

UNIVERSIDADE DE SÃO PAULO



FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

PATRICIA TATEMOTO

**The role of maternal stereotypic behaviour on developmental
outcomes in piglets**

Pirassununga
2018

**The role of maternal stereotypic behaviour on developmental
outcomes in piglets**

Thesis submitted to the Postgraduate Program in
Experimental Epidemiology Applied to Zoonosis of the
School of Veterinary Medicine and Animal Science of the
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CERTIFICADO

Certificamos que a proposta intitulada "O papel do comportamento estereotipado materno na programação fetal", protocolada sob o CEUA nº 6157201114 (ID 0057611), sob a responsabilidade de **Adroaldo José Zanella e equipe; Patricia Tatemoto; Thiago Bernardino de Almeida** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 07/11/2018.

We certify that the proposal "The role of maternal stereotypic behaviour on foetus programming", utilizing 108 Swines (males and females), protocol number CEUA 6157201114 (ID 0057611), under the responsibility of **Adroaldo José Zanella and team; Patricia Tatemoto; Thiago Bernardino de Almeida** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 11/07/2018.

Finalidade da Proposta: **Pesquisa**

Vigência da Proposta: de **04/2016** a **07/2016**

Área: **Epidemiologia Experimental Aplicada As Zoonoses**

Origem:	Animais de proprietários		
Espécie:	Suínos	sexo:	Fêmeas
Linhagem:	Topgen - largewhite x landrace	idade:	9 a 36 meses
		Peso:	120 a 200 kg
Origem:	Animais de proprietários		
Espécie:	Suínos	sexo:	Machos e Fêmeas
Linhagem:	Topgen - largewhite x landrace	idade:	1 a 2 meses
		Peso:	8 a 20 kg

Local do experimento: Na fase I do experimento, em que coletaremos informações das fêmeas, o experimento será realizado na Fazenda Araporanga - Jaguariá/VA - PR. Já na fase II, os leitões serão transportados para o campus da USP - Pirassununga e mantidos até o fim do experimento, que será aos 30 dias após o desmame.

São Paulo, 07 de novembro de 2018

Profa. Dra. Anneliese de Souza Traldi

Presidente da Comissão de Ética no Uso de Animais

Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes

Secretária

Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo



São Paulo, 09 de novembro de 2018
CEUA N 6157201114

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

Título da proposta: "O papel do comportamento estereotipado materno na programação fetal".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ (ID 004027)

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 Notificação (versão de 09/novembro/2018) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Favor alterar o atual título: "O papel do comportamento estereotipado materno na programação fetal" "The role of maternal stereotypic behaviour on foetus programming" Para uma nova versão que mais se adequa ao projeto. "O papel do comportamento estereotipado materno no desenvolvimento de leitões" "The role of maternal stereotypic behaviour on developmental outcomes in piglets".

Comentário da CEUA: "Solicitada mudança no título da tese de doutorado para "O papel do comportamento estereotipado materno no desenvolvimento de leitões" - "The role of maternal stereotypic behaviour on developmental outcomes in piglets".

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo

Roseli da Costa Gomes
Secretária
Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo



CERTIFICADO

Certificamos que a proposta intitulada "O IMPACTO DA REDUÇÃO DA FOME NAS FÊMEAS SUÍNAS DURANTE A GESTAÇÃO, COM INDICADORES DE SAÚDE E BEM ESTAR DOS LEITÕES", protocolada sob o CEUA nº 3606300114 (ID 000849), sob a responsabilidade de **Adroaldo José Zanella** e equipe; *Thiago Bernardino de Almeida* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 15/10/2014.

We certify that the proposal "título em inglês", utilizing 272 Swines (112 males and 160 females), protocol number CEUA 3606300114 (ID 000849), under the responsibility of **Adroaldo José Zanella** and team; *Thiago Bernardino de Almeida* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/15/2014.

Finalidade da Proposta: **Pesquisa**

Vigência da Proposta: de 07/2014 a 11/2015

Área: **Epidemiologia Experimental Aplicada As Zoonoses**

Origem:	Não aplicável biotério	sexo:	Fêmeas	idade:	a	N:	36
Espécie:	Suínos			Peso:	a		
Linhagem:	TopGen Afrodite®						
Origem:	Não aplicável biotério	sexo:	Machos	idade:	a	N:	112
Espécie:	Suínos			Peso:	a		
Linhagem:	TopGen						
Origem:	Não aplicável biotério	sexo:	Fêmeas	idade:	a	N:	112
Espécie:	Suínos			Peso:	a		
Linhagem:	TopGen						
Origem:	Não aplicável biotério	sexo:	Fêmeas	idade:	a	N:	12
Espécie:	Suínos			Peso:	a		
Linhagem:	Mista						

Local do experimento:

São Paulo, 09 de novembro de 2018

Profa. Dra. Anneliese de Souza Traldi

Presidente da Comissão de Ética no Uso de Animais

Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

EVALUATION FORM

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Title: The role of maternal stereotypic behaviour on developmental outcomes in piglets

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.

Date: ____/____/____

Committee Members

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

I dedicate this study to all animals, charming beings that inspire and motivate my life. Above all, those who are exploited, who have their rights forgotten, who are born only for our use and are target of our distorted concepts about respect for other lives. In particular, I dedicate this study to all the pigs that were part of the project, which filled me with joy and love, which inspired me and, somehow, built an eternal bond with my trajectory.

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“If a lion could talk, we could not understand him”.

Ludwig Wittgenstein

ABSTRACT

TATEMOTO, P. **The role of maternal stereotypic behaviour on developmental outcomes in piglets**. 2018. 163 p. Thesis (Doutorado em Ciências) – School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, 2018.

Stereotypic behaviour is considered an indicator of compromised welfare. We have conducted studies to test the controversial hypothesis that stereotypic behaviour helps animals to cope with challenges. We proposed that animals that do not express stereotypies could be under more compromised welfare, than the ones showing the behaviour, when exposed to difficult situations. Rather than asking to the animal if stereotypies are indicating good welfare, we assessed the effects on foetal programming. This study investigated the outcomes of stereotypies measured in sows (*Sus scrofa*) during gestation in shaping their offspring's phenotype measuring both behavioural and physiological indicators of welfare. Environmental enrichment is an efficient protocol, acting in the causal factors of stereotypies, such as motivational systems, to reduce repetitive, invariant behavioural patterns, defined as stereotypies. We demonstrated that stereotypies in pregnant sows are related with a reduction in fear indicators in their offspring. Then, we showed that environmental enrichment, in the last third of gestation, improved the welfare of sows and also the welfare in their offspring. Comparing the brain of the offspring of sows kept in enriched and non-enriched environments and comparing non-enriched sows performing stereotypies with non-enriched sows not showing stereotypies, we identified eight genes related with neuroplasticity and psychiatric diseases, which were differentially methylated. The main contribution of this study is that maternal stereotypic behaviour during gestation decreases fear indicators and alters the neuroepigenome of the limbic system of the offspring. As far as we know, this is the first evidence showing that stereotypies expressed by the mother during gestation did affect offspring's emotionality, in which the mechanism were epigenetic changes in the brain.

Keywords: emotionality; epigenetics; prenatal; stereotypic behaviour; welfare.

RESUMO

TATEMOTO, P. **O papel do comportamento estereotipado materno no desenvolvimento de leitões**. 2018. 163 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

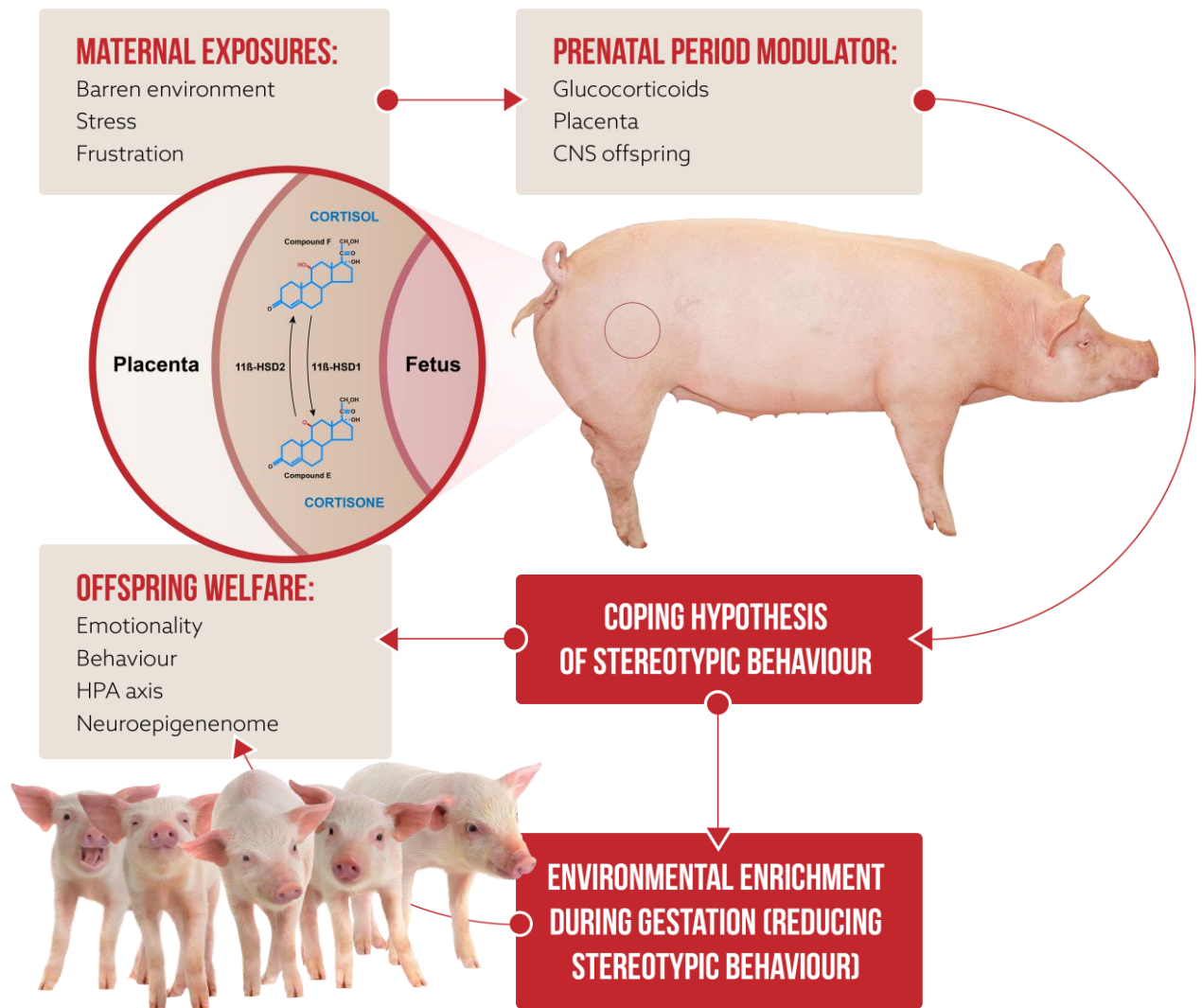
O comportamento estereotipado é considerado um indicador de comprometimento do bem-estar. Nós conduzimos estudos para testar a controversa hipótese de que o comportamento estereotipado ajuda os animais a lidar com desafios. Propusemos que animais que não expressam estereotipias possam estar sob um bem-estar mais comprometido do que aqueles que demonstram o comportamento, quando expostos a situações difíceis. Ao invés de perguntar ao animal se as estereotipias estão indicando um bem-estar adequado, avaliamos os efeitos na programação do feto. Este estudo investigou os resultados de estereotipias em fêmeas suínas (*Sus scrofa*) durante a gestação no fenótipo da prole, acessando indicadores comportamentais e fisiológicos. O enriquecimento ambiental é um protocolo eficiente, atuando nos fatores causais de estereotipias, como os sistemas motivacionais, para reduzir padrões comportamentais repetitivos e invariantes, definidos como estereotipias. Nós demonstramos que estereotipias em suínas prenhes estão relacionadas com uma redução nos indicadores de medo na prole. Em seguida, mostramos que o enriquecimento ambiental, no último terço da gestação, melhorou o bem-estar das porcas e também o bem-estar de seus filhos. Comparando o cérebro da prole de porcas mantidas em ambientes enriquecidos e não enriquecidos e comparando porcas não enriquecidas realizando estereotipias com porcas não enriquecidas sem estereotipias, foram identificados oito genes relacionados com a neuroplasticidade e doenças psiquiátricas, que foram diferencialmente metilados. A principal contribuição deste estudo é que o comportamento estereotipado materno durante a gestação diminui os indicadores de medo e altera o neuroepigenoma do sistema límbico da prole. Até onde sabemos, esta é a primeira evidência mostrando que estereotipias expressas pela mãe durante a gestação afetaram a emocionalidade da prole, na qual o mecanismo era uma mudança epigenética no cérebro.

Palavras-chave: bem-estar; emocionalidade; epigenética; pré-natal; comportamento estereotipado.

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THESIS SCHEMATIC DIAGRAM



Introduction

Stereotypic behaviour is considered an indicator of compromised welfare. This study investigated the outcomes of stereotypic behaviour of sows (*Sus scrofa*) during gestation in shaping their offspring's phenotype. We also tested the hypothesis that stereotypies in the sow shape the phenotype of the offspring (behavioural and physiological indicators of welfare), by mitigating stereotypic behaviour through environmental enrichment. Stereotypic behaviour (or stereotypies) has been widely used as an indicator of animal welfare, but the data regarding the association between the behaviour and welfare outcomes, are generally not consistent. If we consider two equally challenged individuals, the one who expresses stereotypic behaviour may actually be better adjusted compared to the animal that not expresses the behaviour. It is possible that an animal that does not express is in more compromised welfare than the one that expresses it. The hypothesis is that the maternal stereotypic behaviour expression affects foetus programming.

In pregnant sows, there is a wide range of studies demonstrating the expression of stereotypic behaviours (BERGERON et al., 2000; BLACKSHAW; MCVEIGH, 1984; SPOOLDER et al., 1995; TERLOUW et al., 1991; ZANELLA et al., 1996). It is suggested that the lack of complexity in their physical environment and their individual variation are common factors for the emergence of stereotypies (LIU; DUAN; WANG, 2017; MASON et al., 2007; MASON, 1991; MEAGHER; MASON, 2012). This behaviour is developed in chronic stress or in situations of frustration, but can also persist from a situation that happened in the past or from an unsolvable conflict (DANTZER, 1991; MASON, 1991). Stereotypic behaviour has been described in a wide range of species living in artificial environments, but the scientific data are not consistent. However, the expression of stereotypies is often considered a welfare indicator since it is commonly triggered to respond to frustration (DANTZER, 1991; MASON, 1991; ZHANG et al., 2017), and its performance can provide

information about psychological states of animals. In this entire document, the words stereotypies and stereotypic behaviour are used as synonymous.

Although there is an association with individual variation (IJICHI; COLLINS; ELWOOD, 2013; JOSHI; PILLAY, 2016), stereotypic behaviour becomes more consistent in a population when the stressor is persistent or increases in intensity. Some stressors may have a cumulative effect, such as recurrent and long-lasting environmental constraints that increases the frequency of stereotypies. Pigs have a complex behavioural repertoire and they are especially motivated to explore. As a consequence, in order to increase the welfare of pigs attending their motivational needs, straw is often used to promote environmental enrichment (BULENS et al., 2016; DOUGLAS et al., 2012; REIMERT et al., 2014; STATHAM; GREEN; MENDL, 2011).

Poor or barren environments often become a challenge for confined animals. The reduction of environmental stimuli or invariability has been considered a stressor for animals kept in artificial environments (MORGAN; TROMBORG, 2007; VAN ROOIJEN, 1991). The main challenge is due to the discrepancy between the environment in which living beings have evolved and the housing systems where they are kept for commercial purposes. The natural environment is rich and complex, in which motivational systems have been shaped throughout time to maintain life. In a simple approach, stereotypies could be just the appetitive part in the behaviour, and it becomes repetitive due the absence of consummatory behaviour. In this sense, environmental enrichment enables the completion of the appetitive and consummatory phases of behaviour. Some enrichment can enable the performance of highly motivated behaviours. Motivation can be defined as a process by which the animals integrates information based on different stimulus sources, internal and external, and translates into a behaviour (JENSEN; TOATES, 1997).

Environmental enrichment, in addition to allowing the expression of natural behaviours, increases brain plasticity (EBBESSON; BRAITHWAITE, 2012; WILLIAMS et al., 2001); alters the HPA axis activity (GRO et al., 2013; KOTRSCHAL; TABORSKY, 2010; LARSSON; WINBLAD; MOHAMMED, 2002); and reduces methylation in hippocampal and frontal cortex genes (MYCHASIUK et al., 2012). Furthermore, environmental enrichment affects the expression of genes in the brain, especially those involved in neuronal structure, synaptic signalling and plasticity (BARONCELLI et al., 2009). Some genes have been known to be associated with learning and memory (RAMPON et al., 2000). Moreover, enrichment increases brain weight, promotes an increase arborisation and density of dendritic spines (LEGGIO et al., 2005), modulates neurogenesis in the hippocampus (SEGOVIA et al., 2006), and promotes positive affective states (DOUGLAS et al., 2012).

In this context, rather than asking to the animal if stereotypies are indicating better welfare, we asked to the offspring, considering that the prenatal environment shapes the foetus programming during gestation. The prenatal environment has the potential to adjust the offspring's phenotype and shape individuals to the environment that they will be inserted, in order to prepare them to cope with challenges. This concept comes from the "Thrifty Phenotype Hypothesis", in which neurodevelopment reprogramming induces alterations to cope with the initial environment (HALES; BARKER, 2001). The environment in which an animal is maintained during gestation may result in changes to several offspring parameters (BAXTER et al., 2016; BRAASTAD, 1998; DARNAUDÉRY; MACCARI, 2008; MEYER; FELDON; FATEMI, 2009; RUTHERFORD et al., 2014; URAKUBO et al., 2001). By this mechanism, features such as emotional reactivity, responsiveness to stressors and cognition can be modulated by challenges in the prenatal and neonatal periods (BAXTER et al., 2016; HILD et al., 2011; POLETO et al., 2006; RUTHERFORD et al., 2014; WEINSTOCK,

2008). Although some studies have shown that stress during pregnancy or prenatal stress can generate changes that are not necessarily pathological (BRAASTAD, 1998), the excess of glucocorticoids that the offspring receives can affect important brain structures and generate negative effects (BAXTER et al., 2016; COULON et al., 2013; RUTHERFORD et al., 2014). Glucocorticoids are important stress hormones in adult animals but they have multiple functions in the foetus that are dependent on gestational age, severity and duration of the exposure (FOWDEN et al., 2016). The effects of prenatal stress on brain structures such as the hippocampus and amygdala may generate changes in offspring's emotionality (ABE et al., 2007; NOLVI et al., 2016).

In foetal programming, the placenta has a protective role during the prenatal period. Therefore, it modulates stressful events experienced by the maternal organism by acting as a buffer (JANSSON; POWELL, 2007). In mammals, the 11β -hydroxysteroid dehydrogenase enzyme type 2 (11β HSD2) in the placenta forms a barrier that protects the foetus from high levels of maternal cortisol because it oxidizes the biologically active form of cortisol into cortisone (JANSSON; POWELL, 2007; SECKL, 2004). Chronic stressful situations have the potential to inhibit the capacity to up-regulate the type 2 enzyme activity and the capacity to adapt placental 11β HSD2 is greatly reduced by previous exposure to chronic stress (WELBERG; THRIVIKRAMAN; PLOTSKY, 2005), then reducing the protective capacity of the placenta.

However, the main mechanism associated to prenatal environment and foetal programming is epigenetic, that are basically chemical modifications to chromatin that regulate genomic transcription. The modifications could be stable and cross to the next generations. Furthermore, these modifications are dynamic and change in response to environmental stimuli (CAO-LEI et al., 2017). Changes in methylation status at selected genomic loci may affect social cognition (ISLES; DAVIES; WILKINSON, 2006), learning and memory

(GRÄFF; MANSUY, 2008; MILLER; SWEATT, 2007), and stress response. Furthermore, it is associated with dysregulated gene expression in a range of psychiatric disorders (BURNS et al., 2018; DUDLEY et al., 2011; MILLAN, 2013), such as autism (CATTANE; RICETTO; CATTANEO, 2018; DALL'AGLIO et al., 2018), schizophrenia (CATTANE; RICETTO; CATTANEO, 2018), depression and Alzheimer's disease (CHOULIARAS et al., 2010; GRINÁN-FERRÉ et al., 2016, 2018).

Heritable epigenetic changes facilitate rapid adaptation to adverse environmental conditions, but may also result in a mismatch of physiological profiles to later-life challenges, thus increasing disease risk (CAO-LEI et al., 2017). The effects of prenatal stress on brain structures such as the hippocampus and amygdala generate changes in offspring's emotionality (ABE et al., 2007; NOLVI et al., 2016). Neuroepigenetics is a research field that has expanded massively and it is now clear that epigenetics processes, such as DNA methylation, are mechanisms involved in pathological brain function (DELGADO-MORALES; ESTELLER, 2017).

Improving the mother's welfare during pregnancy can lead to positive changes in the offspring. In this study we addressed maternal stereotypic behaviour acting in the offspring neurodevelopment, when sows were challenged in a barren environment. Then, we addressed another study mitigating the expression of stereotypies by using environmental enrichment. It is possible that stereotypic behavior is helping the sow to cope with stress, and during gestation may change the phenotype of the offspring. Rather than ask to the individual what it means to express stereotypic behavior, we addressed this issue studying the impact in the offspring. Furthermore, we asked which are the consequences to the offspring when the environment is attending the motivational systems, and reducing the expression of stereotypies. Are there any epigenetics changes in the offspring during foetal programming

and in the neonatal period, in response to the sow performance of stereotypies and exposure to environmental enrichment?

This thesis is organized into six papers to explore the consequences of maternal stereotypies of sows during gestation in their offspring, including consequences related to the mitigation of this behaviour. The first paper is a literature review including the main subjects studied in this thesis. The second paper is about productivity outcomes related with stereotypic behaviour expression by the sows, since this is a widely used welfare indicator. The third and fourth papers are exploring the effects of maternal stereotypies in their offspring emotionality, behaviour, HPA axis, and glucocorticoids in placental tissues. The fifth paper addresses the effects of environmental enrichment during gestation in welfare indicators in the offspring, since it is consistent in the current literature that an enriched environment reduces stereotypies. Lastly, the sixth paper investigated the mechanisms by which the phenotype is possibly changed during foetal programming. So, the last paper is an attempt to understand the epigenetic changes in the limbic system of offspring born from sows kept in enriched and poor environments and from sows with high or low levels of stereotypies.

We have shown that stereotypic behaviour during gestation affects positively offspring's emotionality, increasing their welfare. In order to elucidate the consequences when stereotypic behaviour is reduced, we looked for the consequences of mitigating stereotypies using environmental enrichment. We demonstrated that environmental enrichment during gestation in sows alters behavioural and physiological welfare indicators in their offspring, both indicating increased welfare outcomes. Focusing on the mechanism by which phenotype shaping occurred we analysed the epigenome of the limbic system of piglets. The genes that were differently methylated, were involved in neuroplasticity and psychiatric diseases. We also demonstrated that stereotypic behaviour expression does not affect productivity parameters. We argue that this outcome could be a result of years of

genetic improvement that has selected animals with high productivity rates, making these variables more consistent within the group. Stereotypic behaviour is widely considered a welfare indicator in which is assumed that when an individual is expressing it, indicates poor welfare compared with animals that do not express the behaviour. However, the data presented in this thesis support the hypothesis that stereotypic behaviour helps animals to cope with challenges, in which individuals that do not express the behaviour could be under more compromised welfare and as a consequence, affects foetal programming.

The role of maternal stereotypic behaviour on foetal programming

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Abstract

Stereotypies comprises a wide range of repetitive and apparently functionless patterns that often develop in environments that are likely to be associated with poor welfare. However conflicting data are available making it unclear the definitive role of stereotypies as an animal welfare indicator. In order to reduce stereotypies, it has been shown that environmental enrichment can be an efficient protocol that may act in the causal factors. If a better environment is reducing the occurrence of this undesirable behaviour, it indicates that animals that express stereotypies are in poorer welfare states than the ones that do not show the behaviour. Rather than asking to the animal if stereotypies are indicating something about their welfare, we assessed their effects on foetal programming. Neurodevelopmental programming induces alterations in the central nervous system to facilitate the mechanisms that allow animals to cope with challenges in the postnatal environment.

Keywords: emotionality; gestation; prenatal; stereotypic behaviour.

Introduction

Stereotypic behaviour or stereotypies comprises a wide range of repetitive and apparently functionless patterns that often develop in environments that probably are causing poor welfare (Dantzer 1991a, Mason 1991). The term ‘apparently’ is important once it has been discussed potential functions for the expression of stereotypies. Although the cause and development of stereotypic behaviour is heterogeneous, is necessary to understand what their performance means since it is widely used as animal welfare indicator.

Previous research demonstrated that environmental enrichment can be an efficient tool to reduce stereotypies (Newberry 1995, Douglas et al. 2012, Dandi et al. 2018) and the published results could be interpreted as an effect in the causal factors associated with the development of stereotypies. Enrichment implies an improvement, which is expected to offer conditions for a better biological functioning of captive animals as a result from modifications to their environment (Newberry 1995). Considering these evidences, we can assume that environmental enrichment is attending behavioural and physiological needs of animals and as a result they will not show stereotypies. Moreover, the possibility to interact with more complex environments is in accordance with several motivational systems, which have been selected throughout evolution.

If a better environment is reducing the occurrence of this undesirable behaviour, it indicates that animals that express stereotypies are in poorer welfare states than animals that do not express the behaviour. However, the “Coping Hypothesis” of stereotypic behaviour assumes that its performance reduces the level of stress experienced by the animals (Cooper & Nicol 1993, Rushen 1993). This hypothesis is still controversial, since the scientific data are not consistently corroborating it.

A better approach, rather than asking to the animal if stereotypies are indicating good welfare, is to assess the offspring, considering that the prenatal environment will shape the foetus during gestation. In mammals, the pregnancy has a determinant role on shaping the

organisms trajectory, since mother's environment may have effects on the offspring (Rutherford et al. 2014, Baxter et al. 2016). As a general rule we can state that neurodevelopmental programming induces alterations to anticipate how the animal may cope with challenges in the postnatal environment (Hales & Barker 2001). One of the mechanisms modulating phenotype on foetal programming is epigenetic, in which the modifications could be stable and cross generations.

The effects in the offspring will depend on the causes of the development and expression of stereotypies. If stereotypic behaviour is helping the animals to cope with stressful situations, it should be expected that the offspring born from animals that show the behaviour would have better welfare than the offspring born from animals that did not perform stereotypies. We investigated the role of stereotypic behaviour expression during gestation on some indicators of foetal programming.

