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Lameness in sows and the emotional consequences in the offspring

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Lameness in sows and the emotional consequences in the offspring

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Thank you to BioRender because some figures were made it there.

La utopía – por Eduardo Galeano

Qué tal si deliramos por un ratito
qué tal si clavamos los ojos más allá de la infamia
para adivinar otro mundo posible

El aire estará limpio de todo veneno que no provenga
de los miedos humanos y de las humanas pasiones

En las calles los automóviles serán aplastados por los perros
la gente no sera manejada por el automóvil
ni será programada por el ordenador
ni será comprada por el supermercado
ni será tampoco mirada por el televisor

El televisor dejará de ser el miembro más importante de la familia
y será tratado como la plancha o el lavarropas

Se incorporará a los códigos penales el delito de estupidez
que cometen quienes viven por tener o por ganar
en vez de vivir por vivir no más
como canta el pájaro sin saber que canta
y como juega el niño sin saber que juega

En ningún país irán presos los muchachos
que se nieguen a cumplir el servicio militar
sino los que quieran cumplirlo
Nadie vivirá para trabajar
pero todos trabajermos para vivir
Los economistas no llamarán nivel de vida al nivel de consumo
ni llamarán calidad de vida a la cantidad de cosas
Los cocineros no creerán que a las langostas les encanta que las hiervan vivas
Los historiadores no creerán que a los países les encanta ser invadidos
Los políticos no creerán que a los pobres les encanta comer promesas

La solemnidad se dejará de creer que es una virtud
y nadie nadie
tomará en serio a nadie
que no sea capaz
de tomarse el pelo

La muerte y el dinero perderán sus mágicos poderes
y ni por defunción ni por fortuna
se convertirá el canalla en virtuoso caballero

La comida no será una mercancía
ni la comunicación un negocio
porque la comida y la comunicación son derechos humanos

Nadie morirá de hambre
porque nadie morirá de indigestion

Los niños de la calle no serán tratados como si fueran basura
porque no habrá niños de la calle
Los niños ricos no serán tratados como si fueran dinero
porque no habrá niños ricos
La educación no será el privilegio de quienes puedan pagarla
y la policía no será la maldición de quienes no puedan comprarla

La justicia y la libertad, hermanas siamesas condenadas a vivir separadas
volverán a juntarse bien pegaditas, espalda contra espalda

En Argentina las locas de Plaza de Mayo serán un ejemplo de salud mental
porque ellas se negaron a olvidar en los tiempos de la amnesia obligatoria

La santa madre iglesia corregirá algunas erratas de las tablas de Moisés
y el 6to mandamiento ordenará festejar el cuerpo

La iglesia tambien dictará otro mandamiento que se le había olvidado a Dios:
amarás a la naturaleza de la que formas parte

Serán reforestados los desiertos del mundo
y los desiertos del alma
Los desesperados serán esperados
y los perdidos serán encontrados
porque ellos se desesperaron de tanto esperar
y ellos se perdieron por tanto buscar

Seremos compatriotas y contemporáneos de todos los que tengan
voluntad de belleza y voluntad de justicia
hayan nacido cuando hayan nacido
y hayan vivido donde hayan vivido
sin que importen ni un poquito las fronteras del mapa ni del tiempo

Seremos imperfectos
Porque la perfección seguirá siendo
el aburrido privilegio de los dioses
pero en este mundo
en este mundo chambón y jodido
seremos capaces de vivir cada día
como si fuera el primero
y cada noche
como si fuera la última.

ABSTRACT

Pork is the second most consumed animal protein in the world, and there are important animal welfare concerns for the over 1 billion pigs raised for meat each year. One important animal-based indicator of good welfare in pigs is the absence of lameness, which may affect up to 15% of sows globally. Lameness is an extremely painful and stressful condition which, when not identified and treated correctly, can affect animals in several ways, including through their social behavior, nutritional condition, and overall biological functioning and mental state. During pregnancy, lameness has the potential to alter developmental outcomes of the offspring by transplacental endocrine modification or epigenetic mechanisms. This study initially explored an existing dataset on lameness in sows during pregnancy in which we assessed behavioral and physiological consequences of lameness in sows and their offspring thorough measures of salivary and placental glucocorticoid concentrations in sows, and indicators of agonistic and exploratory behavior in their weaned offspring when mixed and subjected to open field and novel object tests. Follow up studies involved systematic periodical locomotion assessments in pregnant sows on two commercial pig farms, one in Brazil (Farm 1) and one in Italy (Farm 2). A validated 0 to 5 score system was used, with 0 corresponding to easy locomotion and 5 to a downer sow. The studies included 511 pregnant sows (N=397 in Brazil and N=114 in Italy) with at least 3 locomotion evaluations in the final third of gestation to determine prevalence of lameness. A cohort sample of 30 (Farm 1) and 39 (Farm 2) sows were selected and grouped by the severity of lameness, and their productive performance, physiology, and the developmental outcomes in their offspring were assessed. Sows were grouped as not lame (G1; N = 15 – Farm 1; N = 14 – Farm 2), moderately lame (G2; N = 16 – Farm 2), and severely lame (G3; N = 15 – Farm 1; N = 9 – Farm 2). In Brazil sows were grouped only as severely lame or not lame. Productivity data including gestation length, birth weight, total live/stillborn piglets, and piglets dying during the first week post-partum were collected from the selected sows. Glucocorticoid measures were performed on saliva, hair, and placenta of sows from Farm 2. A cohort sample of weaned piglets was selected from Farm 1 (N=90, 3 piglets per sow) and all piglets in the litters from Farm 2 were included. Behavioral data were collected from weaned piglets from Farm 1, skin lesions were counted to assess post-mixing aggression and exploratory behavior was measured using a combination of open field and novel object tests. Nociception threshold assessments were performed in piglets at birth, before and after castration of male piglets from Farm 2, and in weaned piglets from Farm 1. Hair was collected from newborn piglets from Farm 2 to measure cortisol. Salivary cortisol was measured in weaned piglets from Farm 1 before and after transport. After determining the data distributions, parametric and non-parametric statistical tests were used, considering significance when $p < 0.05$ and a tendency when $p = 0.05 – 0.1$. Results from the initial dataset indicated that lameness in pregnant sows reduced piglet weight at weaning and increased agonistic behavior in the offspring. In both the Brazilian and Italian studies, lameness reduced gestation length, whereby the gestation length in G1 sows was longer than G2 sows (Farm 2) and G3 sows (Farm 1). At birth, G1 piglets were heavier than G2 piglets (Farm 2) and G3 piglets (Farm 1). On Farm 2, nociception threshold values at birth and before castration were higher in G3 piglets than G2, and after castration were higher in G1 than G2 piglets. On Farm 1, nociception threshold after weaning was higher in G3 than G1 piglets. On Farm 1, there were fewer skin lesions after mixing in G1 than G3 piglets. On Farm 2, hair cortisol concentrations in G2 sows were higher than G3 sows and tended to be higher in male offspring from G3 than G1 sows. Placental tissue from G1 sows was more efficient in metabolizing cortisol to cortisone that from G2 sows. Finally, in Farm 1, salivary cortisol response to transport was higher in weaned piglets from G3 sows compared with piglets from G1 sows. Here we demonstrated that lameness in sows during the last third of pregnancy altered their physiology and performance and modified the phenotype of their offspring by reducing weight at birth, decreasing response to noxious stimuli, and altering behavioral and physiological responses when piglets were faced with common challenges present in the commercial farming environment.

RIASSUNTO

L'allevamento suinicolo riveste un ruolo economico centrale a livello mondiale in quanto la carne suina rappresenta la seconda proteina di origine animale più consumata globalmente. Di conseguenza, un'attenzione particolare dovrebbe essere rivolta a questo settore per garantire uno stato ottimale di benessere a milioni di animali. Una delle problematiche principali relativa al benessere e alla salute degli animali negli allevamenti suinicoli è rappresentata dalla presenza delle zoppie, che possono andare ad affliggere anche il 15% delle scrofe. La presenza di zoppie, oltre a rappresentare un grave problema, è anche uno dei migliori indicatori *animal-based* per valutare lo stato di benessere degli animali. La zoppia è una condizione estremamente dolorosa e stressante che, se non identificata e trattata, può compromettere gravemente la vita degli animali. Fra le conseguenze principali si possono riscontrare alterazioni del comportamento sociale e delle abitudini nutrizionali, fino ad arrivare alla compromissione non solo dello stato fisiologico ma anche dello stato mentale degli animali. Inoltre, è importante sottolineare come lo stato di zoppia non abbia ricadute solamente sull'individuo stesso ma anche sulla prole. A questo proposito, l'obiettivo del nostro studio era quello di indagare questa problematica. Per indagare l'incidenza della zoppia nelle scrofe gestanti è stato inizialmente analizzato un dataset relativo; successivamente, sono state valutate le ricadute comportamentali e fisiologiche sulla scrofa e sulla prole. Sono state quindi rilevate le concentrazioni di glucocorticoidi salivari e placentari nelle scrofe gestanti e i comportamenti agonistici ed esploratori in un gruppo di suinetti sottoposti ad un *open-field* e *novel-object test.* Inoltre, è stato eseguito uno studio di *follow-up* con una valutazione periodica delle zoppie in scrofe gestanti in due aziende suinicole commerciali: la prima in Brasile (Azienda 1) e la seconda in Italia (Azienda 2). Per eseguire questa valutazione è stato utilizzato un sistema validato di attribuzione dei punteggi da 0 a 5 in base al grado di zoppia: 0 era attribuito animali non zoppi mentre il punteggio 5 ad animali gravemente zoppi. In totale sono state valutate 511 scrofe gestanti (N=397 in Brasile, N=112 in Italia) di cui sono state ottenute almeno 3 valutazioni dello *score* di mobilità durante il terzo finale della gestazione per poter determinare la prevalenza delle zoppie. Un campione di scrofe (N=30 - Azienda 1; N=39 - Azienda 2) è stato selezionato e gli animali sono stati raggruppati in base alla severità del grado di zoppia (non zoppie: G1, N = 15 – Farm 1; N = 14 – Farm 2; moderatamente zoppe: G2, N = 16 – Farm 2; gravemente zoppe: G3. N = 15 – Farm 1; N = 9 – Farm 2). In Brasile gli animali sono stati classificati solamente come non zoppi oppure gravemente zoppi. Per ogni scrofa sono stati registrati i seguenti dati produttivi e riproduttivi: la durata della gestazione, il peso alla nascita, il totale di nati vivi e nati morti, il numero di suinetti morti durante la prima settimana di vita. La concentrazione dei glucocorticoidi è stata valutata nella saliva, nel pelo e nella placenta delle scrofe coinvolte nell'esperimento in Italia (Azienda 2) e nei suinetti svezzati (Azienda 1 – N=30, 3 suinetti per scrofa; Azienda 2 – tutta la nidiata). Inoltre, sono stati registrati i dati comportamentali nei suinetti svezzati dell'Azienda 1: sono state valutate le lesioni cutanee per valutare le aggressioni dopo il mescolamento, i comportamenti esploratori e gli animali sono stati sottoposti a test etologici (*open field* e *novel object)*. La soglia di nocicezione è stata misurata nei suinetti alla nascita, prima e dopo la castrazione dei maschi nell'Azienda 2 2 e nei suinetti svezzati nell'Azienda 1. Un campione di pelo è stato raccolto dai suinetti dell'Azienda 2 appena nati per valutare il cortisolo mentre il cortisolo salivare è stato valutato nei suinetti svezzati dell'Azienda 1 prima e dopo il trasporto. Dopo aver determinato la distribuzione dei dati, sono stati eseguiti dei test statistici parametrici e non parametrici, considerando un $p<0.05$ significativo e una tendenza alla significatività quando il valore di p era compreso fra 0.05 e 0.1. I risultati iniziali del dataset indicano che lo stato di zoppie nelle scrofe gestanti riduce il peso allo svezzamento ed aumenta la manifestazione dei comportamenti agonistici nella prole. Sia nello studio in Brasile che in Italia, la presenza di zoppie ha causato una riduzione della lunghezza della gestazione in quanto la lunghezza della gestazione delle scrofe del gruppo G1 era maggiore di quelle del gruppo G2 (Azienda 2) e G3 (Azienda 1). I suinetti di scrofe G1 erano più pesanti dei suinetti di scrofe G1 (Azienda 2) e G3 (Azienda 1). Differenze sono state osservati anche per quanto riguarda la soglia di nocicezione alla nascita e

prima della castrazione poiché tale soglia risultava più elevata elevati nei suinetti di madri G3 a suinetti di madri G2. Suinetti di madri G1, inoltre, avevano una soglia nocicettiva maggiore di suinetti di madri G2 anche dopo la castrazione nell'Azienda 2. Nell'Azienda 1, dopo lo svezzamento la soglia nocicettiva era più elevato nei suinetti di scrofe G3 rispetto a quelli da scrofe G1. Nell'azienda 1, le lesioni cutanee erano minori in suinetti provenienti da scrofe G1 piuttosto che quello provenienti da scrofe G3. Per quanto concerne il livello di glucocorticoidi, le scrofe G2 presentavano valori più elevati in tutte le matrici rispetto alle scrofe G3. Inoltre, i tessuti placentari delle scrofe G1 erano più efficienti nell'inattivazione del cortisolo a cortisone rispetto alle scrofe le scrofe G2 nell'Azienda 2. Il cortisolo pilifero nei suinetti neonati era più elevato nei suinetti maschi di scrofe G3 rispetto a quello di suinetti di scrofe G1 nell'Azienda 2. Inoltre, la risposta del cortisolo salivare al trasporto era più elevata in suinetti di scrofe G3 piuttosto che di scrofe G1 nell'Azienda 2. Con il seguente studio, abbiamo dimostrato che la zoppia durante l'ultimo trimestre di gestazione nelle scrofe altera non solo le loro performance fisiologiche ma modifica anche il fenotipo della prole, riducendo il peso alla nascita, la soglia nocicettiva e alterando le risposte fisiologiche e comportamentali alle normali sfide che gli animali si trovano ad affrontare negli allevamenti suinicoli commerciali.

RESUMO

A carne suína é a segunda proteína animal mais consumida no mundo, e há preocupações importantes com o bem-estar animal para mais de 1 bilhão de suínos criados para a produção de carne a cada ano. Um importante indicador animal de bom bem-estar em suínos é a ausência de claudicação, que pode afetar até 15% das matrizes globalmente. A claudicação é uma condição extremamente dolorosa e estressante que afeta os animais de várias maneiras, incluindo seu comportamento social, condição nutricional e todo seu funcionamento biológico e estados mentais, quando não é identificada e tratada adequadamente. Durante a gestação, a claudicação, como uma condição estressante, tem o potencial de alterar os resultados de desenvolvimento da prole por modificação endócrina transplacentária ou mecanismos epigenéticos. O estudo inicialmente explorou uma base de dados existente relacionada à claudicação em fêmeas suínas durante a gestação, no qual avaliamos as consequências comportamentais e fisiológicas da claudicação nas matrizes e nas suas proles, através da mensuração da concentração de glicocorticóides em saliva e placenta, e indicadores de comportamento agonístico e exploratório em suas leitegadas desmamadas, misturadas e testadas nos testes de campo aberto e objeto novo. Estudos subsequentes envolveram avaliações periódicas sistemáticas de locomoção em matrizes prenhes, em duas granjas comerciais de suínos, uma no Brasil (Fazenda 1) e outra na Itália (Fazenda 2). Foi utilizado um sistema de pontuação validado, variando de 0 a 5, sendo 0 um animal com fácil locomoção e 5 um animal que não caminha. Os estudos brasileiro e italiano incluíram 511 matrizes gestantes (N = 397 no Brasil e N = 114 na Itália) com pelo menos 3 avaliações de locomoção no terço final da gestação, para determinar a prevalência de claudicação. Uma amostra de 30 (Fazenda 1) e 39 (Fazenda 2) matrizes foi selecionada e agrupada por severidade de claudicação, para avaliar seus desempenhos produtivos, fisiológicos e resultados de desenvolvimento de suas proles. As matrizes foram agrupadas como “sem claudicação” (G1; N = 15 - Fazenda 1; N = 14 - Fazenda 2), “claudicação moderada” (G2; N = 16 - Fazenda 2) e “claudicação severa” (G3; N = 15 - Fazenda 1; N = 9 - Fazenda 2). No Brasil, as matrizes foram agrupadas apenas como severamente claudicantes ou não claudicantes. Dados de produtividade como duração de gestação, peso ao nascer, total de leitões vivos / natimortos e mortos até a primeira semana de idade foram registrados, a partir das matrizes selecionadas. As medidas de glicocorticóides foram realizadas na saliva, pelo e placenta das matrizes estudadas na Fazenda 2. Uma amostra de leitões desmamados foi selecionada na Fazenda 1 (N = 90, 3 leitões por fêmea) e todas as leitegadas foram utilizadas na Fazenda 2. Dados comportamentais foram coletados em leitões desmamados da Fazenda 1, lesões cutâneas foram contadas para avaliar agressão após a mistura dos leitões e o comportamento exploratório foi mensurado usando uma combinação de testes de campo aberto e objeto novo. Avaliações do limiar de nocicepção foram realizadas em leitões ao nascimento, antes / após a castração de leitões machos da Fazenda 2 e em leitões desmamados da Fazenda 1. O pelo foi coletado de leitões recém-nascidos da Fazenda 2 para medir o cortisol. Cortisol salivar foi medido em leitões desmamados da Fazenda 1, antes e depois do transporte. Após a determinação da distribuição dos dados, foram utilizados testes estatísticos paramétricos e não paramétricos, considerando significância quando $p < 0,05$ e tendência quando $p = 0,05 - 0,1$. Os resultados do conjunto de dados inicial indicaram que a claudicação em matrizes prenhes reduziu o peso ao desmame e aumentou o comportamento agonístico da prole. Tanto no estudo brasileiro quanto no italiano, a claudicação reduziu o tempo de gestação sendo mais longo nas matrizes G1 do que nas matrizes G2 (Fazenda 2) e G3 (Fazenda 1). Ao nascer, os leitões do G1 eram mais pesados do que os leitões do G2 (Fazenda 2) e os do G3 (Fazenda 1). Na Fazenda 2, os valores do limiar de nocicepção ao nascimento e antes da castração foram maiores nos leitões do G3 do que no G2, e após a castração os leitões do G1 apresentaram valores nociceptivos maiores do que os leitões do G2. Na Fazenda 1, o limiar nociceptivo após o desmame foi maior no G3 que nos leitões do G1. Na Fazenda 1, houve menos lesões cutâneas após a mistura no G1 do que nos leitões do G3. Na Fazenda 2, as concentrações de cortisol capilar nas matrizes do G2 foram maiores do que nas do G3 e tenderam a ser maiores nas crias machos do G3 do que nas do G1. O tecido placentário das matrizes G1 foi mais eficiente em metabolizar cortisol

em cortisona do que as matrizes G2. Finalmente, na Fazenda 1, a resposta do cortisol salivar ao transporte foi maior em leitões desmamados de matrizes G3 em comparação com leitões de matrizes G1. Aqui, demonstramos que a claudicação durante o último terço da gestação alterou sua fisiologia e desempenho e modificou o fenótipo de sua prole, reduzindo o peso ao nascer, diminuindo a resposta a estímulos nocivos e alterando as respostas comportamentais e fisiológicas quando os leitões enfrentam desafios comuns presentes no ambiente da suinocultura comercial.

SUMMARY

1. INTRODUCTION

Pig production is an important economic activity representing the second most consumed animal protein in the world. The social contribution of pig and poultry production, in Brazil, is worth mentioning as it involves more than 100 thousand families, primarily small producers (ABPA, 2020). The largest global pig producers are China, European Union, United States and Brazil (FAO, 2020). Pork meat is an important source of animal protein, however, it cannot be lost of sight that as sentient animals, production systems must be constantly evaluated, and it is highly relevant to propose changes to improve animal welfare, as scientific knowledge on the biological needs becomes available. The present study was carried out in Brazil and Italy, countries in which swine production represents more than 20% of income within animal production (Canali et al., 2020).

Pigs are animals of enormous behavioral and social complexity. In free life, boars only socialize during the mating season, while sows live in groups and take care of their offspring together. As omnivorous animals, they feed on various resources, including roots or tubers found underground, so rooting is one of their most characteristic exploratory behaviors (Stolba and Wood-Gush, 1989). Wild sows give birth to six piglets on average per litter and hours before parturition they isolated themselves from the group to build a nest and thus provide the necessary thermal conditions and neonatal care to their piglets (Wischner et al., 2009). In contrast, commercial productive conditions frustrated in most cases behaviors such as rooting, foraging for food or nest building, compromising the welfare of animals (Wischner et al., 2009).

The prevailing commercial breeding conditions do not guarantee the expression of biologically relevant behaviors, and consequently their physical and emotional welfare is compromised. One of the best animal-based indicators to determine the welfare state in pigs is the absence of lameness (Whay et al., 2003). Lameness is a painful condition that alters gait and posture in many production animals, including breeding sows and fattening pigs (Ison et al., 2016; Nalon et al., 2013). In the specific case of sows, lameness prevalence reported in the literature has a wide range between 8% to 65% (Heinonen et al., 2013, 2006; Kramer and Alberton, 2014; Pluym et al., 2013, 2011) and the causes of lameness in pigs are multifactorial, including group handling, nutrition, flooring and genetic factors among others (Nalon et al., 2013; van Riet et al., 2013). There are several negative consequences related to lameness in sows, such as considerable economic losses due to treatment costs, impact of reproduction or early culling (Anil et al., 2009; Pluym et al., 2013); or welfare consequences since it is a painful condition its occurrence impairs the normal

development of social, feeding or exploratory behavior of the sow, increasing the risk of hunger, thirst and social stress (Heinonen et al., 2013).

Good reproductive performance is the main demand for sows on commercial farms, so compromised welfare during gestation due to lameness and its consequences have the potential to alter the offspring development. Stress during gestation with the release of glucocorticoids and other biomarkers, may affect fetal development, growth, and maturation (Robertson et al., 2018; Seckl, 2004). Fetus exposed to high levels of glucocorticoids for long periods, may alter programming of the hypothalamic–pituitary–adrenal (HPA) axis, with consequences for behavioral development and welfare outcomes (Moisiadis and Matthews, 2014). Placental tissue is a protective organ, which responds to maternal stress, primarily through the enzyme 11β-hydroxysteroid dehydrogenase type 2 that can be up and down regulated, pending on the demands to inactivate glucocorticoids and thus control their access to the fetus (Welberg et al., 2005). Consequentially, lameness as a stressful condition during pregnancy have the potential to reprogram brain structures in the offspring and alter their behavioral and emotional response to common challenging situations, such as castration, mixing or transport.

