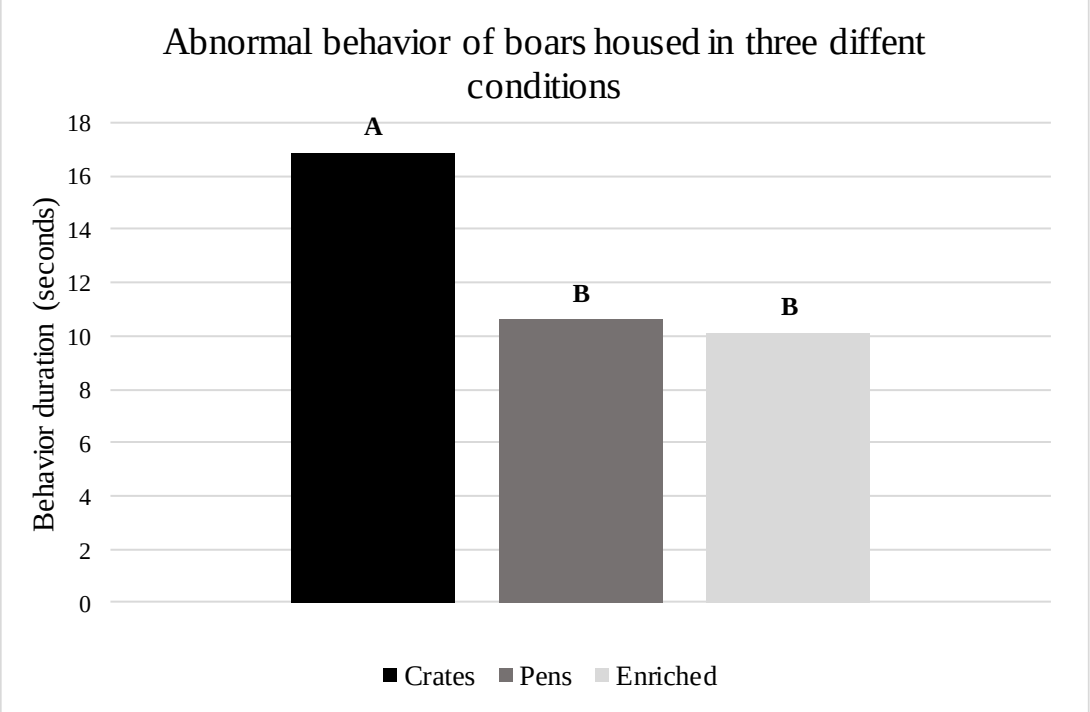
For the behavior Licking Empty Trough, we found an interaction among treatment and time (p value = 0.0011). We found that the boars housed in crates showed this behavior for longer periods at week 8, after treatment allocation. In addition, we did not identify differences between the treatment pens and enriched pens (Figure 1).

Figure 1 – The treatment and time (week) interaction for licking empty through behavior for boars housed in three different conditions. Different letters mean difference among treatments ($p < 0.05$), and no letters mean no difference.



We found a treatment and time effect for abnormal behavior, but no interaction. The boars housed in crates showed longer abnormal behavior when compared with boars housed in pens and enriched pens (Figure 2).

Figure 2 – Abnormal behavior duration of boars housed in three different conditions, during 8 weeks interval. Equal letters mean no difference among treatments. X axis is represented in seconds.



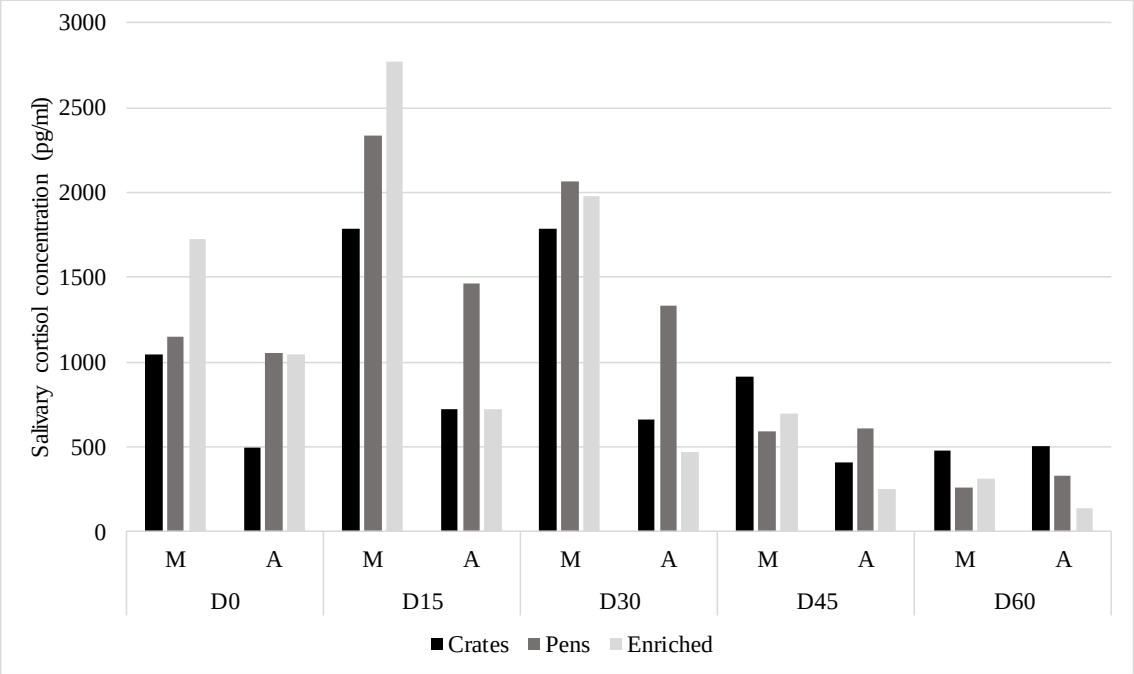
The principal effects of our model in salivary cortisol concentration are presented in table 2. We identified a week, time, and interaction of treatment with week and time effects. The cortisol concentration data is summarized in figure 3.

Table 2 – p value of principal effects of the treatment, week, time, and the interaction among factors.

Effect	**P value**
Treatment	0.4876
Week	<0.0001
Treatment*week	0.0095
Time	<0.0001
Treatment*time	0.0008
Week*time	<0.0001

Treatment*week*time	0.5490

Figure 3 – Salivary cortisol concentration of boars kept in three different housing conditions, over a period of 8 weeks, morning, and afternoon sampling.

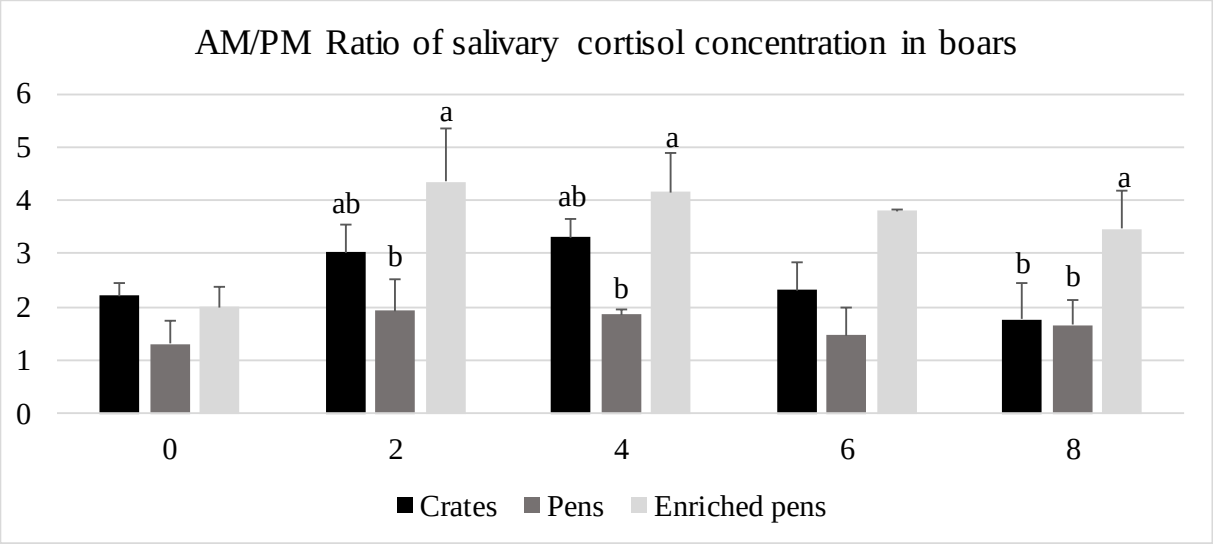


After the Tukey-Kramer test, for the interaction between treatment and week, we found that the salivary cortisol concentration did not differ among treatments at week 8, irrespective of the time of collection (morning or afternoon). For the interaction between week and time, we found that only at week 8, there was no difference among time. In other words, the results from morning and afternoon were not different. For all other weeks, the data from morning samples were different from afternoon, as expected. As we did not find a treatment or a treatment, week, and time interaction, these results will not be detailed discussed.

According to our methodology, the AM/PM ratio showed intriguing results. The AM/PM salivary cortisol concentration was not different in the sampling before

treatment allocation (figure 4). Boars housed in enriched pens showed a higher salivary cortisol concentration ratio.

Figure 4 – AM/PM ratio of salivary cortisol concentration in boars kept in three different housing conditions. Different letters mean $p < 0.05$.



3.4.DISCUSSION

We observed that boars housed in crates licked the empty through longer at 8 weeks after treatment allocation (see figure 1), when compared to other treatments. Moreover, when we created the combined variable abnormal behavior and contrasted it, we identified that during the whole experimental period, boars housed in crates performed more abnormal behavior than penned and enriched penned boars. The performance of abnormal behaviors is reported as a consequence of the inability to carry natural behaviors, associated with frustration, feed restriction, and others (MASON, 1991; D'EATH et al., 2009; MACHADO et al., 2017; ZHANG et al., 2017b), and it is considered a welfare indicator (MASON, 1991; ZHANG et al., 2017b) which could

reflect the psychological state of an animal (TATEMOTO et al., 2019c). Therefore, we can consider that the boars housed in crates manifested a behavioral pattern which indicated compromised welfare. This was observed during the entire studied period, as shown in Figure 2.