Stereotypic behaviour as animal welfare indicator

Stereotypies are defined as repetitive, invariant and apparently functionless patterns that develop in environments that compromises welfare (Mason 1991). It develops in animals kept in environments with few stimuli, physical restraint, that causes fear or frustration (Mason 1991), and has been described in a wide range of species in artificial environments. The occurrence of stereotypies may suggest frustration in response to the inability to perform highly motivated behaviours, which may be tentatively expressed even in the absence of appropriate stimulus. However, the expression of stereotypies cannot be attributed only to the environment, once there is variation among individuals kept in similar environments, whereas animals may or may not express such behaviours.

Stereotypic behaviour expression is often considered a welfare indicator (Dantzer 1991b, Mason 1991, Broom & Johnson 1993, Zhang et al. 2017). However, this specific behavioural

indicator is not consistent with increased levels of cortisol (Terlouw et al. 1991), a widely used physiological welfare indicator. Some scientists argue that there is no reason to suppose that all stereotypies are a response to stress, or that the performance of all stereotypies will affect physiological systems associated with stress (Rushen 1993). In general, situations inducing or exacerbating stereotypies also decrease welfare (Mason & Latham 2004), and the most consistent information in literature is that stereotypies are related with frustration (Dantzer et al. 1987, Dantzer 1991a). Moreover, stereotypies are associated with barren environments, the reduction of stimulus in these conditions, is also related with boredom-like states (Meagher et al. 2017). The predictability of the environment can also be a causal factor triggering stereotypies (Bassett & Buchanan-Smith 2007), which is common component in barren environments.

As neurophysiological markers, endorphins are implicated in these behaviours, since naloxone and opioid antagonist, interrupts stereotypies in sows (Cronin et al., 1985, Rushen et al. 1990, Zanella et al. 1996). Endorphins may be especially an influencer in the stereotypies that are developing, since older stereotypies are not sensitive to naloxone treatment (Cronin et al., 1985). Moreover, endogenous opioids could be involved in the positive feedback that maintains the behaviour and inhibits to switch to others, decreasing behavioural flexibility (Rushen et al. 1990, Savory et al. 1992). In mice, motionless reaction in non-enriched housing was considered an alternative to stereotypic behaviour which could be considered as depression-like states (Fureix et al. 2016). These data support the “Coping Hypothesis”, that is based on the concept that the performance of stereotypies helps animals to cope with stress (Rushen et al. 1990, Cooper & Nicol 1993, Mason & Latham 2004). Perhaps, what is unclear in the existing current knowledge regarding to this hypothesis can be related to the variable time (see Figure 1). In the first stages, stereotypies may be helping animals to cope with challenges. It is likely than the performance of the behaviour can alter the brain, impairing brain function due the absence of

other activities and change brain connections, changes neurophysiology and lastly, changes neuroanatomy (Zanella et al. 1996, Mason & Rushen 2006).

Frustration related to food restriction can trigger oral stereotypic behaviours in sows (Appleby & Lawrence 1987). To facilitate the expression of stereotypies there is also a genetic component (Schwaibold & Pillay 2001, Jeppesen et al. 2004), personality predisposition (Ijichi et al. 2013, Joshi & Pillay 2016), individual variation (Joshi & Pillay 2018), higher susceptibility regarding sex (Hogan & Tribe 2007). Some variables have more impact on triggering stereotypies than others if we consider a range of environmental factors (Liu et al. 2017). Moreover, stereotypies can be confounding factor between others behaviours, such as wheel running in mice, since the motor patterns of this behaviour fill the concept of stereotypy (Joshi & Pillay 2018). Regarding to the affective states, mice with higher levels of stereotypic behaviour made more optimistic choices (Novak et al. 2016).

Stereotypies were considered merely a pathological consequence of dysregulation of dopaminergic system (Nakamura et al. 2000), resulting from an overproduction of dopamine or even an hypersensitivity in the receptors of this neurotransmitter, with unknown causes. Stereotypies may represent the appetitive phase of the motivational systems, in which the restrictive and barren environment does not permit the individual to reach the consummatory part of the behaviour. The absence of completing the phases of the motivational system generates the repetition that defines stereotypies.

Stereotypies in voles also changes their housing preference creating a bias for preference to poor environments. Housing voles in laboratory cages results in highly repetitive locomotor stereotypies, and bias their preference for a less enriched condition when compared to non-stereotypic voles (Cooper & Nicol 1991). Cooper and Nicol (1991), argued that it can be physically easier to perform stereotypies in a barren environment or when the perception about the environments is different.

As far as we know, stereotypic behaviour is a recent mechanism in the evolutionary terms, since it appeared in animals in captivity. So, what are the consequences of stereotypic behaviour expression in the long-term? Males that expressed longer duration of stereotypes in mink had lower success in copulation (Díez-León 2013). Since the comparison consisted in animals developing stereotypes in environmentally enriched conditions (Díez-León 2013), the environment could have affected the ontogeny, physiology of stress, physiological reproductive mechanisms, social behaviour and flexibility, increasing the difference between animals kept in barren and in rich environments.

Opioid antagonists such as naloxone reduce the frequency of stereotypes leading to suggestions that its increases endogenous opioid activity and thus induces a degree of analgesia in animals (Cronin et al., 1985), finding not supported by Lebelt and collaborators (1998), studying horses. Moreover, stereotypes in mink are associated with increased hippocampus neurogenesis (Malmkvist et al. 2012). Since neurogenesis is decreased under chronic stressful conditions (Balu & Lucki 2009, Apple et al. 2017), these outcomes are indicating less stress in animals expressing stereotypes. In horses, the presence of stereotypic behaviour is linked to differences in HPA axis response, which might be an effective coping strategy that helps stereotypic individuals to gain control over stressful situations (Briefer Freymond et al. 2015). Tail-chewing mink are more explorative, a state commonly associated with good welfare (Svendsen et al. 2013). There is also increased reproductive output in females performing stereotypic behaviour (Jeppesen et al. 2004, Jones et al. 2010). In agreement with these studies, positive correlation between fertility and stereotypic behaviour is also associated with bodyweight in mink (Jeppesen et al. 2004). Bodyweight, in mink, was lower in animals not performing stereotypic behaviour (Jeppesen et al. 2004).

Furthermore, some authors considered the possibility that stereotypy in captive animals involves an abnormality within the animal, specifically a brain dysfunction similar to

what is encountered in schizophrenia and autism (Garner & Mason 2002). Tail biting in swine, feather pecking in poultry and tail chewing in mink are sometimes considered stereotypies. However, if the animals are performing the behaviour, in a group situation, causing injuries and pain, the consequences are completely different than performing only self-directed stereotypic behaviour, which involves just the individual. For this reason, we define these behaviours as abnormal instead of naming them stereotypies.

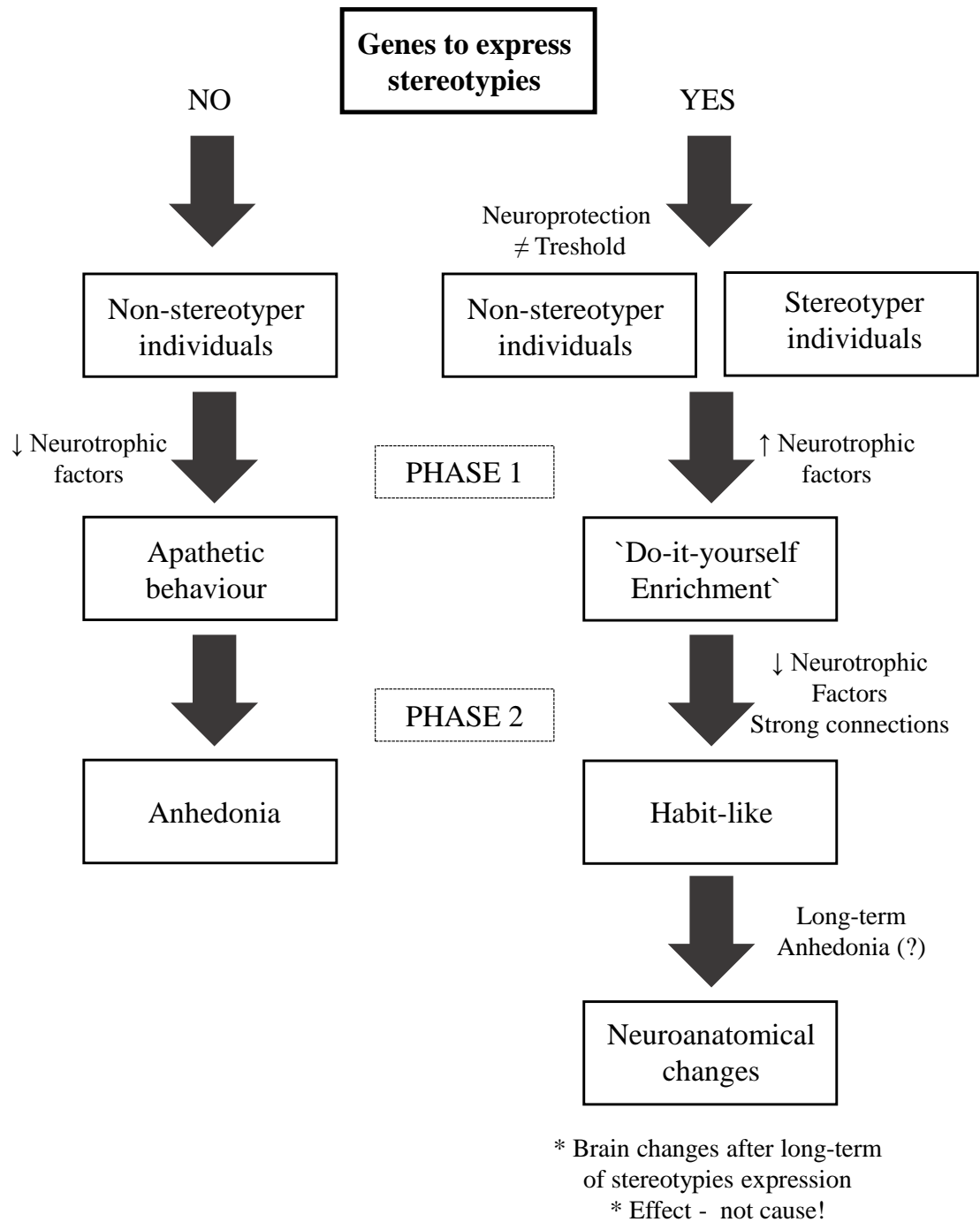


Figure 1. The effect of stereotypic behaviour expression throughout time. Different theories and data regarding to stereotypes are confusing because the comparison has been made without consider the variable time. Probably, positive effects of stereotypes expression are associated with the beginning of stressful situations, helping the animals to cope. After a while, it could be detrimental for reinforcing the same connections, as habit-like or making a `scar` in the brain. Finally, we consider that it is possible that neuroanatomical changes are an effect, instead of cause as has been proposed.

Why environmental enrichment reduces stereotypic behaviour expression?

A wide range of studies shows that environmental enrichment reduces stereotypic behaviour expression (Arellano et al. 1992, Shyne 2006, Mason et al. 2007). The reduction of stereotypies is the main criteria to consider the enrichment successful (Shyne 2006, Fureix et al. 2016). This discussion is consistent with the fact that environmental enrichment improves biological functioning and animal welfare (Newberry 1995). Basically, when enrichment is done well, it tackle the cause of the problem by permitting the environment to attend motivations of the animals (Mason et al. 2007). More studies are necessary considering in the same population the individual variation. Some individuals can benefit from social enrichment, whilst others for physical or sensory stimulation.

Early environmental enrichment “protects” captive-born striped mice against later development of stereotypic behaviour (Jones et al. 2011). However, the idea is not to reduce the performance of stereotypies, but to reduce the frustration that trigger the occurrence of stereotypies. Environmental enrichment, in addition to allowing the expression of natural behaviours, reduces endocrine and behavioural reactivity to stress (Simpson & Kelly 2011), alleviates cognitive and behavioural impairments (Suemaru et al. 2018, Griñán-Ferré et al. 2018), modulates aggressiveness (Kadry & Barreto 2010), alters the HPA axis activity (Larsson et al. 2002, Kotrschal & Taborsky 2010, Simpson & Kelly 2011, Gro et al. 2013), increases brain plasticity (Williams et al. 2001, Simpson & Kelly 2011, Ebbesson & Braithwaite 2012, Dandi et al. 2018), increases performance of hippocampal mediated tasks (Sampedro-piquero et al. 2013, Cortese et al. 2018, Ohline & Abraham 2018), reduces methylation in hippocampal and frontal cortex genes (Mychasiuk et al. 2012a).

Probably, chronic stress and environmental enrichment act on similar mechanisms within the hippocampus, so the manipulations of both components could optimize brain integrity and resilience under chronic stress (Hutchinson et al. 2012). Moreover, plastic

changes in hippocampal astrocytes regarding to environmental enrichment are involved in a brain protection to cope with cognitive impairments related with age, improving spatial memory and inducing accurate spatial strategy (Sampedro-Piquero et al. 2014).

Furthermore, environmental enrichment affects the expression of genes in the brain, especially those involved in neuronal structure, synaptic signalling and plasticity (Baroncelli et al. 2009). Some genes have been known to be associated with learning and memory (Rampon et al. 2000). Moreover, environmental enrichment affects brain weight, promotes an increasing in arborisation and density of dendritic spines (Leggio et al. 2005), modulates neurogenesis in the hippocampus (Segovia et al. 2006), and makes the cognitive bias positive (Douglas et al. 2012, Zidar et al. 2018).

Environmental enrichment also reduces anxiety (Sampedro-piquero et al. 2013, Goes et al. 2015, Lopes et al. 2018, Dandi et al. 2018), and enriched animals are seemingly less emotionally reactive in novel situations, thus enriched animals can explore their environment more efficiently (Simpson & Kelly 2011).

Male mice housed in enriched pens after weaning gained less weight, displayed increased social behaviours, had limited corticosterone and prefrontal IL-1 β elevations in response to a mild social stressor. Moreover, they exhibited reduced TNF- α and increased (brain-derived neurotrophic factor) BDNF expression within the PFC in early adulthood (McQuaid et al. 2018). In pigs, enrichment increased BDNF in blood (Rault et al. 2018), and BDNF has been linked with greatest stress resilience (Mosaferi et al. 2015) and increasing in cognitive functions (Novkovic et al. 2015). These results showed a buffer effect of environmental enrichment when animals are challenged, findings which are in agreement with other studies (Birch & Kelly 2018, Brown et al. 2018, Zidar et al. 2018).

Based on the benefits of sensorial stimulation as enrichment (Wells 2009, Leon & Woo 2018), it arises the argument that it is necessary to stimulate the brain, rather than simply

promote natural behaviours. It is well reported that environmental enrichment induces a number of neuroanatomical, neurochemical and behavioural alterations. The stimulus, operationally performed in enriched environments, are determinant for brain functioning, since brain works in a Lamarck based way: get or lose connections. This is so necessary for health functioning of the brain, and we can predict that loosing connections is deleterious and that evolutionary mechanisms may have evolved to shape the brain to preserve its structure.

Are there consequences in the offspring?

Regarding to the transgenerational effects of stereotypic behaviour, how it changes the offspring phenotype? In mammals, the pregnancy has an important role on shaping the organisms, since maternal environment may have effects on the offspring. This concept comes from “Thrifty Phenotype Hypothesis”, in which the neurodevelopmental reprogramming induces alterations to cope with the early life, anticipating postnatal environment (Hales & Barker 2001). In other words, the prenatal environment has the potential to adjust the offspring phenotype and prepare individuals to cope better with challenges. The environment in which an animal is maintained during gestation may result in changes in several offspring parameters (Urakubo et al. 2001, Darnaudéry & Maccari 2008, Meyer et al. 2009, Rutherford et al. 2014, Baxter et al. 2016). By this mechanism, factors such as emotional reactivity, responsiveness to stressors and cognition can be modulated by challenges in the prenatal and neonatal periods (Poletto et al. 2006, Weinstock 2008, Rutherford et al. 2014). Studies have shown that some stressors such as negative interactions with the handler (Coulon et al. 2013, Rutherford et al. 2014, Baxter et al. 2016) and social stress (Rutherford et al. 2014) have altered emotional reactivity, social behaviour, and responsiveness to stressors, cognition and memory in the offspring. Moreover, sows

experiencing less hunger during gestation have shown reduced aggressive behaviour in their offspring (Bernardino et al. 2016).

Furthermore, the events at the beginning of an animal life can shape their phenotype and produce changes in their biological functioning. Prenatal environments are critical for foetal development, including the organization of the central nervous system. In particular, the stress experienced by the mother has a wide range of effects on the offspring physiology and behaviour. The mother environment can indicate important signals to the foetus, enabling some adjustments for the environment that they will be inserted (Weinstock 2008, Mychasiuk et al. 2012a, Mychasiuk 2015, Nolvi et al. 2016). However, the confined and captive animals may give harmful cues to the foetus due to the environment to be highly artificial (Rutherford et al. 2014, Baxter et al. 2016, Cattane et al. 2018).

Although some studies have shown that prenatal stress can generate changes that are not necessarily pathological, the excess of glucocorticoids can affect brain structures and generate disruptive effects in the offspring (Coulon et al. 2013, Rutherford et al. 2014, Baxter et al. 2016). Glucocorticoids are important stress hormones in adult animals but their functions are less known in their range for the foetus (Moisiadis & Matthews 2014, Fowden et al. 2016). The effects are completely different depending on gestational age, severity and duration of the exposure (Fowden et al. 2016). Effects in the early and mid-gestation depends on the level of stress experienced by the mother, which her hypothalamic-pituitary-adrenal (HPA) responses can also affect glucocorticoid permeability of the placenta. Later in gestation when the foetal HPA axis has developed functionally, glucocorticoid concentrations can also works independently of maternal levels (Fowden et al. 2016). Moreover, glucocorticoids acts in a non-linear “U-shape function”, which low or high concentrations can cause negative effects in the emotionality and learning (Lupien & Lepage 2001). Glucocorticoids act directly on the formation and development in the central nervous system, for example in the

hippocampus, which this region of the brain is characterized by a high plasticity and relevant role in behaviour and welfare (McEwen 1997).

The effects of prenatal stress on brain structures such as the hippocampus and amygdala may generate changes in offspring's emotionality (Abe et al. 2007, Nolvi et al. 2016). To access the emotionality of non-human animals, some tests have been validated and discussed (Forkman et al. 2007). These include the open field test and the novel object test (Forkman et al. 2007, Donald et al. 2011, Rutherford et al. 2012, Antonides et al. 2016), in which behaviours such as activity, exploration and vocalization can be used as measures of emotionality (Rutherford et al. 2012).

Placenta has the role to protect the foetus in the prenatal period and therefore modulates stressful events experienced by the maternal organism, acting as a buffer effect (Jansson & Powell 2007). In mammals, the 11β -hydroxysteroid dehydrogenase enzyme type 2 (11β HSD2) in the placenta forms a barrier that protects the foetus from high levels of maternal cortisol because it oxidizes the biologically active form of cortisol in cortisone (Seckl 2004, Jansson & Powell 2007). Chronic stressful situations have the potential to inhibit the capacity to up-regulate the type 2 enzyme activity and the capacity to adapt placental 11β HSD2 is greatly reduced by previous exposure to chronic stress (Welberg et al. 2005), then reducing the protection capacity of the placenta.

In rats, both male and female offspring in the maternal enrichment group showed increased exploratory behaviour (Mychasiuk et al. 2012a). Positive prenatal experiences, in fathers and during development in mothers, decreases global methylation levels in the hippocampus and frontal cortex, changing offspring developmental trajectories (Mychasiuk et al. 2012a). However, it is reasonable to consider that the outcomes may not be a result of prenatal environment, since the effects from the mother-infant relationship and early social context have been previously demonstrated (Poletto et al. 2006, Mogi et al. 2011, Branchi et

al. 2013, Coulon et al. 2013). Environmental enrichment during gestation could change the mothers' behavior, or even alter traits like anxiety. In this sense, the effects could be related to the mothers' behavior during lactation instead of an impact of prenatal exposure. An alternative explanation for the effects in the offspring is the early postnatal period, in which the HPA-axis altered during gestation releases high concentrations of cortisol, and as a consequence changed the brain.

Epigenetics process driving foetal programming

The term epigenetics refers to chemical modifications to chromatin that regulate genomic transcription, in which the Greek prefix “epi” means “on top of”. Epigenetic modifications can be stable and cross generations, but they are also dynamic and change regarding to the environmental signals and stimulus (Cao-Lei et al. 2017). There are three main epigenetic mechanisms that include DNA methylation (covalent modification of cytosine with a methyl group), histone modification (acetylation, methylation, phosphorylation, ubiquitination) and microRNA (small non-coding RNAs with post-transcriptionally regulate gene expression) (Cao-Lei et al. 2017).

Epigenetic modification of chromatin, including DNA methylation at the sites of CpG dinucleotides, is a key regulator of gene expression, growth and differentiation in virtually all tissues, including brain (Tsankova et al. 2007, Siegmund et al. 2007, Stankiewicz et al. 2013, Mychasiuk 2015, Liu et al. 2015, Ju et al. 2018, Dall'Aglio et al. 2018). Changes in methylation status at selected genomic loci might affect social cognition (Isles et al. 2006), learning and memory (Miller & Sweatt 2007, Gräff & Mansuy 2008), and stress response (Cao-Lei et al. 2017, Kader et al. 2018a, DeSocio 2018). However, the transcriptional silencing is considered mainly as an outcome of hypermethylation of gene promoter regions (Zhang & Ho 2011). Furthermore, hypermethylation is associated with dysregulated gene

expression in a range of psychiatric disorders (Dudley et al. 2011, Millan 2013, Burns et al. 2018, Kader et al. 2018b), such as autism (Cattane et al. 2018, Dall'Aglia et al. 2018), schizophrenia (Cattane et al. 2018), depression and Alzheimer's disease (Chouliaras et al. 2010, Griñán-Ferré et al. 2016, 2018).

From an evolutionary perspective, epigenetic changes provides to the organism a mechanism for instantaneous adaptation. Epigenetics permeates all aspects of development, especially in the brain. Although changes in the brain/neuroplasticity can be transient (fluctuations in neurotransmitters) or persistent (alterations to dendritic morphology or synaptic pruning), each modifications requires epigenetic activity (Mychasiuk 2015). Moreover, heritable epigenetic changes facilitate rapid adaptation to adverse environmental conditions, but may also result in a mismatch of physiological profiles to later-life challenges, thus enhancing disease risk (Cao-Lei et al. 2017). The effects of prenatal stress on brain structures such as the hippocampus and amygdala generate changes in offspring's emotionality (Abe et al. 2007, Nolvi et al. 2016). Neuroepigenetics field has expanded and it is now clear that epigenetics processes, such as DNA methylation, are mechanisms involved in normal and pathological brain function (Delgado-Morales & Esteller 2017, DeSocio 2018, Dall'Aglia et al. 2018, Mulligan 2018). In order to perform DNA methylation analyses one current widely used method is by combining a genotyping followed by sequencing (Pétille et al. 2016) with methylated DNA immunoprecipitation (Guerrero-Bosagna & Jensen 2015). This method reduces the genome by digestion at restrict sites unrelated to CpGs and CCpGG, unbiased to CpG islands.

Although there is some data elucidating the transgenerational effect of environmental enrichment on the gene expression of the brain (Mychasiuk et al. 2012b), there is no studies addressing to the stereotypes effects during prenatal programming.

Conclusion

Stereotypic behaviour expressed by the mother could potentially changes the phenotype of their offspring, specially emotionality. Stereotypies are widely used as animal welfare indicator, and the expression of the behaviour can tell us about psychological states in animals. However, it is still controversial if the animal that express stereotypies are better adjusted or under more compromised welfare states than the ones that do not show the behaviour. Environmental enrichment reduces stereotypic behaviour by attending the requirement of some motivational systems, increasing the welfare of the offspring. Finally, we propose that one of the mechanisms changing phenotype of the offspring in response to enrichment and stereotypies is methylation in the limbic system in the brain.

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Does high stereotypic behavior expression affect productivity measures in sows?

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Abstract

The goal of the study was to evaluate the effects of stereotypic behavior on productivity measures in sows. Frequent expression of stereotypies by pigs has been reported, even when pigs are not maintained in crates. This behavior is only observed in animals kept in captivity and is a wide range welfare indicator, since is considered to develop in situations of chronic stress or frustration. When comparing groups of sows showing low or high frequency of stereotypy expression, we found that stereotypic behavior did not affect productivity. Moreover, did not affect physiological welfare indicators, such as salivary cortisol concentration or glucocorticoids in the placenta. There were no differences in productivity parameters, including weight of sows, weight of piglets, number of piglets weaned, or piglet mortality. Our study was conducted in group-housing pens, where the sows were not confined. Even in this condition which is considered by increasing welfare, the barren environment did not meet the behavioral and sensory needs of sows, which were naturally selected to perform complex behavioral repertoire and to spend part of their time engaging in exploratory behavior.

Keywords: glucocorticoids; pigs; placenta; stereotypies; welfare.

Introduction

It has been reported that sows express stereotypic behavior (or stereotypies) when housed in pig production systems. This behavior arises in situations of chronic stress or frustration, but can also result from a past situation or an existing conflict (Dantzer, 1991; Mason, 1991; Lawrence and Terlouw, 1993). Expression of stereotypies is considered a welfare indicator (Dantzer, 1991; Mason, 1991; Zhang et al., 2017), since the performance of such behavior reveals information on the psychological state. This behavior is often observed in farm animals kept in intensive production systems, since their environments cannot satisfy all their needs. Factors such as recurrent and long-lasting environmental constraints (Zhang et al., 2017), as well as genetic predisposition (Schoenecker and Heller, 2000; Schwaibold and Pillay, 2001), may constitute a cumulative cascade of predisposing factors in stereotypic behavior. In some cases, stereotypies are in agreement with other indicators of reduced welfare, including changes in physiological indicators (Zhang et al., 2017).

Stereotypic behavior has been used as an animal welfare indicator in protocols used to assess farm animals (e.g., Welfare Quality® and Animal Welfare Indicators [AWIN] protocols). However, few studies address the effects of stereotypies on productivity outcomes. Such investigations could improve the understanding of the consequences of this behavior and provide an argument for improving animals' environments, thus reducing stereotypies (Borell and Hurnik, 1990; Williams and Randle, 2017).

Cortisol concentration has been widely used as a physiological indicator of animal welfare since chronically elevated levels of this hormone can negatively affect an animal (Koolhaas et al., 2011). This glucocorticoid can be measured in the placenta, possibly causing alterations in brain development of the offspring. One possible cause is the cortisol concentration in the mother and the activity of 11 β -hydroxysteroid dehydrogenase enzyme in the placenta (Abe et al., 2007; Nolvi et al., 2016; Seckl, 2004).

The question is, how does stereotypic behavior affect animal welfare and productivity? It is important that factors scientists consider key to farm animals' welfare coincide with improvements in productivity, making it easier to convince producers to revise their practices. If scientists fail to demonstrate that improvements that enhance animal welfare result in economic gains, the changes will only occur as a result of moral values and ethical requirements. Our goal was to evaluate the physiological welfare indicators and productivity implications of stereotypy expression by sows.