The main objective of the present study was to evaluate behavioral and physiological effects of lameness in sows during the last third of pregnancy on their offspring. The protocols used to evaluate these effects were glucocorticoids measurements, nociception assessment, and behavioral assessments. Additional information regarding the welfare and productive performance of lame sows was investigated.

2. CHAPTER I: **The *in-utero* experience of piglets born from sows with lameness shapes their life trajectory** – Scientific Reports - Published. DOI: 10.1038/s41598-021-92507-2.

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The in-utero experience of piglets born from sows with lameness shapes their life trajectory

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Experiences during gestation can alter the mother's behavior and physiology, thereby potentially affecting the behavioral and physiological development of the offspring. In livestock, one common challenge for pregnant animals is lameness: a multifactorial condition that causes pain, stress, resulting in poor welfare outcomes. Since maternal pain can affect offspring development, we aimed to quantify the behavioral response in 142 piglets born from sows with different degrees of lameness during pregnancy. Gait scores of 22 pregnant group-housed sows were assessed six times at 2-week intervals. Lameness scores varied from 0 (no lameness) to 5 (most severe lameness score). Saliva samples and behavior were assessed in the sows throughout pregnancy. Sows were moved to individual farrowing pens and placental tissue was collected for glucocorticoid assessment. At 28 days of age, piglets were weaned, weighed, and regrouped by body size and sex. Skin lesions were counted for each piglet on days 28, 29, and 30 after birth. During open field and novel object tests on day 30, the vocalization and activity levels were evaluated. Piglet data were grouped by the lameness score of the sows as G1 (without lameness), G2 (moderate lameness), and G3 (severe lameness). Data analysis included ANOVA or Kruskal–Wallis tests and pairwise comparisons which were performed using Tukey and Kramer (Nemenyi) test with Tukey-Dist approximation for independent samples. G2 piglets were heavier than G3 at weaning. G1 piglets had fewer skin lesions at days 28 and 29 than G2 piglets. Moreover, G1 piglets vocalized more than G2 when they were subjected to the combined open field and novel object test. We did not identify differences among sows showing different lameness scores in the concentration of placental or salivary glucocorticoids. Lameness in pregnant sows altered the offspring's weight gain, number of skin lesions and vocalizations, together showing evidence that lameness in sows affect offspring performance and behavior.

Lameness in pregnant sows is a common and painful condition and is one of the most frequent reasons for culling, causing considerable economic losses[1,2]. It is also recognized as a very important indicator of animal welfare[3]. Lameness can be the consequence of several factors including inadequate handling, improper housing conditions, and deficient nutrition. High sow density and poor flooring conditions can trigger lameness which can be exacerbated by post-mixing aggression[4]. Furthermore, nutritional factors such as mineral and vitamin deficiencies may be detrimental to bones, articular cartilages, and claws[5]. Lameness can cause health, behavioral, and physiological alterations in animals. The main health impairments associated with lameness in sows include traumas, fractures, osteochondrosis, and foot lesions[1]. Behavioral modifications involve a decrease in social interactions, exploratory behavior[6], and alterations in feeding and lying behavior[7,8].

The behavioral changes associated with lameness indicate that the condition causes pain[6,9,10]. Objective assessment of lameness is established using scoring systems based on behavioral changes, caused by pain, that can distinguish levels of severity[11]. According to the International Association for the Study of Pain (IASP), pain can be defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage[12]. Physiological responses to painful stimuli can be measured by changes, which are mostly mediated by the sympathetic nervous system and by the hypothalamic–pituitary–adrenal axis (HPA). Measurement of

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catecholamines and changes in autonomic responses, such as respiratory rate, heart rate, blood pressure and body temperature, are indicators of sympathetic system activation. Measures usually used to assess HPA responses, such as production of glucocorticoids[13] are reported in animals experiencing pain. Painful stimuli also leave traces at the molecular level, altering inflammatory markers such as TNFα, C-reactive protein, and several interleukins[14]. Since pain is challenging and can be stressful, this scenario can worsen welfare outcomes in pregnant animals due to physiological and molecular responses that could result in epigenetic changes affecting offspring developmental outcomes. Studies have shown that stress or inflammatory responses during pregnancy alters the development of brain structures in the offspring, mainly those pathways responsible for memory, social behavior and emotions[15,16].

Glucocorticoids play a fundamental role during pregnancy in the normal development of fetal organs. Glucocorticoid increase as a result of adverse situations in pregnant subjects can impact fetal development affecting mainly the hippocampus, HPA axis functions and behavior[16,17]. The main placental protective system against active and high glucocorticoid levels is the placental enzyme 11 beta-hydroxysteroid dehydrogenase (11β-HSD-2), responsible for the inactivation of cortisol by conversion to cortisone. A failure in this system has negative consequences in fetal programming[18].

It has been reported that a balance between embryotrophic and embryotoxic cytokines in the female reproductive tract is determined by several stressful events, impacting embryo implantation, placental development, and fetal growth. These mediate biological effects of embryo programming, embryo plasticity, and adaptation[19,20]. In a gene expression study using mononuclear cells from cows with and without lameness an up-regulation of the GM-CSF-R-alpha gene in lame relative to sound cows was reported[21]. In another study the cytokine GM-CSF was identified as a physiological regulator of fetal growth trajectory and placental morphogenesis[22].

Painful conditions during pregnancy are sources of prenatal stress, which involve a cascade of physiological and molecular responses with potential to reprogram epigenetically genes involved in the development of stress neurocircuitry in the offspring, producing phenotypic modifications such as increased basal glucocorticoid levels, decreased expression of glucocorticoid receptors in the hippocampus and changes in spatial learning or in memory performance[23].

Lameness is a painful and stressful condition for the sow which has the potential to affect fetal development, the changes being mediated by high glucocorticoid concentration, cytokines and other stress biomarkers that could cause epigenetic changes.

Given the impact of maternal pain in modulating coping systems in the offspring brain[24], we aimed to measure developmental outcomes in piglets born from sound and lame sows. We hypothesized that offspring born from sows with lameness during pregnancy would show changes in fear responses to novel situations, and also in aggressive behavior, which can, in turn, affect performance outcomes. Specifically, we hypothesized that during an open field and novel object test, piglets from lame sows would explore the arena less and would show longer latency to explore a novel object when compared with offspring of non-lame sows. Furthermore, we hypothesized that piglets from sows without lameness would cope better with social conflicts, thus having fewer skin lesions when compared with piglets born from lame sows.

Materials and methods

Ethical approval. Data were collected from the experimental pig farm of the University of São Paulo (USP), located at the Campus Fernando Costa—Pirassununga, Brazil, with the approval of the Ethics Committee on the Use of Animals (CEUA) of the School of Veterinary and Animal Science (FMVZ/USP), with the number N° 3606300114, according to the Law 11.794, of October 8, 2008 and Decree 6899 of July 15, 2009 with the rules issued by the National Council for Control of Animal Experimentation (CONCEA)—Brazil. The study was carried out according to the ARRIVE guidelines (https://arriveguidelines.org/). The approval of the ethics committee is placed as Supplementary Information.

Animals, facilities, and handling. The present data were collected during a concomitant experiment which measured the impact of fiber in the diet of gestating sows on their offspring[25].

Data from 22 pregnant sows (F1 Landrace × Large White) and their offspring were studied. The sows were nulliparous and healthy at selection, and subsequently inseminated in the same period of the year with pooled semen from a specified group of boars. After insemination and until day 107 of gestation, sows were housed groups of nine in pens that measured 6.7 m × 4.4 m (3.3 m^2 per sow). Each group was fed in nine individual feeding stalls (1.8 m × 0.55 m) two times per day—morning and afternoon—and water was supplied *ad libitum* through nipple drinkers. Individual food consumption was measured only during gestation, not during lactation.

On day 107 of gestation, the sows were moved from the group pens to individual farrowing pens measuring 4.3 m × 2 m. Connected to the pen, there was a creep-feeding area made of concrete (0.97 m × 2.2 m), where piglets had unlimited access to solid feed from birth. We kept all animals in the farrowing pens until day 28 of lactation. Bedding material was provided for the sows and piglets, composed of dehydrated sugarcane bagasse and hay. Farrowing was monitored with IP video cameras (FOSCAM, Fi9821p HD 720P), with a real-time internet transmission to the experimenters. Farrowing was followed through computers, smartphones, and direct observation. All sows were fed an identical solid lactation diet with *ad libitum* access.

All piglets were weighed on the 1st, 21st, and 27th days of age. In addition, during the first day of life, routine management tasks of the farm were carried out: teeth grinding, administration of iron dextran (100 mg, intramuscular), and identification by an ear notch under local anesthesia with 5% lidocaine cream. Weaning occurred at 28 days of age and the piglets were moved to experimental pens (1 m × 0.75 m) with slatted plastic floors. Weaned piglets were mixed into groups formed from two different litters, allocating four piglets matched

Degree of lameness	Description
0	The animal moves easily with little stimulation and bears weight comfortably of all its legs
1	Minor alterations in the gait. When standing, the sow alternates weight bearing in legs. It still walks easily
2	Locomotor disturbance is perceptible in the gait, shorting the steps. Alters position and support of the legs when standing
3	Supports the limb with difficulty. Shortened stride. Reluctant to bear weight on the affected limb
4	Lameness of one or more limbs, display of compensatory behaviors such as arching of the back and/or squatting of the head. Reluctance to walk, difficult to move from one place to another
5	Try to lie down, get up with difficulty and try not to support the committed leg(s)

Table 1. Locomotion score system to assess gait in sows . adapted from Refs.[26,27].

Behavior	Definition
Sleeping	Sleeping animal
Lying ventrally	Lying with belly facing the ground with all limbs under the body
Lying laterally	Lying sideways, with all the limbs extended laterally
Standing	Body supported by the four limbs
Shame-chewing	Continuous chewing without the presence of visible food in the oral cavity
Rooting the floor	Snout touches the ground followed by head movements
Rooting on the empty feeder	Snout touches the empty feeder followed by head movements
Licking the floor	The tongue touches the floor and is followed by movements with the head
Interacting with mats	Snout or tongue touches mats followed by head movements
Interacting with fences or gate	Biting or nibbling the fence wire or gate

Table 2. Sows behaviors collected on first, second and last third of gestation, before and after feeding. . Adapted from Ref.[31].

by weight and sex per pen (for more details see Bernardino et al.[25]). Food and water were provided *ad libitum*, and the pen was cleaned daily.

Experimental design. To assess the effects on the offspring of lameness during gestation, we assessed lameness scoring 22 sows, six times throughout gestation[26,27]. The behaviors of sows, salivary, and placental glucocorticoid concentration were analyzed[28]. In the offspring (N = 156 piglets), aggressiveness was assessed indirectly by counting skin lesions[25,29] and behavior was assessed with a combination of open field and novel object tests[30]. An explanation of the experimental design can be seen in Supplementary Fig. S1.

Lameness assessment in sows. During gestation, six lameness assessments were performed with intervals of 2 weeks between measures. The lameness score applied was a combination of two validated score systems (Table 1)[26,27].

According to the lameness score, sows were classified into three groups, G1: Sows with a degree of lameness ≤ 1 in all six lameness assessments. G2: Sows with a degree of lameness ≤ 3, with at least one of the six lameness assessments with degree 2 or 3. G3: Sows with a degree of lameness ≤ 5, with at least one of the six lameness assessments with degree 4 or 5.

When lameness was detected, pain treatment was performed according to a standard procedure; Flunixin Meglumine at 2.2 mg/kg was administrated intramuscularly, once a day, for 4 days to sows with locomotion score ≥ 3.

Pregnancy and farrowing. During pregnancy behaviors related to position and activity were collected on days 29, 30, 31, 59, 60, 61, 74, 75, 76, 89, 90, and 91, in four periods per day: before and after feeding in the morning, and before and after feeding in the afternoon. During each period, each animal was observed three times, each lasting 2 min, for a total of 6 min per period and 24 min per day of observation. The behaviors observed were sleeping, lying ventrally, lying laterally, standing, sham-chewing, rooting the floor, rooting on the empty feeder, licking the floor, interacting with mats, and interacting with fences or gates. The details of each behavior are in Table 2.

Saliva samples were collected on the same days that behavioral assessment was carried out, early in the morning (06:00) and in the late afternoon (18:00). These samples were stored at – 20 °C immediately after collection and cortisol was measured with Enzyme Immunoassay (EIA)[32]. The collection methodology used was adapted from Refs.[33,34], using two hydrophilic cotton rolls tied to a dental floss with long tips and presented to each animal.

After farrowing, four placenta samples of the same size were collected from random locations from 19 sows and stored immediately at – 20 °C. Glucocorticoid extraction was performed to measure cortisol and cortisone levels using an EIA[32]. The placentas of three sows were not collected due to unforeseen problems.

Group	Degree of lameness	Number of sows	Number of piglets
G1	0–1	7	52
G2	2–3	10	66
G3	4–5	5	38
Total	–	22	156

Table 3. The number (n) of sows and piglets studied per group.

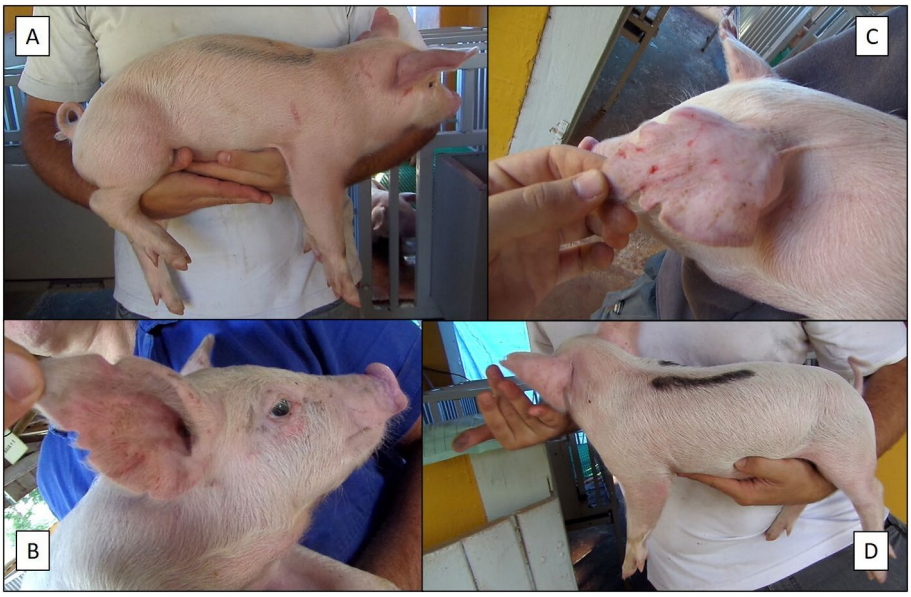

Figure 1. Examples of images used to count skin lesions. (**A**) Right lateral body; (**B**) face and right lateral ear; (**C**) back of the left ear and (**D**) left lateral body.

Additional data collected, included pregnancy length, the total number of piglets born, total piglets born alive, total litter weight at farrowing, total litter weight at 21 and 27 days of age, average daily litter weight gain, average daily weight gain per animal and number of crushed piglets.

Post-mixing aggression score. Data from 156 piglets from the 22 sows were collected and analyzed according to the score of lameness measured in the sow. The number and distribution of the individuals in each group are shown in Table 3.

Pre- and post-mixing aggression was assessed, based on validated methodology[25,29]. Photographs and videos of each piglet were taken daily at 28, 29, and 30 days of age (see Fig. 1). Six piglets from each sow (three males and three females) were used for the evaluation of skin lesions. Two independent evaluators, blind to the treatments, counted skin lesions using the photographs and videos.

Open field and novel object test. A combination of open field and novel object tests were performed on 142 piglets at the end of the experiment, when pigs were 30 days of age using the methodology previously described[30] to assess activity levels, exploratory behavior, and vocalizations (Table 4). The tests were carried out in a pen with the floor marked with squares (see Fig. 2). To carry out the tests, the piglets were taken randomly, alternating between males and females. Due to unforeseen difficulties, the tests could not be done on all piglets; only 142, out of the 156 piglets, were assessed in both tests.

The open-field test consisted of positioning each piglet at the same starting point in the pen, in order to assess the time taken to move, time spent walking, and time remaining in peripheral or central squares. Immediately following the open field test, the novel object test was carried out, and involved introducing an unknown object into the pen to assess latency, exploratory behavior, and proximity to the object. A yellow empty polypropylene bucket with a capacity of 20 L was used as an unknown object. To avoid visual contact between the piglets and the experimenter, a pulley mechanism was used to introduce the bucket in the pen. In both tests, all types of vocalization were counted. For each individual the tests lasted 10 min: 5 min for the open field test followed by 5 min for the novel object test. To reduce the possible chemical signals present in the environment, the pen was always washed with water prior to each piglet assessment.

Test	Measure	Description
Open field test	Latency	Time in seconds between piglet entering in the pen and walking
	Activity	Time in seconds spent walking
	Quadrants accessed	Time in seconds spent in central and lateral quadrants (quadrants on the edge of the pen)
	Vocalizations	A count of all types of vocalization
Novel object test	Latency	Time in seconds between the bucket being placed in the pen until animal interaction with the object (close to and with the head toward to the object)
	Near to the object	Time in seconds the animal spent close to the object (in quadrants that surround the object)
	Quadrants accessed	Time in seconds spent in central and lateral quadrants (quadrants on the edge of the pen)
	Vocalizations	Number of all types of vocalizations

Table 4. Description of data collected during open field and novel object test.

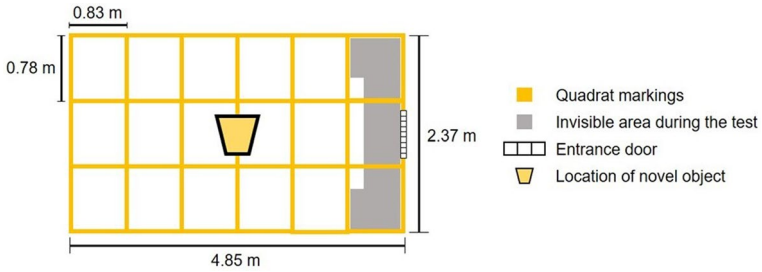

Figure 2. Graphic representation of pen used to perform the open field and novel object tests.

Open field and novel object tests were recorded with a digital camera (Samsung WB250F Smart Wi-Fi Digital).

Statistical analysis. Multiple correspondence analysis (MCA), a type of multivariate analysis, was employed to construct relationships among piglets variables (weight at birth, skin lesions, latency/vocalizations in the open field test, latency/vocalizations/exploration in the novel object test) or sows variables (born piglets, alive born piglets, average weight at birth, average daily weight gain, sow weight, salivary cortisol ratio at pregnancy, placental cortisone and cortisol concentration), the presence of high/low fiber diet of sows (used in the first experiment[25]) and its association with the different lameness groups. The associations were confirmed using a Chi-square test.

To determine the residual distribution was used Shapiro–Wilk test with all variables, when the result was < 0.05 a non-parametric test was used, and when it was > 0.05 a parametric test was used, always considering the number of groups to be compared. The variables used to perform a Shapiro Wilk test were in sows: performance data, ratio between morning/afternoon saliva cortisol concentration and placental cortisol/cortisone concentrations; in piglets: skin lesions number, weight at birth, 21 and 27 days of age, open field, and novel object tests data.

To analyze saliva cortisol at 75 and 90 days of pregnancy, we calculated the ratio between morning and afternoon of each day, ratio was calculated dividing morning cortisol concentration into afternoon cortisol concentration. Subsequently, a Kruskal–Wallis test was used to compare the ratio between groups. Placental cortisol and cortisone were examined in an intraspecific and interspecific way.

Intraspecifically, a T-test was performed, comparing placental cortisol with placental cortisone from the same group. Interspecifically a One-way ANOVA was performed, comparing placental cortisol or placental cortisone from different groups. We calculated the ratio between placental cortisol and cortisone from each sow, dividing cortisol concentration into cortisone concentration and subsequently a One-way ANOVA was performed to compare it between groups.

Weight and skin lesions were compared independently for each day between groups using Kruskal–Wallis test or ANOVA.

A significance level of 5% was considered. All analyses and graphs were performed using the free software environment for statistical computing R (R version 4.0.5)[35]. The MCA was performed using the R packages "FactoMineR"[36] and "factoextra"[37]. To calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended was used the package "PMCMRplus"[38].

Results

After performing an MCA and confirming it with a Chi-square, no associations were found between previous nutritional treatments in sows and the results of the current study (Chi-squared test, p-value = 0.31; see Supplementary Figs. S2, S3 for details).

Variable	Age days	Test	Mean			p-value	Post-hoc p-value		
			G1	G2	G3		G1–G2	G1–G3	G2–G3
Weight (kg)	27	Kruskal–Wallis	8.00	8.45	7.95	0.022	0.08	0.93	0.04
Skin lesions	28		2.69	5.08	2.95	0.003	0.01	0.99	0.02
	29		24.79	32.49	30.58	0.026	0.02	0.26	0.70
Open field test vocalizations	30	ANOVA One way	219	170	183	0.044	0.04	0.35	1.00
Novel object test vocalizations		Kruskal–Wallis	221	160	178	0.002	0.001	0.12	0.51

Table 5. Significant results of weight, number of skin lesions and number of vocalizations during open field and novel object test. The post-hoc test used to ANOVA One way was a Tukey and for Kruskal–Wallis was a Nemenyi test. Degrees of freedom always were 2.

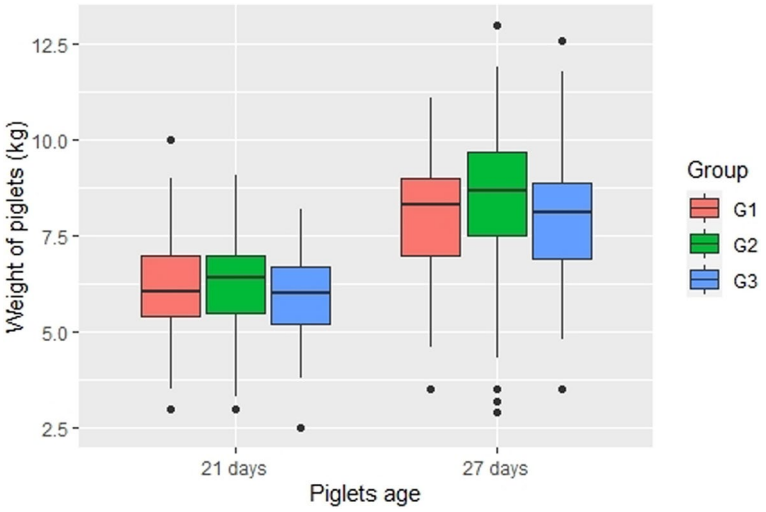

Figure 3. Boxplots to represent the weight of individual piglets at 21 and 27 days of age divided in three groups, according to sow lameness score (G1: lameness score 0–1; G2: lameness score 2–3; G3: lameness score 4–5). This figure was performed in the programming language R using the package ggplot2[35].