A healthy cortisol circadian pattern for pigs, as diurnal animals, consist in higher concentration in the morning, decreasing during the course of the day (RUIS et al., 1997; KOOPMANS et al., 2005). We observed this pattern for the studied period, except for the week 8. All animals from all treatments showed higher salivary cortisol concentrations in the morning compared with the afternoon sampling.

We studied breeding males, which is a topic with few reports regarding cortisol patterns for this category. It was already demonstrated that males showed a different hair cortisol concentration than females (BERGAMIN et al., 2019). The AM/PM ratio is a reflection of a proper function of the HPA axis, associated with health outcomes (STONE et al., 2001; ADAM; KUMARI, 2009; ŠUPE-DOMIĆ et al., 2016). In the AM/PM ratio, as observed in figure 4, boars from enriched pen showed a higher AM/PM ratio in the week 2, 4, and 8. These results indicate that these animals had a well-defined circadian rhythm, since they showed higher levels of cortisol concentration in morning sampling and lower concentration in the afternoon. One of the possible explanations for this finding is because every day in the morning and afternoon, we provided the enrichment protocol for treated boars. Brushing, short baths, and hay could play an important role in the organization of the circadian rhythm, added to higher levels of a positive human-animal interaction during the enrichment protocol. Also, the differences observed in the behavior could be a consequence of this organized circadian rhythm, since the cortisol rhythm is correlated with others hormones rhythms and

behaviors (RUIS et al., 1997; STONE et al., 2001; KOOPMANS et al., 2005; HAN et al., 2019).

3.5.CONCLUSION

When combining, the behavioral and salivary cortisol concentration results, show how the treatments impacted these parameters. Boars housed in crates showed more abnormal behaviors than penned or enriched penned boars. Boars housed in enriched pens showed a more organized cortisol circadian rhythm. Environmental enrichment with brushing, short bath, and hay can impact the behavioral and the cortisol circadian rhythm in breeding boars, and should be considered as a protocol to improve their welfare.

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4. Chapter 3 – Poor welfare compromises testicle physiology in breeding boars

4.1.INTRODUCTION

In commercial pig breeding farms, boars are often exposed to stressful situations, such as individual housing, inadequate environmental temperature, food restriction, lameness, and diseases, among others. The consequences of these welfare challenges are largely unknown and, eventually, could affect semen quality. Previous work demonstrated that housing boars in crates is stressful (FLOWERS, 2015). Boars housed in crates can present reduced opportunities for testicular thermoregulation, due to limited locomotion, stress and limited physical space. There is scientific evidences demonstrating that poor thermal environment could compromise testis physiology and semen quality (CORCUERA et al., 2002; PETAK et al., 2010; FLOWERS, 2015). Moreover, it is already known that the paternal environment can influence the offspring's behavior and metabolism (TRIANTAPHYLLOPOULOS; IKONOMOPOULOS; BANNISTER, 2016).

In pregnant sows, the impact of housing conditions on their welfare has been extensively reported. Housing pregnant sows in crates was prohibited in 2013 in the European Union, (EU Directive 2008/120 EC), as well as in some states in the USA. There is recent legislation in Brazil addressing housing systems for sows, as well as for boars (MINISTÉRIO DE AGRICULTURA PECUÁRIA E ABASTECIMENTO, 2020). In the recently published IN113, adult boars cannot be housed in pens smaller than 6 m^2, this is going to be effective from the year 2045. The concern about the welfare of breeding boars is extremely limited over the globe. One of the reasons could be justified for the number of boars in pig farms which is extremely small, when compared with the number of sows, piglets, weaners, and fatteners. This scenario could partially explain why there are so few research groups investigating boar welfare. In addition, there is limited information about the impact of housing conditions on boar's health and welfare. On the other hand, the number of descendants that each boar can generate in a

year is much higher than individual sows, since artificial insemination increases the reproduction efficiency of males. If one boar mates 3 sows a week, in natural mating, it could father over 2,100 pigs in a year. If the semen will be used in artificial insemination, depending on the technique (intracervical, post cervical, or intrauterine), a single ejaculate can produce 20-60 inseminating doses (KNOX, 2016). This number represents 20 times the production of o boar used in natural mating. Artificial insemination is one of the most efficient breeding method for modern pig production (KNOX, 2016).

Any factor that affects testis health can compromise semen quality in boars. It has been demonstrated that boars with clinical and reproductive health assured, had their sperm quality and high fertility rates guaranteed, which are demanded by the competitive pig industry. Several protocols used to evaluate testicles involve manipulation, invasive techniques, or uncomfortable handling of the animals. Thermography and ultrasonography are noninvasive tools used to evaluate testicular health. The thermography was used for pigs to detect skin surface temperature, heat stress, and to diagnose joint inflammation (PETR; IVANA, 2012). However, we did not find studies using thermography to assess health indicators in boar testis.

Another highly sensitive, easily available and non-invasive tool to measure blood flow is the use of Doppler ultrasonography (LAM et al., 2005; BALOGH et al., 2018; PASCHOAL et al., 2019), which can measure testicular parenchyma and pampiniform plexus vascularization and the resistance index of the pampiniform plexus arteries (ALVES, 2014; BATISSACO et al., 2014).

In this study, we hypothesized that the housing condition can have an effect on testis health and sperm production. Our aim was to investigate the reproductive impact of housing young boars in crates, pens and enriched pens.

4.2.MATERIALS AND METHODS

This study was approved by the Committee on Ethics in Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science (FMVZ-USP), under protocol no. 3612010616.

4.2.1. Experimental Design

According to the experimental protocol, 3 distinct groups were formed. Crates: 9 animals housed in gestation crates, measuring 197 x 76 cm; pens: 9 animals were housed in pens, measuring 241 x 376 cm; and enriched pens: 9 animals housed in enriched pens, measuring 241 x 376 cm. For the enriched pens, environmental enrichment was offered twice daily, one hour after feeding represented by brushing the animals for two minutes using a broom, showering the animals with water for 30 seconds, and hay was provided as rooting material.

4.2.2. Animals

For this study 27 hybrid boars (F1 large white x landrace) with 10 months of age were, initially, housed individually in pens, measuring 3.85 meters x 1.2 meters. Animals were fed twice daily, at 07:00am and 13:00pm, with 2.8kg of concentrate per day, and had *ad libitum* access to a nipple drinker. All animals were kept in pens until the end of semen collection conditioning. Afterwards, animals were allocated to three housing conditions.

4.2.3. Treatments allocation

The distribution of the boars was performed based on their seminal quality and they were homogeneously distributed in the three treatments. The methodology used

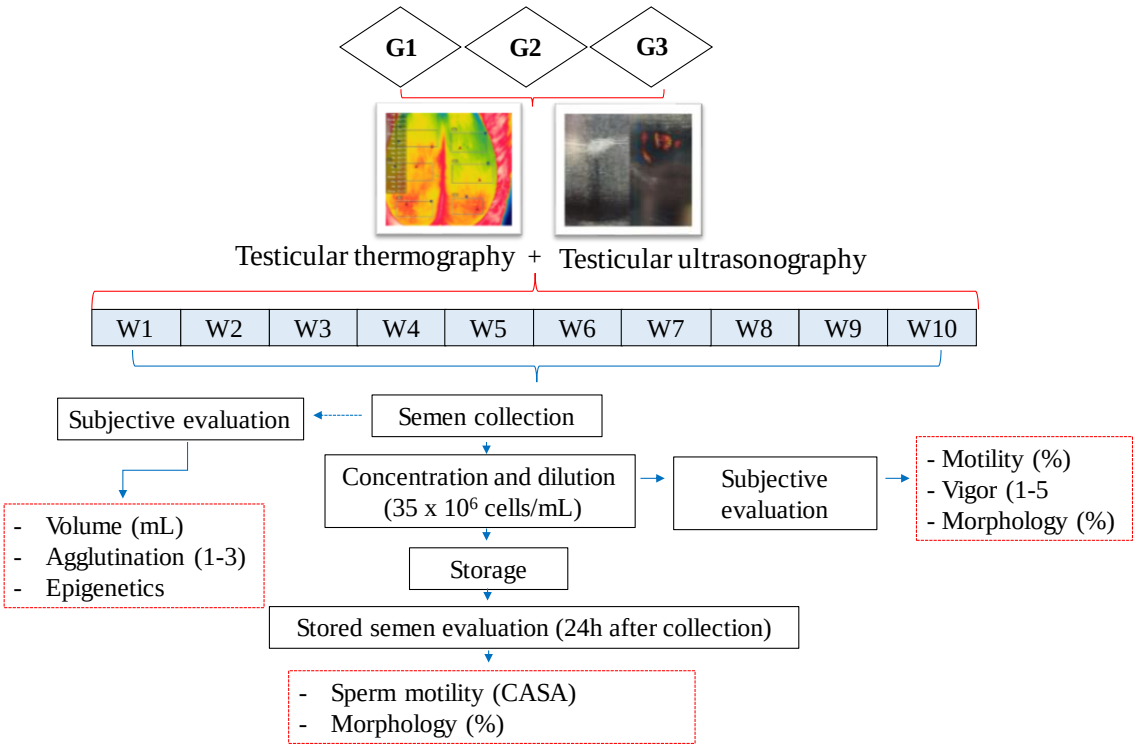
was adapted from Alves (2019) and consisted in a formula taking in account the total major defects, total minor defects, and progressive motility. This approach was chosen in order to guarantee animals with good, medium, and low semen quality in all groups. After the establishment of the semen quality parameters we performed a random assignment for the groups in the respective treatments. We did a random distribution of the groups in the treatments.

In the formula, PROG was progressive motility, MAJ was major defects, and MIN was minor defects.

Boar score = (1xPROG) + (3 x MAJ) + (2 x MIN).

For the 10 weeks after treatment distribution, testicular (scrotum area) and body (eye area) temperature were measured using thermography (T620 Flir Systems), with subsequent assessment of the testicular hemodynamic characteristics using Doppler ultrasound (Mindray, model M5Vet, Digital Diagnostic Imaging System; 6LE5Vs Vet probe model) of the testicular parenchyma and pampiniform plexus. A first measure was performed before treatment allocation.

Figure 1 – A representative diagram of experimental designs, sampling routine, and evaluated parameters.