Material and Methods

This study was approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (CEUA/FMVZ; protocol number 3606300114). The study was conducted in an experimental pig farm at Campus Fernando Costa, School of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga, Brazil.

A total of 28 pregnant sows from a group of 36 animals were used for this study (8 sows returned to oestrus). The sows were TopGen Afrodite® sows of nulliparous lineage (Farm Araporanga, Jaguariaíva, Paraná, Brazil). A parallel experiment involving the same animals evaluated the effect of dietary fiber. The animals were distributed by weight into two treatments (diets with high or low fiber). The sows were inseminated with pooled semen. The insemination protocol comprised three inseminations: one at the time of estrus identification, the second at 24 h after the first insemination, and the third at 36 hours after the first insemination. The average age of animals at the first insemination was 291 (\pm 21) days.

Sows in both groups were kept in a group-housing system during pregnancy, with nine animals per pen, individual feeders, and water *ad libitum*. Immediately prior to feeding, there was a sound stimulus to reduce the anticipatory responses related to food and to the presence

of humans. Animals were confined in individual crates during feeding. Sows had no access to water for 20 minutes during feeding because of a shared food–water connection that could have mixed water with the food. Thirty minutes after the start of feeding, all the animals were released. The pen was 6.7 m wide by 4.4 m long, totaling 29.48 m² (3.27 m² per animal), excluding the area of the feeders. Each pen had nine individual gestation crates measuring 1.80 m by 0.55 m each, with masonry feeders and nipple-like drinkers for *ad libitum* access. The total area per animal was 4.38 m². The floors of the pens were solid concrete, and pen was covered with 12 rubber mats measuring 100 cm² and 30-mm high (EBV 30-Vedovati®). The pens were divided by a straight line consisting of 7 non-electrified wires, with an access gate for each pen. Pens were cleaned each morning. The mean temperature during gestation was 21.1 °C (maximum: 38.9 °C, minimum: 8.4 °C), and the mean humidity was 61.91%; rainfall during this period totaled 534.6 mm (March 26 through October 31, 2015). The average temperature during the lactation period was 22.7 °C (maximum: 38.9 °C, minimum: 8.5 °C). The mean humidity during the lactation period was 61.15%, and rainfall totaled 522.6 mm (August 1 through November 30, 2015). Meteorological data were obtained from the Fernando Costa Campus station, which is located approximately 1100 m from the pig farm.

To assess the stereotypic behavior (sham-chewing) of individual sows, behavioral observations were conducted throughout the pregnancy. There were gradients in expression of stereotypies by sows. To understand our data, we divided the sows into two groups: low level of stereotypical behavior and high level of stereotypic behavior (sham-chewing - Fig. 1). The sows (N = 28) were ranked by high and low rates of stereotypic behavior (N = 14 in each group); welfare indicators such as sow behavior, hypothalamic–pituitary–adrenal axis activity, and feeding motivation were analyzed. Moreover, production parameters were evaluated, including weight of sows at the end of gestation and during lactation period, piglet mortality, and piglet weight. Placenta glucocorticoid (cortisol and cortisone) levels were assessed

because this indicator can provide information on piglet development and possible fetal exposure to glucocorticoids.

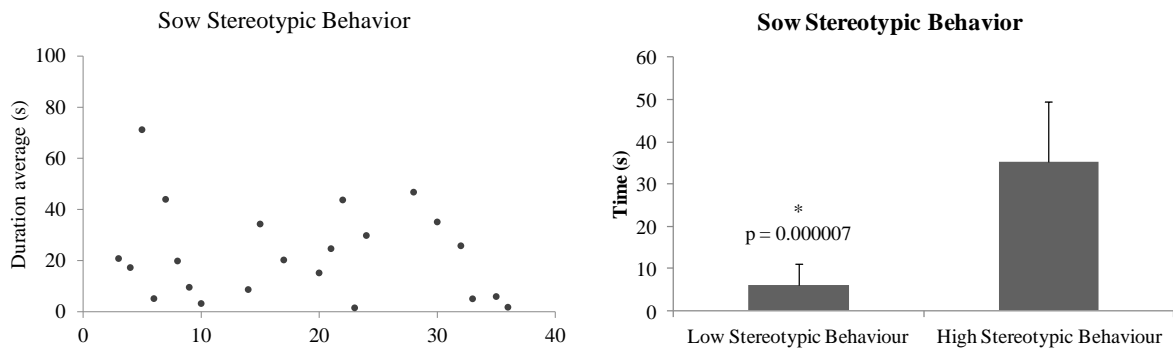


Figure 1. Sow stereotypic behavior expression (sham-chewing). N = 28 (pregnant sows group housed). (A) Sow distribution in the group indicating the individual variation of the expression of stereotypies, which was ranked from low to high rate; (B) difference between sows by level of stereotypic behavior (Mann-Whitney U Test: $p = 0.000007$; $Z = -4.50$).

An ethogram was adapted (Table 1; it was included different behaviors - Zonderland et al., 2004) for collection of behavioral data. Behavioral measures of sows were obtained by direct observation on days 29, 30, 31, 59, 60, 61, 74, 75, 76, 89, 90, and 91 for each group. Behavioral assessments were performed by direct observation at 1 hour before and 1 hour after the two feeding times, totaling four moments of observation by day. Five observers were previously trained to standardize the methodology and avoid bias in data collection. Observations were carried out using a combination of methods for behavioral measures; the process started with a scan sample, followed by continuous observation of a focal animal. At each observation episode, each animal was observed uninterrupted three times for 120 seconds at each period, totaling 6 minutes per animal per observation episode (before and after feeding), with a total of 24 minutes per day of observation. The observations were conducted over three consecutive days to avoid possible interference by stressful events (e.g.,

days 29, 30, and 31 during the first third of pregnancy, following the same observation protocol on three consecutive days).

TABLE 1 - Definition of behaviours for behavioural observation of pregnant gilts.

Behaviors	Definition
Sleep	Animal sleeping
Lying ventrally	Lying with the belly facing the ground with all the limbs under the body
Lying laterally	Lying sideways, with all the members extended laterally
Standing	Body supported by the four limbs
Sham chewing	Continuous chewing without the presence of visible food in the oral cavity
Rooting the floor	Snout touches the ground followed by head movements
Licking the floor	Tongue touches the floor and is followed by movements with the head
Interacting fence or gate	Biting or nibbling the fence wire or gate
Interacting with mats	Snout or tongue touches mats followed by head movements
Bites (E)	Bite on any parts of the body (tail, vulva, ear, body)
Facing (E)	Face to face, with fixed view to the other animal
Pushing (E)	Pushing another animal using the head or the muzzle
Vocalization (E)	Sound emission emitted by the animal
Rest	The sum of sleep, lying ventrally and lying laterally
Oral	The sum of sham chewing, rooting the floor, licking the floor, interacting with fence or gate and interacting with mats

The caption E indicates behaviours that were measured only the events and not the duration adapted from Zonderland et al. (2004).

A food motivation test was performed (based on Souza da Silva et al., 2013) in the final third of gestation. Initially, 2 kg of food was provided for each animal, and small extra portions of food were supplied gradually when almost all food had been consumed, with a

limit of another 2 kg of food. Therefore, the total amount of each test consisted of 4 kg of feed for each animal. However, to avoid the effect of palatability or volume, all animals, irrespective of treatment group, received 2 kg of mixed conventional and high-fiber feed. The test location was the usual feeding area, and feeding lasted for 45 min. The final weight of the second portion was measured to obtain the total weight of food consumed. Because of the shared food–water connection, access to water during the test was prohibited so accurate food measurements could be obtained.

Saliva collection was performed on the same days of the behavioral evaluation - that is, in first third of gestation (days 29, 30, 31), middle third (days 59, 60, 61), and last third (days 74, 75, 76, 89, 90, and 91). These days corresponded with the average gestational age, and the three consecutive days were used to avoid having possible undesirable variables interfere with the response. Two samples were collected per animal on each of these days: the first between 6:00 a.m. and 6:30 a.m., and the second between 6:00 p.m. and 6:30 p.m.; this timing followed the circadian rhythm of cortisol (based on Siegford et al., 2008). Samples were collected using two roller-shaped units of hydrophilic cotton (Apolo®) tied to a dental floss (DentalClean®) and presented to each animal. The animal chewed the cotton until it was saturated with saliva. The first sample collected was discarded; we repeated the protocol to collect only recently produced saliva. After the second sample was collected, it was introduced into a 15-mL falcon tube (Kasvi®). Subsequently, the tube was packed a cooler until the end of the collection, sent to the laboratory, and then frozen at -20°C until processing. Thawing took place in a container filled with ice in a temperature-controlled room. After complete thawing of the sample, it was centrifuged for 10 minutes at a rate of $1000 \times g$ (Celm Combate®), and then the supernatant was aliquoted into microtubes (Kasvi®) and again frozen at -20 °C until analysis. This process assisted in the removal of mucins and other components that could interfere with the analysis protocol. A 50 μ l sample was

analyzed by enzyme immunoassay in duplicate for each sow, pooling each gestation period and without mix the morning and afternoon collections (e.g., samples from 29, 30, and 31 gestation days in the morning collection). For sample analysis, 50 μ l of saliva was analyzed with a cortisol enzyme immunoassay (EIA – based on Cooper et al., 1989; Palme and Möstl, 1997) in duplicate for each sow, with a pool of each gestation period, without mixing the morning and afternoon collections (e.g. with samples from 88 and 89 gestation days in the morning collection). The sensitivity of the EIA was 0.2 pg/well.

The gilts were moved to the farrowing pens at, on average, 107 days of gestation. Farrowing occurred in individual pens measuring 4.3 m by 2.0 m each that contained material for building nests (hay and sugarcane bagasse) and iron bars to optimize protection of piglets from crushing. The creeps were made of concrete, and each was 0.9 m by 2.2 meters; pigs kept in the creeps had access to solid food from birth. Creep floors were lined with sugarcane bagasse, and heat was supplied with 100-W incandescent bulbs. Deliveries were monitored by video cameras with internet access (allowing observers to monitor the beginning of each delivery), followed by direct observation. Interventions were performed only when needed, following a pre-established protocol enabling standardized management measures. Assistance consisted of palpation when the interval exceeded 1 hour and administration of injectable synthetic oxytocin when absence of observed contractions exceeded 1 hour (Placentex® 2 mL subcutaneous). Piglets had their teeth ground and ears notched under local anesthesia in order to minimize pain. Iron application, ear notching, and individual weighing were performed when piglets were 2 days old.

The placentas were collected from four piglets per sow; four random pieces of each placenta were cut and frozen at -20 °C. All placentas from each sow were macerated together to prepare a pool representing the sow. Since the placenta was powdered, 0.1 g was placed in a 1.5-mL microtube; 200 μ L of ultrapure water was then placed in the tube, and the mixture

was homogenized in a vortex for 15 s. After vortexing, 20 μ L was placed in another similar tube for total protein analysis; 1 mL ethyl acetate was placed in this tube with the water and placenta, vortexed for 15 seconds, and centrifuged for 15 minutes at 4 °C. Following centrifugation, 400 μ l of supernatant was placed in a new 1.5-mL microtube, and a second (duplicate) sample was placed in another tube. All samples were dried overnight in a hood until all volume disappeared. To analyze glucocorticoid levels, all samples were re-suspended with the same volume using assay buffer. The analyses were performed with the same enzyme immunoassay protocol for saliva cortisol (Section 2.5).

Residual normality of data was determined by Shapiro-Wilk test. Statistical tests are specified in the respective figures and significance was set at $p < 0.05$. Data that did not show normality was tested with a corresponding nonparametric statistical test. Mann–Whitney U test was used to analyze sow stereotypic behavior, saliva cortisol, piglet weight, and piglet mortality. Tukey’s test was used for post-hoc analysis of piglet weight. The T test was used to assess feeding motivation of sows and their weight. We also performed correlation tests between stereotypic behavior and saliva cortisol concentration, and between stereotypic behavior and other behaviors. The difference between treatments were assessed according to the following model:

$$y_{ij} = \mu + T_i + a_j + e_{ij},$$

Y_{ij} is the dependent variable, μ is the mean, T_i is the fixed effect of treatment (low or high rate of stereotypic behavior expression), a_j is the random effect of sow, and e_{ij} is the residual error with mean 0 and variance σ^2 .

Results

There were gradients in expression of stereotypes by sows. To understand our data, we divided the sows into two groups: low level of stereotypical behavior and high level of stereotypic behavior (sham-chewing - Fig. 1). There was no difference in sow feeding between low- and high-stereotypic behavior sows (Fig. 2A); hunger can be a main trigger of sham-chewing (Appleby and Lawrence, 1987). At the beginning of gestation, sows with high levels of stereotypical behavior had higher cortisol levels (Fig. 2C); however, the difference was observed only in the morning collections. During the other gestation periods, the cortisol concentration in saliva was the same in both groups; there were no differences in afternoon samples (Fig. 2D). There was no difference between stereotypy groups regarding placenta glucocorticoid concentration (Fig. 2B); sow weight at the end of gestation; sow weight at the lactation (Fig. 3A); number of weaned piglets (Fig. 3B); or piglet mortality (Fig. 3C), or piglet weight before weaning at days 1, 21, and 28 of life (Fig. 3D).

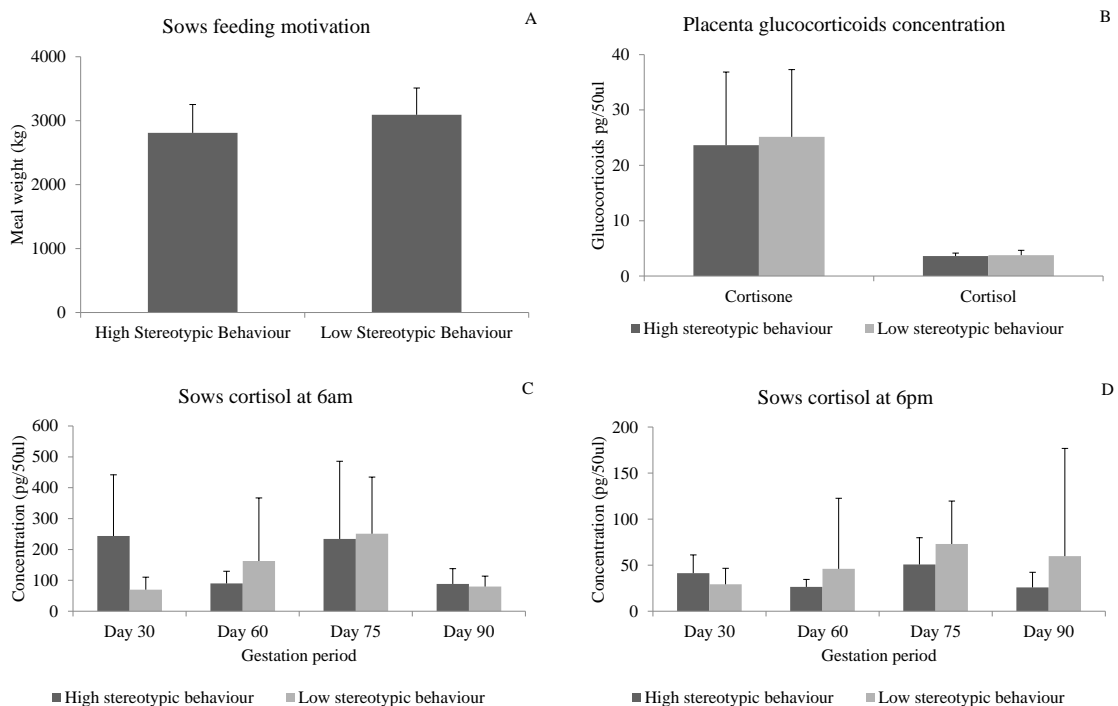


Figure 2. Sow welfare indicators. N = 28 (pregnant sows). * indicates difference in salivary cortisol between treatments on gestation day 30, at 6:00 am. T test: sow feeding motivation ($p = 0.09$; $F = 1.11$); placenta cortisone ($p = 0.63$; $F = 2.43$); placenta cortisol ($p = 0.55$; 1.01). Salivary cortisol data were analyzed using Mann-Whitney U test: there was a significant difference in sow cortisol at 6:00 am on day 30 ($p = 0.01$; $Z = 2.38$) and a tendency at 6:00 pm ($p = 0.07$; $Z = 1.77$). There were no differences in the morning on day 60 ($p = 0.36$; $Z = -0.89$); day 75 ($p = 0.58$; $Z = -0.55$); or day 90 ($p = 0.90$; $Z = -0.12$). There were no differences in the afternoon on day 60 ($p = 0.71$; $Z = 0.36$); day 75 ($p = 0.19$; $Z = -1.28$); or day 90 ($p = 0.52$; $Z = 0.63$).

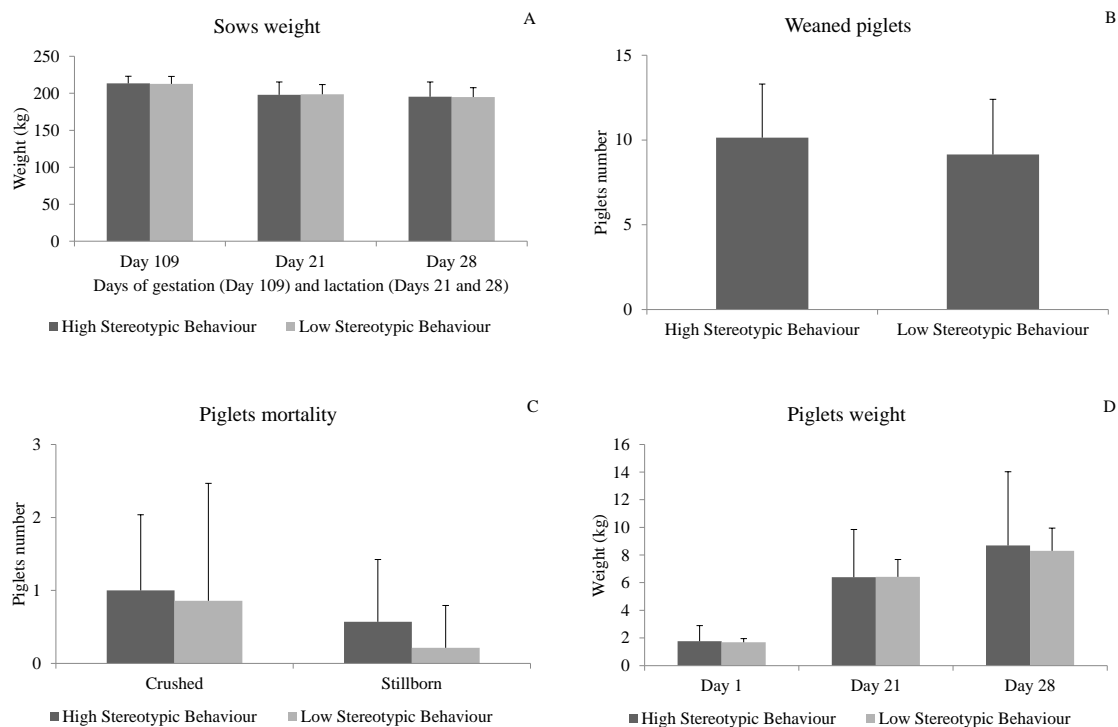


Figure 3. Productivity indicators. N = 28 (pregnant female housed in collective pens) and their respective litters. The sows' weights were the same on day 109 of gestation (T test: $p = 0.90$; $F = 1.06$) as on day 21 of lactation (T test: $p = 0.32$; $F = 1.75$) and on day 28 of lactation (T test: $p = 0.15$; $F = 2.40$). There were no differences in the number of weaned piglets (T test: $p = 0.91$; $F = 1.06$), piglet mortality by crushing (Mann-Whitney

U Test: $p = 0.38$; $Z = 0.94$), or stillborn piglets (Mann–Whitney U Test: $p = 0.12$; $Z = 1.53$). There was no difference in piglet weight on the first day of life (Mann–Whitney U Test: $p = 0.19$; $Z = -1.31$); on day 21 (Mann–Whitney U Test: $p = 0.34$; $Z = -0.93$), or on day 28 (Mann–Whitney U Test: $p = 0.68$; $Z = -0.40$).

There was no correlation between stereotypic behavior and salivary cortisol concentration (Spearman correlation: $p = 0.76$; $r = 0.021$) during gestation, in any period. A correlation analysis was performed between stereotypic behavior duration and each behavior. There was a correlation only with inactivity ($p = 0.05$; $R^2 = 0.13$), but not with activity ($p = 0.46$; $R^2 = 0.02$), foraging ($p = 0.55$; $R^2 = 0.01$), exploratory behaviors ($p = 0.86$; $R^2 = 0.001$), or agonistic behaviors ($p = 0.81$; $R^2 = 0.002$).

Discussion

We showed that stereotypic behavior does not affect physiological welfare indicators or productivity. However, we showed that expression of these behaviors does not affect the production outcomes we analyzed (only one delivery). Contrary to our data, there are suggestions in the livestock industry associating stereotypic behavior expression and reduced economic value of animal production (Borell and Hurnik, 1990). Stereotypic expression by sows during gestation has been associated with reproductive fertility, in which an increase in occurrence of stereotypic behavior is linked with a reduced number of piglets born (Borell and Hurnik, 1990). Despite this, few studies have evaluated this issue (Williams and Randle, 2017).

Due to the fact that the stereotypic behavior we analyzed was an oral one, we considered it could be associated with food motivation. In this case, we would expect that sows with lower levels of satiety would express more stereotypic behaviors (Appleby and

Lawrence, 1987), as frustration outcome of not reached motivation. However, we did not find any effect of stereotypic behavior on food motivation. The meal that sows received during pregnancy represented only 50% to 60% of their *ad libitum* consumption capacity (Lawrence et al., 1988).

There was no difference in placenta glucocorticoids between stereotypic behavior groups. During stress responses, endocrine mechanisms are activated to enhance an individual's fitness, activating primarily the release of glucocorticoids and catecholamines (Möstl and Palme, 2002). Cortisol is a glucocorticoid hormone frequently used as an animal welfare indicator because it is secreted by the adrenal glands as a response to adverse situations (Möstl and Palme, 2002). Cortisone was measured by the activity of the enzyme 11 beta-hydroxysteroid dehydrogenase, which in turn converts cortisol into the inactive biologically molecule cortisone (Seckl, 2004; Jansson and Powell, 2007). The presence and activity of this enzyme have been related to maternal exposure to stressors during pregnancy (Jansson and Powell, 2007). Thus, if stereotypic behavior is an indicator of compromised welfare, we would expect to find differences in the concentrations of at least one of these glucocorticoids in the placenta (indirectly indicating the activity of the 11 beta-hydroxysteroid dehydrogenase enzyme). Moreover, stereotypic behavior may be associated with other physiological mechanisms, including opioids (Cronin et al., 1985; Zanella et al., 1996).

The absence of significance differences in placental glucocorticoid concentrations is consistent with salivary cortisol concentrations during gestation. On day 30 (days 29, 30, and 31) of gestation, there was a difference in salivary cortisol concentration. On that day, sows that expressed more stereotypic behavior had higher concentrations of salivary cortisol ($p = 0.05$); a tendency was observed in the afternoon samples ($p = 0.07$). The concentrations did not differ between groups for the rest of gestation. A possible explanation for this result is fear because there was intense interaction with humans in the first saliva collection. After that, the

concentrations were the same due to habituation resulting from frequent contact with humans. These physiological responses in which cortisol concentrations were higher in sows expressing more stereotypic behavior may be related to individual variation (Ijichi et al., 2013; Joshi and Pillay, 2016). However, there was no correlation between stereotypic behavior and salivary cortisol concentrations throughout the pregnancy, with differences in concentrations observed only in the initial third of gestation. The possibility that stereotypic behavior reduces stress would be corroborated if the difference in concentrations persisted. However, these data were consistent with previous studies that showed expression of stereotypic behaviors does not alter cortisol concentrations (Terlouw et al., 1991).

In terms of productivity data, there was no difference in the weight of the sows at the end of gestation (day 109) or in lactation (days 21 and 28). In addition, there was no difference in the number of weaned piglets, piglet mortality, or piglet weight. Thus, we showed that the expression of stereotypic behaviors does not compromise productivity parameters in pigs. However, genetic improvement has selected maternal breeding with characteristics based on productivity; consequently, although conditions are not optimal, the sows exhibited high productivity indexes. Another factor that may interfere with the results is the age of the animals. Some studies have shown that older sows have higher concentrations of serum cortisol, cytokines, and sham-chewing behavior (Zhang et al., 2017). However, the sows in our study were homogeneous in age, lineage, and body weight. Moreover, experiences during development were similar, such as environment in which their mothers were maintained during gestation, weaning, handling, and transport.

Scientific evidence to date does not fully elucidate what it means, in terms of animal welfare, to express stereotypies. Our questions were based on whether we consider animals equally challenged (excluding the fact that environmental challenges affect each individual differently) or whether the one who expresses stereotypic behaviors is worse than the one who

does not. On the other hand, is the expression of these behaviors a way of mitigating and dealing with external stressors? Alternatively, are stereotypic behaviors merely a way of dealing with the input reduction of stimuli to the sensory system? We believe that expression of stereotypic behaviors is a strategy to deal with environmental challenges and that this expression improves welfare. In this sense, stereotypic behavior is a sign that basically tells us there is a high likelihood that welfare is compromised because the trigger (challenges) exists. Although only some animals use this strategy, those that express stereotypic behavior fare better than those that do not express.

There is concern that if we cannot convince producers that economic losses are related to stereotypic behavior and that changes are needed for profitable reasons, we will rely only changes made for ethical reasons. This takes much longer because it requires moral concern from consumers and society. Apart from this, our work was conducted in group-housed pens, where the sows are not as confined as in gestation crates. However, even though the sows were housed in better conditions, the poor environment does not meet the sensory needs of these animals that have been naturally selected to perform much more complex activities and to spend part of their time in exploratory behaviors. Above all, some of these systems have sows spend the first month confined in crates that have since been abolished and then regrouped with unknown sows because they are selected by body score, ultimately resulting a high frequency of agonistic behaviors. Although pig production systems have advanced in terms of animal comfort, they still do not meet the animals' needs.

Conclusion

Stereotypic behavior does not affect physiological welfare indicators or productivity. However, genetic improvement has selected maternal breeding with characteristics based on productivity; consequently, although conditions are not optimal, the sows exhibited high

productivity indexes. We believe that expression of stereotypic behaviors is a strategy to deal with environmental challenges and that this expression improves welfare. In this sense, stereotypic behavior is a sign that tells us that welfare is compromised because the trigger (challenges) exists. Although only some animals use this strategy, those that express stereotypic behavior fare better than those that do not express. Based on these considerations, studies are still needed to optimize the way pigs are raised, especially in cases such as breeding sows where they will be kept for a long period of life. Moreover, the association between stereotypic behavior and welfare remains unclear; there is a possibility that animals that express stereotypic behaviors are coping better with a low-stimulus environment than are animals that do not express such behaviors.