Data from sows and offspring before weaning. Descriptive measures of saliva cortisol, placental cortisol/cortisone, and performance data can be found in Supplementary Tables S1, S2, and S3, respectively.

On day 75 and 90 of pregnancy, we did not find differences between lame and non-lame sows when comparing salivary cortisol ratios (Kruskal–Wallis test; $p > 0.05$).

No difference was found in placental cortisol and cortisone concentration between G1, G2, or G3 (One-way ANOVA test; $p = 0.681$ for placental cortisol, and $p = 0.457$ for placental cortisone), and when comparing placental cortisol with placental cortisone in each group, concentration of cortisone was always higher than cortisol (T-test; p-value ≤ 0.05; see Supplementary Table S2 for details). We did not find differences when comparing placental cortisol/cortisone ratios between sow groups (One-way ANOVA; $p > 0.05$). The results of performance data showed no differences when comparison was carried out between groups G1, G2, and G3 ($p > 0.05$).

Body weight and skin lesions in piglets. We did not identify any weight difference at birth or at 21 days of age between piglets from groups G1, G2, or G3. However, weight at 27 days old was different between the groups (Kruskal–Wallis test; $p = 0.02$), with G3 piglets being lighter than G2 piglets (see Table 5 for details). A box plot of the weight of the piglets at 21 and 27 days old can be found in Fig. 3 (see Table 5 for details).

Regarding the number of skin lesions, we found differences at day 28 and 29 (Kruskal–Wallis test; $p < 0.05$; see Table 5 for details). The Fig. 4 is a box plot of the number of skin lesions of the piglets at 28 and 29 days of age. On day 28, piglets from group G1 had fewer skin lesions than piglets from G2, and piglets from G3 had fewer skin lesions than piglets from G2 (see Table 5 for details). Additionally, we did not find differences between piglets from group G1 and G3. On day 29 after farrowing, we identified fewer skin lesions in piglets from group G1 when compared with piglets from G2 and no difference in the remaining comparisons (see Table 5 for details). On day 30, no difference was found between groups.

Descriptions of weight and skin lesion measures can be found in Supplementary Table S4.

Open field and novel object test. In the open field test, we did not identify differences in latency (Kruskal–Wallis test; $p = 0.751$), activity (One-way ANOVA test; $p = 0.823$), access to peripheral (Kruskal–Wallis test; $p = 0.931$) or central quadrants (One-way ANOVA test; $p = 0.374$). However, the number of vocalizations

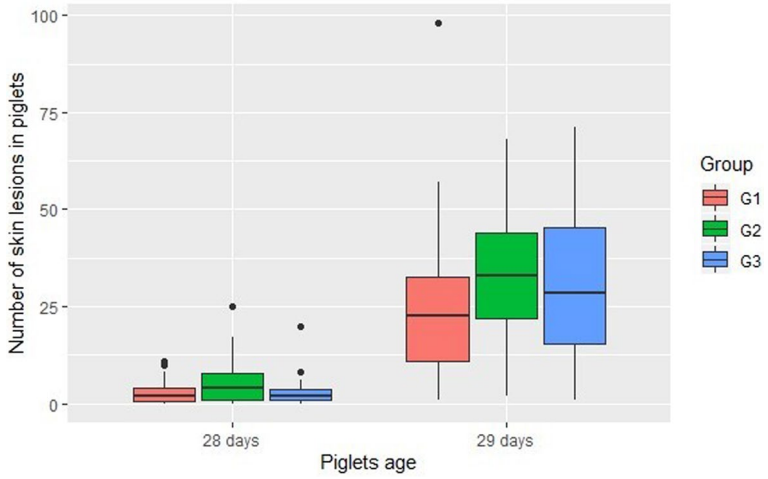

Figure 4. Number of skin lesions in piglets with 28 and 29 days of age, divided in three groups, according to sow lameness score (G1: lameness score 0–1; G2: lameness score 2–3; G3: lameness score 4–5). This figure was performed in the programming language R using the package ggplot2[35].

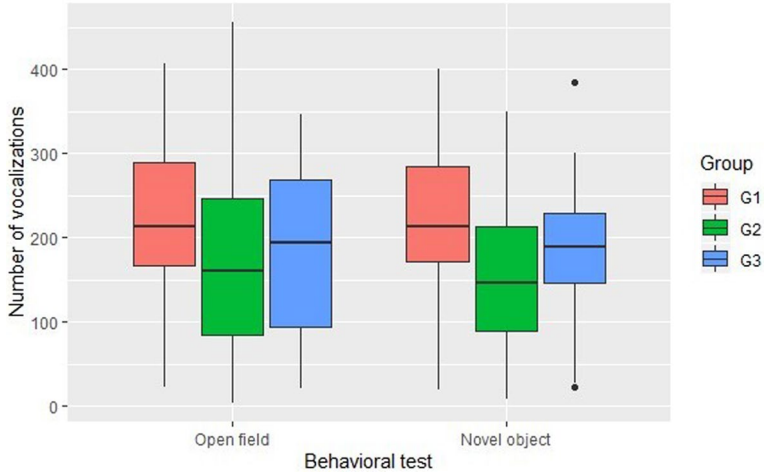

Figure 5. Number of piglet vocalizations during the open field and novel object test, divided in three groups, according to sow lameness score (G1: lameness score 0–1; G2: lameness score 2–3; G3: lameness score 4–5). This figure was performed in the programming language R using the package ggplot2[35].

was higher in piglets from group G1 (offspring of non-lame sows) compared with G2 (One-way ANOVA test; $p < 0.05$; see Table 5 and Fig. 5 for details), G3 was positioned between them without differing from either G1 nor G3.

Similar to the results of the open field test, we found that only the number of vocalizations differed in the novel object test, with a higher number of vocalizations recorded in piglets from group G1 compared with G2 (Kruskal–Wallis test; $p < 0.005$; see Table 5 and Fig. 5 for details), while G3 had fewer vocalizations than G1 but more than G2, without significant differences. We did not see a difference in latency (Kruskal–Wallis test; $p = 0.884$), object exploration (Kruskal–Wallis test; $p = 0.641$), or proximity to the novel object (Kruskal–Wallis test; $p = 0.254$).

Descriptive measures from the variables analyzed during open field and novel object test can be found in Supplementary Table S5.

Discussion

In this study, we have tested the hypothesis that the offspring born from sows with lameness, a painful condition experienced during pregnancy, are affected by their in-utero or neonatal experience. Here we showed that lameness, especially moderate lameness, in pregnant sows has effects on their offspring, increasing number of skin lesions, affecting weight at weaning and vocalizations during open field and novel object tests. The inconsistency of the responses observed, which did not show graded effects with the severity of the lameness, could

be associated with the treatment option that our ethical review board requested, and we promptly applied to the affected animals. The use of flunixin meglumine as an analgesic treatment could potentially have mitigated the negative impact of pain on severely lame sows. Even considering the impact of the therapeutic interventions on the study, we could not let severely lame animals without treatment, for ethical reasons. The administration of flunixin meglumine, in healthy pregnant sows, as a potential control, would be of limited use, given the complex nature of the inflammatory processes associated with lameness.

Surprisingly, there were no differences in glucocorticoid concentrations in saliva or placenta among the groups indicating that other mechanisms may be associated with the reported changes in the offspring.

It has already been reported in several species that an inadequate function of the enzyme 11β-HSD-2 or high glucocorticoid concentrations during pregnancy could decrease the weight and size of the offspring[39–42]. According to the results obtained from the analysis of cortisol and cortisone concentration in the placenta, the absence of difference between the sow groups (G1, G2, and G3) showed no evidence of the role of glucocorticoid-mediated maternal stress. We anticipate that sows had effective action of the enzyme 11β-HSD-2[18], or as the salivary cortisol data demonstrated, glucocorticoids were not the main mechanism responsible for the behavioral changes reported in this study. These findings suggests that other mechanisms could be responsible for the changes found in the offspring of sows with lameness. This could be mediated by other mechanisms associated with longer term stress that have the potential to alter fetal development[43], or the levels of embryo toxic cytokines present in inflammation events during pregnancy[19].

We propose that since lameness is a chronic condition[44], and it could affect inflammatory biomarkers[45,46], they should be evaluated in future studies. Measurement of biomarkers of chronic stress could have given a more comprehensive view of the stress that lame sows experienced, as it has been reported in cows[21].

Moderate lameness and severe lameness during pregnancy did not affect litter performance data before 27 days of age. In the case of prolificacy, lameness has not been reported as a relevant factor that alters reproductive variables such as the number of live piglets[47]. However, individual piglet weight at 27 days of age was different between groups, being significantly lower in piglets G3 compared to G2 and showing a similar tendency in comparisons with G1. This result could have several explanations from the standpoint of nutritional, behavioral, hormonal, or metabolic mechanisms. When nutrition is not ideal during pregnancy, the metabolism of the fetus can be altered during the neonatal period. In our experiment, sows were fed individually during pregnancy, which meant that no effect of lameness on the ability of the sow to compete for food could account for the lower piglet body weight at weaning[25]. Feeding regime during lactation was the same for all the sows, but consumption was not controlled so if lame sows consumed a lesser quantity of food, it could, potentially affect milk production[48,49]. The aspect of a possible difference in food consumption, together with increased lying behavior reported in lactating lame sows[7], is a plausible alternative explanation for the reduced piglet weight at 27 days of age, in the offspring of lame sows. This does not involve the in-utero experience. Shoulder ulceration is common in lactating sows housed in farrowing stalls producing decrease in nursing frequency[50]. In our data, none of the sows showed shoulder ulcers, however we did not collect data on frequency of nursing. It is important to mention that the measures of lameness were collected during pregnancy and at the time of parturition they were resolved. Certainly, measures of nursing events, milk intake and milk quality would be important to characterize the nutritional impact of lameness on the offspring. Another explanation is that glucocorticoids or cytokines intervene in a catabolic way in growth processes[51], and in previous studies, where individuals have been treated with glucocorticoids during pregnancy, it was found that their offspring had lower weight at birth[52]. In our study, we did not find evidence of a higher concentration of glucocorticoids generated by stress or pain in lame sows. In addition the proper functioning of the enzyme 11β-HSD-2 might be also affected, allowing a greater passage of glucocorticoids to the fetus without prior inactivation[43,53]. We did not measure glucocorticoids in placental tissue during pregnancy[54], and our data on the relationship between placental cortisol and cortisone at farrowing suggest that the placenta was efficient in inactivating cortisol to cortisone, but it is important to mention that sows where moved from group housing to individual farrowing pens and as result the placental concentration of cortisol and cortisone may not reflect the events when they did show lameness. Other possibilities are that the metabolic costs associated with coping with lameness that could have compromised the offspring during the prenatal and early postnatal period. In humans, low weight at birth is associated with morbidity in adulthood[55], which leaves us with an open research window to conduct experiments that monitor morbidity in animals born from females with a high or low degree of prenatal stress, especially lameness. The placental concentration of cytokines, which are released in response to inflammatory processes, during pregnancy appears to be higher in smaller piglets when compared with large animals[56]. More studies should be conducted to elucidate the causal mechanisms that may affect the performance of piglets.

As proposed in our hypothesis, a lower number of skin lesions was recorded in piglets from G1 than G2 on days 28 and 29 of age. This result indicates that piglets born from sows without lameness cope better with challenging social situations, probably avoiding agonistic interactions, when compared with the offspring from sows with moderate lameness. No differences were found relating to skin lesions involving piglets from sows with severe lameness, most likely due to the pain relief offered to this group of sows which probably attenuated the effects of severe lameness. Piglet aggression has been associated with compromised memory processes, resulting from the disruption of stress-responsive genes in prematurely weaned pigs[57,58]. We did not measure whether memory processes varied between G1, G2, and G3, piglets, but this would be interesting to explore.

Vocalization was more frequent in piglets from G1 for both open field and novel object tests. These tests are recognized as fear tests, because they impose on the animals a novel and open area, in social isolation, and also a novel object, so that conflicting motivations such as avoidance and exploratory behavior can be measured[59]. Vocalizations, according to their characteristics, are considered as an indicator of negative or positive emotions in different species, including domestic pigs[60–63]. In our results, the emotional valence is difficult to determine since we do not know the characteristics of the vocalizations. Vocalization in piglets could have a beneficial

evolutionary role when exposed to social isolation or situations that represent a negative emotional valence[64,65]. Nevertheless, it would be worth comparing vocalizations in piglets exposed to fear tests in other contexts to make a better comparison, also adding analysis of the acoustic characteristics of these vocalizations, to better analyze associated emotions.

To our knowledge, this is the first study investigating the effects of lameness in sows during gestation on developmental outcomes in the offspring. Here we demonstrated that lameness in pregnant sows, especially moderate lameness, has negative effects on the offspring affecting weight gain and increasing number of skin lesions. We also demonstrated altered reactivity during fear tests, indicated by a decreased vocalization in piglets from sows with moderate lameness. Additionally, since there were no differences in cortisol concentration in saliva or placenta tissue, we suggest that other mechanisms, such as cytokines or epigenetics markers, may be involved in the phenotypic outcomes that we demonstrated, and this needs further investigation. Finally, it is worth emphasizing the consequences of ethical concerns to reduce the pain and suffering in lame animals in our care, especially in contexts where effects may pass to the next generation, particularly when we have the knowledge to assess and mitigate this condition in sows.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M.P.S. contributed to the writing of the manuscript, preparation of figures, analysis, and interpretation of data. T.B. contributed to data acquisition, interpretation of data, discussion of results, and preparation of Fig. 1. P.T. contributed to the data acquisition, interpretation of data, and discussion of results. G.P. contributed to the analysis of data, manuscript draft, and preparation of figures. A.J.Z. contributed with the idea conception, design of the work, interpretation of data, and draft of the manuscript. All the authors contributed to the revision of the final version.

Competing interests

The authors declare no competing interests.

Additional information

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3. CHAPTER II: **Lameness in pregnant sows altered their performance and stress response**

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ABSTRACT

Pregnant sows from commercial pig farms may experience painful states, being lameness the most prevalent one. In this study, we aimed to identify performance and physiological stress responses in sows with lameness during the last third of pregnancy. Periodic locomotion assessments were carried out in 582 (Farm 1) and 171 (Farm 2) pregnant sows from two commercial pig farms, in Brazil and Italy, using a validated score system, from 0 to 5, being 0 an easy locomotion and 5 a downer sow. Sows with at least three assessments before farrowing were selected and grouped as no lame (G1; N = 15 – Farm 1; N = 14 – Farm 2), moderately lame (G2; N = 16 – Farm 2), and severely lame (G3; N = 15 – Farm 1; N = 9 – Farm 2). Productive data as gestation length, type of birth, piglets birth weight, total live/stillborn piglets, and dead piglets during the first week of age were registered from the selected sows. Glucocorticoid measures were carried out in saliva, hair, and placenta of sows from the Farm 2. Descriptive, parametric, and non-parametric statistics were performed to compare data between groups of sows considering significant result when $p < 0.05$ and a tendency when $p = 0.05$-0.1. Main results showed that lameness during pregnancy is highly prevalent in both farms, whereas 41.7% of sows showed moderate lameness – score 2, and 28.2% were with severe lameness – score ≥ 3. Sows with severe lameness have a higher average of live and stillborn piglets than no lame sows; additionally, sows with moderate or severe lameness have higher rate of piglet mortality on the first week ($p = 0.07$) and higher cortisol concentration in hair ($p = 0.05$) than sows without lameness. Sound, no lame sows have more days of gestation ($p < 0.05$), higher average weight of their piglets at birth, and increased placental efficiency to inactivate glucocorticoids than sows with moderate or severe lameness (ratio cortisol/cortisone; $p = 0.02$). The sduty demonstrated that lameness during the last third of pregnancy in sows altered their HPA stress response, reflecting in productive effects and thus negatively compromising sows and offspring welfare.

INTRODUCTION

Lameness in pregnant sows is a common and extremely painful condition, being one of the most frequent reasons for culling, leading to important economic losses and compromised welfare (Dewey et al., 1993; Heinonen et al., 2006). It has been reported that the main productive effect of lameness is regarding the sow's longevity because about 9% of all removals in sows´ herds is due to lameness or foot lesions (Anil et al., 2009; Engblom et al., 2007). Heinonen et al. (2013) reported low number of piglets born alive and an increase of mummified fetuses as a productive consequence of lameness in sows.

Lameness has a multifactorial origin, all combined factors having an important role to account for the high prevalence of lameness in commercial pig farms (Pluym et al., 2017). Among the causes it could be mentioned, inadequate housing conditions represented by high animal densities in a small and barren environment which may increase injuries caused by post-mixing aggression. Along with the established causes, abrasiveness flooring and nutritional factors such as mineral imbalances could be detrimental to bone, articular cartilage, and hoof quality (van Riet et al., 2013).

Lameness is an important welfare problem due primarily to the induced pain and the high prevalence of this condition in commercial pig farms (Pluym et al., 2011). It is a consensus among experts that lameness is one of the best “iceberg” animal-based indicators of welfare in pigs (Dalmau et al., 2009; Whay et al., 2003).

Pain activates the sympathetic/autonomic nervous system and the hypothalamic – pituitary – adrenal axis (HPA) (Sneddon et al., 2014). Physiological changes associated to the HPA activation are good biomarkers to assess animal pain and glucocorticoids are the main stress hormones used as pain and stress indicator (Ison et al., 2016; Sneddon et al., 2014). During pregnancy, glucocorticoids are important for development and maturation of organs to prepare the fetus for the extrauterine environment, but in high concentrations, when produced in response to stress or inflammation, they have negative consequences in the fetal development (Seckl and Holmes, 2007).

Placenta has mechanisms to protect the fetus from maternal glucocorticoids, this involves the release of 11 beta-hydroxysteroid dehydrogenase type 2. This enzyme is responsible for the inactivation of maternal glucocorticoids, transforming cortisol to cortisone, thus reducing the fetal prenatal exposure to cortisol, mitigating their negative effects to the offspring (McTernan et al., 2001). It has been reported that sows that received oral administration of cortisol had shorter duration of gestation (Kranendonk et al., 2005). Environmental enrichment during the last third of pregnancy altered salivary cortisol levels in sows and reduced aggressive behavior in their offspring (Tatemoto et al., 2019), thus showing the effects that the welfare state produces on individual sows with consequences for their offspring.

The high prevalence of lameness in commercial pig farms and their consequent welfare effects supports the importance of knowing more about their effects (Ellingson et al., 2012; Kramer and Alberton, 2014; Pluym et al., 2011, 2013; Sobestiansky et al., 1989). Our research objective was to identify the effects of lameness in pregnant sows on their performance and stress physiology.

MATERIALS AND METHODS

Two experiments were carried out in two different countries – Brazil and Italy, to analyze performance and physiological consequences of lameness in pregnant sows. Each experiment was submitted to the Ethics Committee on the Use of Animals, the first one at the School of Veterinary Medicine and Animal Science (FMVZ/USP), Brazil with the protocol number N° 9870211117; and the second one, protocol number N° 677/2020-PR to the University of Teramo, Italy.

Data from pregnant sows were collected in a Brazilian pig farm, in the state of Parana, and in an Italian pig farm in the region of Abruzzo, both studies carried out during the summer season.

Animals, handling, and locomotion assessment

In the Brazilian pig farm (Farm 1) pregnant sows, TOPGEN® Landrace/Large white, were housed in collective gestation pens that measured 6 x 4 m (2.7 m^2 per sow) with walls of 0.9 m high with a solid/slatted concrete floor area, 4m/2m of length, respectively. Sows were fed with a liquid gestation diet twice daily (06:00h and 15:00h) in collective feeders (5 m long and 0.4 m wide). One week before the expected farrowing date all sows were allocated in individual farrowing crates that measured 2.6 x 1.6 m. During lactation, all sows were fed with lactation diet four times per day (07:00h, 11:00h, 16:00h and 21:00h). Quantity or frequency of food intake in sows during gestation or lactation phase was not collected. Births were always assisted by farm technicians.

In the Italian pig farm (Farm 2) pregnant sows, TOPIGS® TN60, were housed in collective gestation pens that measured 6 x 7m (2.8 m^2 per sow). They were fed twice daily (07:00h and 15:00h), with conventional dry food. One week before the expected farrowing date all sows were transferred to farrowing crates that measured 1.6 x 2.4 m. During lactation they were fed three times per day – at 07:00, 12:00 and 16:30h. Always in both farms water was supplied to sows *ad libitum* by a nipple drinker. Quantity or frequency of food intake in sows during gestation or lactation phase was not collected. Births were assisted by vets only in the daytime.

In both farms the same validated locomotion score system was used, ranging from 0 to 5, being 0 an animal that walks easily and 5 an animal that cannot walks (D'Eath, 2012), see Table 1 for details about each score. On Farm 1 locomotion assessment was carried out biweekly for four months in 582 multiparous pregnant sows, and on Farm 2 locomotion was assessed weekly for two months in 171 multiparous pregnant sows. In both farms the assessment was performed in the collective pens by the same trained person. Sows with locomotion assessment ≥ 3 were treated with 1.1 mg/kg of flunixin meglumine intramuscular for three days, as requested by the ethics committee.

Score	Label	Description
0	Normal	Even strides, rear end sways slightly while walking, pig is able to accelerate and change direction rapidly. Stands normally.
1	Stiff	Abnormal stride length, movements no longer fluent, pig appears stiff. Pig still able to accelerate and change direction. Stands normally
2	Slight lameness	Shortened stride, lameness detected, swagger of rear end while walking, no hindrance in pig's agility. Uneven posture while standing.
3	Lame	Pigs slow to get up (may dog sit), shortened stride, minimum weight-bearing on affected limb (standing on toes), swagger of rear end while walking. May still trot and gallop.
4	Limping	Pig reluctant to get up, holds limb off floor while standing, avoids placing

		affected limb on the floor while moving.
5	Downer	Pig unresponsive: does not move and struggles to stand when encouraged to do so.

Table 1: Locomotion score system used to assess lameness in pregnant sows taken from D'Eath (2012).

Only sows with at least three locomotion assessments in the last third of pregnancy were selected (Farm 1, N = 397; Farm 2, N = 114) and divided in groups according to their scores, G1 (no lame), G2 (moderate lame) and G3 (severe lame). On Farm 1, only G1 and G3 sows were selected, being G1 sows with all locomotion scores recorded as 0 or 1 and G3 were sows with at least one locomotion score ≥ 3. On Farm 2 it was used a sum of the last five locomotion assessments to group the animals, being G1 sows with a sum between 0 to 6; G2 with a sum between 7 to 11; and G3 with a sum > 12.