4.2.4. Semen sampling

Prior to the experimental study, six semen collections, with an average interval of 7 days between them including all animals were carried out. Semen collection, semen analyses, and testicular thermography were performed weekly for 63 days, totaling 1.5 spermatogenesis and 6 epididymal transits. Thus, we analyzed 10 ejaculates for each of the 27 boar (n = 270), with a 7 days interval. Previously to moving the animal to the collection room, the boar was placed in a specific crate, and had the preputial area cleaned, with dry paper and the hairs on the prepuce were trimmed if necessary. The semen was collected manually, by the gloved-handed method. We used a static mannequin, and the ejaculate was collected in a thermic recipient (Equittec ®), previously coated by a non-spermicide plastic collector, and a filter to separate the

gelatinous fraction, which was discarded. Afterwards, the semen was taken to the laboratory attached to the collection room, and the volume was measured in a graduated beaker after taking samples for further analyzes.

4.2.5. Semen analyses

The first observation was regarding the agglutination. This feature was classified according to the methodology described by Martín (2010), and consisted in a score from 0 to 3, where 0 was no agglutinated cells and 3 represented more than 25% of agglutinated sperm cells. Afterwards, semen aspect was observed (appearance, color, smell). Next, the sperm concentration (sperm per milliliter) and volume was evaluated.

Immediately after collection, the semen was filtered to separate the sperm rich fraction from gelatinous fraction. After, the sperm rich fraction was diluted with specific diluent (Androstar ® plus, Minitube, Germany), the concentration adjusted to 35 x 10^6 sperm cells/mL. The next step was to analyze subjective sperm features, motility (%), vigor (1-5), and morphology (%). In order to do these analyses, aliquots were placed on a preheated slides and counted in a phase microscopy (100x magnification).

Sperm concentration (10^6/mL) was determined after a dilution in 1:100 of semen in formaldehyde phosphate buffered saline (DPBS, Biodux®, Brazil; formaldehyde 4%). The counting was performed in a hemocytometer (Neubauer) chamber with a 22x22 millimeter coverslip and the number of cells per quadrant (5 on each side of the chamber) were measured and the concentration determined in optical microscopy (400x magnification).

Sperm morphology was evaluated to guarantee that all samples showed a satisfactory pattern. The samples were diluted in pre-heated (37 ºC) formaldehyde phosphate buffered saline (DPBS, Biodux®, Brazil; formaldehyde 4%), and evaluated

under differential interference contrast microscopy – DIC (Nikkon, Model Eclipse 80i, Tokyo, Japan) with 1000 magnification (BLOM, 1973). 200 cells were evaluated per sample.

The semen was analyzed fresh, in the same day, and aliquots were frozen for epigenetic analyses. The animals were randomly selected within the treatments and the collection time, thus minimizing the effect of the time on the evaluated parameters. After preliminary analyzes, the diluted semen samples were kept at 17 ºC, in a transport container BotuFlex® (Botupharma, Botucatu-SP).

4.2.6. Computer Assisted Semen Analyzes

For the evaluation of motility, the computerized system (CASA, HTM-IVOS-Ultimate Hamilton Thorne Biosciences, Beverly, MA, USA) was used. The analyzes were carried out at the Semen Biotechnology and Andrology Laboratory (LBSA), in the Department of Animal Reproduction (VRA) of the School of Veterinary Medicine and Animal Science (FMVZ) from the University of São Paulo (USP). For this evaluation, Leja® slides chamber (IMV-Technologies International Corp.) was used, placing 20µl of diluted semen preheated in dry water bath, and inserted in the IVOS equipment (Version 12.3, Hamilton-Thorne Bioscience®, Beverly, USA). This equipment analyze the semen by a computerized system (CASA), which captures images from the semen samples through a microscope attached to the computer and transfer the data for analysis of the sperm movements through the *Animal motility* program, which was previously adjusted for porcine sperm analysis.

The CASA, collected data and analyzed it with at least 5 different microscope fields. The following characteristics were analyzed: total motility (MT,%), progressive motility (PM, %), average path velocity (VAP, µm/s), straight line velocity (VSL,

μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), straightness of the average path (STR, %), linearity of the curvilinear path (LIN, %), percentage of rapid cells (RAP, %). All the CASA analyses were performed one day after sampling.

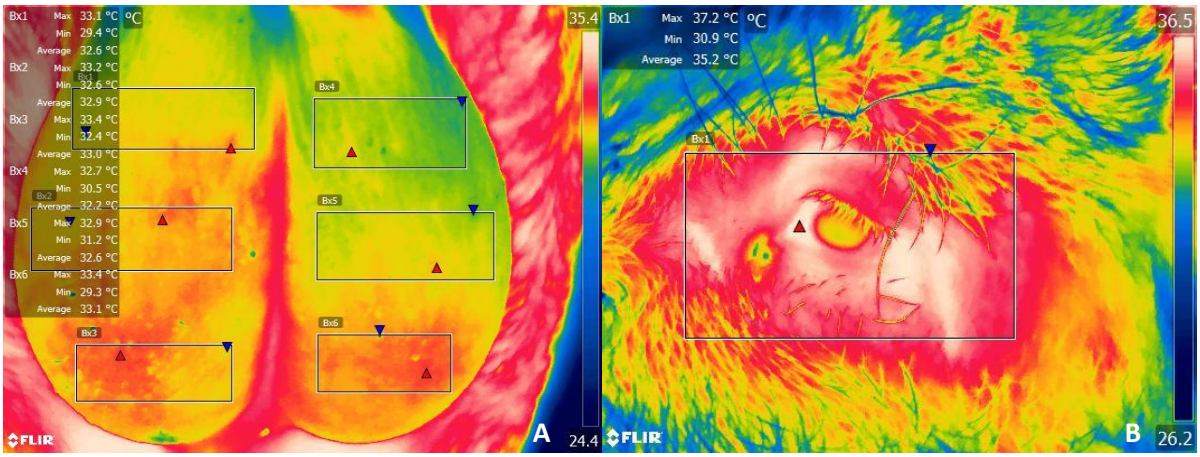
4.2.7. Testicular Thermography

All the thermal pictures were collected using a T620 thermography camera (Flir Systems, USA). The distance between the animal and the camera was standardized for 1 meter and the camera emissivity was adjusted to 0.98. Prior to the pictures collection, each animal was stimulated to stand up, had their scrotal area cleaned with a paper towel (when necessary) and animals were maintained standing up for at least 10 minutes. The scrotal skin was not touched for at least 1 hour prior to image collection. Moreover, in order to keep the animals standing still, a small amount of their regular food was provided for each thermal image sampling. This approach allowed us to collect good quality pictures, inside their home pens, avoiding any kind of physical or pharmacological restraint.

This first measure allowed us to identify potential preexisting differences prior to the treatment allocation. Afterwards, we collected thermal images weekly, one day prior semen collection, in the morning (between 9:00 and 10:15h), in the same day from all animals. The environmental temperature and humidity were measured immediately before the thermal image, with a data logger (OPUS 20 THI, 8120.00; Lufft, Germany). We used the FLIR Quick Report Software (FLIR Systems, USA) to analyze the images, separating the dorsal, medial, and ventral region of the right and left testicles and also to measure the eye area temperature (figure 2-B) (ALVES et al., 2016). This approach measured every single pixel of the image as one information. We used the mean

temperature taking into account every pixel from the selected area (figure 2-A). Additionally, we evaluated the mean temperature from the superficial scrotal temperature, through a fixed central spot (FCP), given by the camera in the moment of the image collection.

Figure 2 – A representative thermal image from the scrotum superficies, with separated areas (A), and from the eye (B). Red triangles indicate the highest temperature and the blue triangles indicates lowest temperature of the selected area.



4.2.8. Testicular Ultrasonography

The testis ultrasound scanning followed a validated methodology (BATISSACO et al., 2014). The evaluations of all boars were performed on the same weekday, in the morning. Initially, the boars were removed from their home pens, and placed in a crate, since it is necessary at least 3 minutes to complete the evaluation. We performed one scanning prior to housing the animals in the experimental treatments. Afterwards, we did the scanning of the testicles every two weeks until the end of the experiment in all boars. The testicles were evaluated separately, left and right. We used the duplex B-mode (grey scale) to measure the parenchyma homogeneity, color-Doppler flow mode

and spectral mode functions, using a 6 MHz convex transducer probe (Mindray, model M5Vet, Digital Diagnostic Imaging System; probe model 6LE5Vs Vet) with a topically use of a water-soluble contact gel.

To classify the ultrasound data, we used the methodology from Alves (2014) and Kahwage (2017). For the homogeneity of the parenchyma (HOP) we classified on a scale from 0 to 2: 0 = homogeneous parenchyma, with no pathological anechoic points; 1 = few pathological anechoic structures; 2 = heterogeneous parenchyma. With this strategy, the lower score of a boar indicated a more homogeneous and healthier testicle.

Moreover, in order to analyze the vascularization of the testicular parenchyma and the pampiniform plexus, we used the same methodology from Alves (2014). Briefly, we used a scale for testicular parenchyma ranging from 0 to 4: 0 = no vascularization; 1 = presence of 1 or 2 vascularization points, with small-caliber vessels; 2 = presence of 3 or 4 vascularization points, with small-caliber vessels; 3 = more than 2 points of vascularization points, with large-caliber vessels, which was seen in 1/3 of the screen; 4 = more than 2 vascularization points, with large-caliber vessels, which was seen in 2/3 or the screen. For pampiniform plexus we used a scale from 1 to 5, which is summarized in table 1.

Table 1 – Score used to evaluate pampiniform plexus of boars housed in three different conditions

Area	**Score**	**Percentage (%)**
Pampiniform plexus area filled with vascularization	1	01 - 20
	2	21 - 40
	3	41 - 60
	4	61 - 80
	5	81 - 100

All the measures were performed by the same person, an experienced veterinarian with PhD in animal reproduction. The ultrasound scanning aimed to verify the presence of alterations in the morphology of the parenchyma tissue, the blood flow in the parenchyma, and the blood flow in the pampiniform plexus.

4.2.9. Statistical Analyses

All the data were analyzed with the package Statistical Analysis System 9.4 (SAS Inst., Inc., Cary, NC). Initially, the data were checked for the presence of discrepant information (outliers) and we verified the residual normality, through the Shapiro-Wilk test. After those approaches, the data were analyzed by ANOVA with PROC GLIMMIX of SAS using a treatment as principal effect over the time and a block (seminal quality) as random effect. In addition, the command REPEATED was added to the command, in order to analyze the time effect. Thus, we had the effect of treatment, the effect of time, and the interaction between these two factors.