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Stereotypic behaviour in sows modifies emotionality in the offspring

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Abstract

Some effects of expressing stereotypic behaviour have not yet been elucidated. During gestation, the environment has the potential to interfere with offspring development and to have prenatal or longer-term consequences. We tested the hypothesis that the occurrence of stereotypic behaviour during gestation could affect the phenotype of the offspring. Twenty-eight pregnant sows were studied, by comparing two groups differing in the amount of stereotypy shown. We analysed emotionality in the offspring from sows showing high or low stereotypy frequency using open field and novel object tests. In the open field test, piglets from sows with a high rate of stereotypies walked more in central quadrants ($p < 0.0001$) and lateral quadrants ($p = 0.04$) than piglets from sows with low rate of stereotypies. In the novel object test the offspring from low stereotypy sows vocalized more ($p = 0.008$). We demonstrate for the first time that stereotypic behaviour by the mother during gestation changes the phenotype of the offspring, in particular their emotionality.

Keywords: emotionality; fear tests; gestation; piglets; prenatal; stereotypic behaviour.

1. Introduction

Stereotypic behaviour, or stereotypy, is repetitive and apparently functionless and often develops in environments that cause poor welfare (1). This behaviour develops in animals kept in environments with few stimuli, physical restraint, fear or frustration (1). In farm animals this behaviour can be widely observed, since the environment does not provide for their needs. The occurrence of stereotypies suggests frustration of highly motivated behaviours, components of which may occur even in the absence of the appropriate stimulus. However, the expression varies among individuals kept in a common environment. Stereotypic behaviour has been described in a wide range of species in artificial environment. Furthermore, the stereotypic behaviour expression is often considered a welfare indicator (1–3), since it can tell us about psychological states that are difficult to evaluate. However, this specific behavioural indicator does not correlate well with cortisol (4) which is mainly an indicator of short-term welfare problems.

In order to use stereotypies as indicator, it is necessary to understand the causal factors. Frustration, associated with motivation to show a behavioural or physiological change that is blocked in some way, is one of the most consistent causal factors. Furthermore, the kind of stereotypy can be associated with specific frustration. For example, frustration related to food restriction can trigger oral stereotypies in sows (5). There is also a genetic component (6) and a personality predisposition (7,8) for expressing stereotypies. In this sense, there is a synergic effect of internal and external variables that trigger this strategy for coping with problems. Some variables have more impact on triggering the stereotypy than others if we consider a range of environmental factors (9).

What is the consequence to the stereotypy, in the long-term for the animals? Male mink that showed more stereotypy had lower success in copulation (10). However, when animals were developing in environmentally enriched conditions, the environment could have affected the ontogeny, physiology of stress, physiological reproductive mechanisms, social behaviour and flexibility (10), increasing the difference from animals kept in a barren environment and showing stereotypies.

Regarding to the long-term effects of stereotypy, how does it change the offspring phenotype? In mammals, pregnancy has an important role in shaping the organisms. The mother's environment may have effects on the offspring. This concept comes from the “thrifty phenotype hypothesis”, in which the neurodevelopment reprogramming induces alterations to cope with the initial environment, anticipating postnatal environment (11). In other words, the prenatal environment has the potential to adjust the offspring phenotype and prepare individuals for the environment into which they will be inserted so that they are prepared to cope better with the challenges. The environment in which an animal is maintained during gestation may result in changes in several offspring qualities (12–17). By this mechanism, factors such as emotional reactivity, responsiveness to stressors and cognition can be modulated by challenges in the prenatal and neonatal periods (16,18,19). Studies have shown that some stressors such as negative interactions with the handler (16,17,20) and social stress (16) have altered emotional reactivity, social behavior, and responsiveness to stressors, cognition and memory in the offspring. Moreover, sows experiencing less hunger during gestation have offspring with reduced aggressive behavior (21).

One of the mechanisms that can affect the emotionality of the offspring during development is the maternal excess of glucocorticoids which can affect important brain

structures and generate negative effects (16,17,20). Glucocorticoids are important stress hormones in adult animals but also have other functions in both adult and fetus. Fetal effects are completely different depending on gestational age, severity and duration of the exposure (22). The effects of prenatal stress on brain structures such as the hippocampus and amygdala may generate changes in offspring's emotionality (23,24). To access the emotionality of non-human animals, some tests have been validated and discussed (25). These include the open field test and the novel object test (25–28), in which behaviours such as activity and vocalization can be used as measures of emotionality (27). Our goal was to evaluate the consequences of stereotypies during gestation for the emotionality of the offspring. As far as we know, this is the first approach relating stereotypic behaviour in the mother during gestation to the phenotype of the offspring.

2. Material and Methods

2.1. Animals

The study was conducted in the production unit of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo, at the Fernando Costa's Campus, in Pirassununga. This study was approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (CEUA / FMVZ - protocol number 3606300114).

Twenty eight pregnant sows were used, from a group of thirty-six animals, nulliparous TopGen Afrodite® (Farm Araporanga - Juaguariaíva-PR). A parallel experiment involving the same animals, evaluated the effect of dietary fiber during gestation on their welfare and on the offspring. The animals were distributed by weight into two treatments (diets with high or low fiber). The sows were inseminated with pooled semen with an age average of 291 days.

Sows were group-housed in pens, with nine animals per pen, with individual feeders and water provided *ad libitum*. Immediately prior to feeding, there was an auditory signal to reduce anticipatory response related to food and to the presence of humans. During the feeding time animals were confined in individual crates. During feeding, sows had no access to water for 20 minutes due the fact that the feeders had communication among them, and water would mix different types of food. Thirty minutes after the beginning of feeding all the animals were released. They were fed twice daily, at 8:00 and 15:00 hours. The pen was 6.7 meters wide by 4.4 meters long, measuring 29.48 m² (3.27 m² per animal), disregarding the area of the feeders. The total area per animal consisted of 4.38 m². The floor of the pens was solid concrete and covered by 12 rubber mats with 100 square centimeters and 30 mm high (EBV 30 - Vedovati®).

2.2. Experimental Design

To assess the effects of stereotypic behaviour throughout pregnancy in the offspring, the sows were ranked from high to low rate of stereotypies (N = 28 sows). The 28 sows were divided in two groups N = 14 each group, which 14 sows showed low rate of stereotypic behaviour and 14 sows showed high rate of the behavior. For this, the sows were ranked in the average duration of expression of stereotypies, and the 14 first sows in the ranking were considered “low rate”, the others 14 sows in the ranking were considered “high rate”. The results of piglets in relation to emotionality was analysed, based on the behavioural results of the open field and novel object tests of four couples of piglets per sow. Principal Component

Analysis (PCA) was performed in order to guarantee that there was no effect of the kind of diet on the data considering stereotypic behaviour.

2.3. Behavioural data

In order to collect behavioural data, an ethogram was adapted (from (29)). Behavioural measures of sows were obtained by direct observation on days 29, 30, 31, 59, 60, 61, 74, 75, 76, 89, 90 and 91, consisting of the average gestational age for the sows kept in the same pen in each period. The behavioural assessments were performed by direct observation of the two different moments, one hour before and one hour after feeding, totalling four periods of observation each day. Five observers were previously standardized to avoid bias in data collection. Observations were carried out using a combination of methods for behavioural measures, which started with a scan sample, followed by focal animal with continuous observation. At each time of observation, each animal was observed three times per uninterrupted 120 seconds, totaling 6 minutes per animal per observation time (before and after feeding), with a total of 24 minutes per observation day. The collection periods were conducted over three consecutive days to avoid possible interference of stressful events (e.g. 29, 30 and 31 in the evaluation of the first third of pregnancy, following the same protocol on two consecutive pregnancy period).

2.4. Farrowing

Parturition happened in individual pens, measuring 4.3 by 2 meters each, with available material for nest-building (hay and sugarcane bagasse) and iron bars, at the perimeter of the pens, in order to optimize the protection of piglets against crushing. Pens had a creep made of concrete, measuring 0.97 by 2.2 meters each, where the piglets had access to solid food since birth. The creep also had a bed composed of dehydrated sugarcane bagasse and the heat source was a 60 watt incandescent lamp. Farrowings were monitored using video cameras, with access via the Internet, followed by direct observation after the onset of farrowing. Interventions were performed only when necessary, following a pre-established protocol, allowing standardization of management procedures. The assistance consisted of palpation when the interval between births exceeded one hour and administration of injectable synthetic oxytocin when absence of contractions observed exceeded one hour (Placentex® - 2 ml intramuscular). Piglets had their teeth ground and ears notched with local anesthesia in order to minimize pain, as it was a standard operation procedure at the farm. Iron application, ear notching and individual weighing were performed when piglets were one-day-old.

2.5. Weaning and fear tests in piglets

Piglets were weaned at 28 days of age. At weaning, they were kept in four suspended pens, each pen consisted of four animals: a pair of male and female from each sow, totaling eight piglets per sow being used in post-weaning studies divided into four pens. Piglets had access to food and water *ad libitum*. Each animal was individually identified, using a marker of non-toxic and non-permanent ink. The observer who collected and registered piglet behaviour was not aware of the treatment of the mother sows.

Tests for assessing emotionality were conducted by a combination of open field and novel object test (30,31), that can indicate the levels of fear and exploratory motivation of each animal. The tests were performed at 30 days of age, four pens being tested on the same day. Piglets were tested one by one, returning them to their home pen, being removed one by

one sequentially from the pens, so that the absence of an individual of the group remained balanced over time. The combination of tests allowed a prior habituation of piglets in the test arena, wherein the open field test preceded the novel object test. The test arena (2.37 m x 4.85 m) contained soil demarcations forming quadrants throughout the pen. Each test lasted five minutes totaling 10 minutes per pig. Each piglet was gently placed in a predetermined location in the test arena and recorded during the test period. The behaviours that were quantified were: latency to walk, number of central and lateral quadrants crossed, time in activity (walking), time in freezing and vocalizations (events). After this test, a novel object was inserted (empty polypropylene yellow bucket) by a pulley system in the center of the arena. Subsequent behaviours were recorded for five minutes. In this test, we evaluated the latency to walk, time close to the object (in the quarters surrounding the object), time exploring the object (near to the object with the head turned to it), time in freezing and vocalization (events). Although in our study vocalizations have not been analysed with appropriate bioacoustical equipment, recordings were short low intensity vocalizations, that is completely different to that long and loud ones, that we can usually observe during painful procedures (such as castration). After each animal had been tested, the pen was washed with water to reduce and remove possible chemical cues and feces and urine of the animals. The pen was also washed before the first test, in order to standardize the entries of the piglets in the wet test arena.

2.6. Data analysis

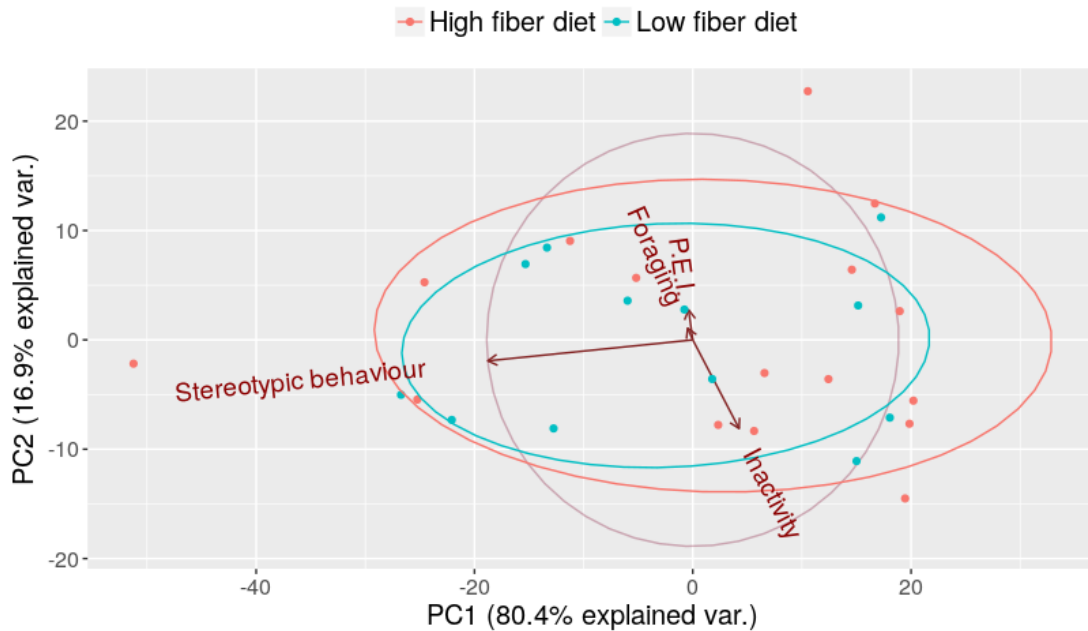
Behavioural units were grouped in order to create categories for the sows, as follows: inactivity (sleeping, lying ventral and lying laterally); foraging (licking the floor, rooting empty feeder); physical environment interaction (rooting floor, interacting with the fence and the gate, interacting with rubber mats); stereotypies (sham-chewing). Within these categories, we performed the Principal Component Analysis (PCA) to verify which of them explained more of the variance in the data, ensuring that the different diet in the parallel experiment was not affecting the groups. Based on this, we selected the category "stereotypic behaviour" and analysed the effect of its variation in the emotionality of the piglets. We tested data normality by the Komogorov-Smirnov test. Statistical tests are specified in the respective figures and the critical level was $p \leq 0.05$. Data that did not show normality were tested with a corresponding nonparametric statistical test.

3. Results

The PCA shows 97.3% of the variation within the first two principal components, being 80.4% of the explained variation in principal component 1 (PC1) and 16.9% at PC2 (Figure 1). Stereotypies was the variable that most explained individual variation, 99.7% of the variation in PC1 and PC2 (Table 1). The biggest vector also shows that stereotypies explained the individual variation and this evidence reinforce the importance of these behaviours as indicators of animal welfare. As seen in Figure 1 the points correspond to the PC1 and PC2 scores of each sow and the ellipses indicate 68% of each fiber diet. One ellipse is inside the other indicating that the low fiber diet and high fiber diet did not influence the behaviours. It is relevant to indicate that the parallel treatment does not split the groups of sows regarding with the diet they received during gestation.

TABLE 1. The explanation proportion of the behaviours in the variation of pc1 and pc2.

Variables	PC1 (%)	PC2 (%)	Total
Inactivity	4.9	84.2	89.1
Foraging	0.1	1.5	1.6
Physical environmental interaction	0.0	9.6	9.6
Stereotypic behavior	95.0	4.7	99.7

**Figure 1. Principal Component Analysis.** PCA evaluated in relation to behavioural categories. (N = 28 pregnant sows group housed; P.E.I. corresponds to physical environmental interaction).

In the open field test (Figure 2) there was difference in the number of central quadrants ($p = 0.000$) and lateral quadrants ($p = 0.04$) in which the piglets from sows with high rate of stereotypies walked more over quadrants in relation to piglets from sows with low rate of stereotypies. In both tests, no piglets showed freezing behaviour.

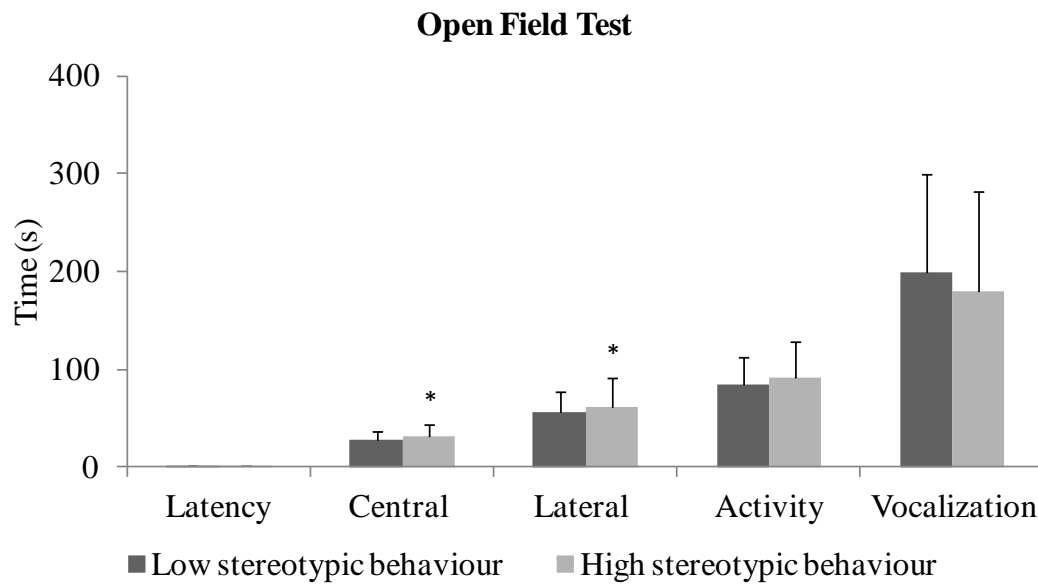


Figure 2. Open field test in the piglets. (N total = 142 N = 76 piglets from sows with low rate of stereotypy and N = 66 piglets from sows with high rate of stereotypy). * indicates difference that is in central quadrants (T test; $p = 0.000$; $F = 0.00$) and lateral quadrants (T test; $p = 0.04$; $F = 0.00$). There is no difference in the latency (Mann-Whitney U test; $p = 1.00$; $Z = 0.00$); activity (T test; $p = 0.54$; $F = 0.00$) and vocalization (T test; $p = 0.34$; $F = 0.00$). The variable time considered in the y axis is the average of duration in each behavior. The vocalization is indicated by the frequency.

In the novel object test (Figure 3) piglets from sows with low rate of stereotypies vocalized more ($p = 0.008$) than piglets from sows with high rate of stereotypies. The other variables did not differ significantly.

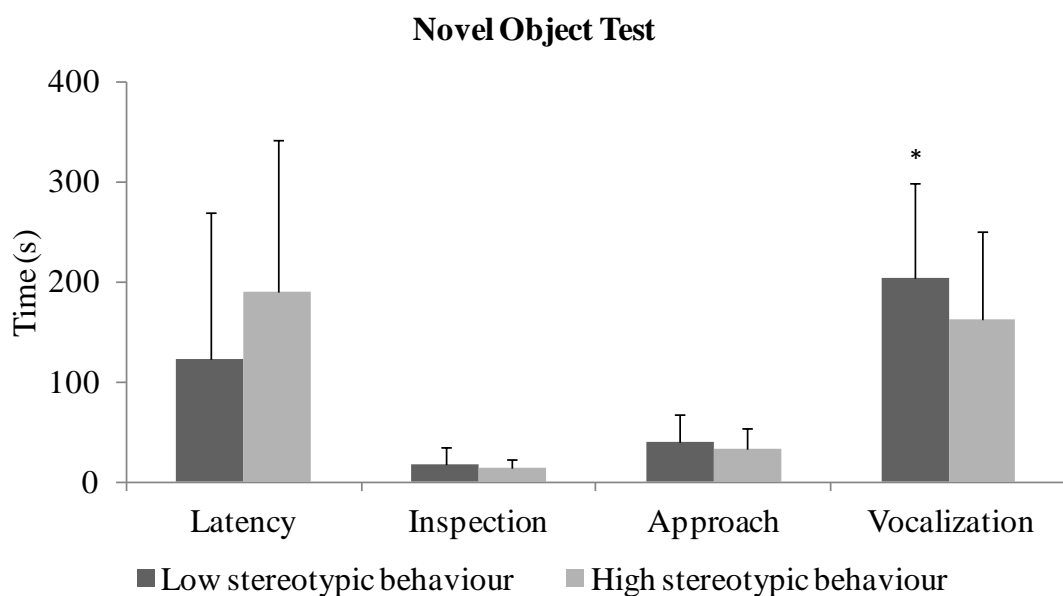


Figure 3. Novel object test. (N total = 142; N = 76 piglets from sows with low rate of stereotypy and N = 66 piglets from sows with high rate of stereotypy) * indicates

difference on vocalization (T test; $p = 0.008$; $F = 1.16$). There is no difference on latency (Mann-Whitney U test; $p = 0.17$; $Z = -1.34$); inspection (Mann-Whitney U test; $p = 0.10$; $Z = 1.61$) and approach (T test; $p = 0.08$; $F = 1.56$). The variable time considered in the y axis is the average of duration in each behavior. The vocalization is indicated by the frequency.

4. Discussion

In this study we showed that there is a relationship between stereotypies expressed by the sow and emotionality in their offspring. As far as we know, this is the first evidence that stereotypies expressed by the mother during gestation can affect offspring emotionality, changing their phenotype. We demonstrated that the stereotypies represented a factor which explained the individual variation in response to situations of challenge in the sows, such as barren environment. This may be due different strategies that animals adopt to cope with the monotony and reduced complexity of the environment, common factors in commercial production environments, plus the fact that individuals may require different levels of environmental stimulation (32). In monotonous environments, the low complexity may not meet the biological needs for some animals and they may develop stereotypies, while others may direct their motivation to the environment (physical environment interaction).

In the open field test, we have shown that piglets from sows with high rate of stereotypic behaviour walked more in the central and lateral quadrants. This increased movement in the test could be an indicator of more encouraged and explorative animals, showing a potential benefit for the offspring from sows with high rate of stereotypies when gestation occurred in challenging environments. An alternative explanation is that, it may be a response to increased anxiety in relation to the novel social isolation. While there are clear definitions of fear and anxiety, both emotions have a close relationship and can be easily confused. Fear is defined as a reaction to the actual perception of danger, while anxiety is defined as a reaction to a potential hazard (33). In terms of evolution, emotions are highly adaptive. In the case of fear, individuals can be prepared with a cascade of physiological and behavioural responses to deal with danger, while anxiety can be considered as a previous step to prepare for the potential loss of integrity of the individual (34,35). From the evolutionary point of view, these reactions promote fitness and can increase the life expectancy since both emotions modulate individual's life to prevent exposure to risk. It also enables the individual to optimize the ability to assess the costs and benefits of certain exposures, which is related to emotional learning, one of the functions of the amygdala (36). However, although these emotions have been strategically selected in the course of evolution, when in excess, it can lead to chronic stress and difficult adjustment of the individual to the environment, and thus reduce its welfare (25). In addition, excessive fear and anxiety can disrupt the expression of a range of desirable behaviours and reduce productivity outcomes in animal production systems (25). Fear can be related to the biology of the species, especially for prey species. Genetic components, previous experience, ontogenetic factors, prenatal and neonatal environment can have effects.

In the novel object test we showed that piglets from sows with low rate of stereotypies vocalized more. In pigs, it has been argued that the vocalization is strongly associated with excitement levels (37). Thus, the vocalization is considered a useful tool for assessing the welfare of an individual (38), but it should be used carefully. Apparently, the low intensity vocalizations are used to maintain social contact, while durable and high intensity

vocalizations are more related to individual mental states (39). The vocalizations are considered to indicate stressful attempted to find characteristics associated with such situations. In some studies, handling situations considered clearly stressful were used to clarify what each vocalization means for the welfare of an animal. For example, studies with social isolation (40,41), castration (42) and weaning (43) reported high rates of high frequency vocalization (> 1 kHz) when the piglets were challenged. Although in our study vocalizations were not analysed with appropriate bioacoustical equipment, recordings were similar to the short, low-intensity vocalizations described in situations of social isolation and used in an attempt to maintain social contact with conspecifics.

The types of vocalization, which may include grunting or screams, are the result of different situations and contexts (37). Some studies suggest that high frequency and long-lasting vocalizations indicate more severe stress, but this has not been validated with physiological indicators. Few studies in swine have analysed the relationship between vocalizations and physiological responses such as release of stress hormones, for example. It was demonstrated that endocrine responses (e.g., release of adrenaline) can be accompanied by different types of vocalization (41), indicating a response of common origin in the central nervous system (37). In this way, we can understand that vocalizations may indicate emotional states of an individual. Social isolation was the biggest stressor in both tests due to the high social motivation of the pig, which can be increased by separation anxiety. However, behavioural responses that indicate emotionality can be considered indicative but not direct measurements.

We propose that further studies are needed to elucidate the relationship between stereotypies expressed by the mother during pregnancy and the emotionality and welfare of the offspring. Although there are many studies elucidating issues about stereotypies, not all of the various aspects of what this indicator tells us about the welfare of an animal and its offspring are clear. We do not know whether the differences in the emotionality of the offspring are a consequence of the genetic (6) or personality (7,8) predisposition associated with stereotypies in the sows. However, this new information helps to build knowledge about the consequences of stereotypic behavior for animal welfare. Since this behaviour can indicate frustration, an affective state, it is useful to understand the consequences in the offspring.

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6. Conflict of Interest

The authors declare that they have no competing interests.

7. Author Contributions

Conceptualization: PT AJZ

Formal analysis: PT MRQ

Funding acquisition: AJZ

Investigation: PT TB BM

Methodology: AJZ PT TB

Project administration: PT TB BM AJZ

Resources: AJZ

Supervision: AJZ

Writing ± original draft: PT AJZ

Writing ± review & editing: PT TB BM MRQ AJZ

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Stereotypic behavior in sows during gestation decreases fear in their offspring

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Abstract

The hypothesis is that stereotypic behavior is helping to cope with stress, and during gestation change the phenotype of the offspring. Rather than ask to the individual what it means to express stereotypic behavior, we addressed this issue to fetus programming. The goal was to assess the effects of maternal stereotypic behavior on offspring welfare indicators, such as behavior and cortisol. Sows that exhibited consistent stereotypic behavior on least two of six days of observation ($N = 7$) were compared with sows that never exhibited the same behavior in these six days ($N = 4$). Salivary cortisol from sows and piglets were analyzed for assessing hypothalamic pituitary adrenal (HPA) axis activity., and placental tissue Piglets' behaviors and fear tests were performed after weaning (one couple per sow). In the open field test, piglets born of sows that did not exhibit stereotypic behavior demonstrated higher latency and lower activity, indicating more fear. Moreover, glucocorticoid concentration was different in placental tissue only for cortisol, the levels of which were higher in sows that exhibited stereotypic behavior. We demonstrate that maternal stereotypic behavior is related to less fear in their offspring. Although stereotypic behavior has been studied for decades, attention has not been devoted to the effects of the prenatal period in considering a fetal reprogramming approach.

Keywords: cortisol; emotionality; gestation; prenatal; stereotypies; stereotypic behaviour.

1. Introduction

Stereotypic behaviors or “stereotypies”, are a wide range of repetitive and apparently functionless patterns that often develop in environments that likely contribute to poor animal welfare (1). This behavior often develops in animals housed in environments with few stimuli, or involve physical restraint(s), fear, and/or frustration (1). There is also a genetic component (2) and personality predisposition (3,4) involved in the expression of stereotypic behaviors. Stereotypies may also be an inherited pattern for some species, e.g. courtship. In this sense, there is a synergetic effect of internal and external variables that trigger strategies for coping with stress. There is little doubt that some variables have more impact on triggering stereotypic behavior than others when the diverse range of environmental factors are considered (5). On this study, we considered the stereotypy sham-chewing. Although oral stereotypies have been discussed as a beneficial strategy against gastrointestinal acidity due low-fiber diets, it seems that might be more related to natural foraging (6). Its motor behavioral patterns could be a response to frustration for natural foraging (6), or just the appetitive behavior without the consummatory phase.