Productive data collection

After farrowing productive data were collected from 30 sows on Farm 1 (G1 = 15; G3 = 15) and from 39 sows on Farm 2 (G1 = 14; G2 = 16; G3 = 9). Productive measures included the number of gestation days, type of birth (normal, induced or dystocic), birth duration in minutes, number of piglets born alive and stillborn, sex, litter weight and mortality during the first week.

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Glucocorticoids measures

Hair, saliva, and placental samples were collected from sows on Farm 2 to measure glucocorticoids. Hair samples were collected, cleaned and cortisol extracted before assay (Stubsjøen et al., 2015). Placental samples were homogenized, glucocorticoids extracted using ethyl acetate (Tatemoto et al., 2019) and protein was measured (Bradford, 1976) before cortisol and cortisone assay (Palme and Möstl, 1997; Rettenbacher et al., 2004). The EIA protocol used to measure glucocorticoids from saliva, placenta and hair was based in the methodologies reported by Palme and Möstl (1997).

Hair samples were collected from the neck region (see Fig. 1 for details) seven weeks before farrowing (called as C1 from now on), and four weeks after birth (called as C2 from now on), always in the same area. Each sample was stored in plastic bags without direct light and in a dry place. The protocol to clean and extract cortisol was adapted from Stubsjøen et al. (2015). Samples were weighed an aliquot of 0.2 g was placed in glass vials from each hair sample. Each aliquot was degrassed adding 7 ml of n-Hexane, mixing in a hand vortex for one minute, subsequently the solvent was everted, and each sample was dried under a hood. To extract glucocorticoids, hair

aliquots were weighed 0.1 g (0,100 ± 0,0005 g) and placed in a new glass vial, 5 mL of methanol 100% was added, vials were plug tightly, placed in a water bath with agitation at 37°C for 24 h, centrifuged for 15 minutes at 3000g, and finally 2.5 mL of the methanol extract were transferred into a new glass vial and dried down at 50°-60°C, under a stream of nitrogen or air. Each glass vial was redissolved in 500 µl of EIA buffer, taken to shaker for 30 min and 50 µl were used to perform the cortisol EIA (Palme and Möstl, 1997).

During three days before saliva collection, each sow was habituated to oral manipulation of cotton rolls impregnated with sugar tied to dental floss. Samples of saliva were collected in the week before farrowing, one sample per day for three days at 13:00h. The procedure used to collect saliva samples was described by Siegford et al. (2008) presenting to each sow cotton rolls tied to dental floss to chew them until they were saturated with saliva in two consecutive times, the first one was to stimulate the animal to produce fresh, and the second one was stored in a falcon tube of 15 ml at 20°C until EIA to measure cortisol concentration. On the day of the analysis, all samples were defrosted at controlled temperature, centrifuged for 10 minutes at 1000 x g, and the content was transferred to a 1.5 ml microtube. It was used 50 µl of saliva to perform the cortisol EIA (Palme and Möstl, 1997).

After farrowing three random placenta samples were collected from each sow, always from the same place (see Fig. 1 for details about collection) and stored in plastic containers at 20°C. For homogenization, the three samples from each sow were mixed and macerated in a porcelain mortar with pestle handle without allowing it to defrost using liquid nitrogen until it was transformed into powder, samples were stored in falcons of 50 ml at 20°C. For glucocorticoids extraction an aliquot of 0.1 g of each homogenized sample was weighed in a 1.5 ml microtube, 200 µl of ultrapure water was added and mixed in a hand vortex for 15 seconds, 1 ml of ethyl acetate was added, mixed again in a hand vortex for 15 seconds and placed in a centrifuge at 4°C, at a speed of 4000 x g for 20 minutes. After centrifuged the supernatant was delicately removed and placed in a 1.5 ml microtube at 20°C until freezing, the pellet was discarded. Finally, the microtubes were removed from the freezer and 500 µl of the supernatant was collected, since ethyl acetate does not freeze at this temperature. Samples were placed in a 1.5 ml microtube and left open until it dried under a hood. Each dry microtube was redissolved in 500 µl of EIA buffer, taken to shaker for 30 min and 50 µl were used to perform the cortisol/cortisone EIA.

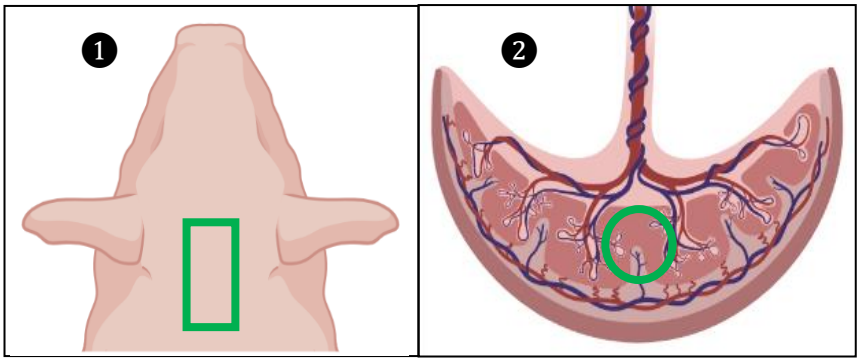

Figure 1: Illustrations of the regions used to collect hair ❶ and placenta samples ❷ from sows on Farm 2.

Protein concentration was determined using an adaptation of the Bradford (1976) technique. Placental tissue samples (0.1 g) were homogenized in lysis buffer containing 150 mM NaCl; l% protease inhibitor cocktail (Sigma Aldrich, Poole, UK). Briefly, to lyse the tissues completely, homogenization was carried out using a sonicator for 9 cycles of 10 sec pulse with 10 sec gaps in between at 35% amplitude. The homogenates were centrifuged for 15 min at 14000 x g at 4 °C, and the resulting supernatant fractions were assayed for protein concentration using Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein concentration of unknown samples was established thank to a standard curve generated using Bovine Serum Albumin (BSA). Each sample was analyzed in duplicate and was read at 595 nm by a microplate reader to measure the optical density (OD) values.

Statistical analysis

In both farms, information from sows with at least three locomotion assessments in the last third of pregnancy were used (Farm 1, N = 397; Farm 2, N = 114). To describe data distribution by percentages of sows divided by locomotion scores as follows: score 0 = sows with all scores 0; score 1 = sows with at least one score 1 and the others ≤ 1; score 2 = sows with at least one score 2 and the others ≤ 2; score 3 = sows with at least one score 3 and the others ≤ 3; score 4 = sows with at least one score 4 and the others ≤ 4; and score 5 = sows with at least one score 5.

Shapiro-Wilk test was used in all variables to determine the residual distribution, when the result was > 0.05 a parametric test was used and when was < 0.05 a non-parametric test was performed, always considering the number of groups to be compared. The variables used to perform a Shapiro-Wilk test were number of gestation days, type of birth, birth duration in minutes, total of life born and stillborn, sex, litter weight, mortality during the first week and glucocorticoids concentrations. Grubbs's test was performed to detect outliers and when found they were removed. Productive data were described also using rates and averages.

Concerning hair cortisol concentrations two approaches were carried out. First, comparing by sows' groups cortisol concentrations from sample one (C1) or from sample two (C2); and second, it was assessed the ratio between hair cortisol concentrations from C1 and C2. Placental cortisol and cortisone concentrations were corrected using protein measures – dividing cortisol or cortisone concentration in protein concentration for each sample. A ratio was made using corrected values between placental cortisol and cortisone to compare by groups. In all comparisons it was used an ANOVA One-way test and a T-test as *post hoc*.

For saliva cortisol concentration, an average of three samples from each sow were made and this value was used to compare between groups using Kruskal-Wallis test and Wilcoxon test as posthoc. A significance level was considered when p value was < 0.05 and was considered a tendency when p value was between 0.05 and 0.1. All analyses were performed using the free software environment for statistical computing R (RStudio Team, 2020).

RESULTS

Locomotion assessment and productive data

The percentage of lame sows from both farms (N = 511) according to locomotion scores are presented in Fig. 2.

Figure 2: Distribution of lame sows by locomotion scores from both commercial pig farms expressed in percentage. Being 0 an animal that walks easily and 5 an animal that cannot walks (D'Eath, 2012).

Gestation length was different between groups, being G1 longer than G2 (Farm 2; Wilcoxon test; p-value = 0.008) and G3 (Farm 1; Wilcoxon test; p-value = 0.027). See Fig. 3 to observe distribution of gestation length in days divided by sows' groups from Farm 1 and Farm 2. Was removed an outlier in the Farm 2 from G3 sows that corresponded to 111 days.

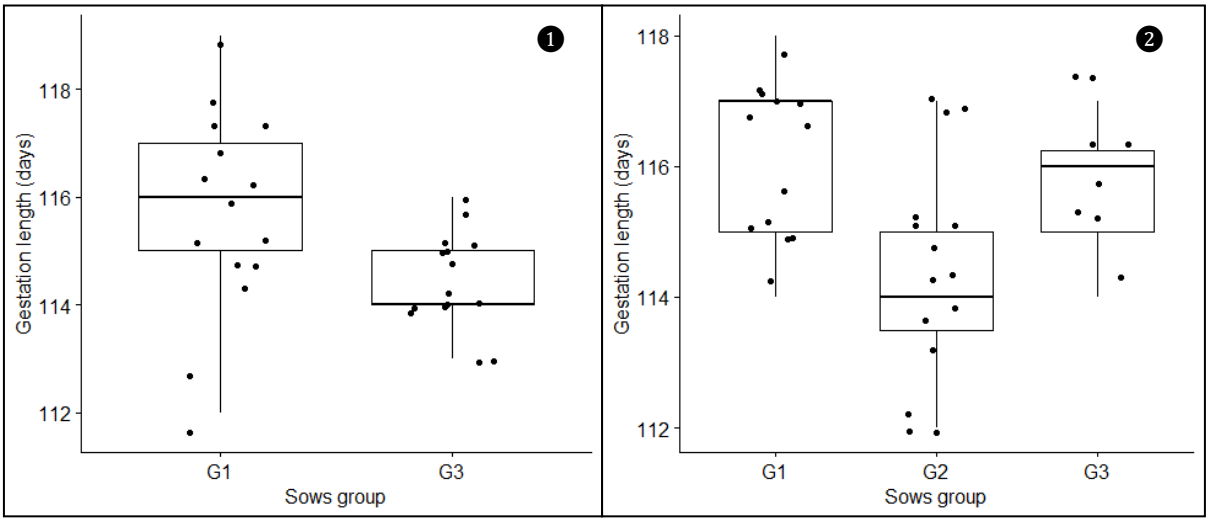

Figure 3: Boxplots to show data distribution regarding gestation length in days from Farm 1 ❶ and Farm 2 ❷ divided by sows' groups: G1 (no lame), G2 (moderate lame) and G3 (severe lame). Only in Farm 2 was collected data from sows with moderate lameness.

Lameness scores had no relation with the number of live born and stillborn piglets, no statistical differences were found in any of the farms ($p > 0.05$). Average of live born and rate of stillborn piglets can be seen in Fig. 4 accordng to severity of lameness of sows from both farms.

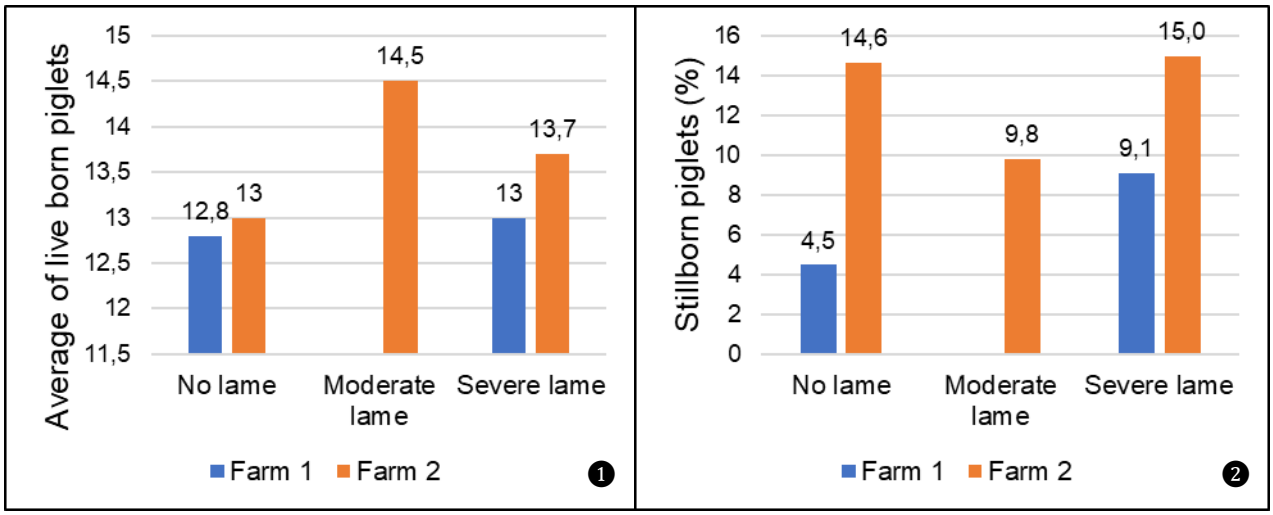

Figure 4: Descriptive means of live born piglets ❶ and rate of stillborn piglets ❷ from Farm 1 and 2 divided by sows' severity of lameness. Only in Farm 2 data were collected from sows with moderate lameness.

No statistical differences were found when comparing average birth weight of piglets from sows of different groups G1, G2 or G3 in any of the farms ($p > 0.05$; Fig 5).

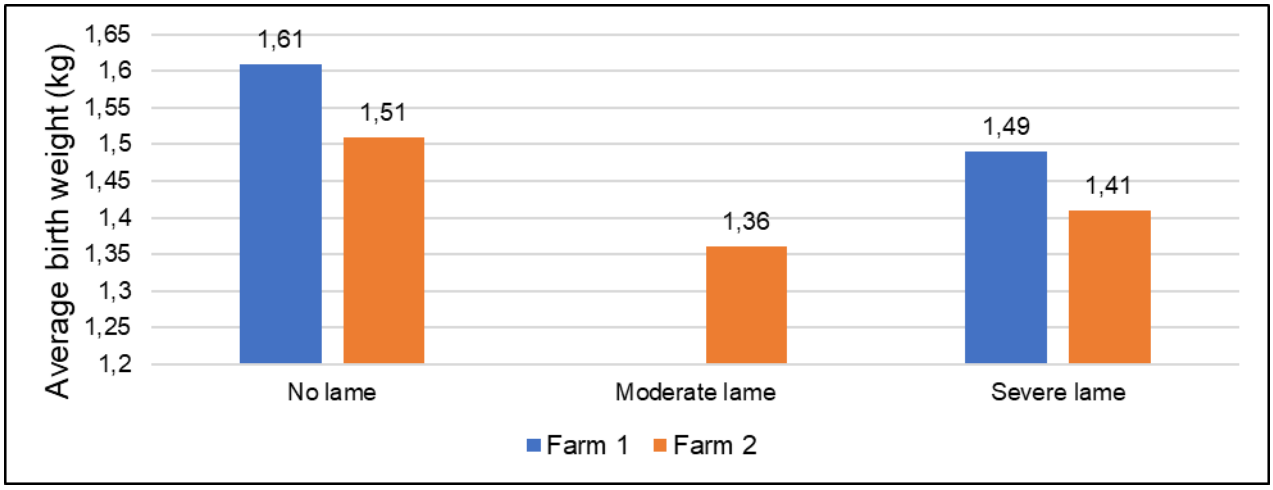

Figure 5: Average of piglets' weight at birth from Farm 1 and 2 divided by sows' severity of lameness. Only in farm 2 was collected data from sows with moderate lameness.

A tendency was found in the number of dead piglets during the first week of age, being less piglets dead from G1 sows than G2 sows (Wilcoxon test; p-value = 0.069). See Fig. 6 to observe number of dead piglets on first week expressed in percentage divided by sows' groups.

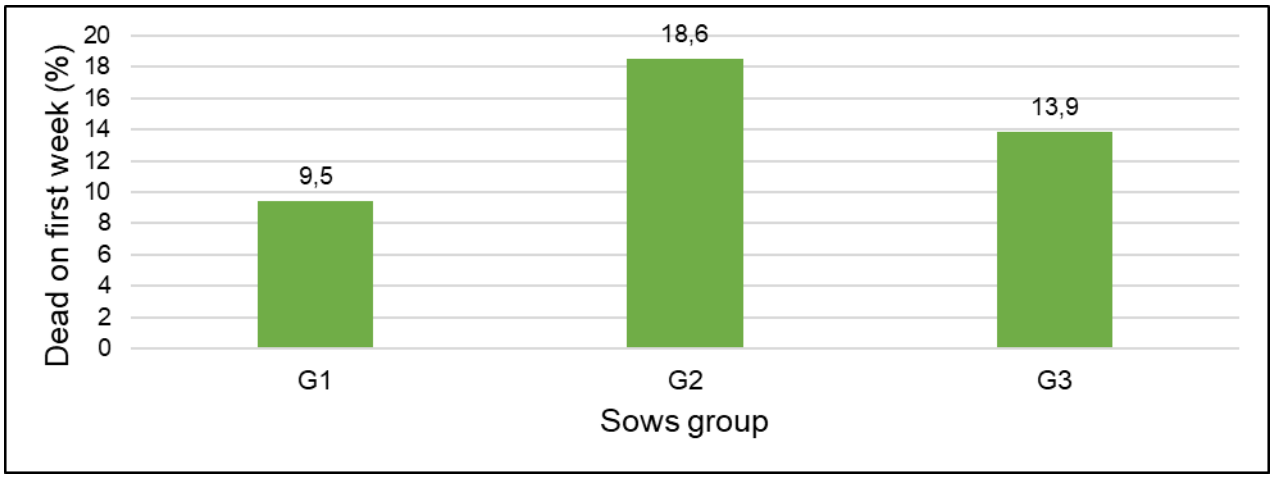

Figure 6: Percentage of dead piglets on first week of age from Farm 2 divided by sows' groups: G1 (no lame), G2 (moderate lame) and G3 (severe lame).

No significant differences or tendencies were found in the remaining comparisons. Descriptive data and results of all variables compared can be seen in the supplementary Table S1.

Glucocorticoids measures

When compared ratio of placental cortisol/cortisone concentrations between groups a tendency was detected (ANOVA One-way; p = 0.079). A significant difference was found when compared ratio between G1 and G2 sows (T-test; p-value = 0.019), being G1 lower than G2 (average G1 = 0.69; and G2 = 1.07). An outlier from G1 sows was removed, corresponding to ratio = 1.4. See Fig. 7 for details about ratio distribution by sows' groups.

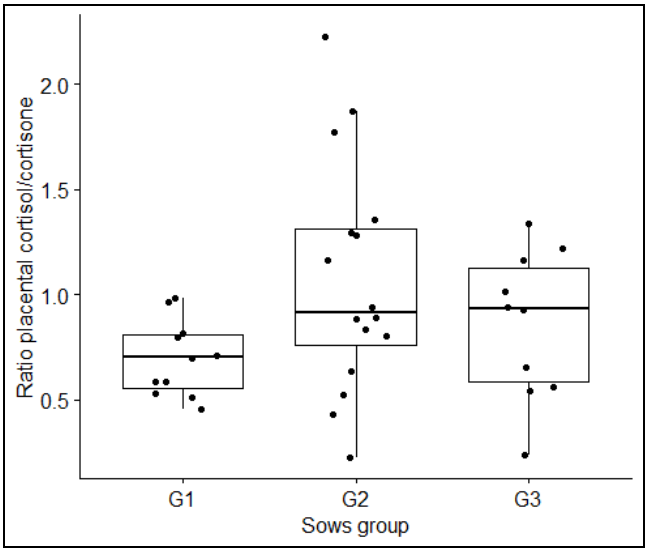

Figure 7: Ratio of placental cortisol/cortisone concentrations accoding to sows' groups: G1 (no lame), G2 (moderate lame) and G3 (severe lame).

When compared hair cortisol concentrations from the second hair sampling (C2) by sows' groups, a tendency was found (ANOVA One-way; p value = 0.055) and a significant difference when compared G2 with G3 (T-test; p value = 0.012), being G2 showing higher cortisol concentrations than G3 (average G2 = 4.26 pg/10 µl; average G3 = 2.46 pg/10 µl). No differences or tendencies were found in the remaining comparisons, first hair sample (C1) nor for the ratio between C1/C2 by sows´ groups. Outliers were removed from C1-G1 (20.23 pg/10 µl) and C2-G3 (5.54 pg/10 µl). No differences were observed when compared mean salivary cortisol concentrations by groups.

DISCUSSION

We found that lameness during the last third of pregnancy is highly prevalent, altering productive and physiological parameters in sows. From locomotion assessments carried out in both farms we observed that 69.9% of sows had lameness, being 41.7% with moderate lameness – score 2, and 28.2% with severe lameness – score ≥ 3. It means that a high number of pregnant sows are experiencing pain, a condition that compromises their welfare. Numerically, sows with severe lameness had a higher average of live piglets and rate of stillborn piglets than no lame sows; additionally, sows with moderate or severe lameness have higher rate mortality on the first week. Sows with no lameness have more days of gestation, lower mortality rate in the first week and increased placental efficiency to inactivate glucocorticoids than sows with moderate or severe lameness.

Despite having found that length of gestation was significantly shorter in sows with severe lameness in the Farm 1 and moderate lameness in the Farm 2 than sows without lameness, mean values are on the physiological range for the specie (Cox, 1967). In the studies of Kattesh et al. (1980) and Ashworth et al. (2011) it was reported the effect of stress during pregnancy on the gestation length in sows, and only in the study carried out by Kattesh et al. (1980) a difference was found that altered negatively the pregnancy length in sows stressed during mid-gestation, 112.3 days in stressed sows and 115.3 days in control sows. However, in the study carried out by Ashworth et al. (2011), despite not finding differences, the average days of gestation in stressed sows was similar to what we reported (see supplementary Table S1 for details).

No significant differences were found related to number of live piglets when groups were compared in both farms. However, in numerical terms, average of live born piglets was higher in sows with moderate or severe lameness than no lame sows; in numerical compensation, rate of stillborn piglets was 4.6% and 0.4% lesser in no lame sows than severe lame sows from Farm 1 and 2, respectively. Curiously, the lowest numeric rate of stillborn piglets in Farm 2 was from sows with moderate lameness, being 4.8% and 5.2% lesser than no lame and severe lame sows, correspondingly. Despite that no significant differences were found, stillborn piglet rates were higher in Farm 2 than Farm 1, probably due to different handling conditions, specifically related to birth care in the Farm 2.