For all analyses, among the 15 different covariance structures were tested and the one that best fits the statistical model was chosen, based on the lower value of the Akaike Index correction criterion (AICC) (WANG; GOONEWARDENE, 2004). When necessary, for a post-hoc test, the Tukey's test was used. The means and the standard error mean (SEM) were obtained from raw data. For all statistical analyses performed, a 5% level of significance was adopted.

4.3. Results

The time effect without interaction with treatment was not part of our experimental design in this study. Therefore, we will not discuss the time effect for all variables.

Analyzing the data related to Computer Assisted Semen Analysis (CASA), we found a treatment effect for the variable curvilinear velocity (VCL), which the boars housed in crates showed higher values, indicating that the sperm from these animals moved more in a circular way when compared with other treatments ($p = 0.046$). For many variables we found a time effect (progressive motility, average path velocity, straight line velocity, curvilinear velocity, beat-cross frequency, and linearity). Another important finding was regarding agglutination. We found that the boars housed in crates showed more agglutination in the semen samples compared with boars housed pens ($p = 0.0431$) and with boars housed in enriched pens ($p = 0.0259$). There was no difference among boars housed in pens and enriched pens ($p = 0.9441$) (table 2).

Table 2 – Data of sperm agglutination from boars kept in three different housing condition (n=9 animals par treatment)

Variable	**Mean score**	**Treatment**	**Week**	**Interaction**
Left testicle	0.3431	0.1475	0.155	0.8562
Right testicle	0.3628	0.6595	0.218	0.3425
Mean	0.7059	0.2025	0.0147	0.2518

The sperm morphology data showed no treatment effect. Many variables (abaxial tail implantation, acrosome defect, bent tail, major and minor defects, bent tail with droplet, total defects, distal droplet, and middle piece droplet) showed time effect. There was not a significant influence of treatment on morphology data.

The animals housed in crates showed higher temperature in the fixed central spot (FCS) than the animals housed in pens and enriched pens. No differences were observed among treatments in the week before housing them in the treatment (week -1), and in the week immediately after the treatment allocation (week 0).

Figure 2 – Superficial scrotal temperature (ºC) from boars housed in crates, pen, and enriched pen. The data were collected weekly over the course of 10 weeks, since one week before housing until 8 weeks after. * p value <0.05

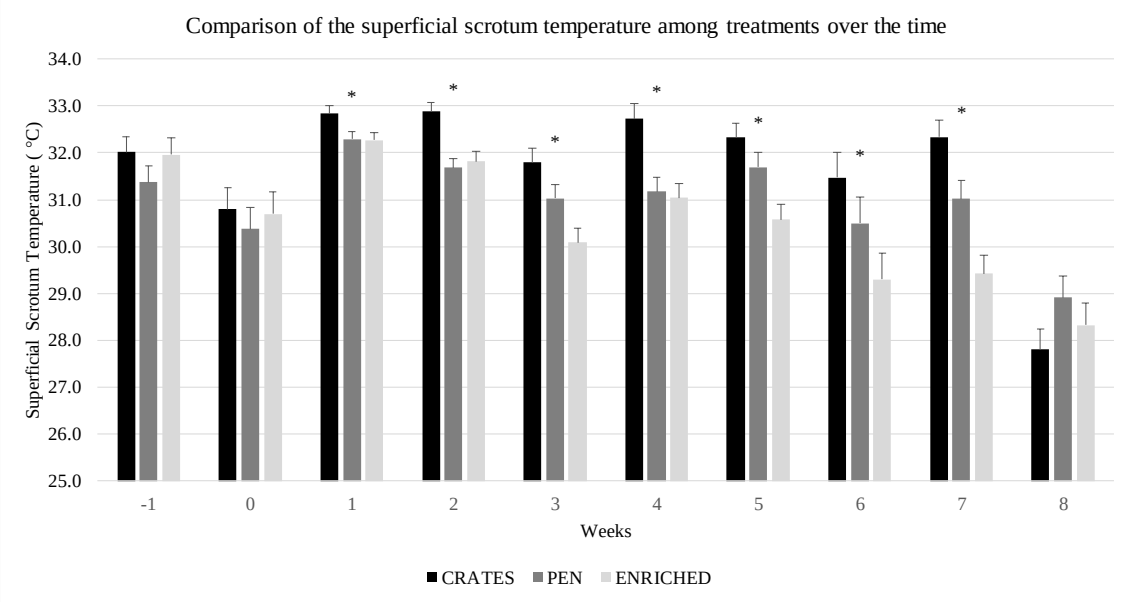
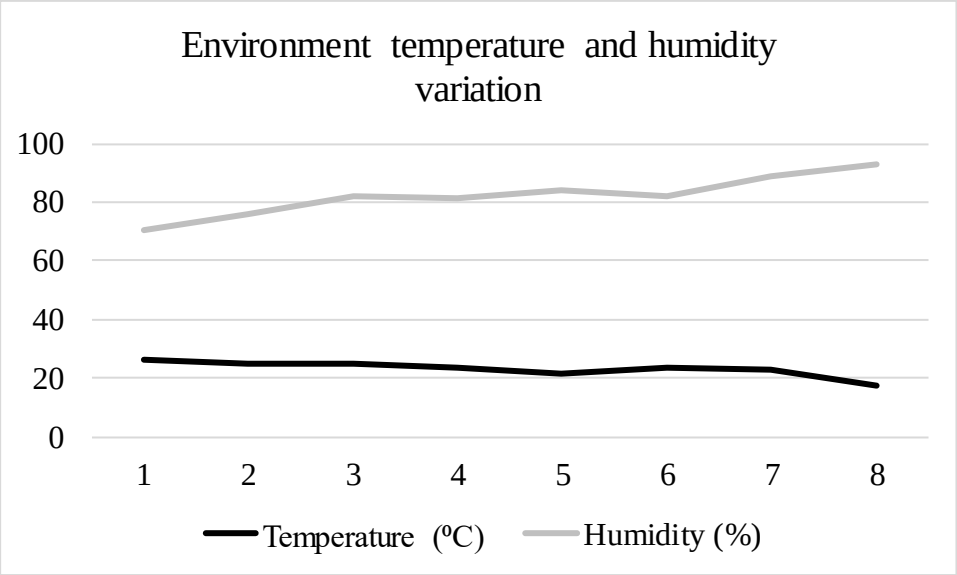


Figure 3 – Mean environment temperature and mean humidity collected by a data logger (OPUS 20 THI, 8120.00; Lufft, Germany) at the moment of thermal pictures collection.



When we compared the separated regions from the testis (top, medium, and bottom area, from right and left test), the data showed a similar pattern. For all comparisons, the animals housed in crates showed higher superficial scrotal temperature than the animals housed in pens and enriched pens.

The summarized data from thermal images, from the different regions and from both testis, are presented in table 3.

Table 3 – Mean temperature (ºC) and standard error mean (SEM) of different areas of testicles from boars housed in three different conditions.

	Mean of the superficial scrotal temperature (ºC)			
Treatments	Top area	Medium area	Bottom area	**SEM**
Pens	30.15	30.26	30.64	0.19
Crates	31.09	31.04	31.15	0.20
Enriched pens	29.61	29.84	29.75	0.23

The data from duplex B-mode contrast ultrasound evaluation is summarized in table 4. The homogeneity was evaluated by a score that varies from 1 to 2.

Table 4 – The mean score and p value of all data obtained from the duplex B-mode ultrasound data.

Variable	**Mean score**	**Treatment**	**Tim**	**Interaction**
Left testicle	0.3431	0.1475	0.155	0.8562
Right testicle	0.3628	0.6595	0.218	0.3425
Mean	0.7059	0.2025	0.0147	0.2518

We did not identify difference between treatment and time for homogeneity evaluation. The data from color Doppler ultrasound evaluation is summarized in table 5. The tissue perfusion data was evaluated by a score that varies from 1 to 4 for parenchyma evaluation and 0 to 5 for pampiniform plexus.

Table 5 – The mean score and p value of all data obtained from the color Doppler ultrasound data.

Variable		**Mean score**	**Treatment**	**Time**	**Interaction**
Left testicle	Parenchyma perfusion	2.27	<0,01	0.38	0.18
	Pampiniform plexus	2.72	0.68	0.20	0.41
Right testicle	Parenchyma perfusion	2.19	<0,01	0.18	0.01
	Pampiniform plexus	2.80	0.64	0.92	0.04
Mean parenchyma		2.23	0.01	0.09	0.01
Mean pampiniform plexus		2.75	0.90	0.51	0.14

There was no difference in the score of the pampiniform plexus. We found a difference in the parenchyma perfusion, in which the boars housed in crates showed higher score when compared with the boars housed in pens and enriched pens, and thus, more vessels were identified in the parenchyma of boars kept in crates. After a Tukey-

Kramer test, we identified that the boars housed in crates were different from boars housed in pens ($p = 0.0009$) and from boars housed in enriched pens ($p = 0.0030$). There were no difference comparing boars house in pens and enriched pens ($p = 0.9152$).

4.4. Discussion

The most relevant finding of the semen analyses was the VCL and agglutination results. The VCL is relevant because it is inserted in the formula to obtain the linearity (LIN = VSL/VCL). Although we did not identify differences between progressive, total, and linear motility, the VCL can compromise all these parameters. Maybe the number of animals and their age contributed to the small difference among treatments. Besides, agglutination can compromise the motility, fertility, litter size, and, sometimes, could be a result of a tight adhesion of bacteria to the sperm cell (MAROTO MARTÍN et al., 2010; GĄCZARZEWICZ et al., 2016). Thus, when an ejaculate is going to be used in an artificial insemination protocol, it is not recommended to use a highly agglutinated sample. The difference among treatments could have an impact on boar fertility indicators.

As we observed in the figure 2, we identified that the SST were higher in boars housed in crates, when compared with the animals housed in pens and enriched pens. One of the possible reasons for this relevant result is the position that the boars showed when resting, which appears to be affected by the space available. The animals housed in crates have severe limitations in their movements which can cause abnormal behaviors (TERLOUW; LAWRENCE; ILLIUS, 1991). In chapter one we reported the occurrence of abnormal behaviors and resting position of the experimental animals.