Stereotypic behavior is often considered to be an indicator of welfare (1,7,8) because it can provide clues about psychological states that are difficult to assess. However, this specific behavioral indicator sometimes is not consistent with cortisol levels (9), a physiological welfare indicator involved in the stress cascade. It has been considered that stereotypies expression indicated compromised welfare. However, considering one population, the individual that is expressing is better adjusted, compared with the one that is not expressing? The “Coping Hypotheses” of stereotypic behaviour assumes that the performance reduces the level of stress experienced (10,11). This hypothesis is still controversial, since the scientific data are not consistently corroborating it.

To our knowledge, stereotypic behavior is a recent mechanism in the evolutionary context since it appeared in animals in captivity. Nevertheless, the long-term consequences of stereotypic behavior remain unclear. Addressing this issue in sexual selection, male minks that exhibited more stereotypic behavior demonstrated lower success rates in copulation (12) in a comparison between enriched and barren environments. It remains unclear how the long-term effects of stereotypies impact phenotype in offspring. In mammals, pregnancy has an important role in shaping the organism, given that the mother's environment may affect the offspring development during gestation. This concept has emerged from the “thrifty phenotype hypothesis”, in which neurodevelopment reprogramming induces alterations to cope with challenges in anticipating the postnatal environment (13). In other words, the prenatal environment has the potential to impact the offspring phenotype and prepare individuals for the environment that they will be inserted in order to prepare them to better cope with challenges. The environment in which an animal is maintained during gestation may result in changes in several offspring parameters (14–19). By this mechanism, factors such as emotional reactivity, responsiveness to stressors and cognition, can be modulated by challenges in the prenatal and neonatal periods (18,20,21). Studies have reported that some stressors, such as negative interactions with the handler (18,19,22) and social stress (18), can alter emotional reactivity, social behavior, and responsiveness to stressors, and cognition and memory in the offspring. Moreover, offspring of sows that experienced less hunger during gestation have exhibited reduced aggressive behavior (23).

Although some studies have demonstrated that stress during pregnancy or prenatal stress can generate changes that are not necessarily pathological (14), an excess of

glucocorticoids that the offspring are exposed can affect important brain structures and lead to negative effects (18,19,22). Glucocorticoids are important stress hormones in adult animals, but their functions are more diverse in the fetus, and their effects may be completely different depending on gestational age, and the severity and duration of the exposure (24).

The placenta has a role in protecting the fetus in the prenatal period and, therefore, modulates stressful events experienced by the maternal organism and acts as a buffer (25). In mammals, 11 β -hydroxysteroid dehydrogenase enzyme type 2 (11 β HSD2) in the placenta forms a barrier that protects the fetus from high levels of maternal cortisol because it oxidizes the biologically active form of cortisol in cortisone (25,26). Chronic stressful situations have the potential to inhibit the capacity to up-regulate type 2 enzyme activity, and the capacity to adapt placental 11 β HSD2 is significantly reduced by previous exposure to chronic stress (27), thus reducing the protective capacity of the placenta.

The effects of prenatal stress increase cortisol concentration, which can cross the placenta and affect brain structures such as the hippocampus and amygdala, and may generate changes in offspring's emotionality (28,29). To access emotionality of non-human animals, some tests have been validated and discussed (30). These include the open field test and the novel object test (30–33), in which behaviours such as activity and vocalization can be used as measures of emotionality (32). Our goal was to evaluate the consequences of stereotypic behavior in sows during gestation in the welfare of their offspring. Rather than ask to the individual what it means to express stereotypic behavior, we addressed this issue to fetus programming. The hypothesis is that stereotypies are helping the sows to cope with stress, so it should affect fetus programming, promoting increased welfare compared with sows which do not express stereotypies.

2. Material and Methods

2.1. Animals and housing conditions

The study was performed at the Araporanga Farm, at Jaguariaíva, Paraná, Brazil. This study was approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (CEUA/FMVZ, protocol number 6157201114).

The analysis was performed in sows in a parallel experiment that consisted of 36 pregnant sows (TopGen Afrodite®), distributed according to body condition score in enriched and conventional pens, totaling 6 pens (10 sows per pen, in which only 6 were considered in the parallel experiment). The distribution according to body condition was previously made by the workers in the farm considering size and weight of the sows. The sows were from 2nd to 7th parity ($p > 0.05$; [T test]). Three pens had straw (hay) as substrate from the 90th day of gestation, while control pens (without straw) were maintained with a concrete floor (i.e., conventional pens). Only sows in the control pens were analyzed, for which we analyzed four sows that never exhibited stereotypic behavior (not express) and seven that consistently exhibited stereotypic behavior (express - on at least 2 of 6 days of observation), divided in three pens with mixed treatment.

A meal was offered at two times, 07:00 am and 11:40 am, and the animals had *ad libitum* access to water. Each pen was 6 m long \times 3.86 m wide, with a solid/slatted concrete

floor area of 3.97 m in length and pen walls that were 0.85 m high. The feeder was 5 m long and 0.37 m wide. However, the slatted floor area was covered with plywood boards to trap straw to avoid harm to the manure management system. The control pens were also fitted with plywood boards to avoid differences in relation to the microclimate inside the pens, which were cleaned every day.

2.2. Experimental design

To assess the effects of stereotypic behavior on the offspring during gestation, outcomes of sows that exhibited consistent behavior on at least two of six days of observation were compared with those that did not exhibit consistent behavior. The welfare of sows and piglets was assessed by behavior and salivary cortisol analysis. Piglets from each sow (one couple per sow) were evaluated after weaning in relation to the behaviors including aggressiveness, nosing, emotionality. In addition, the glucocorticoids in the placental tissue were accessed.

2.3. Sow behavioral data

To collect behavioral data, an ethogram was adapted (34). Behavioral measures of sows were obtained by direct observation on days 88, 89, 91, 92, 106 and 107, which represent the final one-third of the gestational period. The collection periods were conducted over two consecutive days to avoid possible interference by stressful events. The behavioral assessments were performed by direct observation at two feeding times, 1 h before and 1 h after, and a final at 17:30, totaling five of periods per day. For this experiment, only the data collection at 17:30 was evaluated to mitigate the interference expected due to motivational feeding by the sows. Two observers were previously standardized to avoid bias in data collection. Observations were performed using a combination of methods for behavioral measures, which started with a scan sample, followed by a focal animal with continuous observation. At the time of each observation, each animal was observed three times per uninterrupted 120 s period, totaling 6 min per animal per observation time, which in the six days of observation totaled in 36 min per animal

TABLE 1 - Definition of behaviors for behavioral observation of pregnant gilts.

Behaviors	Definition
Sleep	Animal sleeping
Lying ventrally	Lying with the belly facing the ground with all the limbs under the body
Lying laterally	Lying sideways, with all the members extended laterally
Standing	Body supported by the four limbs
Sham chewing	Continuous chewing without the presence of visible food in the oral cavity
Rooting the floor	Snout touches the ground followed by head movements

Licking the floor	Tongue touches the floor and is followed by movements with the head
Interacting fence or gate	Biting or nibbling the fence wire or gate
Interacting with mats	Snout or tongue touches mats followed by head movements
Bites (E)	Bite on any parts of the body (tail, vulva, ear, body)
Facing (E)	Face to face, with fixed view to the other animal
Pushing (E)	Pushing another animal using the head or the muzzle
Vocalization (E)	Sound emission emitted by the animal

The caption E indicates behaviours that were measured only the events and not the duration.

2.4. Salivary cortisol and enzyme-linked immunosorbent assay

Saliva collection was performed on the same days of the behavioral evaluation; more specifically, on days 88, 89, 91, 92, 106, and 107. Two samples were collected per animal, at 06:00 am and 18:00 pm, to follow the circadian rhythm of cortisol, and the effect of stereotypic behavior on hypothalamic pituitary adrenal (HPA)-axis activity. The saliva was collected using hydrophilic cotton on two roller-shape units tied to dental floss with long tips that were presented to each animal. The animal chewed the cotton until it was saturated with saliva. The first sample collected was discarded; the collection was repeated to collect only recently produced saliva. Once the second sample was collected, it was placed into a 15 mL tube. Subsequently, the tube was packed in an ice box until the end of the collection and the sample frozen at -20° C until processing. Thawing was performed in a box containing ice in a temperature-controlled room. After complete thawing, the sample was centrifuged for 10 min at 1000 × g; the supernatant was aliquoted into microtubes and again frozen at -20°C until analysis. This process assisted in the removal of mucins and other components that may interfere with analysis. For sample analysis, 50 µL of saliva was analyzed using enzyme-linked immunosorbent assay (ELISA) in duplicate for each sow, with pooled samples from each period, without mixing the morning and afternoon collections (e.g., with samples from days' 88 and 89 gestation in the morning collection).

2.5. Farrowing

Sows were moved to the farrowing pens at 108 days of gestation. The deliveries were monitored and occurred in conventional farrowing crates. At birth, each piglet had its umbilical cord tied with string kept in antiseptic solution and dipped in iodine (10%) for 5 s. The piglets were then cleaned with paper towels, assigned a number for the order of birth on their back with a stick marker, and were passed through antiseptic powder to reduce body moisture. After this initial management, the piglets were placed to ingest colostrum. On the first day of life, the piglets had their teeth grinded, tail cut, ears notched, and individual weight recorded. Dextran iron supplementation was administered the day after delivery.

2.6. Placenta collection and glucocorticoid analysis

The placenta was collected from four piglets per sow, in which on standardized (size and location) piece for each placenta were cut then frozen in a -20°C freezer. All placentas from each sow were macerated together to prepare a pooled sample. Once the homogenized placenta was powder-like, 0.1 g was placed in a 1.5 mL microcentrifuge tube and 200 μL of ultrapure water was added; the suspension was vortexed for 15 s. Subsequently, 20 μL was placed in another similar tube for total protein analysis (performed in triplicate for each sample, using the Bradford protocol (35)). One milliliter of ethyl acetate was added to the tube with water and placenta, vortexed for 15 s and centrifuged for 15 min at 4°C . Supernatant (400 μL) was transferred to a new 1.5 mL microtube; the second (duplicate) was transferred to another tube. All samples were dried overnight in a hood until all the liquid volume evaporated. For glucocorticoid analysis, all samples were re-suspended in the same volume using assay buffer. The analysis was performed using the same ELISA protocol for salivary cortisol (see section 2.4).

2.7. Weaning and emotionality tests on piglets

The piglets were weaned at 28 days of age, vaccinated (vaccines against porcine circovirus, *Streptococcus suis*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*) and transported from the Araporanga Farm in Jaguariaíva-PR (where the first stage of the experiment was performed) to the Fernando Costa Campus of the University of São Paulo in Pirassununga-SP, which involved approximately 8 h of travel. One couple of piglets per sow was used for the second part of the experiment. During the trip, each couple was placed in a transport box (73.5 cm long \times 53 cm wide \times 21 cm high), making it impossible for aggressive interaction between different litters, and were lined with straw (hay).

After weaning, the animals were housed in suspended pens, with six litters housed in the same pen. Each pen consisted of 12 animals: a pair from each sow, totaling six pens. Piglets were grouped according to their mothers during gestation treatment, which means that they were grouped with piglets born from sows that were grouped with their mothers. The groups were balanced, since the stereotypies expression were mixed in the pens. The piglets had *ad libitum* access to food and water. Throughout the experimental period, each pen was monitored for further analysis of behaviors, such as aggressiveness and nosing. In this study it was considered nosing in any part of pen mates' body (duration) and aggressiveness (duration), although it has been characterized for threatening, pushing, head-knocking, biting, chasing and avoiding (36–38), it was considered only in the behaviors indubitable by video recording. It was not considered threatening and avoiding. The duration of each agonistic interaction considered started and ended with one of the cited behaviors.

In relation to fear tests, a combination of open field and novel object tests (39) was performed to access fear behavioral indicators or exploratory motivations of each animal. The tests were conducted at 41 days of age. The piglets were tested individually, and returned to the pen immediately after the test. The combination of tests enabled a previous piglets' habituation in the arena test, in which the open field test preceded the novel object test. The animals were individually tested in the arena (243 cm \times 194.5 cm), which contained demarcations on the ground forming quadrants. The duration of each test was 5 min, totaling 10 min. The piglets were gently placed in a predetermined location in the arena and recorded during the test period. From the recording, the behaviors were analyzed and the latency to walk was quantified, as well as the number of central and lateral quadrants accessed, walking

time, freezing time, and vocalizations (events). After this test, a novel object was inserted (traffic cone) using a pulley system in the center of the pen. Subsequent behaviors were recorded for 5 min. In this test, the latency for walking, time near the object (quadrants surrounding the object), time exploring the object (near the object with the head facing the object), freezing time, and vocalization (events) were evaluated. After each animal was tested, the pen was washed with water to reduce possible chemical clues, as well as to remove feces and urine. The pen was also washed before the start of the first test of the day to standardize the entry of piglets in the arena test.

TABLE 2 - Definition of behaviors for fear tests in piglets.

Behaviors	Definition
Latency to walk	Duration to start to walk
Number of quadrants	Quadrants accessed in numbers
Walking time	Duration of walking to any direction
Freezing time	Duration spent without any movements
Vocalizations	Number of vocalizations
Near to object	Duration in quadrants (eight) surrounding the cone
Exploring the object	Duration near the object with the head facing it

2.8. Saliva collection from piglets

Similar to the sows, saliva collection in piglets aimed to access the activity of the HPA axis in relation to cortisol. The samples were collected on days 28, 29, 35, and 36, with two samples collected individually at 06:00 am and 18:00 pm. The collection of saliva was performed using the same methodology used for the sows (see section 2.4). Because these were piglets and did not produce significant amounts of saliva, the first cotton swabs were considered, and each sample was placed into a collection tube, placed in a box with ice, and shipped to the laboratory where they were frozen at -20°C until processing. The ELISA protocol followed the same performed for the sows (section 2.4).

2.9. Data analysis

Data were initially tested for normality using the Shapiro-Wilk test. Statistical tests were performed using R studio software and are specified in the respective figures; differences with $p < 0.05$ were considered to be statistically significant. The non-parametric Mann-Whitney U test was used in the analysis of sows, since the number of replicates was < 5 in one of the two groups.

3. Results

Sows that did not exhibit stereotypic behavior demonstrated higher levels of cortisol in the morning of days 91 and 92 of gestation ($p = 0.02$; $Z = 2.19$ [Mann-Whitney U test]) (Fig. 1). However, closer to delivery, sows that exhibited stereotypic behavior demonstrated a tendency for higher concentration on days 106 and 107 of gestation ($p = 0.05$; $Z = -1.90$ [Mann-Whitney U test]) (Fig. 1). There was a difference in cortisol concentration in placental tissue, in which sows that exhibited stereotypic behavior demonstrated higher concentrations ($p = 0.04$; $Z = 2.00$ [Mann-Whitney U test]) (Fig. 2) compared with sows that did not exhibit stereotypic behaviors.

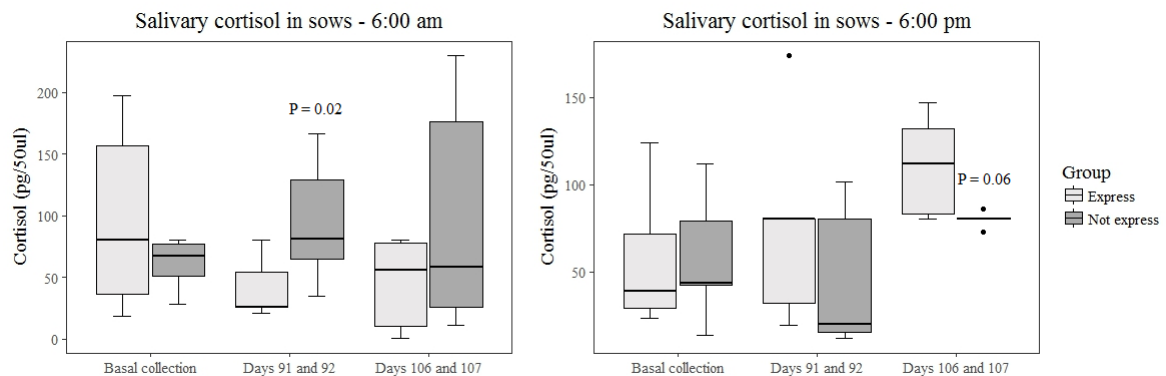


Figure 1. Salivary cortisol concentration in sows. There was a difference on the morning of days 91 and 92 ($p = 0.001$; $Z = 3.20$ [Mann-Whitney U test]), and a tendency toward higher cortisol levels on the afternoon of days 106 and 107 ($p = 0.06$; $Z = 3.99$ [Mann-Whitney U test]).

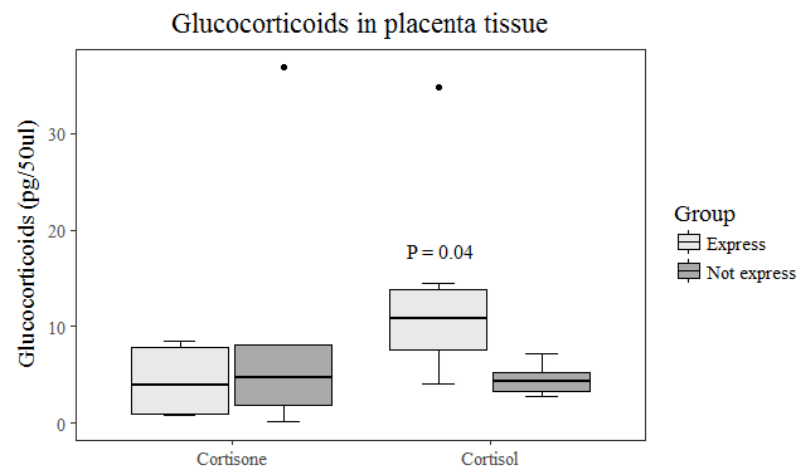


Figure 2. Glucocorticoid concentration in placental tissue. Cortisone and cortisol concentrations in sows that did and did not exhibit stereotypic behavior ($p = 0.85$; $Z = 0.18$ [Mann-Whitney U test]); $p = 0.04$; $Z = 2.00$ [Mann-Whitney U test]), respectively.

In the piglets, there was no difference in salivary cortisol concentration at weaning or at 35 days of life (Fig. 3). In addition, there was also no difference in aggressive behavior between piglets from sows that did or did not exhibit stereotypic behavior ($p > 0.05$). However, in the nosing behavior, piglets from sows that exhibited stereotypic behavior spent more time performing nosing on day 4 ($p = 0.03$; $Z = -2.08$ [Mann-Whitney U test]) and day 6 ($p = 0.05$; $Z = 1.91$ [Mann-Whitney U test]). In the fear tests, piglets from sows that did not

exhibit stereotypic behavior demonstrated higher latency ($p = 0.04$; $Z = 2.04$ [Mann-Whitney U test]) and less activity ($p = 0.01$; $Z = -2.38$ [Mann-Whitney U test]), indicating more fear.

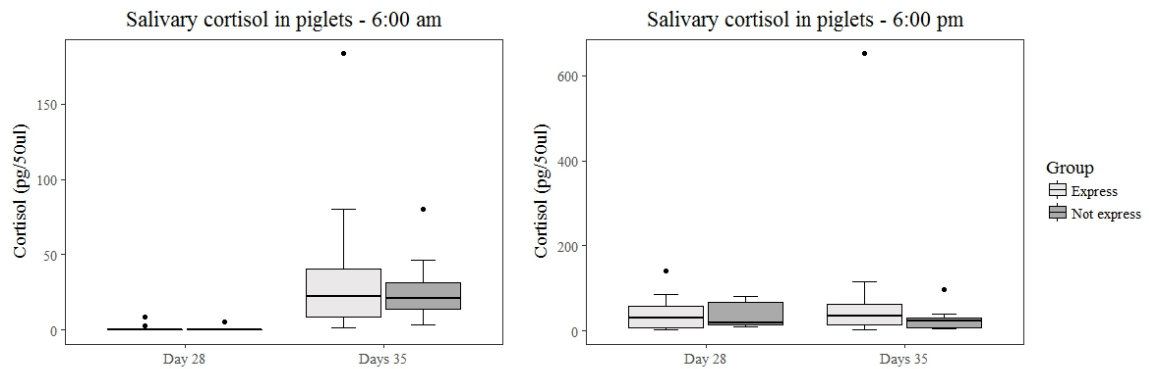


Figure 3. Salivary cortisol levels in piglets. There was no difference in salivary cortisol levels in piglets at weaning (day 28) and at 35 days of life.

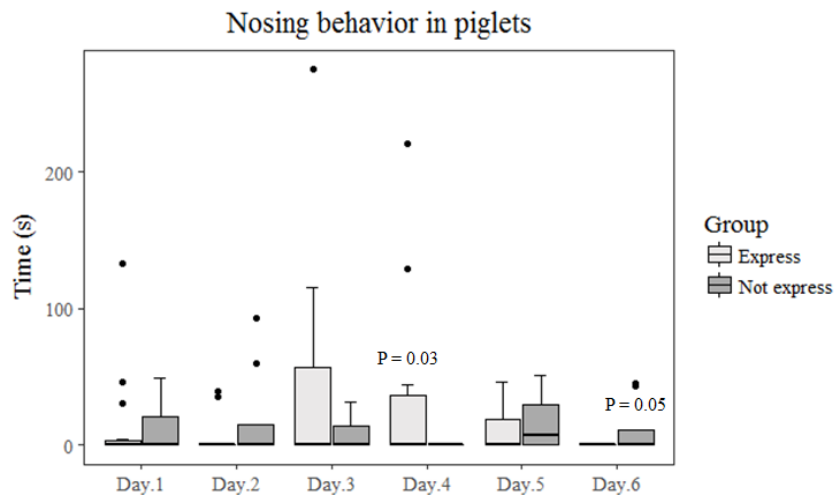


Figure 4. Nosing behavior in piglets. Nosing behavior (average duration) was higher on day 4 ($p = 0.03$; $Z = -2.08$ [Mann-Whitney U test]) and day 6 ($p = 0.05$; $Z = 1.91$ [Mann-Whitney U test]) in piglets born from sows that do not exhibited stereotypic behavior.

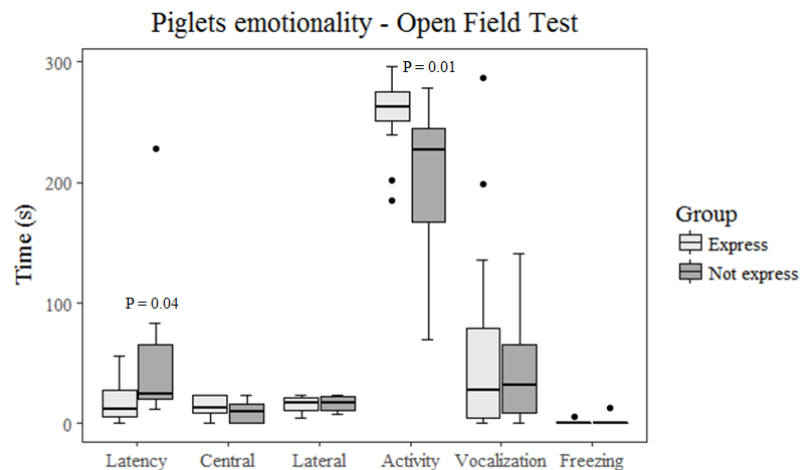


Figure 5. Open field test in piglets. Piglets from sows that did not exhibit stereotypic behavior demonstrated higher latency ($p = 0.04$; $Z = 2.04$ [Mann-Whitney U test]) and less activity ($p = 0.01$; $Z = -2.38$ [Mann-Whitney U test]). The duration average by piglets in each group is indicated by the variable time in the y axis. The vocalization is the indicated by the frequency.

4. Discussion

We demonstrated that stereotypic behavior exhibited by sows was associated with less fear in their piglets. Since it is still controversial if the individual that express stereotypies is in a more compromised welfare, we addressed this study to fetal programming. Moreover, although stereotypies have been studied for decades, attention has not been devoted to the consequences on fetal programming and long-term consequences, considering next generations.

Differences in cortisol concentration in sows that did not exhibit stereotypic behavior were also consistent with emotionality in the offspring outcomes, considering the potential effects of glucocorticoids on brain development (18,19,22), which was higher in sows that did not exhibit stereotypic behavior. Although the association between cortisol concentration and stereotypic behavior is a controversial indicator (9), we found that sows that did not exhibit had higher concentrations on days 91 and 92 of gestation. On days 106 and 107 of gestation, there was a tendency toward higher concentration in sows that exhibited stereotypic behavior. However, these measures were collected close to delivery (predicted to occur on day 114). In this sense, when delivery is imminent, a cascade starts in offspring cortisol concentration to trigger proper physiological responses (40), overlapping with the stereotypy effect.

Moreover, there was a difference in the placental tissue, but only in cortisol concentration, which was higher in sows that exhibited stereotypic behavior. Because this outcome is not congruent with salivary cortisol levels, we can argue that there is difference in 11β HSD enzyme activity, which oxidizes the biologically active form of cortisol in cortisone (25,26). Chronic stressful situations have the potential to inhibit the capacity to up-regulate type 2 enzyme activity, and the capacity to adapt placental 11β HSD2 is greatly reduced by previous exposure to chronic stress (27), thus reducing the protection capacity of the placenta. Furthermore, is possible that cortisol is concentrated in the placental tissue instead of crossing

and reaching the fetus' brains. In other words, the higher cortisol concentration in sows that exhibit stereotypic behavior could result for a placenta holding this glucocorticoid and protecting the brain development in their offspring. We have also some evidence that the epigenome in the limbic system of these piglets is differentially methylated (Tatemoto et al., in preparation).

There was no difference in salivary cortisol concentration at weaning or at 35 days of life in the piglets (Fig. 3). Additionally, there was no difference in aggressiveness between piglets from sows that did or did not exhibit stereotypic behavior. However, in nosing behavior (Fig. 4), piglets born of sows that exhibited stereotypic behavior spent more time performing nosing on day 4, but not in day 6, which piglets born of sows that do not exhibited stereotypic behavior spent more time performing nosing behavior. Nosing is an undesirable piglet-directed behavior expressed in piglets after weaning that sometimes can be a trigger for aggressiveness (personal observation) and cause skin lesions in the recipient when persistently performed, as well as belly nosing (41). In this study we considered nosing, since was in any part of pen mates' body. Once this motor behavior pattern precedes suckling and milk intake, it has been suggested that it may be associated with hunger or feeding (42), or even to the artificial early weaning.

In previous studies, there was a negative correlation between suckling behavior in the sow and nosing after weaning (43). Poor-quality diet post-weaning and the presence of milk do not affect the development of belly-nosing in piglets weaned between days 14 and 18 (44). These data support the hypothesis that feeding motivation and hunger is not a causal factor of belly nosing. Moreover, there are differences between nosing (as a piglet-directed behavior) and belly nosing, for which the levels in nosing remain significantly more consistent throughout time and start the first day after weaning, instead of the peak of belly-nosing, which appears only in the second week post-weaning and starts to decrease thereafter (44). Another possibility is that the nosing which we observed is a stereotypic behavior because it is consistent with the definition of repetitive behavior and appears to have no obvious function (1). As a stereotypic behavior, it can be a strategy to cope with artificial weaning in some piglets.