Mortality rate during the first week was 9.1% higher in G2 sows than G1 sows, and 4.4% higher in G3 sows than G1 sows in the Farm 2. Additionally, average birth weight in the same farm differed between groups, whereas piglets from G3 and G2 sows were100 and 150 g lighter, respectively, than piglets from G1 sows, although without significant differences. The same thing

happened on Farm 1, piglets from G3 sows were 120 g lighter than piglets from G1 sows. These findings can be explained from behavioral, nutritional, and physiological perspectives, when considering the biology of the lame sow, including the survival rates in piglets associated with lower birth weight. It has been reported that lame sows alter their feeding behavior, lie down more, stand, and explore less their environment than healthy sows (Ala-Kurikka et al., 2016; Cornou et al., 2008) which together to the painful condition may alter their food/water intake or ability to acquire resources (Heinonen et al., 2013), in consequence, modifying the development and growth of the fetus (National Research Council, 2012).

The study of Bonde et al. (2004) demonstrated that sows with severe and moderate lameness have poor uncontrol on their lying behavior, increasing the risk to crush piglets, then increasing mortality rate before weaning. Also, lighter piglets have less survival rate than heavir piglets before weaning (Hales et al., 2013). Both of these later factors can be related with higher piglet mortality rate in the first week from sows with moderate and severe lameness than from offring from sows without lameness.

From a physiological perspective, in the literature it has been reported that glucocorticoids during pregnancy participate as catabolic agents in growth processes, restraining fetal growth and tissues development (Seckl, 2004). Although no differences were found regarding cortisol concentrations in saliva samples between sows' groups, differences were found in hair cortisol concentrations – in the second sample, and in the placental ratio cortisol/cortisone. These findings indicate that G2 sows were more stressed than G3 sows, based in hair cortisol concentrations, and G1 sows were most efficient in their response to inactivate placental cortisol into cortisone than G2 sows, probably protecting fetuses to the increased exposure to cortisol. We acknowledge that there were inconsistencies in relation to the findings, whereas clear differences were found when comparing no lame sows and sows with moderate lameness, but no differences when comparing no lame sows with sows with severe lameness. We anticipate that these results may be related to the fact that with severe lameness were treated with painkillers – as requested by the ethical committee, decreasing thus their pain and stress.

In our research, we studied the effects lameness as a source of maternal stress during pregnancy. In conclusion, lameness during the last third of pregnancy of sows altered their HPA stress response, reflecting in productive effects and thus negatively compromising sows and offspring welfare. Further studies may contribute with more indicative of the effects of lameness during pregnancy throughout the life trajectory of the offspring.

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Variable	Farm	Mean			p-value	Posthoc p-value		
		G1	G2	G3		G1-G2	G1-G3	G2-G3
Gestation length (days)	1	115.7	-	114.5	0.027 □	-	-	-
	2	116.2	114.4	115.2	0.015 ■	0.008	0.427	0.060
Type of birth (induced = 0; normal = 1)	1	0.6	-	0.27	0.600 ○	-	-	-
	2	1	1	0.9	0.230 ●	-	-	-
Birth duration (minutes)	1	158.3	-	171.4	0.660 Δ	-	-	-
Total born piglets	1	134	-	14.3	0.510 Δ	-	-	-
	2	15.2	16.1	16.0	0.763 ▲	1	1	1
Total life born piglets	1	12.8	-	13	0.890 Δ	-	-	-
	2	13	14.5	13.7	0.408 ■	0.420	0.960	0.650
Total stillborn piglets	1	0.6	-	1.3	0.160 □	-	-	-
	2	2.2	1.6	2.4	0.301 ■	0.320	0.950	0.560
Total females	1	6.8	-	6.1	0.450 Δ	-	-	-
	2	6.5	6.2	6.7	0.887 ▲	1	1	1
Total males	1	6	-	6.87	0.370 Δ	-	-	-
	2	6.1	7.6	6.7	0.236 ▲	0.29	1	1
Average weight at birth (Kg)	1	1.6	-	1.5	0.310 Δ	-	-	-
	2	1.5	1.3	1.4	0.292 ▲	0.37	1	1
Dead piglets (first week of age)	2	1.2	2.7	1.9	0.098 ■	0.069	0.494	0.100

Supplementary table S1: Productive variables with descriptive measures and p-value of all comparisons. Description of each test used follows: T-test = Δ; ANOVA One-way =▲; Wilcoxon test = □; Kruskal Wallis test = ■; Fisher test = ○; Chi-square test = ●. Wilcoxon a T-test were used as posthoc for parametric and no parametric data. G1 (no lame), G2 (moderate lame) and G3 (severe lame).

4. CHAPTER III: **Lameness in sows during the last third of pregnancy modifies pain perception on their offspring**

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ABSTRACT

Lameness in sows is a painful and common condition, affecting more than 15% of sows. Stressful and painful experienced by lame sows causes unfavorable scenarios not only for pregnant animals, but also for their offspring, possibly due to glucocorticoid-mediated effects on fetal programming. We aimed in this study to assess nociception and physiologic effects of sow lameness in their offspring. This study was carried out in two trial using the same locomotion score system to assess sows in their last third of gestation, from score 0 to 5, being 0 an animal that walks easily and 5 an animal that cannot walks. In the Experiment 1 (Exp1), 30 sows were selected, 15 without lameness (G1; scores $\leq$ 1) and 15 with severe lameness (G3; scores $\geq$ 3) to study their offspring. In the Experiment 2 (Exp2), 39 sows were selected and divided in three groups, 14 without lameness (G1, when the sum of the last five assessments was between 0 to 6), 16 with moderate lameness (G2, when the sum of the last five assessments was between 7 to 11), and 9 with severe lameness (G3, when the sum of the 5 assessments was between 12 to 16). Sex and weight at birth from each piglet were collected in both experiments. Mechanical nociception threshold was measured using and algometer in piglets at 32 days of age (N=90) in the Exp1; during the first 12 hours of life (N=238), before and after castration of male piglets (N=50) in the Exp2. The body regions assessed were left and right plantar pad (LP and RP); and left and right leg (LL and RL). Additionally, in the Exp2 the time response in seconds was registered. Normality was tested with Shapiro-Wilk and based on the result was used a parametric or non-parametric tests. Significance was considered when $p \leq 0.05$ and was considered a tendency when $p \geq 0.05$ - 0.1. In the Exp1 the nociception values were lower in G1 piglets than G3 when used mean values of all regions ($p=0.004$), and when considered left side measurements ($p=0.008$). In the Exp2 during the 12 hours of life, G3 piglets had higher nociceptive thresholds than G2 piglets in LP region ($p=0.03$) and also in their time reaction in the other regions: LL, RL, LP, left and right sides and mean of all regions ($p<0.01$). Before castration nociception values of G2 were lower than G3 in LP and right-side regions ($p<0.05$); and after castration G1 nociception values and time reaction were higher than G2 in RP region ($p<0.05$). From our results we can conclude that lameness during the last third of pregnancy alter nociceptive threshold in the offspring, regardless of piglets age.

INTRODUCTION

Lameness is the most important pain condition in pigs (Ison et al., 2016), especially in sows, where the prevalence has been reported of more than 15% in some studies (Heinonen et al., 2006; KilBride et al., 2009). Like in other species, lameness in sows has a multifactorial origin (Blowey, 2005; Gelasakis et al., 2019; Nalon et al., 2013), ranging from unsuitable handling conditions, nutritional factors, joint disorders, among many others (Nalon et al., 2013; Liesbet M. Pluym et al., 2013; van Riet et al., 2013). Regardless of the causes, it is indisputable that lameness is a painful condition (Ison et al., 2016; KilBride et al., 2009), and just like the causes of lameness, its consequences are innumerous as well, but studies mostly have been focused on the immediately effects for the sows and not for their offspring (Heinonen et al., 2013, 2006).

Epidemiological studies in humans and lab animals (e.g. mice) since the 90's showed associations between birth weight and several adulthood diseases, leading to postulate that exists a type of programming during the embryonic and fetal environment, capable of determining

functional and metabolic aspects of extrauterine life (Lau and Rogers, 2004). The review work by Rutherford et al. (2012) showed also numerous studies on fetal programming in farm animals with the focus on the offspring effects in terms of nutrition, husbandry, health state, or stress during pregnancy. However, few studies have evaluated the effects of stress during pregnancy on the perception of painful stimuli in the offspring from pigs (Rutherford et al., 2009; Sandercock et al., 2011) and more information on this is required to improve swine welfare.

Discussing about pain is more complex than just labelling it as an unpleasant experience. During the course of the evolution of the species pain has played a fundamental role, co-evolving according to the environmental challenges of the species (Walters and Williams, 2019), meaning that in healthy conditions its function is always protective against potentially noxious stimuli (Sneddon et al., 2014). Additionally, the neural nociception and pain process involves several brain structures like the amygdala (Ji and Neugebauer, 2009), limbic system, hypothalamus, thalamus, insula, dorsal horn in the spinal cord, somatosensory cortices, prefrontal cortex, among others (Ong et al., 2019; Sneddon, 2018). The function of these structures goes beyond the managing of adverse and noxious information from the environment, participating in the processing of emotions, homeostasis, behavior, problem solving, social control and more (Ong et al., 2019; Roxo et al., 2011; Shin and Liberzon, 2010; Xie and Dorsky, 2017).

Given the fact that lameness is a common condition in sows, neglected most of the time, that activates the pain system in pregnant animal, the development of resilient offspring could be compromised, and in consequence, their welfare too. In this study, we propose that a challenging gestation environment during the last third of pregnancy, caused by lameness in pregnant sows like a model, have the potential to program the perception to noxious stimuli in their offspring. The main hypothesis is that piglets from lame sows are more tolerant of noxious stimuli than piglets from sows without lameness.

MATERIALS AND METHODS

Experiment 1

All procedures were carried out upon approval by the Ethics Committee on the Use of Animals (CEUA) of the School of Veterinary Medicine and Animal Science (FMVZ/USP), N° 3606300114. Data from sows were collected in a commercial pig farm in the state of Paraná, and from the piglets were collected in the experimental pig farm at the University of São Paulo (USP), Campus Fernando Costa - Pirassununga, SP-Brazil.

Animals and handling

Locomotion assessment was performed in 582 multiparous pregnant sows TOPGEN® (pure breed Large White and Landrace) fortnightly over a period of four months using a validated scoring system (D'Eath, 2012) from 0 to 5, being 0 a sow without lameness and 5 a sow with severe lameness (see Table 1 for details). Sows with locomotion assessment ≥ 3 were treated with 1,1 mg/kg of flunixin meglumine intramuscular for three days, as requested by the ethical committee. Locomotion assessment was performed always by the same trained person in pens with animals kept in groups. Each pen measured 23.16 m^2 (6 m x 3.86 m) with a solid/slatted concrete floor area (3.97 m in length) grouping nine sows per pen. The animals were fed twice daily with gestation diet – at 07:00h and 11:40h – and water was offered *ad libitum* by nipple drinkers. According to the final third of gestation locomotion assessments, 30 animals were selected, 15 without lameness (G1: all assessments with scores 0 or 1) and 15 with severe lameness (G3: with at least on time with scores 3 to 5) to study their offspring. One week before the expected farrowing date all sows were housed in individual farrowing crates that measured 2.6 x 1.6 m. During lactation, all sows were fed four times per day (07:00h, 11.00h, 16:00h and 21:00h) and water was supplied *ad libitum* by a nipple. Data on piglet sex and weight at birth from each litter were collected.

Score	Label	Description
0	Normal	Even strides, rear end sways slightly while walking, pig is able to accelerate and change direction rapidly. Stands normally.
1	Stiff	Abnormal stride length, movements no longer fluent, pig appears stiff. Pig still able to accelerate and change direction. Stands normally
2	Slight lameness	Shortened stride, lameness detected, swagger of rear end while walking, no hindrance in pig's agility. Uneven posture while standing.
3	Lame	Pigs slow to get up (may dog sit), shortened stride, minimum weight-bearing on affected limb (standing on toes), swagger of rear end while walking. May still trot and gallop.
4	Limping	Pig reluctant to get up, holds limb off floor while standing, avoids placing affected limb on the floor while moving.
5	Downer	Pig unresponsive: does not move and struggles to stand when encouraged to do so.

Table 1: Locomotion score system used to assess lameness in pregnant sows taken from D'Eath (2012).

Weaning was carried out at 28 days of age. A cohort sample of three weaned piglets from each sow, one female and two males, were transported to the experimental pig farm at USP, Fernando Costa Campus, in Pirassununga, distant 436 km from the commercial farm to monitor developmental outcomes, such as behaviour, weight gain and nociceptive threshold. Transport always took place at the end of the afternoon and litters were never mixed during transportation.

The characteristic used to choose the animals in the sample was the size of the animal, all those that were of medium size were candidates to be included in the cohort sample.

Piglets were allocated homogeneously in pens by mixing three different litters for a total of nine piglets per pen, always from the same group of sows, G1 or G3. Pens measured 2.4m long x 0.6m wide x 0.9m high, with slatted floor, and a heat floor area (0.9m long x 0.9m wide). Water was provided *ad libitum* by one nipple drinker per pen. Piglets were fed several times per day according to their consumption of a conventional starter diet. Disease events were always attended by a veterinarian.

Nociception assessment

During three days before the nociception assessment, piglets were habituated to a gentle restraint by trained experimenters like it is showed in the Fig. 1. At 32 days of age, nociception assessment was performed in each piglet using an electronic von Frey model EFF 301, Insight (max. 10N) (Insight Equipamentos Ltda - EPP, 2019) in four body regions, left and right leg, adjacent to the tail (LL and RL), and left and right plantar pad (LP and RP) (Fosse et al., 2010). Fig. 2 show the pressure points used. Pressure was applied at a constant rate and the pressure was released when the animal had a reaction to avoid the pressure, like withdrawal of its leg, vocalization, or movements of the tail. The animals had no visual contact with the evaluator and the legs were hanging freely to allow an easy access to the behavioral responses in order to perform the assessment.

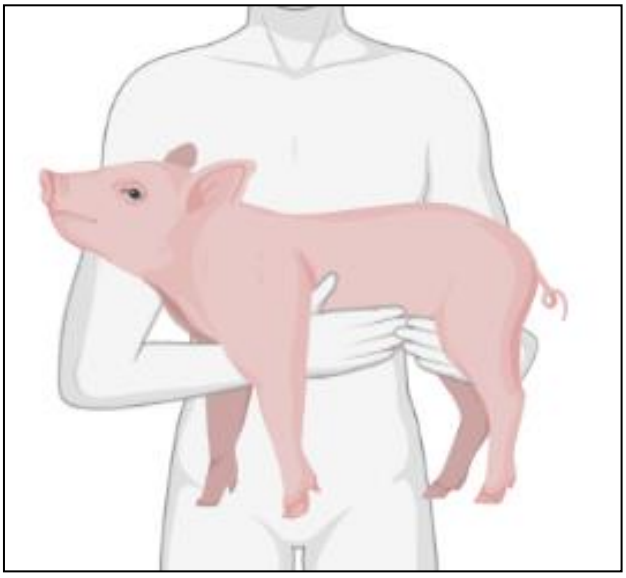

Figure 1: Physical restraint used with piglets for habituation and data collection of nociception assessment during the experiment 1.

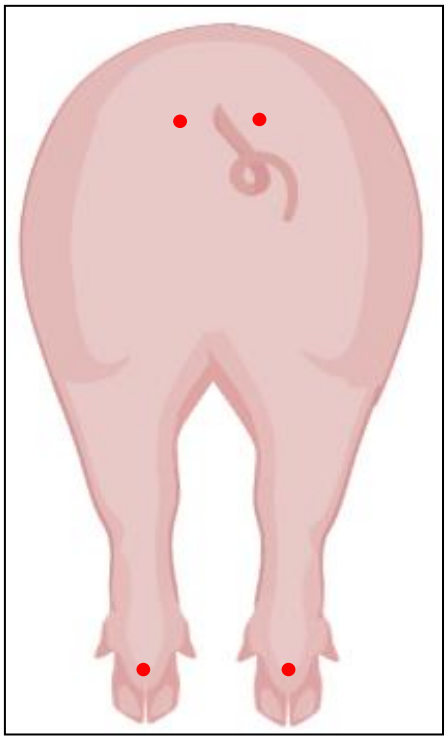

Figure 2: Areas used for nociceptive threshold assessment. The red dots show the pressure points used for nociception assessment in the piglets. Left and right leg, adjacent to the tail (LL and RL), and left and right plantar pad (LP and RP).

Experiment 2

All procedures were submitted and approved by the Ethics Committee on the Use of Animals with the protocol number N° 677/2020-PR to the University of Teramo, Italy. Data from sows and piglets were collected in a commercial pig farm located in the region of Abruzzo, Italy.

Animals and handling

Locomotion assessment was performed in 126 multiparous pregnant sows TOPIGS® TN60 weekly over a period of two months using the same validated score system (D'Eath, 2012) of the experiment 1. Locomotion assessment was performed always by the same trained person in collective pens and sows with locomotion assessment ≥ 3 were treated with 1.1 mg/kg of flunixin meglumine intramuscular for three days. According to the last five locomotion assessments prior to farrowing, 39 animals were selected and divided in three groups, G1 (N=14, when the sum of the 5 assessments was between 0 to 6), G2 (N=16, when the sum of the 5 assessments was between 7 to 11), and G3 (N=9, when the sum of the 5 assessments was between 12 to 16). The grouping of these animals was different from the experiment 1 due to the greater frequency of assessments. Piglet's weight and sex were collected at birth.

The area of pens for gestating sows was 42m^2 (6m x 7m) where they were feeding two times per day with gestation diet 1.3kg/sow/time – at 7:00h and 15:00h – and water supplied by nipple drinkers *ad libitum*. In the last week of pregnancy sows were transferred to farrowing crates, with an area of 3.84m^2 (1.6m x 2.4m), they were feeding three times per day with lactation diet 1.0kg/sow/time increasing 300 g daily until complete 2.6 kg/sow/time. Water was supplied by nipple drinker *ad libitum*.

Nociception assessment

A Pressure Application Measurement device (PAM 38550, high-pressure model for large animals) (Basile, 2020) was used to measure mechanical nociception threshold and reaction time in seconds in three different times, 1) during the first 12 hours after birth – in males and females, 2) 60

minutes before castration, and 3) between 30 to 60 minutes after castration. Surgical castration occurred between four to seven days of age in male piglets without pain relief, which is allowed in commercial settings in Italy. A hammock was used to restrain the animals (see Fig. 3 for details).

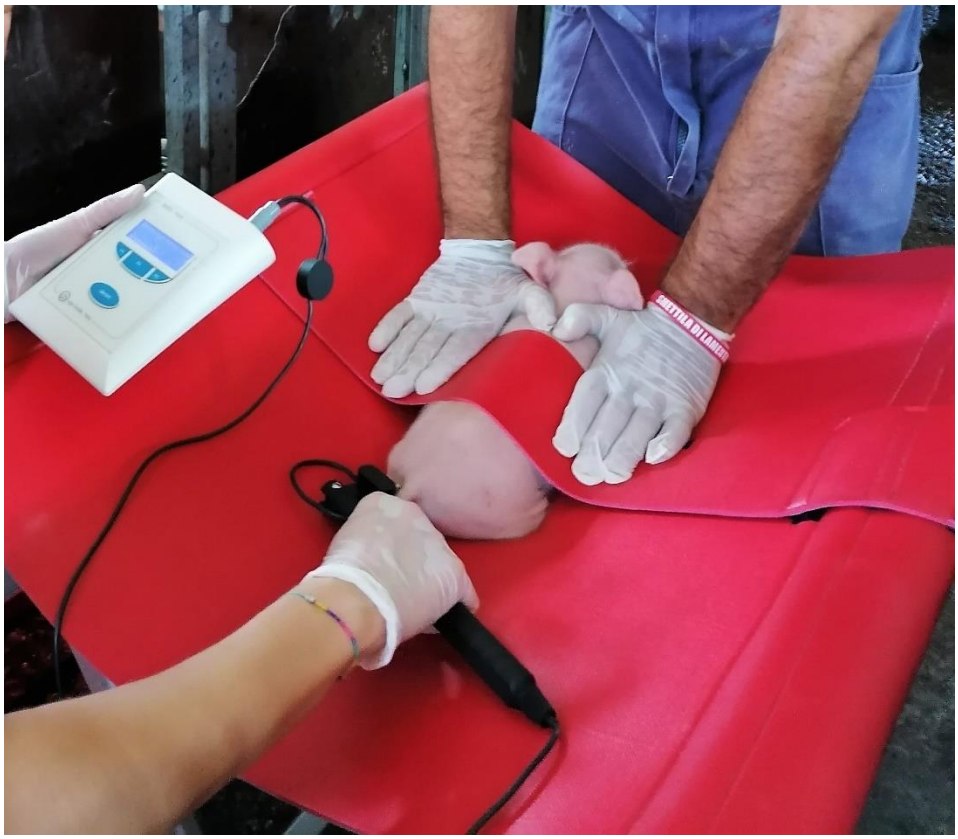

Figure 3: Hammock model used to perform nociception assessment in piglets from the experiment 2.

Behaviour reactions to release the pressure and body regions used to assess nociception were the same performed in the first experiment (see Fig. 2.). Each body region was assessed twice, with an interval of 30 seconds between each of them.

Settings used in the device included a target force rate of 30 grams/force per second (controlled by a ratemeter function), the maximum measure time was 25 seconds, and the procedure was performed with the short filament always by the same trained person. The animals had no visual contact with the evaluator and the legs were hanging freely to allow an easy access to carry out the assessment of behavioral responses.

Statistical analysis

Normality was tested with Shapiro wilk for later use of Wilcoxon test, T test, Kruskal Wallis test or ANOVA. Spearman correlation was performed to look if nociception measures were correlated with piglets' weight at birth or weaning.

In the experiment 1, nociception data was analysed comparing measures between groups, using net values from each body region (LL, RL, LP or RP), mean values of dorsal region (LL and RL), plantar region (LP and RP), left region (LL and LP), right region (RL and RP) and total mean (LL, RL, LP and RP).

In the experiment 2, nociception values and time of reaction were analysed comparing measures between groups using the mean values of the repetitions performed in each body region: LL, RL, LP, RP, dorsal/plantar region, left/right region, and total mean.

Significance was considered when p-value ≤ 0.05 and was considered a tendency when p-value ≥ 0.05 until 0.1. All analyzes were performed in the programming language R (RStudio Team, 2020).

RESULTS

Experiment 1

Overall analysis of the nociceptive threshold data with body weight showed a weak negative correlation when comparing weight at birth with total mean nociceptive measures (Spearman correlation test; p-value = 0.029, r_s = -0.230) and no correlation was found when comparing weight at weaning with total mean nociceptive measures (Spearman correlation test; p-value = 0.819, rho = 0.024).