Previous work demonstrated that crates have a systemic effect in pigs. Sows kept in this restrictive environment showed lower quality of bone mineralization and muscle

health (MARCHANT; BROOM, 1996). Since they have limited movements, the posture changes may also be affected. In a previous pilot study, we observed that the boars housed in crates lie on top of their testis. Because of that, keeping in mind that those animals can reach more than 300 kg of body weight, we hypothesized this behavior could lead to testis damage.

Taking our results into consideration, we can observe that the housing condition can affect the SST, where the animals housed in crates showed a higher temperature. The SST is negatively correlated with sperm quality in bulls (BERRY; EVANS; MC PARLAND, 2011). This testicular temperature imbalance could lead to a pathological condition, testicular degeneration (FERNANDES et al., 2008), which can decrease the semen quality (VAN CAMP, 1997). We observed the same pattern when we analyzed the separated regions of the testis. In pigs, the epididymis cauda is in a dorsal position (BRIZ; BONET; FRADERA, 1993), and in this structure, could lead to sperm modifications, important enough to alter the sperm programming (MORGAN; CHAN; BALE, 2019).

The ultrasound data showed that the animals housed in crates had more perfusion in the parenchyma, in both testis (table 2, mean parenchyma). This result could be an indicator that the boars had an alteration in the testis homeostasis, which increased the blood flow to that region. Note that these alterations followed the same pattern observed in the thermal images data. However, probably due to young age of the boars, we could not observe any disturbances in the homogeneity of the parenchyma. Probably because these types of lesions, observed in the ultrasonography measures, are results from a chronic insult. Maybe, if we kept them longer, we would observe severe alteration, since the insults were maintained.

4.5.Conclusion

Housing conditions can lead to changes in superficial scrotal temperature and blood flow in the testis. Changes in testicle temperature were observed in the whole testis, as well as in the top, medium, and bottom areas. It is important to highlight that this alterations in temperature, could impact in the semen quality, as we reported, and in important molecular mechanism of stress inheritance. Additionally, housing boars in crates, can lead to an acute alteration of the blood flow to the testis parenchyma. Surprisingly, two weeks after the housing treatment, we were able to identify differences through color-Doppler ultrasound. We predict that, after months housed in crates, the results would be much more severe, regarding those variables. In addition, boars housed in pens and enriched pens showed indicators of a better testicular health, better sperm motility features, and no less agglutinated semen than what was observed in boars kept in crates, which can reflect in reproductive performance. To conclude, housing boars in crates is stressful enough to cause changes in testis physiology, which could compromise the sperm quality and the boar's reproductive performance. On the other hand, penned and enriched penned boars showed indicators of better testis health and physiology.

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5. Chapter 4 – miRNAs in the semen of boars: do the welfare of the animals impact their population in the ejaculate?

5.1.INTRODUCTION

In commercial pig breeding farms, boars are often exposed to stressful situations, such as individual housing, inadequate environmental temperature, food restriction, lameness, diseases, among many others challenges. The consequences of those welfare challenges are unknown and, eventually, could have an influence in semen quality. Housing boars in crates, which is common in commercial settings, limits the ability of the animal to perform biologically relevant behaviors, dysregulate the function of the hypothalamic-pituitary adrenal axis and affects markers of semen quality and fertility (Chapter 2 and 3 of the current thesis). Furthermore, researches showed that paternal environment can influence the offspring behavior and metabolism (TRIANTAPHYLLOPOULOS; IKONOMOPOULOS; BANNISTER, 2016).

Epigenetic changes can be caused by exposure to stress or even by the effect of the environment that the animal is exposed to, causing changes to germ cells (SHARMA et al., 2016; CHAN et al., 2020), which allows the transmission if the epigenetic markers, such as miRNAs population, to their offspring, modulating, for example, the vulnerability of the offspring to stress (NESTLER, 2016). Example of such effect was identified by Dietz et al. (2011b), when adult male mice were subjected to a challenge called defeat chronic social stress. They generated an offspring more vulnerable to various stressful stimuli. It is likely that semen can be the transmission route to modulate the susceptibility to stress for the next generations contributing to the modulation of the phenotype of the progeny and future descendants (FRANKLIN et al., 2010; BOHACEK; MANSUY, 2015). Epigenetic changes are found in germ cells,

which can be maintained for generations even if that organism is not exposed to the initial stimulus that caused epigenetic changes (FRANKLIN et al., 2010).

In addition, recently published research showed that early life stress can affect the microRNAs cargo in epididymal extracellular vesicles, in a mouse model (ALSHANBAYEVA et al., 2021).

Micro RNAs are defined as a single stranding non-coding RNA, which regulate gene expression at the post-transcriptional level, and are highly conserved among different species (WU et al., 2013; ALVES et al., 2021). Another function of microRNAs, which are a significant molecules in epigenetic regulation, is to regulate the posttranscriptional gene expression (TAL et al., 2012). A population of microRNAs is involved in transgenerational transmission (BELLEANNÉE, 2015), and may be involved in gene silencing by up to five generations in the paternal line in nematodes (FIRE et al., 1998). Thus, the population of microRNAs, together with other epigenetic changes are probably crucial for the transmission of the consequences of paternal stress, such as the challenge experienced by boars, to their offspring (RODGERS; BALE, 2015). Another study has shown that when a certain population of micro RNAs from stressed animals are inserted into an oocyte, they can alter the phenotype of offspring as well as in natural fertilization (GAPP et al., 2014). Moreover, there is recent evidences that exist some micro RNAs involved in the regulation of spermatogenesis (KOTAJA, 2014) and could be correlated with semen quality (YANG et al., 2020). Beyond its role in sperm cells, micro RNAs from sperm are recognized as important transporters of RNA to the oocyte, showing essential contribution to embryonic development and offspring health (LIU et al., 2012b; RODGERS et al., 2015; ALVES et al., 2019).

Epigenetics is crucial for survival, and it has been reported that often cloned animals present pathologies and inadequate nuclear reprogramming, thus establishing a

relationship between disease and epigenetics (TRIANTAPHYLLOPOULOS; IKONOMOPOULOS; BANNISTER, 2016). Moreover, the population of seminal micro RNAs has a potential to alter intracellular communication even in a mature and inert spermatozoon. DNA, being condensed, can prevent some epigenetic alterations (RODGERS et al., 2015), however is known that extracellular vesicles, present in boars' seminal plasma for example, interact and fuse with the sperm membrane (DU et al., 2016), which has a potential to increase sperm motility (ARIENTI; CARLINI; PALMERINI, 1997).

The impact of progenitor's lifestyle in their offspring has been investigated in the literature. Recently, additional to the above mentioned impact, microRNAs have been correlated with neuropsychiatric disorders, such as autism spectrum disorders, schizophrenia, and anxiety disorders. These type of disorders, are a group of diseases of the central nervous system, which can be characterized by alterations in morphology, connectivity, and function, reflecting in different behavior outcomes (NARAYANAN; SCHRATT, 2020).

Based on the abovementioned information, the goal of this study was to measure, and evaluate the sperm microRNAs population from the boars ejaculated housed in three different housing conditions.

5.2.MATERIALS AND METHODS

This study was approved by the Committee on Ethics in Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science (FMVZ), under protocol no. 3612010616.

5.2.1. Experimental Design

For this study, a cohort sample with 18 boars, from a population of 27 animals, were studied. According to the experimental protocol, 3 distinct groups were included in this study. Out of the 18 boars, 6 were housed in gestation crates, measuring 197 x 76 cm; pens: 6 boars were housed in pens, measuring 241 x 376 cm; and 6 boars were housed in enriched pens, measuring 241 x 376 cm. For the enriched pens, environmental enrichment was offered twice daily, one hour after feeding represented by brushing the animals for two minutes using a broom, showering the animals with water for 30 seconds, and hay was provided as rooting material.

5.2.2. Animals

For this study 18 hybrid boars (F1 large white x landrace) with 10 months of age were, initially, housed individually in pens, measuring 3.85 meters x 1.2 meters. Animals were fed twice daily, 07:00am and 13:00pm, with 2.8kg of concentrate per day, and had *ad libitum* access to a nipple drinker. All animals were kept in pens until the end of semen collection conditioning. Afterwards, animals were allocated in the assigned to three housing conditions.

5.2.3. Treatment allocation

The distribution of the boars was performed based in their seminal quality and they were homogeneously distributed in the three treatments. The methodology used was adapted from Alves (2019) and consisted in a formula taking in account the total major defects, total minor defects, and progressive motility. This approach was chosen in order to guarantee animals with good, medium, and low semen quality in all groups. After the establishment of the semen quality parameters we performed a random

assignment for the groups in the respective treatments. We did a random distribution of the groups in the treatments.

In the formula, PROG was progressive motility, MAJ was major defects, and MIN was minor defects.

Boar score = (1xPROG) + (3 x MAJ) + (2 x MIN).

Thus, for each treatment, we had 6 animals, 2 animals of each quality group (high, medium, and low semen quality).

5.2.4. Semen sampling

Prior to the experimental study, six semen collections, with an average interval of 7 days between them including all animals were carried out. Semen collections were performed before and for 4 weeks after assigning the animals to the housing treatment, totaling 3.5 epididymal transits. Thus, we analyzed 2 ejaculates for each of the 18 boars (n = 36), with 4 weeks interval. This interval of 4 weeks was chosen because at this moment, we used the semen to inseminate 15 gilts with three distinct pools of semen, five gilts were inseminated with each pool, where the three treatments were represented in each pool (Sabei et al., submitted), mimicking the way that the molecular work was carried out.

Previously to moving the animal to the collection room, the boar was placed in a specific crate, and had the preputial area cleaned, with dry paper and the hairs on the prepuce were trimmed if necessary. Semen was collected manually, by the gloved-handed method. We used a static mannequin, and the ejaculate was collected in a thermic recipient (Equittec ®), previously coated by a non-spermicide plastic collector, and a filter to separate the gelatinous fraction, which was discarded. Afterwards, the

semen was taken to the laboratory attached to the collection room, and the samples were frozen at -80 ºC for molecular analyzes. Five replicates of 1.5 ml were collected from each boar. In order to evaluate the presence of somatic cells in the ejaculate, one replicate was used for this purpose. A duplicated smear was prepared and evaluated in a microscopy under 100x magnification. Four fields were evaluated, 300 cells were counted, and no somatic cells were found.