Furthermore, it has been shown that there is a strong genetic basis for the development of stereotypies (2,45). There is not necessarily a congruence between the type of stereotypy exhibited by mothers and offspring (2), although in this sense, it is reasonable to expect stereotypies in offspring that the mothers themselves are also exhibiting. However, the genetic component must interact with environmental conditions to trigger this feature in the offspring. Apart from genetic predisposition, the effects of parental behavior cannot be excluded because the offspring passed the first 28 days with their mothers. Maternal behavior affects litter development, including behavior and emotionality (46–48).

In the fear tests, piglets from sows that did not exhibit stereotypic behavior demonstrated higher latency and less activity, indicating more fear. Fear is the most common emotion investigated in domestic animals (30) and this emotion is related to welfare, since is a negative emotion. However, evolutionary mechanisms are shaping emotions to increase fitness, and fear is a reaction to the perception of actual danger to trigger appropriate adaptive responses (30,49). Although pigs in general have a explorative trait on their genome, it is expected that a piglet kept alone in a novel space, and then faced with a unknown object will experience some level of fear. It should be adaptive this response throughout evolution, since it take risk to be alone, exposed to predators and loose from their mates and mother.

Nonetheless, it is possible to measure only the indicators of fear, once as every emotion, it is a subjective state (30).

We have shown in preliminary results that expression of stereotypic behavior by the mother affects the offspring's emotionality (Tatemoto et al, submitted). However, in that experiment, we considered a gradient of sows, in which we divided 28 sows in two groups, from low to high expression in terms of duration. In contrast, the present study adopted a focused strategy, in which we considered consistent stereotypic behavior expression throughout the days of observation. This approach enabled us to select a desirable profile for answering our question regarding the "thrifty hypothesis". Our data indicate that it is not simply a difference in piglets' emotionality, but that piglets from sows that do not exhibit stereotypic behavior exhibit more fear. To our knowledge, these results are the first to indicate that sows exhibiting stereotypic behavior bear piglets with less fear.

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6. Conflict of Interest

The authors declare that they have no competing interests.

7. Author Contributions

Conceptualization: PT AJZ

Formal analysis: PT

Funding acquisition: AJZ

Investigation: PT TB LA

Methodology: PT AJZ

Project administration: PT

Resources: AJZ

Supervision: AJZ

Writing ± original draft: PT

Writing ± review & editing: PT TB LA AJZ

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**Environmental enrichment for pregnant sows modulates HPA-axis and behavior
in the offspring**

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Abstract

Our goal was to assess the effects of environmental enrichment during gestation on the HPA axis and behavior of offspring. In order to test our hypothesis, we kept 18 sows in straw in the final third of gestation and 18 sows without straw (control). The straw reduced stereotypic behavior in sows kept in the barren environment. There was no difference regarding to the glucocorticoid analysis in the placenta. In piglets, we performed an analysis of behavior for six consecutive days after weaning. We also measured cortisol levels in saliva and performed fear tests to assess emotionality. Environmental enrichment during gestation reduced aggressiveness and nosing in piglets. In addition, salivary cortisol was higher in piglets from sows in barren environments. Salivary cortisol was higher in piglets from sows in environmentally enriched conditions only in the morning on the day of weaning. There was no difference in the piglets' emotionality when we compared groups with both sexes together. However, there was a sex-specific difference, in which females born from sows kept with environmental enrichment explored more and showed less fear. Environmental enrichment changed the offspring HPA-axis activity and behavior, improving offspring's welfare.

Keywords: environmental enrichment; gestation; prenatal stress; glucocorticoids; straw.

1. Introduction

Environmental enrichment allows the expression of natural behaviors, modulates aggressiveness (Kadry and Barreto, 2010), increases brain plasticity (Ebbesson and Braithwaite, 2012; Williams et al., 2001), alters HPA axis activity (Gro et al., 2013; Kotrschal and Taborsky, 2010; Larsson et al., 2002), and reduces methylation in hippocampal and frontal cortex genes (Mychasiuk et al., 2012). Furthermore, environmental enrichment affects the expression of genes in the brain, especially those involved in neuronal structure, synaptic signaling, and plasticity (Baroncelli et al., 2009). Some genes have been known to be associated with learning and memory (Rampon et al., 2000). Moreover, environmental enrichment affects brain weight, promotes an increase in arborization and density of dendritic spines (Leggio et al., 2005), modulates neurogenesis in the hippocampus (Segovia et al., 2006), and makes the cognitive bias positive (Douglas et al., 2012). However, in captivity, animals are usually kept in barren environments. There is a wide range of studies demonstrating barren environments as a trigger of stereotypic behaviors expression (Morgan and Tromborg, 2007; Wemelsfelder et al., 2000), which is considered a welfare indicator.

The effects of environmental enrichment in animals are beneficial, but it is still unclear how it affects fetal programming. In mammals, pregnancy has an important role in shaping the organism. The environment of the mother may have effects on the offspring. This concept is derived from the “thrifty phenotype hypothesis”, in which neurodevelopment programming induces alterations that predict the postnatal environment (Hales and Barker, 2001). In other words, the prenatal environment has the potential to adjust the offspring phenotype, preparing individuals for the environment that they will experience. The environment in which an animal is maintained during gestation may result in changes in several offspring parameters (Baxter et al., 2016; Braastad et al., 1998; Meyer et al., 2009; Rutherford et al., 2014; Urakubo et al., 2001). Through this mechanism, factors such as

emotional reactivity, responsiveness to stressors, and cognition can be modulated by challenges in the prenatal and neonatal periods (Poletto et al., 2006; Rutherford et al., 2014; Weinstock, 2008). Additionally, hunger during gestation increases aggressive behavior in the offspring (Bernardino et al., 2016).

Prenatal stress can generate changes that are not necessarily pathological (Braastad et al., 1998), but the excess of glucocorticoids that the offspring receives can affect important brain structures and generate negative effects (Baxter et al., 2016; Coulon et al., 2013; Rutherford et al., 2014). Glucocorticoids are important stress hormones in adult animals but the functions range more widely in the fetus, in which the effects are completely different depending on gestational age, severity, and duration of exposure (Fowden et al., 2016).

How can environmental enrichment during gestation alter the offspring's parameters? Environmental enrichment has been widely discussed as a tool to improve animal welfare. Improving the mother's welfare during pregnancy can lead to positive changes in the offspring, through the mechanism of the "thrifty hypothesis." Still, how environmental enrichment will act on the HPA axis of the mother and how this is reflected in the offspring HPA axis during development is unknown. In this study, we addressed how the prenatal environment acts on offspring development, in which the placenta can serve as a buffer and mediate the effects on the offspring phenotype.

2. Material and Methods

2.1. Animals and holding condition

This study was approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (CEUA / FMVZ - protocol number 6157201114). The study was performed in the Araporanga Farm, at Jaguariaíva, Paraná, Brazil.

For this study, 58 pregnant sows were used (TopGen Afrodite®). The animals were distributed by body condition score in six gestation pens with ten animals per pen. From this total, 36 sows were studied and they were from 2nd to 7th parity in the treatment and control (T test, $p > 0.05$). Three pens had straw (hay) as substrate from the 90th day of gestation, while control pens (without straw) were maintained with concrete floors (conventional pens). Thus, half of the animals had access to straw, which was replaced daily between 8 h and 11 h. Sows were divided into three blocks, one week apart in relation to the gestational period. In each block, there was a treatment and a control pen, in order to maintain balance.

Food was offered twice daily, at 0700 h and 1140 h. Animals had access to water *ad libitum*. Each pen was 6 meters long and 3.86 meters wide, with a solid/slatted concrete floor area of 3.97 meters in length and walls that were 0.85 meters high. The feeder was 5 meters long and 0.37 meters wide. However, the slatted floor area was covered with plywood boards in order to avoid the straw from becoming trapped and harming the manure management system. The control pens also received the plywood boards, to avoid any differences in relation to the microclimate inside the pens.

2.2. Experimental design

In order to assess the effect of straw at the final third of gestation, 18 pregnant sows were submitted to an environment with substrate. Their behaviors were measured two days before the beginning of the treatment (basal collections: Days 88 and 89 of gestation), early on Days 91 and 92 of gestation, and prior to moving to farrowing crates (Days 106 and 107 of gestation). The control treatment had the same routine behavioral observations. Saliva was collected on the same days as the behavioral samples, at 0600 h and 1800 h for cortisol analysis. To measure the effects of environmental enrichment in the final third of gestation, piglet behavior was evaluated (from each sow, one couple per sow), including aggressiveness

and piglet-directed behavior, emotionality, and salivary cortisol levels. In addition, during farrowing, placental tissues were collected.

2.3. Sow behavioral data

In order to collect behavioral data, an ethogram was adapted (Zonderland et al., 2004). Behavioral measures of sows were obtained by direct observation on Days 88, 89 (basal collection, before environmental enrichment), 91, 92, 106, and 107, in the final third gestational period. The behavioral assessments were performed by direct observation during the two feeding times, one hour before and one hour after, and a final one, at 1730 h, totaling five observations each day. Two observers were previously standardized to avoid bias in data collection. Observations were carried out using a combination of methods for behavioral measures, which started with a sample scan, followed by continuous observation of the focal animal. For each observation, each sow was observed three times uninterrupted for 120 seconds, totaling 6 minutes per animal per observation time (before, after feeding, and at the end of the day), with a total of 30 minutes per observation per day. The collection periods spanned over two consecutive days to avoid possible interference from stressful events (e.g. Days 88 and 89 in the evaluation of the basal behavior).

2.4. Salivary collection and cortisol analysis

Saliva was collected on the same days as the behavioral evaluation, that is, Days 88, 89, 91, 92, 106, and 107. On all of these days, two samples were collected per animal, at 06:00 h and 18:00 h in order to assess cortisol in respect to the circadian rhythm and to assess the effect of enrichment on HPA-axis activity. Saliva was collected using hydrophilic cotton in two roller-shaped units tied to a dental floss with long tips and presented to each animal. The animal chewed the cotton until it was saturated with saliva. The first sample collected

was discarded and the protocol repeated to collect only recently produced saliva. After the second sample was collected, it was placed in a 15-milliliter falcon tube. Subsequently, the tube was stored in ice until the end of the collection, then frozen at -20°C until processing. Samples were thawed on ice. After complete thawing, the sample was centrifuged (10 min., 1000 x g) and the supernatant was aliquoted into microtubes and again frozen at -20°C until analysis. This process removes mucins and other components that may interfere with the analysis. For sample analysis, 50 µl of saliva was analyzed with a cortisol enzyme immunoassay (EIA – based on Cooper et al., 1989; Palme and Möstl, 1997) in duplicate for each sow, with a pool of each gestation period, without mixing the morning and afternoon collections (e.g. with samples from 88 and 89 gestation days in the morning collection). The sensitivity of the EIA was 0.2 pg/well.

2.5. Farrowing

The deliveries were monitored and occurred in conventional farrowing crates. At birth, each piglet had its umbilical cord tied with string kept in antiseptic solution and dipped in iodine (10%) for 5 seconds. The piglets were then cleaned with paper towels, assigned a number for the order of birth on the back with a stick marker, and were passed through antiseptic powder to reduce body moisture. After this initial management, the piglets were placed with their mother to ingest colostrum. On the first day of life, the piglets had their teeth grinded, the tail cut, the ears notched, and individual weight recorded. The dextran iron application was performed the day after delivery.

2.5. Placenta collection and glucocorticoid extraction

The placenta was collected from four piglets per sow; a standardized (size and location) piece for each placenta was cut and frozen in a -20°C freezer. All placentas from

each sow were macerated together in order to prepare a pool. Once the placenta was macerated, 0.1 g of the powder was placed in a 1.5-ml microtube. About 200 µl of ultrapure water was added and the mixture was homogenized by vortexing for 15 seconds. Twenty microliters of this mixture was placed in another similar tube, for total protein analysis (performed in triplicate for each sample, following Bradford protocol (Bradford, 1976). Ethyl acetate (1 ml) was added to this tube with water and placenta, vortexed for 15 seconds, and centrifuged for 15 minutes at 4°C. About 400 µl of supernatant was transferred to a new 1.5 ml microtube; the second (duplicate) was transferred to another tube. All samples were dried overnight in a hood until dry. For glucocorticoid analysis, all samples were re-suspended with the same volume, using assay buffer. The analysis was performed using the same EIA protocol as for salivary cortisol (item 2.4), and cortisone was analyzed by an EIA.

2.7. Weaning and emotionality tests on piglets

The piglets were weaned at 28 days of age, vaccinated (vaccines against Porcine circovirus, *Streptococcus suis*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*), and transported from Fazenda Araporanga in Jaguariaíva-PR (where the first stage of the experiment was carried out) to the Fernando Costa Campus of the University of São Paulo in Pirassununga-SP, with approximately eight hours of travel. One pair of piglets per sow was used for the second part of the experiment. During the transportation, each couple was placed in a box (73.5 cm long, 53 cm wide, 21 cm high) lined with straw (hay).

After weaning, the animals were kept in suspended pens, with six litters kept in the same pen. Each pen consisted of 12 animals: a pair from each sow, grouped according to their mothers during gestation treatment, totaling six pens. The piglets had access to water and food *ad libitum*. The piglets were weighed on Days 28 and 42. Behavioral analysis was performed based on the videos recorded by the camera.

For fear tests, a combination of open field and novel object tests (Zupan et al., 2016) was performed to assess fear levels and exploratory motivations of each animal. The tests were conducted at 41 days of age. The piglets were tested one by one, returned to the pen immediately after the test, and withdrawn one at a time sequentially between the pens, so that the absence of one individual from the group would be balanced over time. The combination of the tests allowed a previous habituation of the piglets in the arena test, in which the open field test preceded the novel object test. The animals were individually tested in the arena (243 cm x 194.5 cm), which contained demarcations on the ground, forming quadrants. Each test lasted five minutes, for a total of 10 minutes. Each piglet was gently placed in a predetermined location in the arena and recorded during the test period. From the recording, the behaviors were analyzed and the latency to walk was quantified, as well as the number of central and lateral quadrants accessed, walking time, freezing time, and vocalizations (events). After this test, a novel object (traffic cone) was inserted by a pulley system in the center of the pen. We recorded subsequent behaviors for five minutes. In this test, the latency for walking, time near the object (quadrants surrounding the object), time exploring the object (close to the object with the head facing the object), freezing time, and vocalizations (events) were evaluated. After each animal was tested, the pen was washed with water to reduce possible chemical cues, as well as to remove feces and urine from the pen. The analyzer was blind to treatment.

2.8. *Skin lesions on piglets*

To evaluate aggressiveness, the number of skin lesions was counted using photographs, (Guy et al., 2009) performed on weaning day (day 28), and later on Days 29, 35, 36, 42, and 43. Each piglet was restrained and individually photographed, in which photos of

the body, back, face, ears, and neck were recorded on both sides, totaling six photos per animal per day of registration. The analyzer was blinded to treatment.

2.9. Piglet saliva collection

As in sows, saliva collections in piglets aimed to assess the activity of the HPA-axis. The samples were collected when the piglets were 28, 29, 35, and 36 days old, with samples collected individually at 0600 h and 1800 h. The collection material and methods were the same as in the sows (see 2.4). The animal chewed the cotton until saliva saturation. Piglets do not accumulate significant amounts of saliva, so the first cotton was used and each sample was placed in a 15-ml falcon tube, placed in a box with ice, and then frozen at -20°C until processing. The cortisol analysis using EIA followed the same protocol as described before (item 2.4).

2.10. Data analysis

For analysis, we first tested the normality of the data using the Shapiro-wilk test. The statistical tests were performed using the software R studio and are specified in the respective figures. The significance level adopted was $p < 0.05$. Data that did not show normality were tested using a corresponding non-parametric test.

3. Results

The environmental enrichment changed positively behavior and HPA axis in the sows, as well as in their offspring. There was difference in the stereotypic behavior expressed between treatments (Fig. 1). There was no difference in the duration of lying down laterally ($p > 0.05$ in all days). There was no difference in the number of piglets born alive (T test; $p = 0.77$; $F = 1.11$), total number of piglets born (Mann-Whitney U Test; $p = 0.40$; $Z = 0.83$), and

farrowing duration (Mann-Whitney U Test; $p = 0.66$; $Z = 0.42$). Saliva cortisol concentrations were the same at each time point, during gestation before enrichment and in the basal sampling (Days 88 and 89), showing that the sows had similar HPA-axis activity. The only difference between groups was a higher cortisol concentration in the afternoon in the control group (Days 91 and 92; Fig. 2). There was no difference in the glucocorticoid concentrations in the placenta tissue (Fig. 3).

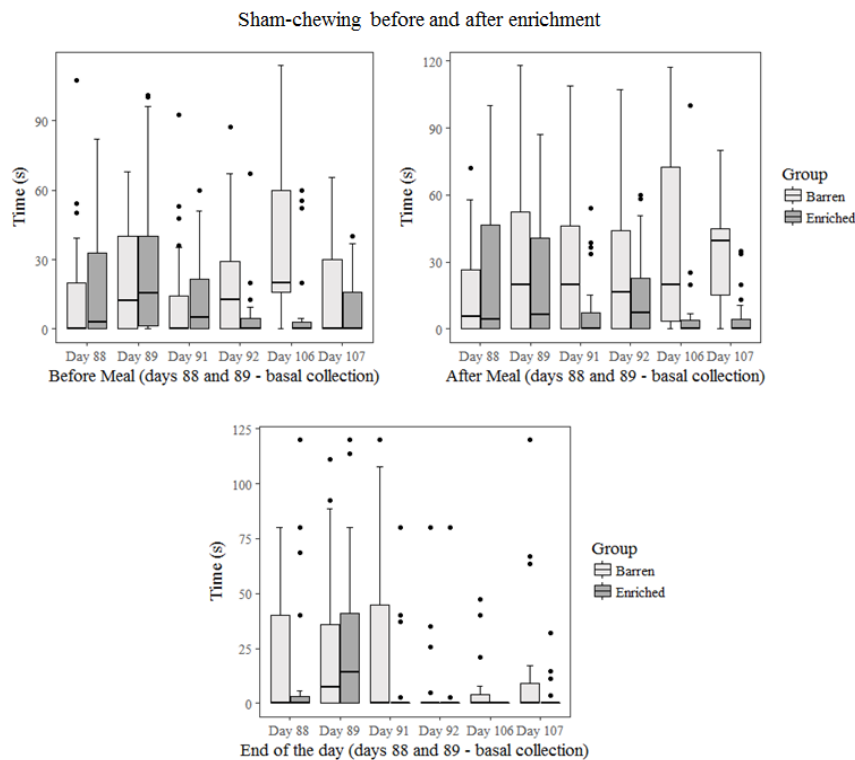


Figure 1. Sham-chewing before and after enrichment. Stereotypic behavior before (basal Days: 88 and 89) and after enrichment (Days 91, 92, 106 and 107). The barren treatment expressed more sham-chewing before meals on Days 92 (Mann-Whitney U Test; $p = 0.001$; $Z = 3.14$) and 106 (Mann-Whitney U Test; $p = 0.00006$; $Z = 4.01$). After meals, they expressed more sham-chewing on Days 91 (Mann-Whitney U Test; $p = 0.004$; $Z = 2.81$), 106 (Mann-Whitney U Test; $p = 0.001$; $Z = 3.20$), and 107 (Mann-Whitney U Test; $p = 0.00006$; $Z = 3.99$). There was no difference in sham-chewing in the afternoon (performed in the end of each day of samples collection).

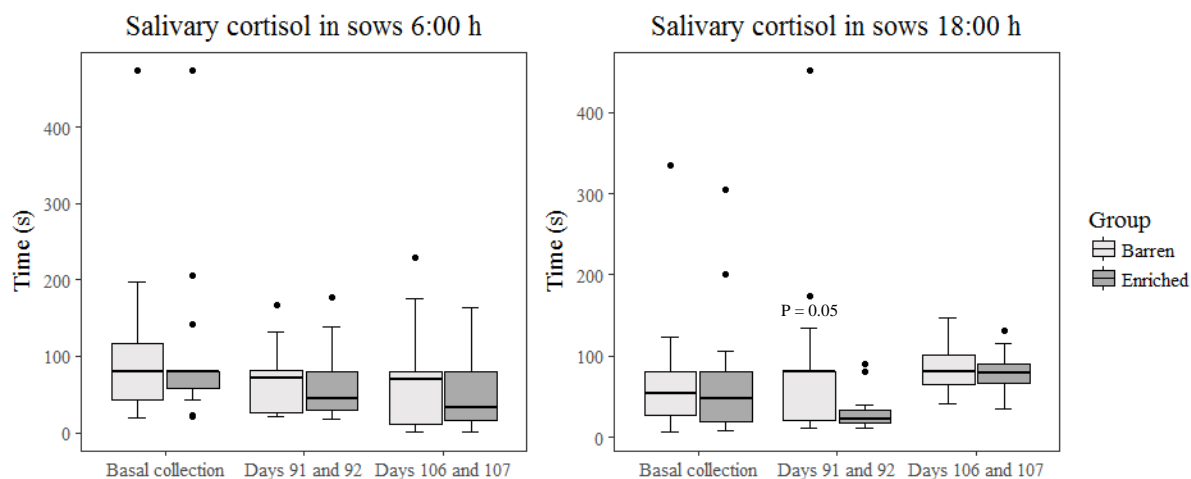


Figure 2. Salivary cortisol in sows. The control group showed higher saliva cortisol concentrations on Days 91 and 92 (Mann-Whitney U Test; $p = 0.05$; $Z = 1.92$) at 6:00 pm. There was no difference in the morning on Days 88 and 89 (Mann-Whitney U Test; $p = 0.75$; $Z = 0.31$), Days 91 and 92 (Mann-Whitney U Test; $p = 0.77$; $Z = 0.28$), or Days 106 and 107 (Mann-Whitney U Test; $p = 0.83$; $Z = 0.20$). There was no difference at 6:00 pm on Days 88 and 89 (T test; $p = 0.80$; $F = 1.05$) or Days 106 and 107 (T test; $p = 0.37$; $F = 1.53$).

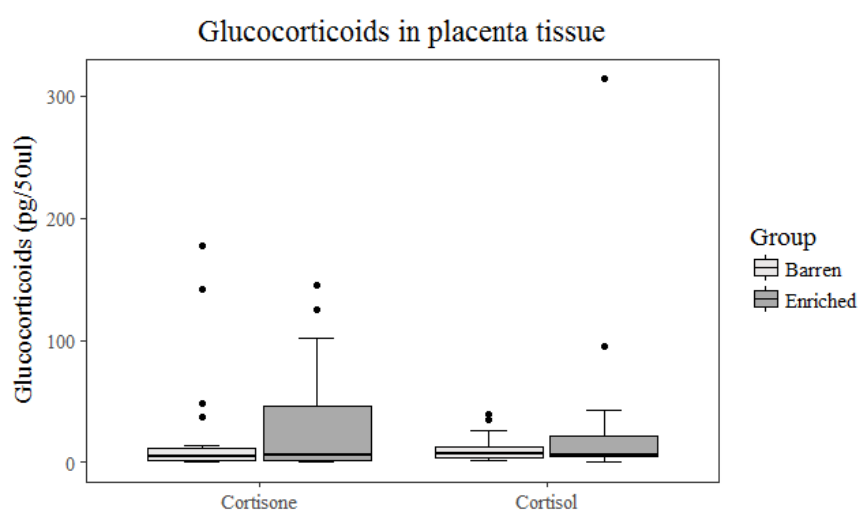


Figure 3. Glucocorticoids in the placenta. Mean (\pm SEM) concentrations of 19 sows in the barren and 15 sows in the enriched treatment. There was

neither a difference in the cortisol (Mann-Whitney U Test; $p = 0.84$; $Z = -0.19$) nor in cortisone concentrations (Mann-Whitney U Test; $p = 0.61$; $Z = -0.50$).

The piglets born from enriched sows had higher cortisol concentrations on the weaning day (Fig. 4). However, on day 35, piglets from sows with environmental enrichment showed lower cortisol concentrations (Fig. 4). In the afternoon, representing lower cortisol levels due to the circadian rhythm, piglets born from sows kept in the enriched environment had lower cortisol levels (Fig. 4). In addition, piglets born from sows kept in the enriched environment spent less time performing nosing on Days 4 and 5 (Fig. 5) and had less aggressive behavior (Fig. 6). Piglets born from sows in the enriched environment had lower skin lesion scores on day 42 (Fig. 7). There was no difference in the piglets' emotionality when we compared males and females. However, when the data were divided based on sex, there was a difference in the females' exploratory behavior (Fig. 8).

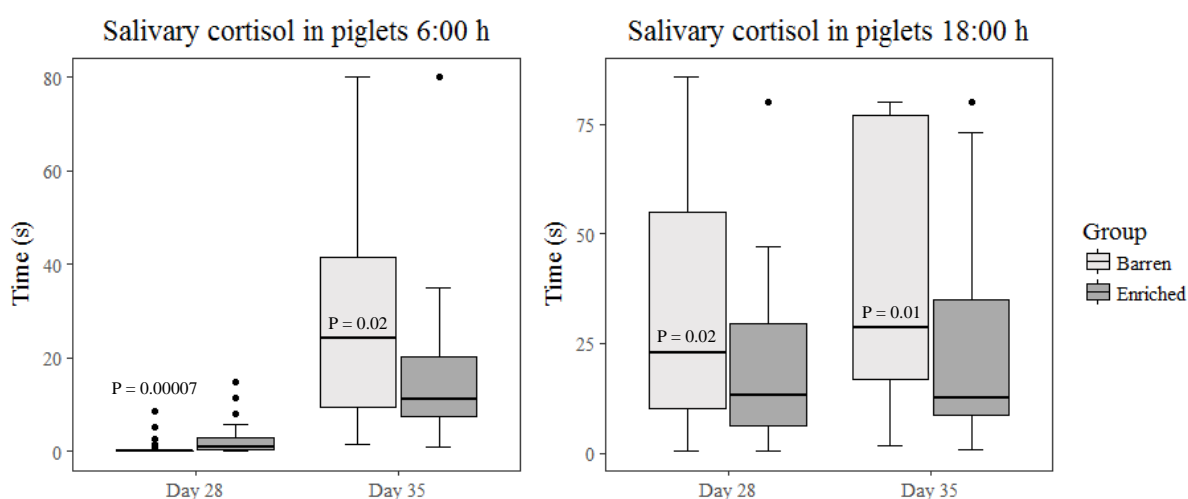


Figure 4. Salivary cortisol concentrations in piglets. In the morning offspring from enriched sows had higher cortisol concentrations on the weaning day (Day 28; Mann-Whitney U Test; $p = 0.00007$; $Z = -3.37$). On day 35, offspring from barren sows had

higher cortisol concentrations (Mann-Whitney U Test; $p = 0.02$; $Z = 2.21$). In the afternoon, offspring from barren sows had higher cortisol concentrations on day 28 (Mann-Whitney U Test; $p = 0.02$; $Z = 2.28$) and on day 35 (Mann-Whitney U Test; $p = 0.01$; $Z = 2.37$).