Details of descriptive measures and p values from comparisons between G1 and G3 piglets concerning nociceptive values, weight at birth and weaning are presented in Table 2.

Measures collected from piglets (g)		Descriptive values from piglets divided by sows' groups				
		G1 (no lame)		G3 (severe lame)		p-value
		Mean	SD	Mean	SD	
Nociceptive value	RP (right plantar pastern)	916	211	997	74.2	0.427
	LP (left plantar pastern)	893	270	1007	69.1	0.076
	RL (right leg)	962	116	974	132	0.286
	LL (left leg)	896	250	982	124	0.146
	Total mean value	917	122	990	50.1	0.004
	Plantar region (LP and RP)	905	183	1002	52.8	0.071
	Dorsal region (LL and RL)	929	150	978	84.8	0.135
	Right region (RL and RP)	939	120	986	76.7	0.067
	Left region (LL and LP)	895	184	995	70.5	0.008
Weight	Birth	1600	386	1470	298	0.076
	Weaning	7370	1460	7620	1360	0.396

Table 2: Descriptive measures and p-value of comparisons between G1 and G3 piglets used on the experiment 1 concerning nociceptive values, weight at birth and weaning. Wilcoxon test was used to compare nociceptive measures, and T-test to compare weight at birth and weaning. Group G1 incorporate data collected from piglets which were offspring of sows with all locomotion assessments with scores 0 or 1; group G3 included data from piglets which were offspring of sows with at least one locomotion assessment with score ≥ 3.

When comparing net pressure nociceptive values from each body region between G1 and G3, a significant difference was found when the total mean from each piglet group was compared (Wilcoxon test; p-value = 0.004; see Fig. 4 for data distribution details). A tendency was observed when comparing LP region between the groups (Wilcoxon test; p-value = 0.077). The mean values from all four regions always were lower in piglets from G1 than G3.

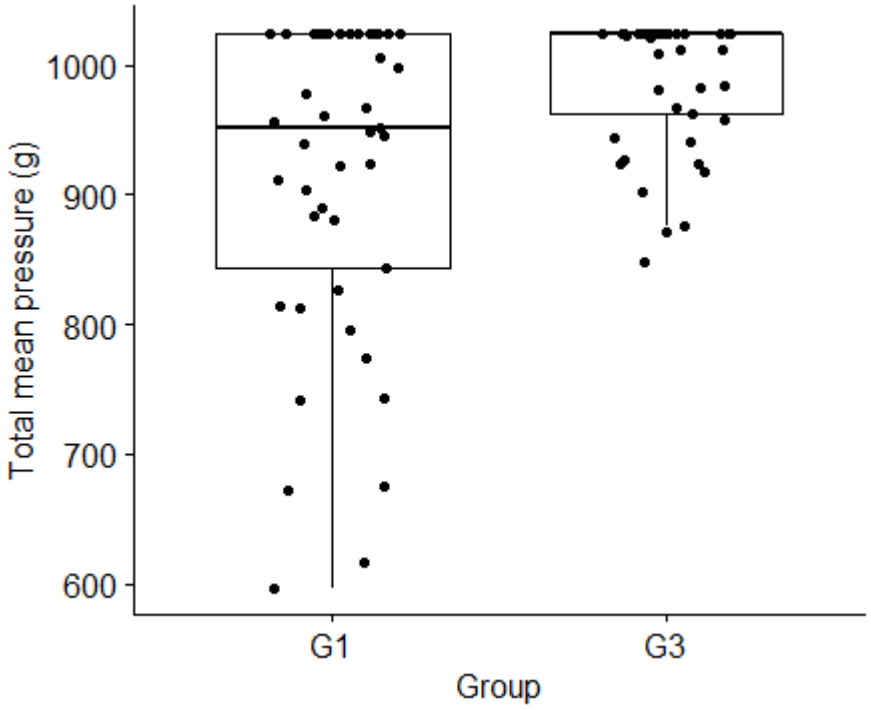

Figure 4: Distribution of total mean nociceptive values from G1 and G3 piglets assessed in the experiment 1. Group G1 represented data from piglets offspring f sows with all locomotion assessments with scores 0 or 1; group G3 data from piglets which were offspring of sows with at least one locomotion assessment with score ≥ 3.

Significant difference was found when comparing left region from G1 and G3 piglets (Wilcoxon test; p-value = 0.008). Nociceptive threshold measures of different body parts, in the same animal appeared to be related. A tendency was observed when comparing right region (Wilcoxon test; p-value = 0.067) and plantar region (Wilcoxon test; p-value = 0.071). Dorsal and plantar region data were not significantly different (p>0.05).

Experiment 2

A weak positive correlation was found when comparing weight at birth with total mean nociceptive measures (Spearman correlation test; p-value < 0.0001; $r_s = 0.25$).

Descriptive measures, number of piglets from each group, and p-values from each comparison for nociceptive threshold values and time of reaction between the groups are presented in Tables 3, 4 and 5.

Region assessed in piglets	Descriptive values from piglets by sows' groups						p-value
	G1 (N=60)		G2 (N=103)		G3 (N=75)		
	Median	SD	Median	SD	Median	SD	
Dorsal (g)	273	109	270	133	306	152	0.108
Left (g)	208	90.1	202	117	264	121	0.096
Left leg (g)	257	145	259	162	283	179	0.393
Left plantar pastern (g)	143	102	127	132	165	131	0.038
Plantar (g)	152	84.2	158	124	182	112	0.397
Right (g)	204	101	236	121	262	131	0.334
Right leg (g)	268	160	262	156	313	179	0.086
Right plantar pastern (g)	151	95.8	174	142	167	127	0.276
Total mean value (g)	231	76.4	228	107	258	109	0.135
Dorsal (s)	6.22	3.16	4.61	3.60	7.09	4.65	<0.0001
Left (s)	4.86	2.44	3.31	2.90	6.36	3.87	<0.0001
Left leg (s)	5.43	4.12	3.83	4.48	6.72	5.58	0.003
Left plantar pastern (s)	2.83	2.80	2.25	3.17	3.90	4.45	0.0003
Plantar (s)	3.51	2.49	2.67	3.23	3.75	3.78	0.008
Right (s)	4.83	2.74	4.09	3.15	5.69	3.97	0.005
Right leg (s)	5.94	3.98	4.70	3.92	7.58	5.17	0.0003
Right plantar pastern (s)	3.88	3.12	2.90	3.97	3.60	4.19	0.344
Total mean value (s)	5.11	2.11	3.96	2.73	6.42	3.47	<0.0001

Table 3: Descriptive measures and levels of significance summarizing the comparisons between G1, G2 and G3 piglets studied on experiment 2 concerning nociceptive values and time of reaction when male and female piglets were assessed in the first 12 h of life. Group G1 are piglets offspring from sows with the sum of the locomotion scores between 0 to 6, classified as no lame. Group G2 are piglets offspring from sows with the sum of the locomotion scores between 7 to 11, classified as moderate lame. Group G3 are piglets offspring from sows with the sum of the locomotion scores between 12 to 16, classified as severely lame.

Region assessed in piglets (g)	Castration	Descriptive values from piglets by sows' groups											p-value
		G1				G2				G3			
		N	Mean	Median	SD	N	Mean	Median	SD	N	Mean	Median	SD	
Dorsal	Before	18	267	240	145	33	284	276	151	18	341	290	181	0.404
Left			218	216	92.4		225	228	112		272	265	124	0.378
Left leg			275	231	160		294	274	169		314	230	199	0.904
Left plantar pastern			162	145	87.5		156	134	100		231	184	134	0.098
Plantar			166	155	74.8		162	126	92.0		247	246	141	0.088
Right			215	213	99.3		222	190	114		315	288	159	0.069
Right leg			259	201	183		275	212	166		369	302	213	0.145
Right plantar pastern			169	153	94.3		169	127	99.9		269	255	184	0.216
Total mean value*			217	229	80.4		223	201	102		294	284	135	0.051
Dorsal	After	16	302	300	144	32	271	241	158	12	222	219	79.6	0.368
Left*			290	303	119		232	200	130		212	207	94.1	0.188
Left leg			360	371	213		289	264	163		223	169	128	0.198
Left plantar pastern			219	199	116		175	135	136		201	161	117	0.247
Plantar			252	241	109		178	138	121		216	198	110	0.046
Right			265	261	84.9		219	173	140		226	206	103	0.160
Right leg			244	251	104		254	211	172		220	189	108	0.846
Right plantar pastern			285	267	133		187	130	155		232	201	127	0.011
Total mean value			277	273	88.5		225	183	127		219	201	84.5	0.151

Table 4: Descriptive measures, number of piglets and significance levels when comparing nociceptive values in grams (g) between the three groups, representing the offspring of sound, moderately lame and severely lame sows, before and after castration. Kruskal Wallis test was used to compare these values, and the regions with the symbol * ANOVA test were used. Group G1 are piglets, offspring from sows with the sum of the locomotion scores between 0 to 6, classified as no lame. Group G2 are piglets offspring from sows with the sum of the locomotion scores between 7 to 11, classified as moderate lame. Group G3 are piglets offspring from sows with the sum of the locomotion scores between 12 to 16, classified as severely lame.

Region assessed in piglets (s)		Descriptive values from piglets by sows' groups												p-value
		G1				G2				G3				
	Castration	N	Mean	Median	SD	N	Mean	Median	SD	N	Mean	Median	SD	
Dorsal			7.19	6.78	4.36		6.88	6.38	4.64		9.36	7.45	5.87	0.345
Left			5.72	5.24	2.69		5.41	4.78	3.60		7.27	6.61	3.96	0.222
Left leg			7.38	6.13	4.80		7.28	6.47	5.11		8.47	6.70	6.08	0.834
Left plantar pastern			4.07	3.50	3.12		3.55	2.40	3.78		6.07	4.61	4.48	0.049
Plantar	Before	18	4.17	3.75	2.50	33	3.82	2.69	3.31	18	6.69	7.13	4.78	0.070
Right			5.65	5.51	3.14		5.30	4.41	3.68		8.77	7.83	5.43	0.061
Right leg			7.01	5.54	5.63		6.48	4.70	5.28		10.2	10.7	6.54	0.078
Right plantar pastern			4.24	4.19	3.16		4.07	2.75	3.38		7.45	6.19	6.31	0.230
Total mean value			5.69	6.25	2.37		5.35	4.64	3.29		8.01	7.54	4.50	0.080
Dorsal			8.22	8.75	4.59		7.13	5.71	5.01		5.55	5.28	3.00	0.412
Left			8.12	8.50	3.77		6.08	5.09	4.28		5.63	5.90	3.24	0.133
Left leg			10.2	10.8	6.55		7.73	7.30	5.12		5.88	4.15	4.46	0.158
Left plantar pastern			6.00	5.30	3.96		4.44	2.55	4.57		5.38	4.09	4.32	0.217
Plantar	After	16	7.27	6.38	3.87	32	4.67	3.09	4.14	12	6.11	4.21	4.77	0.060
Right			7.37	7.16	3.44		5.76	4.16	4.68		6.03	4.66	4.59	0.167
Right leg			6.21	6.06	3.84		6.52	4.06	5.70		5.22	4.11	4.24	0.748
Right plantar pastern			8.54	7.83	4.88		5.08	3.25	5.29		6.84	5.09	5.55	0.027
Total mean value			7.74	7.41	3.23		5.92	4.88	4.16		5.83	4.88	3.57	0.128

Table 5: Descriptive measures, number of piglets and significance levels when comparing time of reaction in seconds (s) between the three groups, before and after castration. Kruskal-Wallis test was used to compare all values. Group G1 are piglets offspring from sows with the sum of the locomotion scores between 0 to 6, classified as no lame. Group G2 are piglets offspring from sows with the sum of the locomotion scores between 7 to 11, classified as moderately lame. Group G3 are piglets offspring from sows with the sum of the locomotion scores between 12 to 16, classified as severely lame.

Nociception assessment during the first 12 hours of life

When comparing the nociception threshold values between the lameness groups of sows, a significant difference was found in the LP region, being the responses of G3 piglets higher than the responses of G2 piglets (Nemenyi post-hoc test; p-value = 0.03). A tendency was found in the RL region, being the responses of G3 piglets higher than the responses of G2 piglets (Nemenyi post-hoc test; p-value = 0.097). A tendency was found also in the left region (p-value = 0.096) without differences in the Nemenyi post-hoc test (p-value > 0.05). No significant differences were found in the other body regions: LL, RP, right region, dorsal/plantar region, and total mean value (p-value > 0.05).

When comparing the time reaction, significant differences were found in several regions and always offspring from G3 sows showed longer responses than the offspring of G2 sows – the p-values presented below correspond to the Nemenyi post-hoc test; LL region (p-value = 0.002), RL region (p-value = 0.0002), LP region (p-value = 0.0003), left region (p-value < 0.0001), right region (p-value = 0.004) dorsal region (p-value < 0.0001), plantar region (p-value = 0.009), and total mean value (p-value < 0.0001). Additionally, tendencies were found when contrasting nociceptive pressure thresholds in the offspring of G1 and G2 sows, being G1 higher than G2, in the next regions: LP region (p-value = 0.082), left region (p-value = 0.061), dorsal region (p-value = 0.069) and total mean value (p-value = 0.059). Only RP region showed no evidence of difference (p > 0.05).

Nociception assessment before and after castration in male piglets

When comparing nociceptive measures in male piglets before castration between the groups, only tendencies were found between the offspring of G2 and G3 sows, being always G2 lower responses than the offspring of G3 sows, detailed values of the post-hoc Nemenyi test and regions are as follows: LP (p-value=0.083), right region (p-value=0.085), plantar region (p-value=0.073) and total mean (p-value=0.083). No differences were found in the following regions: LL, RL, RP, left region, and dorsal region (p>0.05).

Regarding the time reaction measures, a significant difference was found in two regions, whereas the offspring of G2 sows had lower nociceptive pressure thresholds than the offspring of G3 sows, post-hoc test and regions are as follows: LP region (p-value=0.041) and right region (p-value=0.049). Tendencies were found in the next regions, being the responses of the nociceptive pressure threshold of the offspring of G2 sows always lower than the offspring of G3 sows, post-hoc test and regions are as follows: RL (p-value=0.072), plantar region (p-value=0.057) and total

mean (p-value=0.064). No differences were found in the following regions: LL, RP, left region, and dorsal region.

Differences in nociceptive pressure thresholds were observed in two regions after castration, whereas the offspring of G1 sows showed values higher than the offspring of G2 sows after post-hoc Nemenyi test in the region RP (p-value=0.008) and plantar region (p-value=0.04). No differences were found when comparing nociceptive values between the groups in the following regions: LL, RL, LP, left/right/dorsal region, and total mean value (p-value>0.05).

When comparing reaction times, significant differences were noticed in two regions RP and plantar region. After post-hoc Nemenyi test, the time reaction was always higher in the offspring of G1 sows when contrasted with the offspring of G2 sows as follows: RP region (p-value=0.021) and plantar region (p-value=0.048).

DISCUSSION

The main biological function of nociception and pain is that they play a protective role against noxious or harmful stimuli (Sneddon et al., 2014), making them essential evolutionary mechanisms for the preservation and survival of animals. In this paper, we demonstrated that the experiences of the mother, with different levels of pain, modifies the nociceptive thresholds of the offspring. The perception of noxious or potential harmful stimuli are altered in offspring from sows that are diagnosed with pain during the last third of gestation, suggesting that the protective role of pain and nociception could be compromised in the offspring by the experience of the pregnant sow.

Both in experiments 1 and 2, severe lameness during pregnancy of sows resulted in offspring with higher nociceptive threshold in different body regions when compared with weaned offspring from sows without lameness. Or, when compared with offspring from sows with moderate lameness during the first 12 hours of life and before castration, in 4 to 5 days old males. These findings suggest that offspring exposed to a “painful” prenatal environment are less responsive to noxious stimuli. In consequence, they could be more tolerant of pain and perhaps less responsive to harmful situations as a coping mechanism, directly affecting the main function of pain that is to preserve life.

Conflicting results were only found after castration where the higher nociceptive threshold was only observed in the plantar region of male piglets which were offspring from sows without lameness during pregnancy when compared with piglets offspring from sows with moderate lameness. Castration is a painful procedure (Ison et al., 2016), which activates peripheral and central mechanisms. It is possible that the pain response system of the offspring of G3 sows,

measured using the existing protocol, was disorganized when the animal faced a real-life pain inducing event.

Considering that intensive pig production systems were established towards the middle of the last century (Woods, 2012), an explanation of this findings could represent a mismatch between the demands of high welfare standards in commercial pig farms and the negative consequences caused by the prenatal modulation of the pain system. We argue that the differences in pain responses have been recorded in the body region where more lesions are reported in sows. The plantar region – responsible for the high prevalence of lameness in sows (Pluym et al., 2013; Pluym et al., 2011) had altered nociceptive threshold indicating that if animals are more tolerant to plantar pain, they will, likely, showed a delayed behavioural response when claw lesions first appear, compromising early therapeutic interventions

Our results are in agreement with published work studying the effects of stressful prenatal environments on offspring from pigs and mice. Offspring from females kept on a stressful prenatal environment had higher values of basal nociception (Sandercock et al., 2011; Sternberg and Ridgway, 2003), even if the stressors were from a different origin. Regardless of the type of stress during pregnancy, it appears that stress is capable to alter the development of somatosensory, emotional system and learning process in offspring (Sneddon, 2004).

In this paper we discussed only about the effects of prenatal stress on the immediate pain threshold responses in the offspring, but in the study conducted by Ashworth et al., (2011) the authors showed that prenatal social stress and post-natal pain can disrupt the developing reproductive axis, changing their plasma concentrations of reproductive hormones or decreasing the number of primordial ovarian follicles and the weight of the testis. The consequences of stress during pregnancy are innumerous, having the potential to impair animal welfare and productive outcomes.

There are some biological mechanisms proposed to explain the occurred effects that we reported in the offspring. In the revision work by Lau and Rogers (2004) biological mechanisms have been offered for humans and laboratory animal models, involving hormones essentials for fetal development such as glucocorticoids. Other potential mechanisms are represented by epigenetic effects involving on the fetal programming, as recently proposed by Géranton (2019), showing that neonatal stress in mammals predispose them to a chronic pain by a higher expression of the protein FKBP51, responsible for the regulation of the stress axis.

From our results we can conclude that lameness during the last third of pregnancy altered nociceptive threshold in the offspring, regardless of the age of the piglets. Considering that lameness is a prevalent problem in breeding pigs, including in boars (Li et al., 2017) it is worrying

and problematic to continue with the same current housing and management conditions for pigs, because the effects have the potential to program the resilience and environmental perception of future generations. Finally, recent studies tshow the advantages to use pigs as a translational research model (Bassols et al., 2014), our results will contribute to the creation of research opportunities and address hypotheses not previously studied in humans.

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5. CHAPTER IV: **Behavioral, physiological, and productive outcomes in offspring from lame sows**

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ABSTRACT

Lameness is a painful condition that affects the welfare of millions of sows globally, and as stressful condition during pregnancy have the potential to compromise coping systems of the offspring, including fear and aggression. The objective of this study was to assess behavioral, emotional, and physiological outcomes in the offspring from sows with contrasting locomotion scores During the last third of pregnancy, locomotion assessment was carried out in 30 (Brazil) and 39 (Italy) pregnant sows and scored from 0 (normal gait) to 5 (severe lameness). Sows were grouped as no lame (G1; N = 15 – Brazil; N = 14 – Italy), moderately lame (G2; N = 16 – Italy), and severely lame (G3; N = 15 – Brazil; N = 9 – Italy). Data obtained from 90 weaned piglets (Brazil) included body photographs, taken for three days to count skin lesions; behavior during open field and novel object tests; and salivary samples to measure cortisol responses when challenged with transport. In Italy, all piglets from 39 litters were tested (N = 539 piglets), to measure hair cortisol and to assess intrauterine growth restriction (IURG). After determining data distribution, parametric and non-parametric tests were performed. Significance was determined when $p < 0.05$ and was considered a tendency when $p = 0.05 – 0.1$. Skin lesions were significantly different at 28 days of age between groups G1 and G3 inthe left face and head ($p = 0.02$). Latency to approach the novel object was higher in piglets, which were offspring from G1 sows, when compared with piglets offspring from G3 sows ($p = 0.03$). The HPA response in response to the transport challenge was higher in piglets from G3 sows ($p = 0.03$). Hair cortisol concentration in male piglets from G1 was tendentially lower than piglets from G3 sows ($p = 0.094$) and piglets born from G3 sows had a higher IURG score than piglets from G1 sows ($p = 0.05$). Lameness in pregnant sows altered physiological and behavioral outcomes in their offspring.

INTRODUCTION

Welfare of an individual is defined as its state as regards its attempts to cope with its environment (Broom, 1986). Environmental conditions for most pigs housed in commercial farms are challenging, often with no opportunities for real adaptation. Oftentimes, sows are housed in environments poor in stimulus with insufficient resources to meet their biological needs and animals are constantly mixed with unknown individuals (Broom et al., 1995; Tönepöhl et al., 2013). Many of these challenges bring with them behavioral and physiological consequences that can be observed and measured to determine the welfare state of animals subjected to the challenges.

One of the consequences determined as the main animal-based measure to assess welfare of sows is lameness (Whay et al., 2003). Lameness is an extremely painful and stressful condition that alters locomotion in sows, modifying their social, feeding, and maternal behavior (Babenko et al., 2015; Larsen et al., 2015; Nalon et al., 2013; Tönepöhl et al., 2013). Causes of lameness are multifactorial, including poor infrastructure designs and materials used in sow housing systems, which tend to be harmful to their locomotory system. In addition, there are negative consequences associated with the occurrence of agonistic events due to the constant mixing with unknown animals. In summary, a combined effect results in a detrimental outcome to the

productiveperformance added to unacceptable levels ofwelfare (Pluym et al., 2017; Rutherford et al., 2012).

Reproductive performance is the main demand for sows on commercial farms, so it is relevant to investigate the relationship between sow welfare indicators and the potential outcomes for their offspring (Costa, 2014; Rutherford et al., 2012). The beginning of communication, through hormonal signaling, between a pregnant female and her offspring starts from fertilization, each parents' gamete brings with it genetic and epigenetic information that will foster necessary adaptations to maintain a viable pregnancy (Lacal and Ventura, 2018; Nadeau, 2017) and promote the development of most adapted phenotypes. There is evidence that biologically relevant signals received by the fetus, at different stages of gestation may program and reprogram the development of the offspring according to the environmental factors that the mother experiences, to prepare them for postnatal conditions. A stressful prenatal environment could modify brain development in the offspring (Petit et al., 2015; Rutherford et al., 2014) as an adaptative strategy to foster more adequate responses to the extrauterine environment that the offspring will face. However, if the external environment show dissonance from *in-utero* experience, offspring responses may be inappropriate or undesired mainly for production animals (Bale, 2015).