5.2.5. RNA extraction

The initial evaluation was carried out in a raw ejaculate, without any addition of extensors, processing, and/or manipulation. All samples were frozen immediately after the semen collection and stored until RNA extraction.

The RNA extraction was performed with the miRNeasry Micro kit (Qiagen, USA). The sample, which contained the sperm rich fraction of the ejaculate (sperm cells and seminal plasma), was thawed in a styrofoam box contained ice. A 500µl was taken from the sample for RNA extraction and the sample that remained was snap frozen in liquid nitrogen and after redirect to -80 ºC freezer. 700 µl of QIAzol Lysis Reagent (Qiagen, USA) was added to the raw semen and the manufacturer protocol was followed. The protocol consisted in homogenizing the sample for 1 minute and incubate the homogenate for 5 minutes at room temperature. Afterward, 200 µl of chloroform was added. The homogenate was shaken vigorously for 1 minute and incubated at room temperature for 5 minutes. A centrifugation at 12,000x g for 15 minutes at 4 ºC was performed to separate the aqueous phase. The aqueous phase (approximately 600 µl) was then transferred to another tube, and 900 µl of 100% ethanol was added to the

sample and mixed by pipetting up and down several times. 700 µl of the solution was added in the RNeasy MinElute spin column, attached to a 2 ml collection tube. After this step, we centrifuged the tube at 8000x g for 15 seconds at room temperature. This step was repeated until all volume went through the column. All the flow-through was discarded. 700 µl of the Buffer RWT was added onto the column and centrifuged at 8000x g for 15 seconds and the flow-through was discarded. 500 µl of Buffer RPE as added onto the column and centrifuged for 15 seconds and the flow-through was discarded. 500 µl of 80% ethanol as added onto the column and centrifuged for 2 minutes and the flow-through was discarded. We added the spin column to a fresh 2 ml tube, opened the lid, and centrifuged it at 10,000x g for 5 minutes to dry the membrane. The column was placed over a 1.5 ml collection tube. 14 µl of RNase-free water was added directly to the center of the spin column membrane and centrifuged at 10,000x g for 15 seconds. The samples were analyzed using spectrometry (NanoDrop 2000, Thermo Fisher Scientific, Maryland, US) to measure the RNA concentration and quality (280/260 ratio). If the extracted RNA sample showed a 280/260 ratio lower than 1.7, the extraction was carried out again, following the same protocol. The samples were frozen at -80 ºC until further analyses.

5.2.6. Reverse transcription for cDNA synthesis

Before the cDNA synthesis, we created a pool the samples from boars with the same semen quality and from the same treatment, based in the total RNA of the extracted samples. Thus, we had 3 samples, for each treatment in 2 different moments, before and 4 weeks after treatment allocation. We followed the manufacturer instructions, using a commercial miScript II RT Kit (Qiagen, Hilden, Germany). All reactions were standardized for 10 µl and with 100 ng of total RNA. We used 10 µl of

pooled sample, 2 µl of 5x HiFlex Buffer, 1 µl of 10x nucleic acid mix and 1 µl of reverse transcriptase enzyme. The reverse reactions were carried out in a thermocycler (Life Technology, Carlsband, US), at 37 ºC for 60 minutes followed by 95 ºC for 5 minutes (DE ÁVILA et al., 2020).

5.2.7. Reverse transcriptase and quantitative PCR analyses of miRNAs from boar ejaculates

We analyzed all miRNAs reported in the *Sus scrofa* species, totaling 383 miRNAs found in boar ejaculate, through reverse transcription and quantitative RT-PCR analyses. We used a commercial kit miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) and we followed all manufacture's instruction. The reactions performed had a total volume of 6 µl and contained 3 µl 2x QuantiTect SYBR Green PCR Master Mix, 0.6 µl of 10x miScript Universal Primer, 1.37 µl of RNase-free water, 0.03 µl of cDNA, and 1 µl of 10 µM specific forward primer as previously described by de Ávila et al. (2020). We used primers based on the mature miRNA sequences reported at miRBase database for pigs. The amplifications were all carried out on QuantStudio 6 Flex (Thermo Fisher Scientific, Maryland, US). For all reactions we used the polymerase DNA enzyme activation with an initial incubation of 95 ºC for 15 minutes and 45 cycles of 95 ºC for 15 seconds, 55 ºC for 30 seconds, and 70 ºC for 30 seconds.

The cycle threshold (CT) from all samples were normalized using the CT of the miR-99b as previously described by Alves (2019). Normalized data is presented as $2^{-\Delta Ct}$.

We performed a literature search for each microRNA that we found differently abundant in our work. We used the name of the miRNA combined with different key-

words, added the following topics: embryo development, cleavage, inflammatory process, metabolism, infertility, testicular degeneration, and sperm maturation.

5.2.8. Statistical Analyzes

Relative expression of miRNAs in the ejaculated samples of the 3 different groups and two times showed a residual normality distribution. Their average values were compared using ANOVA considering a significance level of 5%. If an interaction was found, we used Tukey-Kramer as a post-hoc test. All analyses were performed in the JMP software.

Markers of semen quality were analyzed using Computer Assisted Sperm Analyses (CASA) and morphology. All data are presented in the chapter 3. Reproductive outcomes, such as number of piglets born alive and stillborn were obtained from the study of Sabei et al, 2021 (submitted), which were paternity allocated to the individual boars after DNA tests, carried out at the end of the trial.

5.3. RESULTS

We studied 266 miRNAs out of 383, since we did not evaluated miRNAs containing missing values. Among these 266 miRNAs studied, we identified 12 differently abundant miRNAs in the treatments ($p<0.05$), which are described in table 1, associated with the p value of the interaction between treatment ant time.

Table 1 – Different abundant miRNAs from the ejaculate of boars housed in three different housing conditions. The column interaction represent the p value between treatment and week, obtained by ANOVA.

miRNA	**Interaction**
Ssc-miR-129a-5p	0.0238
Ssc-miR-148a-5p	0.0101
Ssc-miR-153	0.0136
Ssc-miR-20a-3p	0.0488
Ssc-miR-330	0.0028
Ssc-miR-335	0.0153
Ssc-miR-574-5p	0.05
Ssc-miR-7137-3p	0.05
Ssc-miR-7141-3p	0.0031
Ssc-miR-92b-3p	0.0202
Ssc-miR-9799-3p	0.0459
Ssc-miR-9788-3p	0.0382

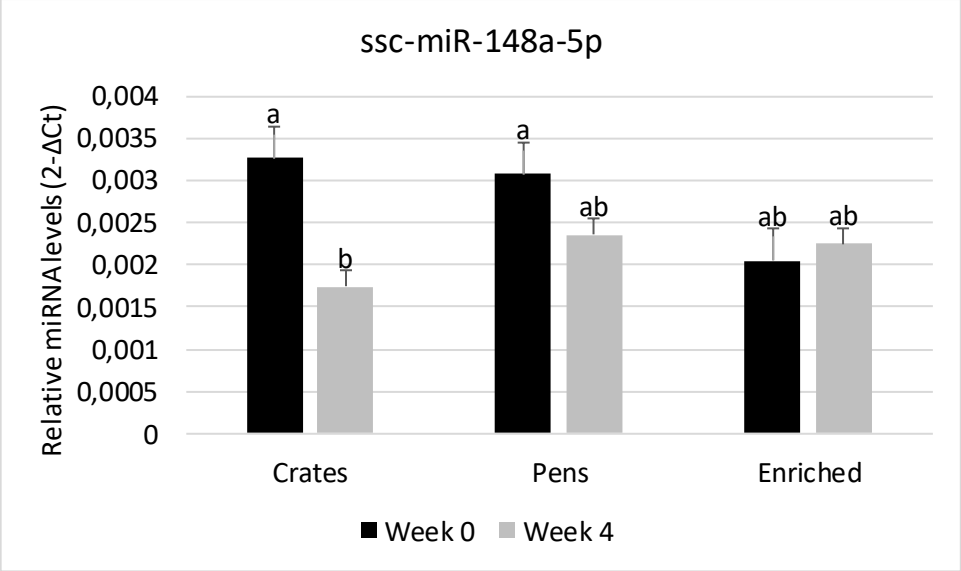
We did not identify exclusive population of miRNAs from ejaculate of studied boars, associated with their treatment assignment.

Regarding the expression of the abovementioned miRNAs, graphs with the qRT-PCR results are presented in figures 1, 2, and 3. We focused on data obtained from the

boars housed in crates and in the boars housed in enriched pens, since they were the ones that showed contrasting results regarding behavior, testicle physiology, and sperm outcomes.

The miRNA ssc-miR-148a-5p, ssc-miR-129a-5p, and ssc-miR-92b-3p decreased the abundancy (were down-regulated) in the boars housed in crates. The ssc-miR-148a-5p from boars housed in enriched pens and in pens did not change over the weeks (see figure 1). Moreover, the miRNA ssc-miR-92b-3p relative expression was the same comparing penned and enriched penned boars and downregulated for boars housed in crates.