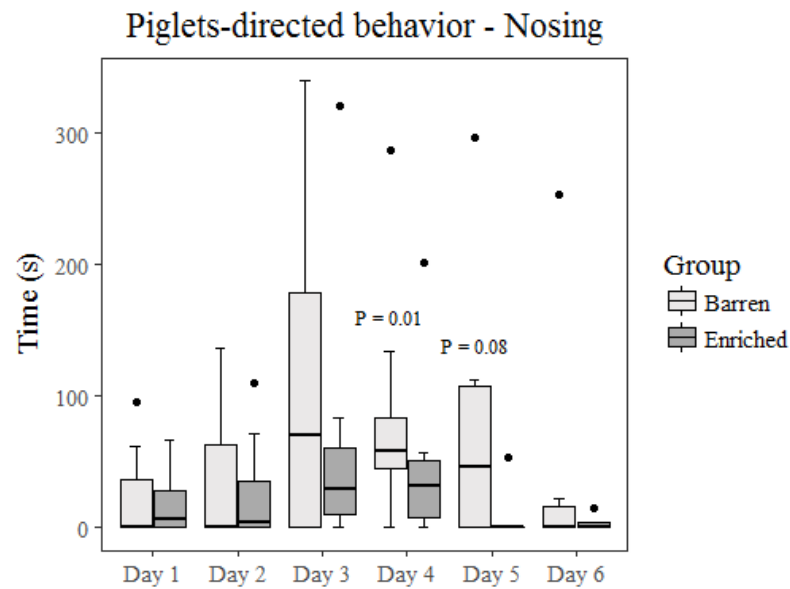


Figure 5. Piglet-directed behavior (nosing). The offspring from barren sows spent more time nosing on Days 4 (Mann-Whitney U Test; $p = 0.01$; $Z = 2.51$) and 5 (Mann-Whitney U Test; $p = 0.08$; $Z = 1.70$). The duration average between piglets in each group is indicated by the variable time in the y axis.

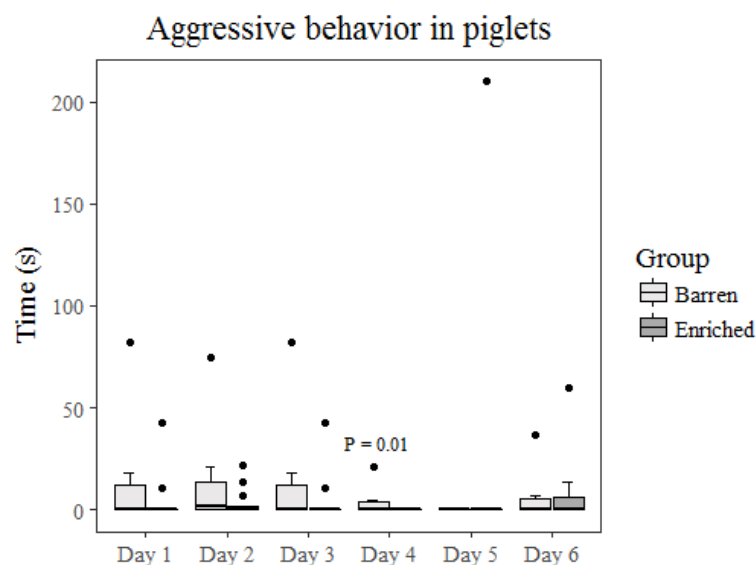


Figure 6. Aggressive behavior in piglets after weaning. The offspring from barren sows spent more time showing aggressive behavior (Mann-Whitney U Test; $p = 0.01$; $Z = 2.42$). The duration average between piglets in each group is indicated by the variable time in the y axis.

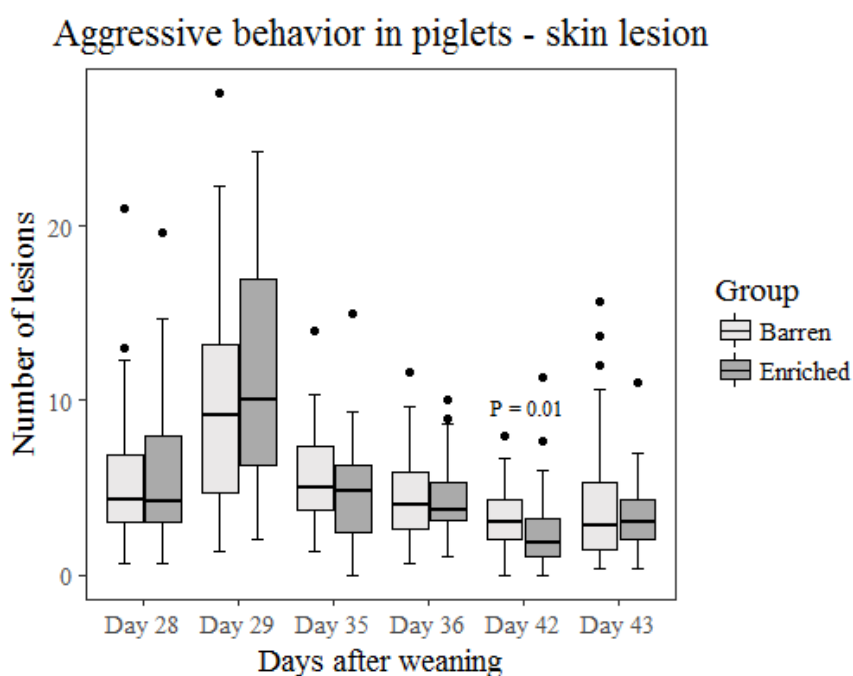


Figure 7. Skin lesion. Piglets from enriched sows had lower skin lesion scores on day 42 after weaning (Mann-Whitney U Test; $p = 0.01$; $Z = -2.48$).

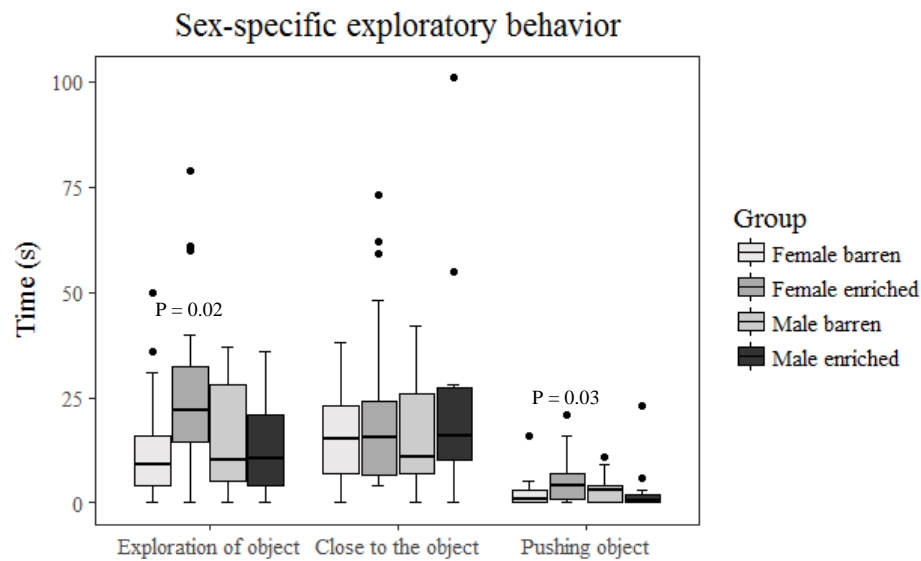


Figure 8. Sex-specific exploratory behavior in the novel object test. The female offspring from enriched sows expressed higher object exploration (Mann-Whitney U Test; $p = 0.02$; $Z = -2.22$), and spent more time pushing the object (Mann-Whitney U Test; $p = 0.03$; $Z = -2.08$). The average duration among piglets in each group is indicated by the variable time in the y axis.

4. Discussion

We showed that environmental enrichment can change the offspring HPA-axis activity and behavior. Providing straw at the end of gestation in sows affected piglet behavior, such as aggression and nosing, which were both higher in the offspring from sows kept in the barren environment. Regarding emotionality, there was no difference when we compared groups with both sexes together. However, there was a sex-specific difference, in which females born from sows kept with environmental enrichment explored more and showed less fear. We also demonstrated that environmental enrichment during the end of gestation could change the HPA-axis in the offspring; higher salivary cortisol concentrations were observed in piglets born from sows kept in the barren environment. In the sows, salivary cortisol was higher only

at the afternoon collection in sows maintained in the barren environment and there was no difference in glucocorticoid concentrations of placental tissue.

In the first part of our study, we showed that environmental enrichment changed sow behavior by reducing stereotypic behavior (sham-chewing). We expected the oral stereotypic behavior reduction from the addition of straw, which is in agreement with other studies (Fraser, 1975; Spoolder et al., 1995). For salivary cortisol concentrations, there was only a difference in one measure (Days 91 and 92 of gestation) in the afternoon, with higher levels in sows kept in barren environment. The morning peak in the cortisol curve is similar between sows, because it plays a role in the circadian rhythm and there was no straw effect. However, in the afternoon, cortisol concentrations were lower in sows subjected to environmental enrichment. As expected, there was no difference in the basal measures (Days 88 and 89 of gestation). This result also suggests that the effect that we showed was a result of the treatment, not to a possible difference in the individuals or groups (e.g. high stress levels in a pen associated with the social context). The absence of differences on Days 106 and 107 of gestation could be due to the physiology in the sows changing to prepare them for delivery, overlapping with the straw effect.

In the placental tissue there was no difference in the glucocorticoid concentrations between sows kept in barren or enriched environments. The placenta protects the fetus during gestation. In placenta physiology, the 11β -hydroxysteroid dehydrogenase inactivates cortisol by converting it into cortisone (and vice versa), thus regulating glucocorticoid availability and their effects on the neural receptors. During development, the amount of glucocorticoids that the fetus receives from the mother has the potential to completely change the trajectory of the offspring (Abe et al., 2007; Fowden et al., 2016; Nolvi et al., 2016; Rutherford et al., 2014). The alterations in the offspring HPA-axis activity in our results, as a reflex of the stress response, indicate that the glucocorticoid-associated mechanisms are altered.

In the piglets, day 28 represented a challenge because it was the weaning day. In the morning, salivary cortisol concentrations were higher in piglets born from sows kept in environmental enrichment. It is possible that their responsiveness to stressful events was more functional and efficient. The combination of weaning and transportation was a huge stressor, which raises HPA-axis activity in order to overcome it. In other words, they were coping properly with stressful events. However, in the afternoon for both measures, during which time cortisol levels are reduced as part of the circadian rhythm, piglets born from sows in the barren environment showed higher cortisol concentrations. In this case when they were not dealing with an acute stressor, the cortisol concentration was an indicator that the HPA-axis in these piglets was more active.

Glucocorticoids are important stress hormones in adult animals but the functions range more widely in the fetus (Fowden et al., 2016; Moisiadis and Matthews, 2014). Glucocorticoids have a non-linear “U-shape function”, in which low or high concentrations can cause negative effects on emotionality and learning (Lupien and Lepage, 2001), and the effects are completely different depending on the gestational age, severity, and duration of the exposure (Fowden et al., 2016). Later in gestation when the fetal HPA-axis has functionally developed, fetal glucocorticoid concentrations can also work independently of maternal levels through cortisol secretion from the fetal adrenal glands. This can occur through HPA-axis activation in response to adverse intrauterine conditions, such as hypoxia and hypoglycaemia (Fowden et al., 2016).

There was a difference in the behavior of piglets with regard to nosing and aggressiveness. Nosing was higher in the offspring born from sows in the barren environment on day four and showed a tendency on day five. Nosing is an undesirable piglet-directed behavior expressed in piglets after weaning that can sometimes be a trigger for aggressiveness (personal observation) and cause skin lesions in the receiver when persistently performed, as

well as belly-nosing (Gardner et al., 2001). When this behavior pattern occurs before suckling and milk intake, it may be associated with hunger or feeding (Gonyou et al., 1998). However, there is a negative correlation between suckling behavior on the sow and nosing after weaning (Torrey and Widowski, 2006). There are differences between nosing (as a piglet-directed behavior) and belly-nosing; the levels in nosing remain much more consistent over time and are seen from the first day after weaning. We consider the nosing we observed to be a stereotypic behavior, since it agrees with the definition of a repetitive behavior without obvious function (Mason, 1991).

Aggressiveness was also higher in the piglets born from sows kept in the barren environment, only on day four of behavioral analysis, and the same result was at day 42 in skin lesion. The stress experienced by the mother could have changed the development of the amygdala, a part of the limbic system. The amygdala is a brain structure that mediates fear, anxiety, aggressiveness, and emotional learning (Balleine and Killcross, 2006; Chiba, 1996).

Emotionality, assessed by fear tests, was not different when we compared males and females in both treatments. However, our results showed that females from sows in enriched conditions explored the novel object more, indicating that they are less afraid and have more exploratory motivations. The placenta works in a different way with respect to sex and the metabolism of glucocorticoids by 11β -hydroxysteroid dehydrogenase type 2, which sometimes auto regulates in females but not in males (Stark et al., 2009). This difference has sex-specific consequences (Mukhopadhyay et al., 2016), in which the placenta can protect the female fetus from excess glucocorticoid exposure, enabling appropriate adrenal responses to physiological stressors (Stark et al., 2009). In our data, we cannot assess the effects of placental glucocorticoids because we pooled samples without regard to sex.

We also have to consider that the effects that we found may not be a result of the prenatal environment, since the effects from the mother-infant relationship have been widely demonstrated (Mogi et al., 2011). Environmental enrichment during gestation could change the mothers' behavior, or even alter traits like anxiety; then the effects that we saw in the piglets could be related to the mothers' behavior during lactation instead of during the prenatal period. An alternative explanation for the effects in the offspring is the early postnatal period, in which the HPA-axis altered during gestation releases high concentrations of cortisol, and as a consequence changes the brain. In both cases, our outcomes would not be related to the prenatal period and we have to consider these possibilities.

In addition, in our study, it was difficult to collect saliva from the animals kept in the enriched pens, especially in the collection performed at 1800 h, because they were apparently in a deeper sleep than the control animals. These observations may be because the animals were more active during the day, interacting with the straw and entering deeper sleep states. Some studies have shown that environmental enrichment may alter the circadian rhythm (De Groot et al., 2000; Mirmiran et al., 2003; Ruis et al., 1997), and may possibly alter the quality of sleep states. Sleep states can be related to metabolism pathways and can change the cortisol circadian rhythm.

Regarding the way that the animals lay down, we did not observe an effect of the substrate in the choice between lying down ventrally or laterally. Pregnant sows fed a higher volume (fiber-rich diets) are more satiated and spend more time lying laterally (Zonderland et al., 2004). These observations are relevant because a possible undesirable interfering variable would be the difference in satiety, since the substrate was consumed. However, we consider the straw to be extremely important because it is biologically relevant to pigs. Straw consumption may be an important factor for animal welfare, which suggests the possibility of controlling some variables in relation to their state. The possibility to interact with the

environment in which the animal is placed increases the likelihood of adjusting to the environment.

Conclusions

In this study, we have shown that environmental enrichment during end of gestation changed the offspring phenotype, making them more robust and adjusted to their environment. The behavioral and physiological indicators were consistent, corroborating our hypothesis. Swine was the model used in this study and we suggest that environmental enrichment should be used during gestation in animals for avoiding or reducing stress.

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Conflict of Interest

The authors declare that they have no competing interests.

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Maternal stereotypic behaviour affects neuroepigenome in the offspring

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Abstract

The goal of the study was to assess the relationship between the performance of stereotypes in late pregnant sows on the neuroepigenome (methylation) in the brain of the offspring. Sows that exhibited consistent stereotypic behaviour on at least two of six days of observation were compared with sows that never exhibited the same behaviour in these six days. We demonstrated in our previous work that stereotypic behaviour in late pregnant sows increased fear responses in their offspring. Environmental enrichment was used to reduce stereotypes, in late pregnant sows, and it improved welfare outcomes in the offspring. Here, we analysed epigenetic changes in the brain, considering structures related with emotionality, such as frontal cortex, hippocampus and amygdala. We focused on the methylation of eight genes, which are involved in neuroplasticity and psychiatric disorders. As far as we know, this is the first study investigating the effects of maternal stereotypic behaviour in the neuroepigenetic programming.

Keywords: emotionality; epigenetic; gestation; prenatal; stereotypic behaviour.

1. Introduction

The lack of complexity in the environment is a common factor associated with the development and occurrence of stereotypic behaviours ^{1,2}. The expression of stereotypies is considered a welfare indicator ^{1,3,4}. Stereotypies are defined as repetitive, invariant and apparently functionless patterns that develop in environments that compromises welfare ¹. It is expressed by animals kept in environments with few stimuli, that causes fear or frustration ¹. However, this specific behavioural indicator is not consistent with elevated levels of cortisol ⁵, a widely recognized physiological welfare indicator. In general, situations inducing or exacerbating stereotypies also decrease welfare ⁶, and the most consistent data available support the fact that stereotypies are related with frustration ⁶⁻⁹.

Stereotypies are often considered one of the most important long-term welfare indicator, usually informing reduced welfare ¹⁰. Considering one given population, the individual that is expressing stereotypies might be better adjusted to that challenging condition, compared with the one that is not expressing the behaviour abnormality, when exposed to the same situation. Based on “The Coping Hypothesis”, the performance of stereotypies helps animals to cope with stress ^{6,11,12}. This hypothesis is still controversial, since the scientific data are not consistently corroborating it.

Learning from data related to sexual selection, male mink that expressed stereotypies had lower success in copulation ¹³. Considering the long-term outcomes associated with maternal programming, we hypothesize that stereotypic behaviour during gestation can affect the offspring’s phenotype. Based on the understanding that it may help to cope with stressors, mothers expressing stereotypies during gestation would provide a more adjusted uterine environment for the development of their offspring .

Environmental enrichment has been used to reduce stereotypies. Recent studies have shown that environmental enrichment, in addition to allowing the expression of natural

behaviours, has a neuroprotective role in brain development and aging ¹⁴; increase brain plasticity ^{15,16}; regulates the HPA axis activity ¹⁷⁻¹⁹; reduce methylation in hippocampal and frontal cortex genes ²⁰. Environmental enrichment affects the expression of genes in the brain, especially those involved in neuronal structure, synaptic signalling and plasticity ²¹. Some genes have been known to be associated with learning and memory ²². Moreover, enrichment affects brain weight, increase arborisation and density of dendritic spines ²³, and modulates neurogenesis in the hippocampus ²⁴.

During gestation, stereotypic behaviour could affect the offspring's programming based on the "Thrifty Phenotype Hypothesis" ²⁵, that is a concept which neurodevelopment programming induces alterations in the offspring in order to help animals to cope with their initial environment, anticipating postnatal environment ²⁵. The prenatal environment has the potential to adjust the offspring for their future environment helping animals to cope better with challenges. Emotional reactivity, responsiveness to stressors and cognition can be modulated by challenges in the prenatal and neonatal periods ²⁶⁻²⁸.

The main mechanism associated to changes in the prenatal environment and foetal programming is epigenetic, in which the modifications could be stable and cross to the next generations. However, these modifications are dynamic and may change in response to environmental stimuli ²⁹. Epigenetic modification of chromatin, including DNA methylation at the sites of CpG dinucleotides, is a key regulator of gene expression, growth and differentiation in virtually all tissues, including brain ³⁰⁻³⁶. Changes in methylation status at selected genomic loci may affect social cognition ³⁷, learning and memory ^{38,39}, and stress response. Furthermore, it is associated with dysregulated gene expression in a range of psychiatric disorders ⁴⁰⁻⁴², such as autism ^{35,43}, schizophrenia ⁴³, depression and Alzheimer's disease ^{14,44,45}.

Heritable epigenetic changes facilitate rapid adaptation to adverse environmental conditions, but may also result in a mismatch of physiological profiles to later-life challenges, thus enhancing disease risk ²⁹. The effects of prenatal stress on brain structures such as the hippocampus and amygdala can generate changes in the offspring's emotionality ^{46,47}. Neuroepigenetics research has expanded and the recent protocols to assess the mechanisms, such as DNA methylation, indicated that they are involved in normal and pathological brain function ⁴⁸. Improving the mother's welfare during pregnancy can lead to positive changes in the offspring. In this study we studied the impact of maternal stereotypic behaviour in the offspring neurodevelopment, comparing these effects with sows kept in enriched environment as an approach to reduce stereotypes. In order to perform DNA methylation analyses we combined a genotyping followed by sequencing ⁴⁹ with methylated DNA immunoprecipitation ⁵⁰. This method reduces the genome by digestion at restrict sites unrelated to CpGs and CCpGG, unbiased to CpG islands.

2. Material and Methods

2.1. Animals and housing conditions

This study was approved by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (CEUA / FMVZ - protocol number 6157201114). The study was performed in the Araporanga Farm, Jaguariaíva, Paraná, Brazil.

For this study, 36 pregnant sows were studied (TopGen Afrodite®), from six pens out of a group of ten animals per pen, in which 6 from each pen were involved in the study. The sows were from 2nd to 7th parity in the enriched and control treatments (T test, $p > 0.05$). Three pens were supplied with hay from the 90th day of gestation, while control pens were maintained with concrete floor (conventional pens). Thus, half of the animals had access to

hay as bedding material, which was replaced daily between 8 h and 11 h. Sows were divided into three blocks, one week apart in relation to their gestational period. In each block, there was a treatment and a control pen, in order to maintain balance in the experimental design.

Food was offered twice daily, at 0700 h and 1140 h. Animals had access to water *ad libitum*. Each pen was 6 meters long and 3.86 meters wide, with a solid/slatted concrete floor area of 3.97 meters in length and walls that were 0.85 meters high. The feeder was 5 meters long and 0.37 meters wide. The slatted floor area was covered with plywood boards in order to avoid the hay from becoming trapped and damaging the manure management system. The control pens also were fitted with the plywood boards, to avoid any differences in relation to the microclimate inside the pens.

2.2. Experimental design

To measure the effects of the prenatal environment we analysed the methylome in brain tissue of weaned piglets, in the amygdala, hippocampus and frontal cortex. First, we compared the neuroepigenome of male piglets from sows kept in enriched environment (N = 9) with sows kept in barren environment, conventional pens (N = 9). As a second comparison we studied just sows kept in the non-enriched environment, comparing brain tissue from piglets born from sows that express stereotypic behaviour (N = 5) with sows that did not express the behaviour (N = 4). Five male piglets were studied from sows that expressed stereotypic behaviour in at least 2 of the 6 days of observation; while 4 piglets were investigated from sows which was never observed showing stereotypies. Observations were carried out in the end of the day, in order to exclude motivation for food since selected stereotypic behaviour was sham-chewing, an oral stereotypy. The criteria for not using all the replicates was the number of available barcodes in our protocol (56 barcodes).

2.3. Farrowing

Parturitions were monitored and occurred in conventional farrowing crates. At birth, each piglet had its umbilical cord tied with string, immersed in antiseptic solution and dipped in iodine (10%) for 5 seconds. The piglets were then cleaned with paper towels, assigned a number reflecting their birth order on the back with a non-toxic marker, and were passed through antiseptic powder to reduce body moisture. After this initial standard management procedure, the piglets were placed with their mother to ingest colostrum. On the first day of life, the piglets had their teeth grinded, the tail cut, the ears notched, and individual weight recorded, all commercial practices in the farm. The dextran iron application was performed the day after delivery.

2.4. Weaning

Piglets were weaned at 28 days of age, vaccinated (vaccines against Porcine circovirus, *Streptococcus suis*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*), and transported from Fazenda Araporanga in Jaguariaíva-PR (where the first stage of the experiment was carried out) to the Fernando Costa Campus of the University of São Paulo in Pirassununga-SP, with approximately eight hours of travel. One pair of piglets per sow was used for the second part of the experiment. During the transportation, the two littermates were placed in a box (73.5 cm long, 53 cm wide, 21 cm high) bedded with hay.

After weaning, the animals were kept in conventional suspended nursery pens, with six litters kept in the same pen. Each pen had 12 animals: a pair from each sow, grouped according to their mothers treatment during gestation, totalling six pens. The piglets had access to water and commercial pig diet *ad libitum*.

2.5. Brain tissue collection and DNA extraction

Transportation, stunning and slaughter was monitored and controlled to assure good welfare practices and to minimize impact of the procedures on brain collection. The brain of each animal was weighted and dissected. Brain tissue from hippocampus, frontal cortex and amygdala of each animal was collected and immediately frozen in liquid nitrogen.

The DNA extraction was performed with Invitrogen® kit for tissue, following the standardized protocol recommended by the company. The digestion was performed with proteinase K under 56°C overnight. The homogenizations were made each 30 minutes on vortex till each sample become completely digested.

2.6. DNA methylation analyses

In order to perform DNA methylation analyses we combined a genotyping by sequencing ⁴⁹ (GBS) with methylated DNA immunoprecipitation ⁵⁰ (MeDIP). This combination was required due to the method developed to assess DNA methylation in reduced genomes. The reduction was carried out through enzymatic digestion targeting restriction sites that contain 5' CCGG 3' sites ⁵¹. The protocol also enabled the use of multiplex immunoprecipitation using of barcode identification system ⁴⁹ (GBS). As mentioned previously, this method reduces the genome by digestion at restrict sites unrelated to CpGs and CCpGG, unbiased to CpG islands. The GBS method uses ligation steps in which a barcode adapter (identifying individual samples) and the adapter for Illumina sequencing barcoding system are linked at each end of the digested DNA fragments ⁵². Because of the barcoding system, GBS technique enables the performance of a sequencing library with DNA pooled of several individuals ^{52,53}. Since the barcodes and adaptors are bonded, PCR is performed by the clean-up of primer dimers and unbound adaptors ^{52,53}. First, we digested the genome with *PstI* (Thermo Scientific). On the DNA fragments which were connected, Illumina adapters and inter individual barcodes were inserted in the ends of each fragment.

These individual barcodes permitted to join all individual samples into a single tube, getting a pool.

DNA fragmentation generated a reduced (approximately 2% of its original size) and enriched genome of fragments in a suitable range for Illumina sequencing (200–500 bp)⁴⁹. The methylated fraction of the samples was captured by an anti-methyl-cytosine antibody (2 µg µl⁻¹; catalogue number C15200006, Diagenode, Denville, NJ, USA) as previously described⁵⁰. Paired-end sequencing was performed with a read length of 100 bp on the Illumina HiSeq2500 platform, at the Animal Biotechnology Laboratory (ESALQ/USP), BR.