The mechanisms proposed in the literature to explain prenatal programming include the influence of glucocorticoids and cytokines during fetal life, and / or epigenetic modifications (Bale, 2015; Robertson et al., 2018, 2015; Tatemoto et al., 2019). In pregnant sows, administration of high levels of ACTH, adrenocorticotropic hormone involved in the adrenocortical synthesis and release of cortisol, has demonstrated an increase in salivary and plasma cortisol concentration in the offspring, and a reduction in the length of gestation (Kranendonk et al., 2005). Furthermore, it has been shown that a wealth of negative and some positive experiences of pregnant sows, such as periodic isolation, high cortisol concentration, environmental enrichment, or food satiety, alters developmental outcomes in the offspring, such as body weight, basal cortisol concentration, HPA (hypothalamic–pituitary–adrenal axis) stress responsiveness, brain neurotransmitters, and aggressive behavior (Bernardino et al., 2016; Fowden et al., 2016; Tatemoto et al., 2019). In relation to the role of cytokines, it has been reported that a balance between embryotrophic and embryotoxic cytokines in the female reproductive tract is determined by several stressful events, impacting embryonal implantation, placental development, and fetal growth, mediating biological effects of embryo programming, embryo plasticity, and adaptation (Robertson et al., 2018, 2015).

To our knowledge, studies in pigs evaluating the effects of lameness during pregnancy on the offspring have not been performed.

Our aim is to assess the HPA and behavioral responses, using cortisol measurements and behavioral tests to assess fear, exploratory behavior, and aggression, when the offspring of lame and sound sows are challenged with common situations present in commercial pig farms. Our hypothesis is that offspring from sows with lameness during late pregnancy have different, less adaptive, behavioral, and physiological responses when faced with challenging events than the offspring of sound sows.

MATERIAL AND METHODS

This research was conducted in two separated, but complementary experiments carried out in Brazil, and in Italy, and always in summer season. In Brazil, the experiment was carried out in a commercial pig farm in the state of Paraná, and followed with the study at the Experimental Swine Farm, School of Veterinary Medicine and Animal Science, University of São Paulo, Campus Fernando Costa, Pirassununga, SP. In Italy, the animal trials were carried out in a commercial pig farm in Abruzzo and followed with studies carried out at the School of Veterinary Medicine, University of Teramo, Abruzzo.

The experiment carried out in Brazil was submitted to the Ethics Committee on Animal Use (CEUA) with the protocol number 9870211117. The experiment carried out in Italy was submitted with the protocol number N° 677/2020-PR to the University of Teramo, Italy.

In Brazil

Animals, handling, and locomotion assessments

Thirty multiparous pregnant sows (TOPGEN Large white and Landrace) kept on a commercial pig farm, were selected using locomotion assessment, and a cohort sample of 90 of their weaned piglets were used to assess behavior and salivary cortisol measures. Sows were housed in collective gestation pens that measured 6 x 4 m (2.7 m^2 per sow) with walls of 0.9 m high, a wood gate and a solid/slatted concrete floor area, 4m/2m of length, respectively. Sows were fed with a liquid diet twice daily (06:00h and 15:00h) using collective feeders without divider (5 m long and 0.4 m wide). Water was provided *ad libitum* by two nipple drinkers per pen.

Sow lameness was evaluated biweekly for four months using a scale which ranged from 0 to 5, being 0 an animal with normal locomotion and 5 a downer animal (D'Eath, 2012). Assessment of locomotion was performed always by the same trained person. Based on the locomotion assessments of the last third of gestation, 30 sows were selected, 15 without lameness (G1: all

assessments with scores 0 or 1) and 15 animals with severe lameness (G3: with at least on time with scores between 3 to 5). Sows with locomotion assessment ≥ 3 were treated with 1,1 mg/kg of flunixin meglumine intramuscular for three days, as requested by the ethics committee.

One week before the expected farrowing date, all sows were housed in individual farrowing crates that measured 2.6 x 1.6 m. During lactation, all sows were fed four times per day (07:00h, 11.00h, 16:00h and 21:00h) and water was supplied *ad libitum* by a nipple drinker. All piglets were weighted at birth and with three days of age they received iron injection and ears were notched, for identification. Management practices such as castration and tail docking were not performed in the animals.

Weaning was carried out when piglets reached 28 days of age. A cohort sample of three piglets – one female and two males – per sow (N = 90) were selected, weighted, and transported for 436 Km the late afternoon from the commercial farm to the experimental farm at the University of Sao Paulo. During transport, the litters never were mixed. At the University Farm, piglets were allocated in pens with slatted floor measured 2.4 m long, 0.6 m wide and 0.9 m high and a heat floor area (0.9m long x 0.9m wide). Nine piglets were housed per pen, from the same locomotion score sow group, and from three different sows, mixed homogeneously using weight and to create homogenous groups. During the following days, feed was offered several times according to their consumption using conventional started diet. Water was supplied by one nipple drinker and an open trough, *ad libitum*. Disease events were always attended by a veterinarian.

Agonistic behavior

On days 28, 29 and 30 of age six photographs from different body regions were taken from each piglet to count skin lesions as an indirect measure of agonistic behavior based on published protocols (Bernardino et al., 2016; Guy et al., 2009; Tatemoto et al., 2019). Body regions assessed were face, behind the ear, and trunk/limbs in the left and right sides (see Fig. 1 for details). Physical restrain was done gently and always in the same way to obtain the photographs as illustrated in Fig. 1. Skin lesions were counted by two independent evaluators who did not know the treatment of each piglet.

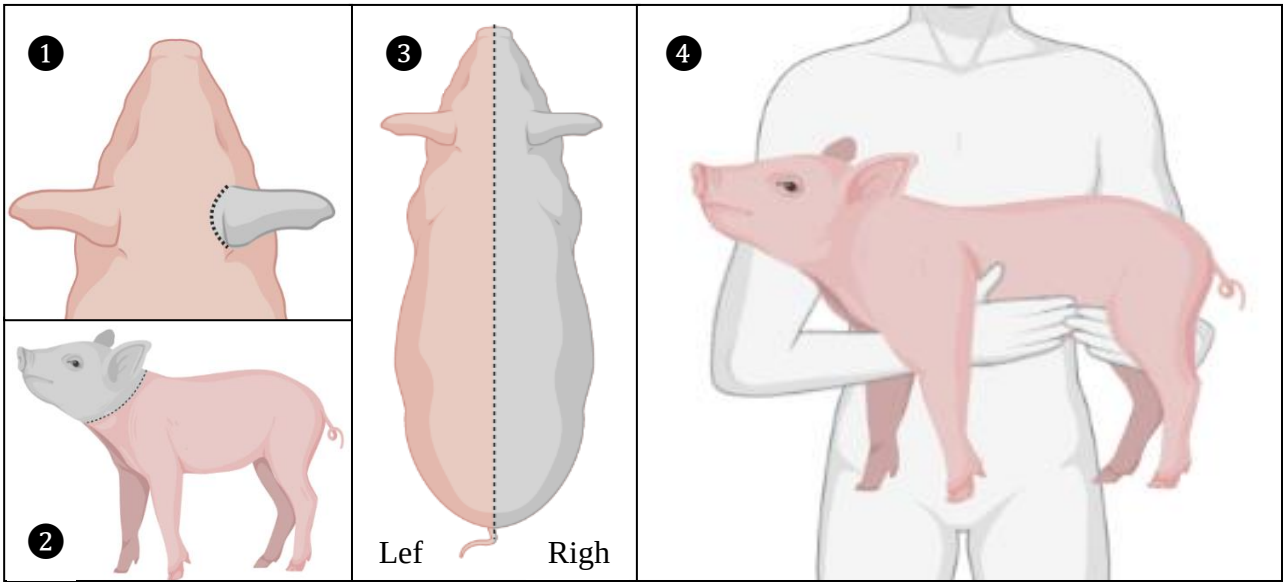

Figure 1: Body regions assessed to count skin lesions (Bernardino et al., 2016; Guy et al., 2009; Tatemoto et al., 2019), behind the ear (1), face (2 – gray region), and trunk/limbs (2 – pink region), in the left and right sides each one (3). Gentle physical restraint was performed to hold piglets during the photographs.

Open field and novel object test

A combination of open field (OT) and novel object (NT) test was performed in all piglets on day 31 of age using previously validated protocols (Bernardino et al., 2016; Puppe et al., 2007; Tatemoto et al., 2020; Zupan et al., 2016). Pigs were tested individually in pens that measured 2.5 m x 2.5 m with white painting lines on the floor forming squares in all their extension and sets of squares were categorized into three regions according to their location within the pen as follows, peripheral (P), middle (M), and central (C) (see Fig. 2 for details).

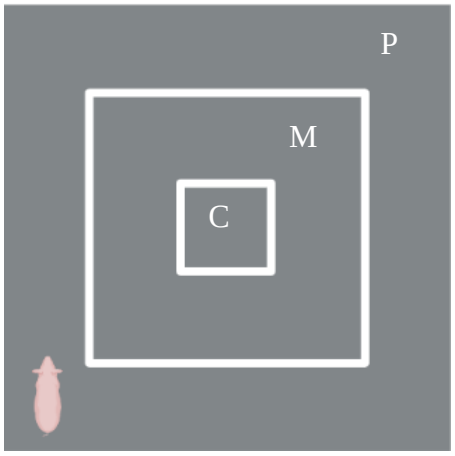

Figure 2: Illustration of the pen used to perform Open field and Novel Object Test in piglets (Bernardino et al., 2016; Puppe et al., 2007; Tatemoto et al., 2020; Zupan et al., 2016). The pink silhouette on the lower left side represents the entry point of each piglet. The mean of each letter is as follows, C: central square; M: middle squares; and P: peripheral squares.

Each test lasted five minutes, starting with OT followed by NT, totaling 10 minutes (Zupan et al., 2016). A traffic cone was inserted by pulleys in the pen as a novel object at the beginning of the NT. Before the entry of each piglet feces were removed and the pen was washed with pressure water to decreased possible chemical clues. Each animal was identified with a non-toxic marker in its back, placed in the pen always at the same starting point, behavior was recorded with a camera HD (infrared camera Multi HD VHD 1220 B G4, Intelbras, Brazil) during both tests, all types of vocalization were counted. Videos were analyzed in the open-source software BORIS (Friard and Gamba, 2016) always by the same trained person who was unaware of prenatal environment experienced by the piglets, represented by the lameness condition of the sow. During OT, the following measures were obtained: the latency to start walking, number of C, M and P squares accessed, time walking, number of escape attempts, number of defecation/urination events and number of all types of vocalization. During NT, the following measures were obtained: the latency to touch the object for the first time, time near the object, time exploring the object, number of escape attempts, number of defecation/urination events and number of all types of vocalization. Time variables were reported in seconds and frequency variables were reported as number of events (see Table 1 for details).

Behavior observed	**Description**
Latency walks ▲	Time between putting the animal in the pen and start to walk.
Center frequency ▲	Events that the subject accessed the central square.
Center time ▲	Time that the subject was in the central square.
Middle frequency ▲	Events that the subject accessed the middle squares.
Middle time ▲	Time that the subject was in the middle squares.
Peripheral frequency ▲	Events that the subject accessed the peripheral squares.
Peripheral time ▲	Time that the subject was in the peripheral squares.
Exploration ▲	Time walking through the arena.
Latency object ■	Time between putting the novel object and interact with it.
Exploring object stand ■	Time interacting with the object without moving it.
Exploring object walking ■	Time interacting with the object by moving it from its position.
Exploring total ■	Sum of *exploring object stand* and *exploring object walking*.
Escape attempts ▲■	Events jumping in the wall.
Urinate frequency ▲■	Events in which the subject urinated.
Defecate frequency ▲■	Events in which the subject defected.
Vocalization frequency ▲■	Events of all kinds of vocal sounds produced by the subject.

Table 1: Behaviors observed during Open Field (▲) and Novel object test (■). Behaviors expressed in *time* were measured in seconds, and behaviors expressed in *events* were measure as number of times that each behavior was observed.

Salivary cortisol before and after transport

Twice a day for three days before saliva collection, a line of dental floss was tied in each pen with several cotton rolls impregnated with sugar and water to habituate 36 piglets to collect saliva (18 from G1 sows, and 18 from G3 sows). On the day of transport, two saliva samples were collected from each piglet using dental floss tied to a cotton roll, the first one was taken in their home pens before the transport, and the second one 20 minutes after arriving at the holding pens, on a journey that lasted 10 minutes. Saliva collection protocol reported by Siegford et al. (2008) was used. Feed was removed 12 h before the first saliva sample as the male piglets were taken to the slaughterhouse and water was always available. Piglets from different pens were never mixed during transport or in the slaughterhouse holding pens.

Each sample was stored in a falcon tube of 15 mL at 20°C until Enzyme Immunoassay (EIA) to measure cortisol concentration. On the day of the analysis, all samples were defrosted at controlled temperature, centrifuged for 10 minutes at 1000 x g, and the content was transferred to a 1.5 ml microtube. The EIA procedure used to measure saliva cortisol concentration was based on the protocols reported by Palme, and Möstl (1997) using 50 µL of saliva from each sample, always placed in duplicate. When the concentration was greater than the EIA could detect, 25µL of the sample was diluted in 100 µL of EIA buffer, using 50 µl of this solution in duplicate.

In Italy

Animals, handling, and locomotion assessments

Locomotion assessment was carried out weekly in the last two months of pregnancy, in a commercial pig farm, monitoring 39 multiparous sows TOPIGS® TN60. The same locomotion system used in Brazil was used in Italy, always by the same trained person in collective gestation pens (D'Eath, 2012). Each pen housed 15 sows, being 2.8 m^2 per sow (6 x 7m). During gestation, all sows were fed twice a day – at 07:00h and 15:00h, with conventional dry food and water was supplied *ad libitum* by two nipple drinkers per pen. One week before the expected farrowing date all sows were transferred to farrowing crates that measured 1.6 x 2.4 m, they were fed three times per day – at 07:00, 12:00 and 16:30h, and water was supplied *ad libitum* by a nipple drinker. Based on locomotion scores all sows were divided in three groups, G1 (N = 14, when the sum of the 5 assessments was between 0 to 6), G2 (N = 16, when the sum of the 5 assessments was between 7 to 11), and G3 (N = 9, when the sum of the 5 assessments was between 12 to 16). Sows with locomotion assessment ≥ 3 were treated with 1.1 mg/kg of flunixin meglumine intramuscular for three days as requested by the ethics committee. All piglets were weighted at birth.

Intrauterine growth restriction and hair samples

After farrowing information about sex ratio and intrauterine growth restriction (IURG) was collected in all litters from the 39 sows (N = 519 piglets). The protocol described by Hales et al. (2013) was used to assess IURG in all piglets to classify them according to three morphological head criteria, 1) steep, dolphin-like forehead; 2) bulging eyes; and 3) wrinkles perpendicular to the mouth. If all 3 criteria applied to a iven piglet, it was defined as IUGR score 3; if 1 or 2 characteristics were identified, it was defined as light IUGR score 2, and if none of the 3 criteria were identified, the pigled was classified as normal, score 1. Observation and classification were carried out always by the same person. See Fig. 3 to details about head characteristics.

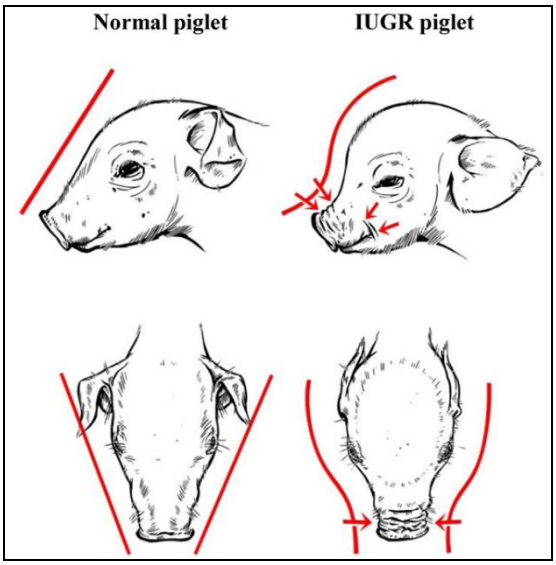

Figure 3: Illustration taken from Hales et al. (2013) to show a normal piglet (left) and a IURG piglet (right). IURG = Intrauterine growth restriction. IURG classification is based on three morphological head criteria, 1) steep, dolphin-like forehead; 2) bulging eyes; and 3) wrinkles perpendicular to the mouth. If all 3 criteria applied to a piglet, was defined as IUGR (score 3); if 1 or 2 characteristics applied, was defined as light IUGR (score 2), and if none of the 3 criteria applied, was defined as normal (score 1).

Hair samples from all piglets were collected between four to seven days of age, always from the back region. The Animals were physically restrained in a hammock to collect the samples like showed in the Fig. 4. Hair samples were stored individually in a plastic container, without direct light contact and stored in a dry place.

Figure 4: Physical restraint method used to collect hair samples in piglets.

Preparation of hair samples and cortisol EIA

The protocol to clean and to extract cortisol from hair samples was adapted from Stubsjøen et al. (2015). To clean the samples, two hair aliquots of 0.2g were weighted in glass vials from each litter separated by gender – males and females. Each aliquot was degrassed adding 7 ml of n-Hexane, mixing in a hand vortex for one minute, subsequently the solvent was everted, and each sample was dried under a hood. To extracted cortisol hair samples were weighed 0.1g (0,100 ± 0,0005 g) from each dry hair aliquot in a new glass vial, 5 mL of methanol 100% was added, vials were plug tightly, placed in a water bath with agitation at 37°C for 24 h, centrifuged for 15 minutes at 3000 x g, and finally 2.5 mL of methanol was transferred in a new glass vial and dried down at 50°-60°C, under a stream of nitrogen.

To measure cortisol an enzyme immunoassay (EIA) was performed. Each glass vial was redissolved in 0.5 mL of EIA buffer, taken to shaker for 30 min and 50 µl were used to perform the cortisol EIA based in the protocols reported by Palme and Möstl (1997).

Statistical analysis

It was used Shapiro-Wilk test in all variables to determine the residual distribution, when the result was $p > 0.05$ a parametric test was used and when was $p < 0.05$ a non-parametric test was used, always considering the number of groups to be compared. The variables used to perform a Shapiro Wilk test were weight at birth, skin lesions at 28, 29 and 30 days of age, behavioral data from open field and novel object test, saliva, and hair cortisol concentrations and IURG scores.

Skin lesions from each day collected were analyzed comparing individual body regions and groups of regions between the sows' groups – G1 and G3. Individual regions assessed were face, behind the ear, and trunk/limbs in the left and right side each one. Group's regions assessed were total right (face, behind the ear, and trunk/limbs in the right side) total left (face, behind the ear, and trunk/limbs in the left side), head (face and behind the ear from both sides), total trunk/limbs (trunk

and limbs from both sides) and total (all individual regions from both sides). In all cases individual or grouped regions were analyzed from each day and sum of all days.

Salivary cortisol concentration was analyzed in two ways, first comparing the concentration of sample 1 (before transport) with sample 2 (after transport) of the same group using Wilcoxon paired test; and second, a ratio between sample 1 and sample 2 was performed for all animals and subsequently a comparison between groups was realized using Wilcoxon test.

To analyze correlation between IURG score and weight at birth was used a Spearman correlation test dividing by groups, G1, G2 and G3. A significance level was considered when p value was < 0.05 and was consider a tendency when p value was between 0.05 and 0.1. All analyses and graphs were performed using the free software environment for statistical computing R (RStudio Team, 2020).

RESULTS

In Brazil

There was a significant difference between groups in birthweight (T-test; $p = 0.01$), whereas G1 piglets were heavier than G3 piglets (G1 mean = 1.53 kg ± 0.03; G3 mean = 1.44 kg ± 0.03). Skin lesions were significantly different at 28 days of age between groups G1 and G3 in the regions named left face and head ($p = 0.02$; see respective boxplot in the Fig 5). Tendencies were found between groups G1 and G3 in the other regions and ages: behind the right ear at 28 days ($p = 0.08$), in the left face at 29 days ($p = 0.09$), in the left side at 28 and 29 days ($p = 0.07$), and total lesions at 28 days ($p = 0.08$). When significant differences and tendencies were reported, the mean value of skin lesions were always lower in G1 offspring than in G3 offspring. In the remaining body regions comparisons and their respective days, no statistical differences were found ($p > 0.05$). Mean values and p values for all regions analyzed are presented on the Supplementary Table S1.

During the open field test, it was found a tendency in the time passed in the middle squares (Wilcoxon test; $p = 0.05$), where piglets from G1 sows spent more time than piglets from G3 sows (G1 mean = 20 sec ± 4; G3 mean = 17 sec ± 5) in the middle squares. No significant differences or tendencies were found with the other variables measured during the open field test (Wilcoxon test or T-test; $p > 0.05$). During the novel object test a significant difference was found in the latency to interact with the object (Wilcoxon test; $p = 0.03$), where piglets from G1 sows took longer to interact with the object than piglets from G3 sows (G1 mean = 53 sec ± 10; G3 mean = 44 sec ± 10). No significant differences or tendencies were found with the other variables measured during the novel object test (Wilcoxon test or T-test; $p > 0.05$).

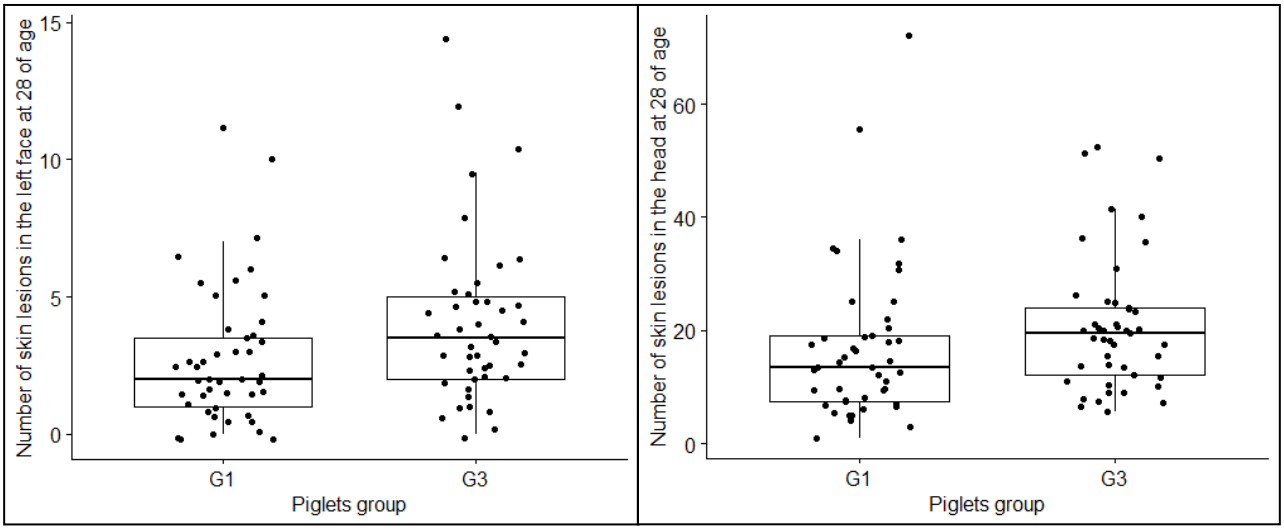

Figure 5: Data distribution according to the number of skin lesions in left face region and head region from piglets with 28 days of age divided by groups: G1 (no lame) and G3 (severe lame).