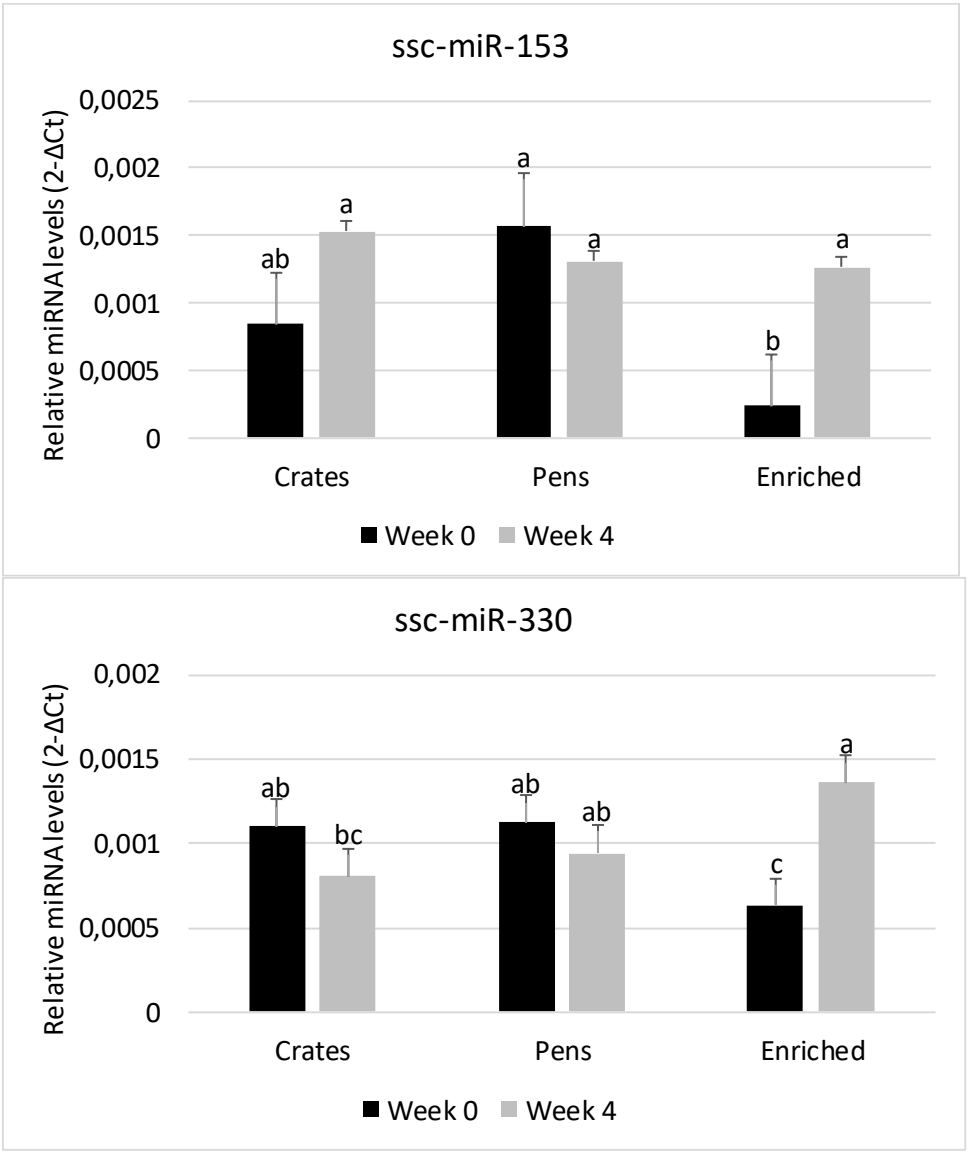
Figure 1 – Relative expression of the miRNA ssc-miR-148-5p of boars housed in three different housing condition in a period of 4 weeks (n=9 boars per treatment)



In the boars housed in enriched pens, we found that some miRNAs enriched their abundance over the weeks. The miRNA ssc-miR-153, and ssc-miR-330, both related to embryo development, increase their abundancy only in boars housed in enriched pens.

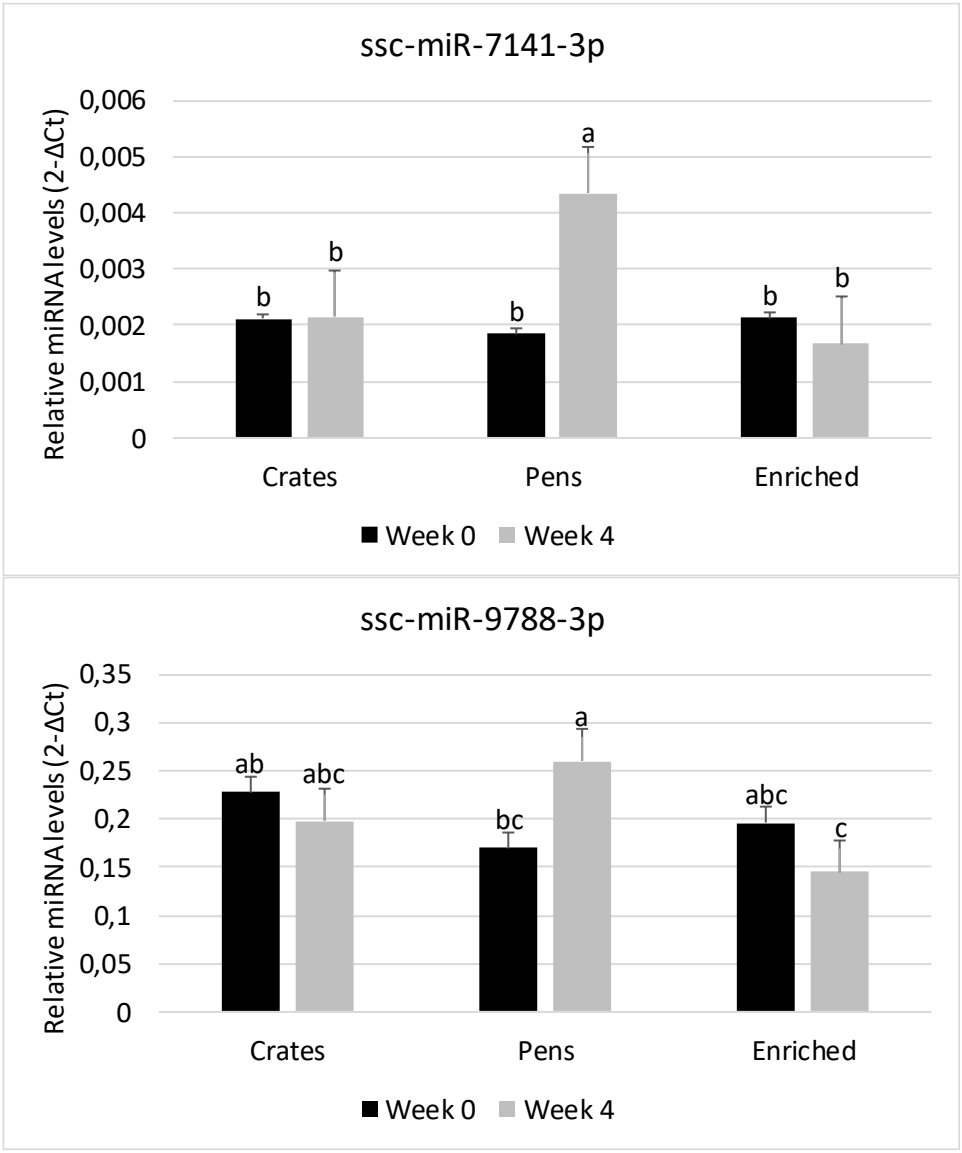
For the boars housed in crates or pens, their abundancy was not different over the weeks (figure 2).

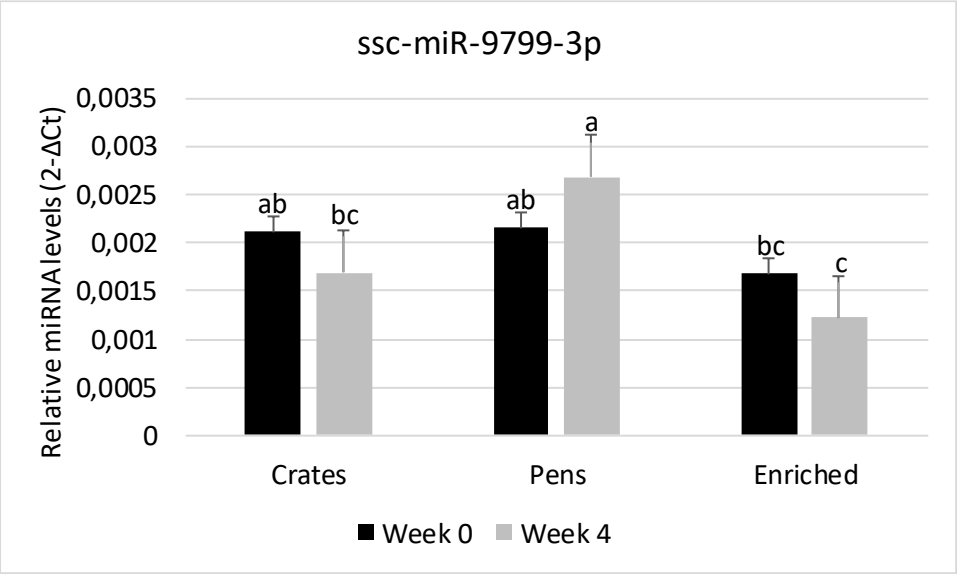
Figure 2 – Relative expression of the miRNA ssc-miR-153 and ssc-miR-330 of boars housed in three different housing condition in a period of 4 weeks (n=9 boars per treatment)



In the boars housed in pens, we found that some miRNAs enriched their abundancy over the weeks. For the animals in this treatment, the miRNAs ssc-miR-7141-3p, ssc-miR-9788-3p, and ssc-miR-9799-3p increased their abundancy (figure 3). For the boars housed in crates or enriched pens, these miRNAs were not different.

Figure 3 – Relative expression of the miRNA ssc-miR-7141-3p, ssc-miR-9788-3p, and ssc-miR-9799-3p of boars housed in three different housing condition in a period of 4 weeks (n=9 boars per treatment)





5.4.DISCUSSION

We studied 266 miRNAs out of 383 analyzes, since we did not evaluated miRNAs containing missing values (no amplification plot on qRT-PCR). It is our goal to further investigate all miRNAs using specific techniques, separating miRNAs from sperm cells, from extracellular vesicles or free miRNAs in the seminal plasma, to better understand the results. Also, 12 miRNAs differently abundant in the ejaculate in our study, hence limited a more comprehensive assessment of their effects in the sperm cells, seminal plasma or even to the embryo development.

The relationship between micro RNAs and semen quality has been previously reported (YANG et al., 2020). In our data, semen from boars housed in crates showed poor fertility indicators, based on data on sperm agglutination and sperm curvilinear velocity, obtained through Computer Assisted Sperm Analysis (CASA). Micro RNA ssc-miR-153 showed an increase in boars kept in enriched pens and could be an interesting candidate to understand the impact of testicular temperature and testicular parenchyma perfusion on semen quality, differences reported in chapter 3 of the current thesis. Interestingly, an upregulation of this miRNAs is related to protective role in the central nervous system, at hippocampal neuron-specific sites (XU et al., 2019) and in

animals exposed to ethanol (TSAI; MIRANDA, 2014). miRNA-153 overexpression or dramatically decreased expression are correlated with movement impairments on zebrafish embryo (WEI et al., 2013). We have data on behavioral developmental outcomes in the offspring of studied boars, showing compromised welfare in the offspring of boars kept in crates (Sabei et al., submitted).