2.7. Bioinformatic analyses

In the methylated DNA sequences, the Stacks v.1.39 program was used for data demultiplexing (Catchen et al., 2011) and quality trimming reads using default parameters. In this procedure, each read stored in a FASTQ file has an identification map key file, a barcode containing matching information for the respective sample. The expected reads begin with one of the individual barcodes and are followed by the cut site remnant for *PstI*, which contains the sequence 5' CTGCA 3'. Fragments are then grouped into individual files, which correspond to individuals identified by their respective barcodes. The option 'very sensitive-local alignment' was used in the Bowtie2 tool v.2.2.5⁵⁴ for the alignment of quality-trimmed reads against the pig reference sequence *Sus scrofa* 11.1 (NCBI). Default parameters for paired-end sequences were used. The coverage depth of each sample was checked using Samtools v.0.1.19⁵⁵ with the 'depth' option. Following read alignment, we merged the sequences of the animals of each group of animals to identify peaks of sequencing coverage between each treated versus control test using the Mac2 (v.2.1.1) program with default parameters. This program generates a .bed file with the positions of each peak that we called "regions of interest" (ROI) to serve as input in the nearby analyses. After this, analyses were

performed using bioinformatics packages from the ‘R’ Bioconductor repository. The BSgenome.Sscofa.UCSC.susScr11 package was uploaded as the reference genome. The MEDIPS R-package was used for basic data processing, quality controls, normalization and identification of differential methylation regions (DMRs). In order to avoid possible artefacts caused by PCR amplification, MEDIPS allows a maximum number of stacked reads per genomic position. This is done by using a Poisson distribution of stacked reads genome-wide. The default parameter of $P = 0.001$ was used as the threshold for the detection of stacked reads. Instead of the default parameter from MEDIPS in which the genome is divided into adjacent windows of pre-defined length size, we used the ROI bed file obtained from Mac2 peaking calling. Mas2 instead of MEDIPS, allows to large methylated regions not to be arbitrarily divided into smaller windows, therefore the analysis is "peak specific" and more powerful. MeDIP-seq data were transformed into genome-wide relative methylation scores by a CpG-dependent normalization method ⁵⁶. This normalization is based on the dependency between short-read coverage and CpG density at genome-wide windows ⁵⁷ and can be visualized as a calibration plot. A calibration plot was generated using one of the individuals of each test to generate a coupling set (object that groups information about CpG density genome-wide) to each specific treated vs. control test. Based on this, a threshold for a minimum sum of counts across all samples per window was defined (minRowSum=10). Sequencing data for each individual were then assigned to one of the experimental groups and differential coverage (i.e. differential methylation) was calculated between each two pre-defined conditions. The ROIs were considered DMRs by defined two thresholds, FDR adjusted P-value < 0.5 for describing genes related to significant DMRs and P-value < 0.05 for exploratory analysis of DRM-gene related enrichment pathways. All the considered DMRs were then annotated against the pig reference genome (*Sus scrofa* 11.1, NCBI) using the Variant Effect Predictor (VEP) tool ⁵⁸.

The internet-based tool used in this study to identify over-represented pathways related to our gene list was Reactome ⁵⁹, which is an open-source curated bioinformatics database of human and other animals pathways and reactions (www.reactome.org). Reactome is capable of accessing a variety of databases that contain previously described biological pathways (e.g. Kegg, Biocarta, Reactome, Wikipathways).

3. Results

We compared the methylome, using combination of the GBS and MeDIP methods, to identify genome-wide variation in DNA methylation between experimental groups, in three structures of the brain, in piglets with different prenatal conditions. First, we compared piglets born from sows kept in environmental enriched pens with sows kept in barren environment. Considering just sows kept in barren environment, we compared piglets from sows that expressed stereotypic behaviour with sows that did not express stereotypies. The reduced-methylated DNA fraction from brain's tissues was sequenced, bioinformatics analyses were performed and filter parameters applied. The location of regions relative to the main genes was analysed varying depending on the comparison (table 1). The chromosomal location is illustrated in the Figure 2.

Table 1. Genes, consequence and function. AC – Amygdala comparison between enriched and barren (+ methylated); CE – Frontal cortex comparison between enriched (+ methylated) and express stereotypy; CEN – Frontal cortex comparison between express (+ methylated) and not express stereotypy; HC – hippocampus comparison between enriched (+ methylated) and barren; HE – hippocampus comparison between enriched (+ methylated) and stereotypy expression; HN – hippocampus comparison between enriched (+ methylated) and not express stereotypy.

Group	Location = DMR	Consequence	SYMBOL	Gene	FoldChange
AC	7:3628423-3628586	3_prime_UTR_variant	FARS2	ENSSSCG00000038717	1,813902125
CE	7:53467012-53467493	intron_variant	FURIN	ENSSSCG00000001817	-4,964525932
CEN	6:85543043-85543203	upstream_gene_variant	RCC1	ENSSSCG00000029097	-3,119373885
HC	11:25218380-25218612	intron_variant	VWA8	ENSSSCG00000029837	-2,119254086
HE	6:42839716-42840010	intron_variant	RHPN2	ENSSSCG00000022312	-3,546216205
HN	1:272992482-272992594	upstream_gene_variant	SURF4	ENSSSCG00000040710	-4,881282418
HN	1:272992482-272992594	intron_variant	STKLD1	ENSSSCG000000031407	-4,881282418
HN	1:272992482-272992594	upstream_gene_variant	STKLD1	ENSSSCG000000031407	-4,881282418
HN	3:110388578-110388953	intron_variant	TRMT61B	ENSSSCG00000030147	-4,865506906

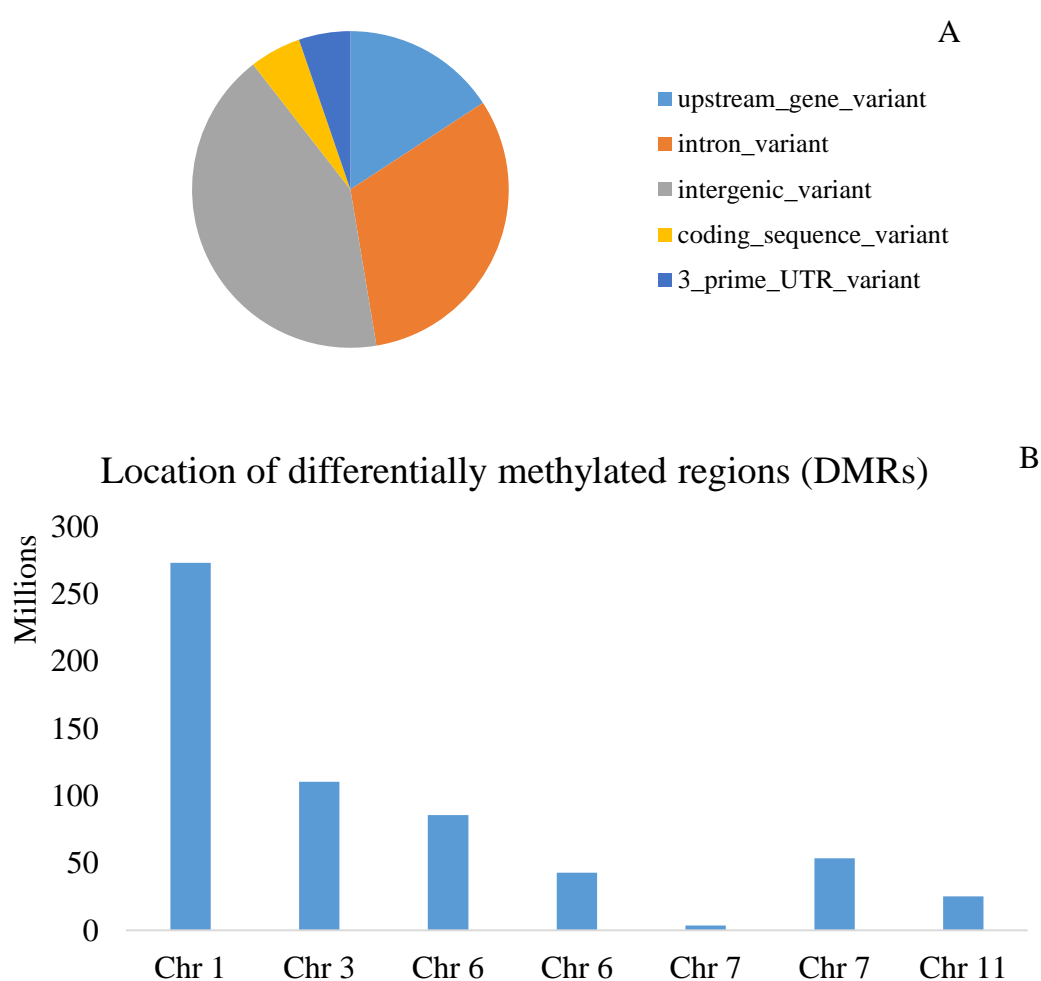


Fig 2. Location of differentially methylated regions (DMRs). (A) Location of DMRs relative to genes. (B) Chromosomal location.

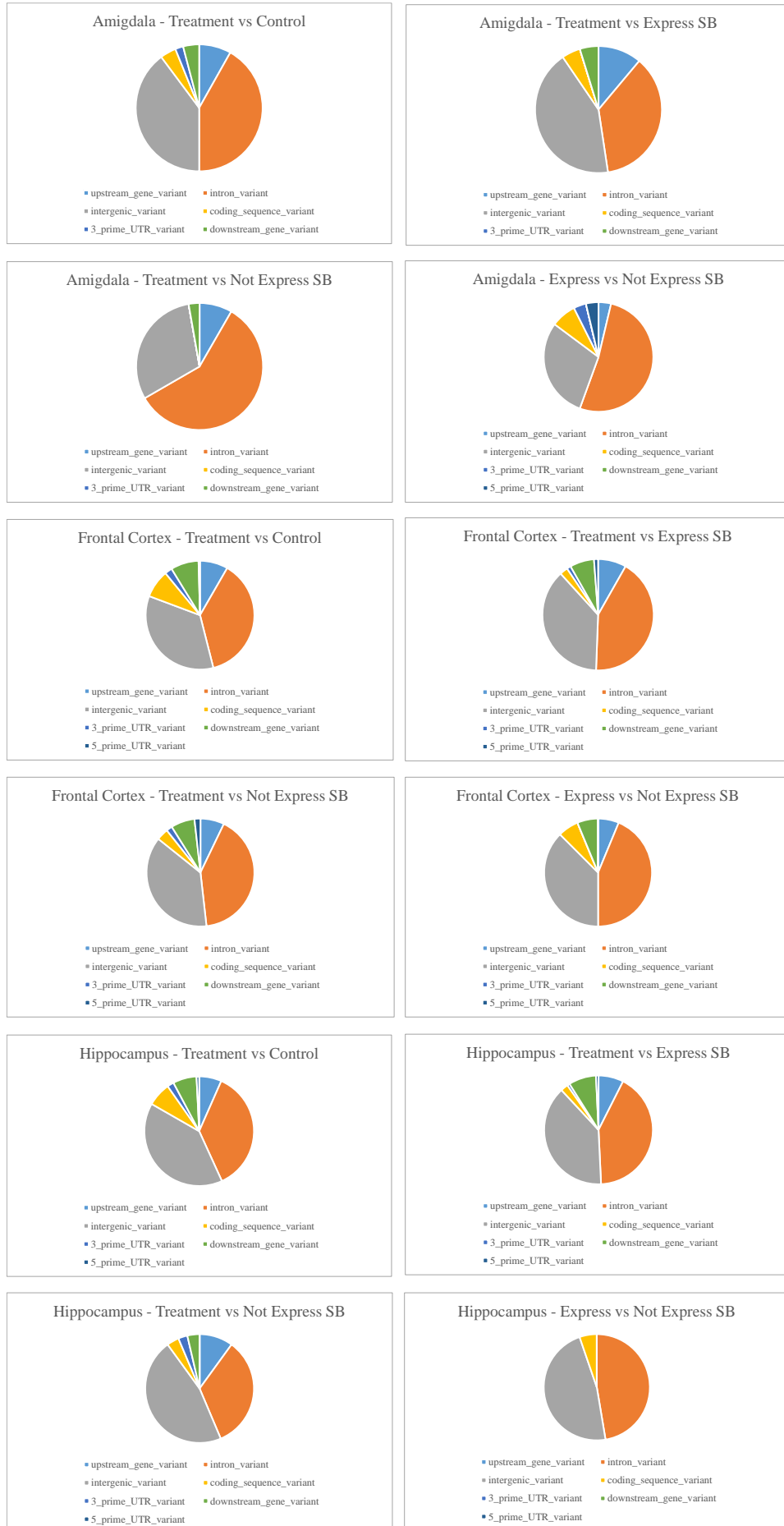


Fig 3. Location of differentially methylated regions (DMRs) in each brain tissue and comparison, showing specific differences in the location of the DMR depending on the group and brain tissue.

FURIN gene was more methylated in the frontal cortex of piglets from sows kept in enrichment when compared with piglets from sows kept in the barren environment that expressed stereotypic behaviour. The gene FURIN is precursor of BDNF, the most common neurotrophin involved in neurogenesis and neuroplasticity, in which FURIN inhibition reduced BDNF maturation and secretion ⁶⁰. FURIN promotes dendritic morphogenesis, learning and memory in mice ⁶¹. These mice exhibited enhanced long-term potentiation, spatial learning and memory performance, without alterations of miniature excitatory and inhibitory postsynaptic currents ⁶¹. Moreover, FURIN promoted spine morphogenesis ⁶¹. In normal tissues, FURIN has been described to be expressed at very low levels, but elevated expression of this convertase has been reported in human cancers ⁶⁰. It has been suggested that FURIN plays a role in homeostasis as well as in diseases including Alzheimer's, cancer, anthrax, Ebola fever, and schizophrenia ⁶².

FARS2 was more methylated on amygdala of piglets from non-enriched pens when compared with piglets from mothers kept in the enriched group. FARS2 gene encodes a protein that transfers phenylalanine to its cognate tRNA. This protein localizes to the mitochondrion and plays a role in mitochondrial protein translation. FARS2 encodes mitochondrial phenylalanyl transfer RNA synthetase (mtPheRS). This gene is associated with infantile epilepsy and abnormal brain MRI findings ⁶³, and progressive myoclonus with compound heterozygous FARS2 mutations ⁶⁴. FARS2 gene has been reported as a causal link with early-onset epileptic encephalopathy ⁶⁵, providing a plausible explanation for pathogenicity of the FARSs mutations. Moreover, the same gene contributes to hereditary

spastic paraplegia, which is a clinically and genetically heterogeneous group of neurodegenerative disorders characterized by spasticity of the lower limbs due pyramidal tract dysfunction ⁶⁶.

Considering the gene RCC1, piglets born from sows expressing stereotypic behaviour, when compared with piglets from sows that did not expressed stereotypies, showed difference in the methylation on the frontal cortex. This difference was reported as higher methylation on frontal cortex in piglets from sows that expressed stereotypic behaviour, when compared to the offspring of sows that did no show the behaviour. RCC1 is a regulator of chromosome condensation ⁶⁷. Proteins with HECT domains have been described to function as ubiquitin ligases, and those that contain RCC1-like domains have been reported to functions as GTPases regulators ⁶⁷. These two activities are essential in a number of important cellular processes such as cell cycle, cell signalling, and membrane trafficking ⁶⁷. Mutations affecting these domains have been found associated with retinitis pigmentosa, amyotrophic lateral sclerosis, and cancer ⁶⁷.

VWA8 gene was more methylated in the frontal cortex of piglets born from sows kept in enriched environments when compared with the offspring of non-enriched sows. VWA8 is associated with ATPase activity in mitochondria, and its presence exclusively in mitochondria raises the possibility that this protein has a role in metabolic regulation or bioenergetics events ⁶⁸. Genome-wide associated studies have linked human VWA8 to neurological and oncological pathologies, which include autism, bipolar disorder, comorbid migraine, and acute myeloid leukemia ⁶⁹. High methylation and low expression of this gene is linked with better outcomes in acute myeloid leukemia ⁷⁰. This gene also appears as a possible signal in three genome wide association studies, for serum calcium concentrations, autism, bipolar disorder with retinitis pigmentosa, and myeloid leukemia ⁶⁸.

The gene RHPN2 was more methylated on hippocampus of piglets born from sows kept in enriched pens when compared with piglets born from sows kept in non-enriched pens, with expression of stereotypic behaviour. RHPN2 is a RhoA-binding protein that modulates cytoskeletal organization and is a critical determinant of tumor invasion in malignant gliomas and cellular changes ⁷¹. When upregulated by either gene amplification or mutation, RHPN2 induces aberrant RhoA-GTP activity, which dramatically reorganizes the actin cytoskeleton to promote tumor invasion and changes in cellular morphology ⁷¹.

SURF4 is a gene that was more methylated in the hippocampus of piglets born from sows kept on enriched environment compared with piglets born from sows kept in non-enriched pens which did not express stereotypic behaviour. This gene may play a role in the maintenance of the architecture of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC)-53 and p24 proteins. Silencing SURF4 together with ERGIC-53 or silencing p24 family member p25 induced an identical phenotype characterized without effect on anterograde transport ⁷².

The gene STKLD1 was more methylated in the hippocampus of piglets born from sows kept in enriched pens when compared with piglets born from sows kept in control, non-enriched environments, the differences were more accentuated in the offspring of sows that did not express stereotypic behaviour. This gene is related to transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity. Protein kinases mediate most of the signal transduction in eukaryotic cells, by modification of substrate activity, protein kinases also control many other cellular processes, including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, and differentiation ⁷³.

TRMT61B was a gene more methylated in piglets born from sows kept in enriched environment in the prenatal period when compared with piglets born from non-enriched sows,

particularly when the sows did not express stereotypic behaviour. Differential expression of TRMT61B in astrocytes was associated with Alzheimer's. Moreover, mitochondrial 16S rRNA is methylated by tRNA methyltransferase TRMT61B in all vertebrates, revealing a conserved mechanism of rRNA modification that has been selected instead of DNA mutations to enable proper mitochondrial ribosome function.

4. Discussion

Here we showed the effects of stereotypic behaviour and environmental enrichment during gestation in sows on the neuroepigenome in their offspring. We have shown before (Tatemoto et al., under review) that both conditions appeared to improve the welfare of pregnant sows, having a positive effect on the welfare of their offspring. In this study we focused on the methylation of eight genes, which are involved in neuroplasticity and psychiatric disorders. A common factor among the eight genes evaluated is the type protein coding. The pathways identified were involved in basic cell functions, increasing the effects on the neurophysiological functioning and diseases risk.

Although stereotypic behaviour is widely used as welfare indicator, it is still not clear what the behaviour may be informing about animal welfare. Based on “The Coping Hypothesis”, the performance of stereotypies helps animals to cope with stress ^{6,11,12}. Our data, from this and previous work (Tatemoto et al. under review) supports the hypothesis that mothers expressing stereotypies during gestation would provide a more adjusted environment to favour their offspring development.

We could speculate that stereotypies are an attempt to complete both the appetitive and the consummatory phases of behavioural sequences, in a restricted, barren environment. The performance is likely to reduce arousal, promoting a better prenatal environment for the offspring. Previous studies demonstrated a time-based shift in the control systems for

stereotypies. In the beginning, stereotypies are helping animals to cope with challenges, than they appear to create alternative pathways in the brain (habit-like), changing neurophysiology and lastly, neuroanatomy. Based on this, we hypothesized that the effects of stereotypies on animal welfare will depend on the developmental phase that the mother is expressing the behaviour.

Appropriated environments during gestation are mandatory for an adequate organization of the central nervous system during development. Epigenetics changes in the brain, by DNA methylation is one of the mechanisms in which the offspring were shaped during prenatal environment, in our study. This plastic response allows the environmental regulation of physiological and behavioural systems. It is an overall adaptive response, since some alterations during development result in changes that confer adjustment to circumstances that the offspring may encounter in adulthood ^{74,75}. However, in artificial environments sometimes there is a mismatch between the developmental cues and postnatal environment, leading to harmful consequences to the strategies favoured as a result of the offspring prenatal adjustment. For instance, sows that have concentrated and limited meals in order to control their body condition score during gestation, showed hunger. This cue is not informative or relevant for the offspring, since piglets in production settings have *ad libitum* access to food. This mismatch causes higher aggressive behaviour in piglets, even before weaning ⁷⁶.

Methylation of the brain's epigenome increases with age ³⁰. Neuroplasticity refers to changes in neural pathways and synapses, which occur in response to changes in behaviour, environment, and other neural processes ³³. The understanding regards to changes in the brain throughout life is changing from a previous understanding that the brain is a physiologically static organ, to the current consensus that the brain remains plastic throughout life ³³. Although changes in the brain/neuroplasticity can be both transient (i.e., fluctuations in

neurotransmitters) or persistent (i.e., alterations to dendritic morphology or synaptic pruning), each modification requires epigenetic activity³³.

Interestingly, environmental enrichment has been associated with increased neuroplasticity in different levels, such as synapses²¹, spine dendritic²³, and then weight of the brain. Environmental enrichment reduces methylation in hippocampal and frontal cortex genes²⁰. Moreover, the enrichment provided for animals could be the operational way to increase the stimulus that they receive. Stereotypies have also been cited as a way animals, including humans, in a not-conscious way attempts self-stimulation (do-it-yourself enrichment)⁶, in order to keep the brain active. In this sense, increasing stimulus could be the common factor between prenatal conditions studied here, in which both enrichment and stereotypies in barren environments are improving welfare outcomes in the offspring.

It is reasonable to consider that the outcomes may not be a result only of the prenatal environment, but it might be influenced by the postnatal environment. The effects from the mother-infant relationship and early social environment have been widely demonstrated^{26,77–79}. Environmental enrichment during gestation could change the mothers' behaviour, or even altered traits such as anxiety^{80,81}. The effects observed could be related to the mothers' behaviour during lactation. An alternative explanation for the effects in the offspring is the prenatal and early postnatal period, since that we demonstrated changes in the HPA-axis activity when comparing the offspring of enriched and non-enriched sows, and the offspring of high stereotypic sows with low stereotypic sows kept in non-enriched pens (Tatemoto et al., under review). Based on this, the HPA-axis altered during gestation could be associated with the release of higher concentrations of cortisol, in the vulnerable offspring, and as a consequence changed their brains.

As far as we know, this is the first study investigating the effects of maternal stereotypies in neuroepigenetic programming. Although there are evidence of foetus

programming and modulation resulting from environmental enrichment ⁸², here we studied enrichment as a protocol to reduce stereotypic behaviour. We have shown before (Tatemoto et al., under review) that enrichment and stereotypies, for non-enriched sows, are improving sow welfare and welfare outcomes in their offspring. We focused on the methylation of eight genes, which were involved in neuroplasticity and psychiatric disease. Meeting the needs of maternal motivational systems, during pregnancy, has a determinant role in the neurodevelopment of the offspring.

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6. Competing interests

The authors declare no competing or financial interests.

7. Author contributions

Conceptualization: P.T., A.J.Z.; Methodology: P.T., A.J.Z, F.P, C.G.B; Formal analysis: F.P.; Resources: A.J.Z; Writing – original draft: P.T.; Writing – review & editing: P.T., A.J.Z.; Supervision: A.J.Z., C.G.B.; Project administration: P.T., A.J.Z.

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Overall Discussion

The main findings of this study is that maternal stereotypic behaviour during gestation decreases fear indicators in the offspring and alters the neuroepigenome of the limbic system. Moreover, we have shown that the performance of stereotypies and environmental enrichment, improved indicators of welfare in sows and improved welfare indicators assessed in their offspring. The changes reported are likely to have altered the phenotype of the offspring during prenatal programming. As far as we know, this is the first evidence showing that stereotypies expressed by the mother during gestation can affect offspring's emotionality, in which the mechanism were epigenetic changes in the brain.

We showed in the second paper that stereotypic behaviour does not affect sows' physiological indicators of stress and productivity measures. Physiological indicators of stress did not show any difference, such as salivary cortisol and glucocorticoids in the placental tissue. In addition, there was no difference in the productivity parameters, such as sows' weight, number of piglets weaned, piglets' mortality, piglets' weight. We argued that the lack of difference could be a result of years of genetic improvement that has selected animals with high productivity rates, making these variables more consistent within the group. If we demonstrated that the expression of stereotypies could compromise livestock production, we could argue that it would be more profitable to improve the welfare in order to avoid stereotypies in sows. Since there is no difference in productivity parameters, it is necessary to foster an ethical approach in order to change the way that we maintain pregnant sows in commercial settings.

In the third paper, we showed that there is a relationship between stereotypies expressed by the sows and emotionality in their offspring. In this approach, we did split the group in a gradient ranging from low to high performance of stereotypies. The results in the open field test indicated that piglets born from sows with high rate of stereotypic behaviour

showed more exploratory behaviour. In the novel object test, the results indicated that piglets from sows with high performance of stereotypies were less stressed because they vocalized less. High frequency of vocalizations is considered to express stressful situations and can be related to poor welfare.

In the fourth paper, we compared sows that did not express stereotypies with sows that expressed the behaviour in at least in two from six days of observation. We demonstrated that stereotypies expressed by sows is associated to reduced fear in their piglets. There was also difference in the cortisol concentration across the placenta, which was higher for sows which did show stereotypies, but no changes were observed for cortisone levels. This finding suggests that the placenta from sows that were expressing stereotypies was maintaining cortisol, instead of making possible for the glucocorticoid to cross the placenta and affected the piglets. We speculated that piglets from sows with high level of stereotypies were less exposed to cortisol. In conclusion, to express stereotypies helps sows to cope with challenges when they are kept in the barren environment, reflecting in positive welfare outcomes in piglets.

In order to elucidate the consequences when stereotypic behaviour is mitigated, we designed one study focusing on the consequences of mitigating stereotypies by using environmental enrichment, in paper five. We demonstrated that environmental enrichment during gestation in sows alters behavioural and physiological welfare indicators in their offspring. Aggressive and nosing behaviour were higher in the offspring born from sows kept in a barren, non-enriched, environment. Regarding to the measures of emotionality, the difference is gender specific, in which females born from sows kept with environmental enrichment explored more and showed less fear. We also demonstrated changes in the HPA axis, which appeared more functional in piglets born from sows kept in environmental

enriched pens. Putting together, these results suggests that when motivational systems are stimulated, with enrichment, it decreases the challenges imposed by artificial rearing of sows.

Finally, we focused in the potential mechanisms that might be associated with the changes in the phenotype observed, analysing the epigenome of limbic system of the piglets. We demonstrated that the behavioural differences of the piglets were affected by the stereotypic behaviour of the sow and also by environmental enrichment. We focused on the methylation of eight genes, which, in the literature, are involved in neuroplasticity and psychiatric disorders. The eight genes were protein coding and the pathways were involved in basic cell role, increasing the effects on the neurophysiology functioning and diseases risk.

We considered that is necessary to understand stereotypies throughout its development and emancipation. The inconsistencies in scientific data can be attributed to the effects of stereotypies, in which we argued that it may help to cope with stressors, but in long-term, it becomes detrimental to the animal. A neuroepigenetic study comparing each phase of stereotypies expression and neuroplasticity would be important in order to elucidate what it means to cope with challenges expressing stereotypies.

Overall, we consider that is reasonable to accept that stereotypies are a welfare indicator. When animals are expressing stereotypies it may indicate that the environment and context in which they are kept is not meeting their needs. However, it is possible that the individuals that are not expressing stereotypies, under the same difficult situation, are experiencing more compromised welfare, as we have shown in our studies investigating the consequences to the offspring.

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