Our data demonstrated that boars housed in enriched pens showed a more organized cortisol circadian rhythm, less abnormal behaviors, lower superficial scrotum temperature, lower testicular parenchyma blood perfusion, and less agglutinated semen. We reviewed 72 scientific publications, in order to obtain the maximum of information in our 12 miRNAs population (see table 2). In our literature review, we were not able to find publications correlating specifically to our reported findings to explain the consequences of the different abundance of specific miRNAs among treatments. However, a recent publication reported an effect-cause relationship with testicular insulation and changes in miRNAs population in sperm and extracellular vesicles (ALVES et al., 2021). The authors argued that a testicular insulation did lead to an increase of the testicular temperature and hypoxia, which could be the responsible for the changes found in the miRNAs profile from extracellular vesicles and from spermatozoa. This was the first report that identified a correlation between testicular temperature and the semen' miRNAs population in farm animals. In addition, the testicular insulation model also increased the testicular temperature, one of the consequences of crates for boars that we reported. We understand that the duration and intensity of the challanges play an important role in the miRNAs population modulation, but maybe, for this high testicular temperature, boars and bulls showed different changes in miRNAs population in sperm.

In a follow up study, we reported different phenotypes of the piglets born from boars housed in crates or in enriched pens (Sabei et al, unpublished data/submitted). An interesting finding, indicated that more piglets were born from boars housed in enriched pens, also showing lower pre-weaning mortality. This was one of the reasons that we included in our literature research the embryo development and cleavage.

In addition, in this reported follow up study, we monitored the behavioral characteristics of the offspring of the studied boars, reporting an impact of the way the boars were housed in the emotionality, performance, aggressiveness and vocalization. We grant that the attempt to relate the microRNA abundance and developmental outcomes is speculative and a better understanding will demand a much more robust and detailed analyses.

Considering that the semen was pooled from boars representing the three different treatments and that paternity was assigned through genetic tests at the end of the trial, the bias represented by the higher number of piglets born from boars housed in the enriched pens, compared with the two other systems, must be explained through factors encountered in the early stages of fertilization or embryo development. It has been reported that the following miRNAs have impact on embryo development: 153, 20a-3o, 330, 335, 92b-3p. Our data shows that ssc-miR-153 and ssc-miR-330 were more abundant in the ejaculate from boars kept in enriched pens. Further studies are needed to understand the potential impact of the identified micro RNAs on embryo development.

We found that the piglets born from boars housed in crates had more skin lesions, an indicator or aggressive behavior (GUY et al., 2009), and they vocalized less during an open field and novel object tests, and showed higher nociceptive threshold (Sabei et al., 2021, submitted).

In a significant number of the differently abundant miRNA, we found some correlations with key important metabolic roles, which could explain the phenotype found in the piglets (see table 2). Many miRNAs are related to psychiatric disorders, such as schizophrenia, autism spectrum disorder, Alzheimer, neuronal function, and major depressive disorder (50% of the miRNAs differently abundant in our study). As observed in the offspring born from hereby studied boars, the piglets showed different outcomes for nociception, vocalization, aggression, and pre-weaning mortality. The correlation between these outcomes and the central nervous system is high, since all of the reported findings could be explained by a morphometric or function brain modification, reflecting in some behavioral changes (NARAYANAN; SCHRATT, 2020). Moreover, the behavior may represents the final output of the central nervous system (HÅNELL; MARKLUND, 2014) and may represent the consequences of the miRNAs difference abundant findings that we reported. Gapp et al., (2014) reported similar results. They showed that the population of miRNAs, specifically miR-375-3p, miR-375-5p, miR-200b-3p, miR-672-5p, and miR-466c-5p, were differently expressed on sperm, serum, and hippocampus of F1 and F2 offspring from stressed males mice.

Regarding the literature research for miRNA role in embryonic development, cleavage, inflammatory process, metabolism, infertility, testicular degeneration, and sperm maturation, we analyzed all miRNAs individually. However, for one miRNA, we were not able to find information in the scientific literature (ssc-miR-9799-3p).

All the information found in the literature search is summarized in the Table 2. In total, we found 72 scientific publications with the mention of the different abundant miRNAs population and our key-words previously selected.

Table 2 – Differential abundant miRNAs found in the ejaculate from boars housed in three different housing conditions and the respective scientific correlations.

Process	**miRNA involved**
Embryo development	153, 20a-3p, 330, 335, 92b-3p
Estrus and/or gestation	335, 574-5p, 7137-3p, 92b-3p, 9788-3p
Immune function	20a-3p,
Infertility	153, 574-5p, 92b-3p
Inflammation	330, 335, 92b-3p
Metabolism	129a-5p, 148a-5p, 335, 92b-3p
Muscle, bone or adipose tissue	153, 20a-3p, 330, 335, 574-5p, 92b-3p
Pig enteric diseases	129a-5p, 92b-3p
Pig respiratory diseases	129a-5p, 148a-5p, 574-5p, 7141-3p
Psychiatric disorders	129a-5p, 153, 20a3p, 335, 574-5p, 92b-3p
Reproduction development	148a-5p, 153, 335
Semen (pig, horse, human)	129a-5p, 148a-5p, 153, 335
Unknown	9799-3p

The scientific literature used for this table were: (NAKANISHI et al., 2009; NIELSEN et al., 2010; WANG; RUAN, 2010; FERNÁNDEZ-HERNANDO et al., 2011; CHEN et al., 2012; LIAN et al., 2012; CHEN et al., 2014; LIU et al., 2012a; MEDRANO et al., 2012; PODOLSKA et al., 2012; ROTLLAN; FERNÁNDEZ-HERNANDO, 2012; WEI et al., 2013; ZHOU et al., 2013, 2020; GOMEZ et al., 2014; COHEN; LEE; FIELDS, 2014; TSAI; MIRANDA, 2014; YANG et al., 2014; ZHANG et al., 2014, 2017a; ZHU et al., 2014; KASIMANICKAM; KASIMANICKAM, 2015; PORTILHO et al., 2015; SCALICI et al., 2015; SUN et al., 2015, 2019; WANG et al., 2015, 2017, 2019, 2020; XU et al., 2015, 2019; YE et al., 2015, 2018, 2020; MUNAUT et al., 2016; TWENTER, 2016; GU et al., 2017; LIAO et al., 2017; TWENTER et al., 2017, 2020; WANG; WANG; QI, 2017; FAFIÁN-LABORA et al., 2017; BELARBI et al., 2018; HAO et al., 2018; HERKENHOFF et al., 2018; HUANG et al., 2018, 2019; KINOSHITA; AOYAMA; NAKAKI, 2018; MENG et al., 2018; OTTON et al., 2018; DAVOLI et al., 2018; FLEMING; MILLER, 2019; KAY et al., 2019; LEE; HEO; KANG, 2019; LI et al., 2019, 2021; LIANG et al., 2019, 2020; LIU; HUANG; KE, 2019; PRESSLAUER et al., 2019; VASU et al., 2019; GLAESEL et al., 2020; KNAPCZYK-STWORA et al., 2020; LIP et al., 2020; PRZYGRODZKA et al., 2020; SUN; TIAN; LI, 2020; BU et al., 2021; HE et al., 2021; PÉRTILLE et al., 2021; DO et al., 2021; DOGHISH et al., 2021).

5.5.CONCLUSION

We found 12 microRNAs differently abundant in the semen, sperm cells and seminal plasma, of boars housed in three different scenarios. The boars housed in crates, the most common housing condition for breeding boars in Brazil and USA and a well know source of stress for pigs, showed indicators of low fertility. Furthermore, the boars housed in enriched pens showed the opposite outcomes, including their reproductive performance, which they produced more piglets compared with boars housed in crates or pens (Sabei et al 2021, submitted). The miRNA ssc-miR-153 and ssc-miR-330 was upregulated on boars housed in enriched pens (see figure 2), which could explain our findings. In our literature search, we did not find a directly correlation for this specific outcomes, however, other studies indicated that the upregulation of these miRNAs is positive, regarding neuroprotection (WEI et al., 2013; COHEN; LEE; FIELDS, 2014; TSAI; MIRANDA, 2014; XU et al., 2019). More studies or more detailed investigations are needed to better understand the role of the hereby related microRNAs and our boar's phenotype findings. In addition, this information has a great potential to contribute to improve boar welfare and boar performance.

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Discussion

Measures of boar behavior and boar welfare are poorly reported on scientific literature. Maybe because the low number of animals in pig farms. However, as previously mentioned, paternal stress and welfare can impact offspring performance and behavior. In Brazil and many other countries, it is often observed pigs housed in crates. This is the most common housing for boars and for pregnant sows. Data are available on the impact of such housing conditions in muscular and bone health in sows (MARCHANT; BROOM, 1996), in brain neurotransmitors, indicating negative mental state (BROOM; ZANELLA, 2004), abnormal behavior (MASON; LATHAM, 2004), and many others negative impacts. Nevertheless, it is rare to find data about confined housing conditions on boar behavior and welfare. Our study is, to our knowledge, one of the few studies which reported the impact of housing boars in crates on their welfare, physiology, behavior, reproductive health, semen features, and in their semen microRNA population.

Our results corroborate the research conducted in sows, which shows that the crate is a source of stress and can compromise boar welfare and performance. In addition, we showed that environmental enrichment, a tactile stimulus represented by brushing, is efficient on mitigating the boar's response to a disease challenge (chapter 1). In a more positive environment, with water baths, brushing, and rooting material (hay), the boars showed less abnormal behavior, lower testicular temperature, and lower testicular parenchyma perfusion (chapter 2 and 3).

In our assessment, the epigenetic data is the most innovative approach to test our hypothesis that poor welfare, represented by housing boars in crates, had a potential impact of their reproductive an, in the life trajectory of their offspring. The idea of this

approach is to better understand the mechanisms which males can contribute in their offspring resilience and welfare. We demonstrated that the housing conditions for boars, an often neglected subject in pig's research, can modulate the microRNAs population in the ejaculate. This epigenetic modulation can explain the differences found in boars' from different treatments.

More studies are needed to help us to have a comprehensive understand of this complex interactions. However, our data corroborate that housing boars in crates can compromise their welfare, their health and performance. We also demonstrated that environmental enrichment can improve the welfare of boars and reproductive outcomes.

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