Regarding salivary cortisol concentrations, no difference was observed when comparing basal and post-transportation levels in G1 piglets (Wilcoxon paired test; $p > 0.05$; mean sample 1 = 21.6 ± 3.1 pg/50µl; mean sample 2 = 48.4 ± 19.2 1 pg/50µl). A significant increase between basal and post-transportation samples were observed in G3 piglets (Wilcoxon paired test; $p = 0.01$; mean sample 1 = 33.1 ± 9 pg/50µl; mean sample 2 = 64.9 ± 15.5 pg/50µl; an increase of 50,95%). No differences were found when compared ratios between G1 and G3.

In Italy

Weight at birth was different between groups (ANOVA One-way; $p < 0.001$); G1 piglets were significantly heavier than G2 (T-test; $p < 0.001$) and G3 piglets, being a tendency in the latter (T-test; $p = 0.06$). Mean weights of piglets were G1 = 1.5 ± 0.03 kg, G2 = 1.3 ± 0.02 kg, and G3 = 1.4 ± 0.04 kg.

A tendency was found regarding IURG scores between G1 and G3 piglets (Wilcoxon test; $p = 0.05$) being G1 lesser than G3 (G1 mean = 1.98 ± 0.06; G3 mean = 2.14 ± 0.06). No differences were observed in the remaining comparisons ($p > 0.05$). A point error plot about IURG scores divided by groups could be seen in the Fig. 6.

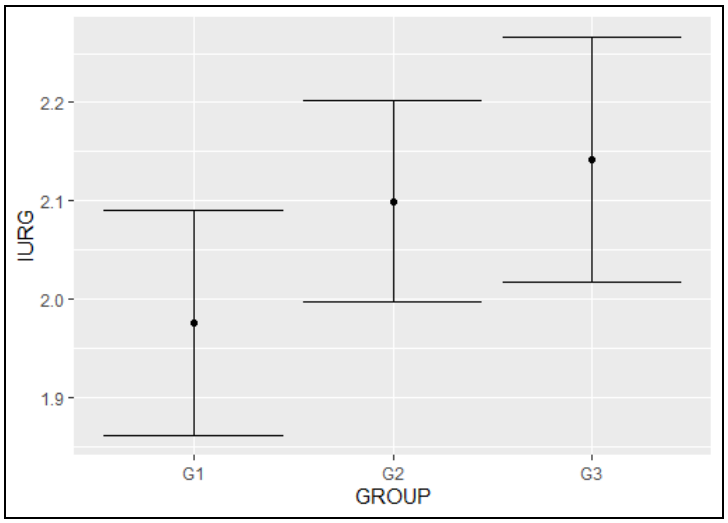

Figure 6: Point error plot to show the distribution of IURG (Intrauterine growth restriction) scores between groups G1 (no lame), G2 (moderate lame) and G3 (severe lame). IURG assessment was performed using the protocol reported by Hales et al. (2013).

Moderate negative correlations were found between IURG score and weight at birth for all groups (Spearman correlation test; $p < 0.0001$; G1 rho = -0.624; G2 rho = -0.618; G3 rho = -0.623).

Regarding hair cortisol, only a tendency was found when comparing male piglets from G1 group with male piglets from G3 group (T-test; $p = 0.09$) being less concentrated in piglets G1 than G3 (G1 mean = 8.13 ± 0.81 pg/10 µl; G3 mean = 11.18 ± 1.49 pg/10 µl). There were no differences in the remaining comparisons ($p > 0.05$).

DISCUSSION

Our data supports previous results where lameness in sows during late pregnancy alters the phenotype of the offspring (Sarmiento et al., 2021). Piglets from lame sows responded behaviorally and physiologically differently when subjected to social challenges or unknown situations than piglets from sows without lameness.

Piglets from lame sows were lighter at birth in the experiments carried out in Brazil and in Italy, probably due to the behavioral changes of lame sows, the metabolic challenges caused by pain and inflammation or by the stress and poor welfare all factors previously reported in the literature (Ala-Kurikka et al., 2016; Bale et al., 2010; Heinonen et al., 2013). Lame sows were less active, lie down more, explored less their environment, and modified their feeding behavior (Ala-Kurikka et al., 2016; Heinonen et al., 2013) directly affecting the nutritional intake and consequently, the nutritional contribution for the development of the offspring. Our findings associated to low weight at birth and higher IURG scores in piglets from lame sows are factors directly related to piglet

survival (Amdi et al., 2013; Hales et al., 2013; Pedersen et al., 2011). It has been reported previously a positive relationship between increased birth weight with decreased of risk of crushing (Pedersen et al., 2011) and survival (Hales et al., 2013). IURG piglets have been described as piglets that ingest insufficient amounts of colostrum further decreasing their survival outcomes (Amdi et al., 2013; Hales et al., 2013). We can then infer that from a productive perspective lameness in sows is disadvantageous for the offspring.

According to our findings measuring the outcomes of agonistic behavior, piglets from sows without lameness evaluated different their environment, our conclusions are based on the lesser number of skin lesions, recorded by experimenters unaware of the treatments, in the offspring of sows that did not show lameness. Aggressive behaviors are performed by animals predominantly to acquire or defend limited resources (Jalabert et al., 2018) and in our experiment all weaned piglets had access to the same conditions, including an abundant offer of food and water. Additionally, Turner et al. (2006) related that heaviest animals have advantages in fights and this variable was controlled in our experiment, mixing litter homogeneously according to weight and gender thus, our findings are robust and indicative of a real effect of lameness on agonistic behaviors. If piglets from sows without lameness got involved in fewer fights after mixing with unknown piglets it suggests that they, perhaps, "analyzed" better the costs and benefits of competing for resources. Avoiding fighting implies a lower use of energy and a decrease in physical injuries, in benefit of their performance and welfare, since using aggressive behavior when coping with difficulties may be associated with pain and suffering (Broom, 1991).

We found a trend in relation to the lameness score of the sows in the open field test related to the time and location in the arena where the piglets stayed during the test. The context in which these data has been collected has not been reported in the literature as an indicative of different emotional states to novel situations in pigs, and the only information reported as reliable data in this test is activity (Forkman et al., 2007), in our experiment overall activity was not different between the piglets from sows with or without lameness.

The findings from the novel object test are very interesting and demonstrated that *in-uterus* experience was sufficient to influence the response to the test, either making piglets from lame sows more motivated to explore, more curious or less concerned with potential threats, than piglets, offspring from sows without lameness. We use the same argument as stated for the open field test results, because it is difficult to determine if the novel object was perceived by piglets as a threat or as a neutral, attractive novelty (Forkman et al., 2007).

We propose that piglets from sows without lameness during gestation coped different with the social and behavioral challenges due to the reported physiological findings, since when

challenged to respond to a stressful practice common in pig farming – transport, they had lower increase in salivary cortisol concentration after the transport, when compared with piglets from lame sows. Piglets from sows without lameness are likely to show lower activation of the HPA axis than the offspring from lame sows.

Lameness impact sow welfare directly causing pain, stress and suffering, altering their movement and decreasing their capacity to compete for resources suchas water or food, increasing in consequence the risk to signal hunger and thirst to the offspring, in the uterus (Heinonen et al., 2013). The direct welfare challenges caused by lameness altered the physiology, the behavior, and some productive parameters in their offspring. Despite having carried out the experiment using different genotypes and in different countries, our results are comparable and complementary, concluding that lameness compromised not only the welfare of sows, but also the welfare of their offspring.

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Supplementary material

Body regions	Side	Age in days	Mean skin lesions		p-value
			G1	G3	
Trunk and limbs	Right	28	2.27	1.87	0.55
		29	2.60	2.16	0.94
		30	2.88	1.99	0.24
	Left	28	1.81	1.58	0.96
		29	1.94	2.21	0.16
		30	2.37	2.03	0.86
Behind ear	Right	28	5.04	6.31	0.08*
		29	7.06	7.70	0.58
		30	9.14	9.29	0.82
	Left	28	6.11	7.20	0.13
		29	7.40	7.84	0.35
		30	9.00	8.84	0.95
Face	Right	28	2.98	3.36	0.47
		29	4.74	4.91	0.55
		30	5.81	5.44	0.75
	Left	28	2.78	3.99	0.02**
		29	4.24	4.97	0.09*
		30	4.97	5.11	0.67
Right side	Right	28	10.29	11.53	0.16
		29	14.13	14.77	0.48
		30	17.83	16.72	0.63
		Total	42.26	43.02	0.88
Left side	Left	28	10.70	12.77	0.07*
		29	13.28	15.02	0.07*
		30	16.02	15.99	0.73
		Total	40.00	43.78	0.18
Head	Left and Right	28	16.91	20.86	0.02**
		29	23.43	25.42	0.21
		30	29.02	28.69	0.89
		Total	69.15	74.96	0.34
Body		28	4.08	3.44	0.80
		29	4.59	4.37	0.27
		30	5.24	4.02	0.44
		Total	13.63	11.83	0.79
Total		28	20.99	24.30	0.08*
		29	28.02	29.79	0.22
		30	34.26	32.71	0.76
		Total	82.76	86.80	0.41

Table S1: Mean skin lesions for all body regions of each group and p values of the comparisons between G1 and G3 divided by days of age. The comparisons realized with the sum of all days are named as Total in the third column. ** p value considered significant. * p value considered as tendency. For all comparisons was performed Wilcoxon test.

6. DISCUSSION

With the present study, we demonstrated that different degrees of lameness during the last third of pregnancy in sows altered their placental physiology and performance, modifying the phenotype of their offspring, changing weight at birth, perception to noxious stimuli, and behavioral/physiological responses facing common challenges that are present in commercial pig farming environment.

Placental and HPA axis responses were altered in lame sows and in their piglets depending on the degree of lameness. Cortisol concentration measured in hair samples that grew during the period of data collection, between the locomotion assessments, demonstrated that sows with moderate lameness showed higher levels than sows with severe lameness; antiinflamatory was used in severe lamed sows. Furthermore, the placenta collected from sows with moderate lameness appeared to be less efficient in inactivating cortisol to cortisone, indicating that, possibly, their piglets were exposed to higher concentrations of cortisol during gestation, than piglets from sows which did not show lameness. Hair cortisol in neonatal male piglets from sows with severe lameness tended to have higher levels that in hair samples collected from piglets which were the offspring from sows without lameness, suggesting that male piglets from lame sows were more exposed to cortisol during gestation. Finally, weaned piglets from severe lame sows showed higher increase in cortisol levels in response to transportation stress than offspring from sows without lameness during gestation.

It has been reported previously that males have more risk to be affected by maternal stress, including sex-specific changes in placental tissue (Mueller and Bale, 2008). To our knowledge, no previous study included the measurement of hair cortisol in neonatal piglets to indirectly assess the dam's stress determined by the exposure to intrauterine cortisol of the offspring. Measures of hair cortisol collected at birth have already been used in other species, including humans, to investigate fetal experiences related to acute or chronic maternal stress (Kapoor et al., 2016; Romero-Gonzalez et al., 2018), reporting direct effects of the mother's stress on hair cortisol levels in their offspring. Regarding the higher HPA axis responses to transport in the piglets born from lame sows, when compared to the offspring of not lame animals, this is very relevant as it is a common situation throughout pig production to transport animals. Our results are consistent with that reported in the literature, where stress during pregnancy increased the hormonal response linked to stress in their offspring, to other challenges (Bale, 2015; Kapoor and Matthews, 2005; Tatemoto et al., 2019). Although our results showed some inconsistencies when comparing the indicators measured in piglets, which were offspring from sows with different levels of severity in lameness assessment, it

is evident that lameness in pregnant sows alterer relevant systems in the offspring. The combined measures, indicating reduced placental protective efficacy that inactivates cortisol to cortisone, changes in hair cortisol concentration of sows and male piglets and increased the HPA axis responses of weaned piglets when subjected to transport, which are routine challenges in pig farms, all indicate a robust impact of lameness on the sow and on their offspring.

Continuing with the physiological findings, we observed that the severity of lameness in sows affected nociception pressure threshold in piglets at birth, at weaning and in response to castration. During the first 12 hours of life and before piglets' castration offspring from sows with moderate lameness were less tolerant and responded faster to noxious stimuli than piglets from sows with severe lameness. Surprisingly, nociception threshold after castration in piglets, offspring from sows without lameness was higher and with a slower response than piglets from sows with moderate lameness. It may indicate that the responses of the pain system to severe challenge herein represented by castration did not appear to be adaptive in the offspring of lame sows, as their responses were exacerbated, after castration. Weaned piglets from sows without lameness are less tolerant to noxious stimuli when compared with piglet's offspring from sows with severe lameness. The studies that evaluated the effects of maternal stress, represented by lameness, on noxious stimuli perception in the offspring supports previous research where social stress was used as a model of a challenging prenatal environment (Rutherford et al., 2009; Sandercock et al., 2011), Sandercock et al. (2011) reported that piglets from a prenatal stressful environment have basal nociceptive thereshold higher, than control animals, and Rutherford et al. (2009) observed that piglets from stressed sows were less tolerant to noxious stimuli after tail docking than control piglets. Our results support previous research indicating that common stressful challenges in sows housed in commercial farms, caused by pain or stressful events, have the potential to re-program the pain perception pathways in their offspring. This reprogramming could be a potential adaptation strategy to environmental adversity or it also may be maladaptation when facing situations that do not involve stress. It is important to mention that the function of nociceptive and painful system evolved to meet the demands of the environment (Sneddon et al., 2014) therefore, disturbances in these systems can put animal welfare at risk.

We propose that the nociception changes observed may play a role as a basis to partially explain our findings on agonistic behavior, because weaned piglets from sows without lameness had fewer skin lesions than piglets from severe lame sows. If piglets from sows without lameness are less tolerant to noxious stimuli, it is possible that due to their greater sensitivity they avoid getting involved in potentially harmful situations such as fights. In studies that evaluated the effects of the prenatal environment on agonistic behavior, it was demonstrated that the number of skin lesions in

offspring from pregnant sows subjected to challenging environments was modified (Bernardino et al., 2016; Provençal et al., 2015; Tatemoto et al., 2019). Thus, the intrauterine environment can influence postnatal social behavior in the offspring according to the mother's experiences.

Piglets from sows with different severity of lameness were lighter than piglets from sows without lameness and had a lower latency to explore a novel object in test conditions. An explanation of these results includes the probable insufficient nutritional intake in lame sows, kept in a competitive group situation, due to behavioral limitations that has been reported (Ala-Kurikka et al., 2016; Cornou et al., 2008). It is possible that the combined challenging social environment, which prevented them from competing equitably for resources with their penmates (Heinonen et al., 2013) may be signalled to the offspring.

We also observed that changes in glucocorticoids are related to lameness condition, both in the sows and in their offspring, suggesting that glucocorticoids may be involved in the mechanism that produced the phenotypic alterations in piglets, reported in this research. Prenatal exposure to stress and glucocorticoids has been associated with changes in the behavior, HPA axis response and development of fetus and neonates, restraining fetal growth or altering development of brain structures responsible for memory, learning or emotional process (Fowden et al., 2016; McEwen, 2007; Moisiadis and Matthews, 2014; Seckl, 2004).

The prevalence of lameness in sows found in the literature seems to be underestimated, at least when compared to our findings. The stressful and painful conditions that millions of sows housed may experience in commercial farms are worrysome and unacceptable from various dimensions. Lame sows are at high risk that several of the five freedoms are compromised (Heinonen et al., 2013) affecting in consequence their welfare.

We demonstrated that lameness in pregnant sows goes beyond the discomfort for the animal itsef, as it reprograms important systems that modulate the perception and relation with the environment of its offspring and with conspecifics, compromising welfare, with the potential to alter, intergenerationally, adaptative systems. The results pointed out in our studies are important at a scientific level. Other data exploration methodologies such as multivariate analysis or linear models can be considered to include the effects of some variables, e.g., sample size, female effect, or litter size. These approaches will be considered for the construction of future publications.

The way in which pigs are housed and managed must change in order to ensure animal welfare not only for sows, but also for their offspring, promoting sustainability in pig production globally.

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APPENDIX – I - Supplementary Information

The *in-utero* experience of piglets born from sows with lameness shapes their life trajectory

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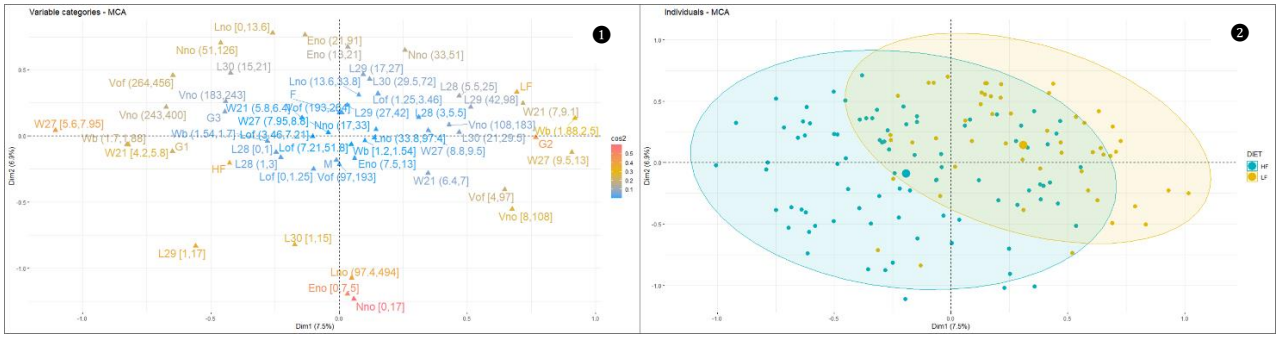
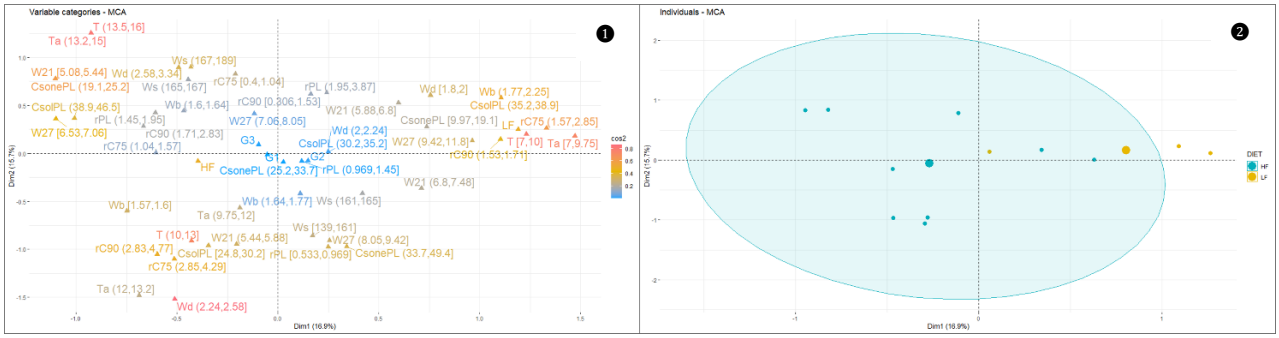
Supplementary Figure S1. Experimental design. Timeline of the experiment, from insemination in the sows to the fear tests in the offspring.

To identify the possible impact of the sow diet in the experiment, a multiple correspondence analysis was carried out and no effects were found between the previous experiment and our current findings. Based in the supplementary Fig. S2 - 1, it is possible to observe an association between the high fiber diet of the mothers and belonging to the group G1. Additionally, with fewer skin lesions at days 29 and 30, birth weight between 1.7-1.88 Kg, weight at day 21 between 4.2-5.8 kg and weight at day 27 between 5.6 -7.95 Kg. Likewise, feeding of sows with low fiber diets is associated with belonging to the G2, with heavier piglets at birth between 1.88-2.5 Kg and at weeks 21 (7-9.1 Kg) and 27 (8.8-13 Kg), greater number of skin lesions on days 28, 29 and 30 and more vocalizations in the novel object test. However, when the relationship is observed at the level of individuals (see supplementary Fig. S2 - 2 for details) a clear differentiation is not observed between the piglets of sows fed with high and low fiber, being noticeable an important overlap.



Supplementary Figure S2. Graphic result of the multiple correspondence analysis to show squared correlations between variables of piglets and the dimensions are used as coordinates identified. The description of each variables abbreviation in the graph **(1)** follows: high fiber (HF) and low fiber (LF) diet; piglets from sows without lameness (G1), from sows with moderate lameness (G2) and from sows with severe lameness (G3); weight at birth (Wb), at 21 (W21) and at 27 (W27) days of age; skin lesions at 28 (L28), 29 (L29) and 30 (L30) days of age; latency (Lof) and vocalizations (Vof) in the open field test; latency (Lno), exploration (Eno), near to the object (Nno) and vocalizations (Vno) in the novel object test. The graph **(2)** shows the relationship between piglet's variables – at the level of individuals, and sow dietary treatments – high fiber diet (HF) and low fiber diet (LF).

According to Supplementary Figure S3 - 1 , in which the squared correlations between sow variables are identified, it is possible to observe an association between feeding with low fiber and some variables such as number of total born piglets and live born piglets, cortisol ratio in the morning and late on days 75 and 90, and average weight on days 21 and 28. However, when the relationship is observed at the individual level (Supplementary Figure S3 - 2) there is no association between the type of diet and the group according to the degree of lameness.



Supplementary Figure S3. Graphic result of the multiple correspondence analysis to shows squared correlations between sow variables are identified. The description of each variable's abbreviation in the graph **(1)** follows: total born piglets (T) and total alive born piglets (Ta); average weight at birth (Wb), at 21 (W21) and at 27 days (W27) of age; average daily weight gain (Wd); sow weight (Ws); salivary cortisol ratio (morning and afternoon concentrations) at 75 (rC75) and 90 (rC90) days of pregnancy; placental cortisone (CsonePL) and cortisol (CsolPL) concentration; and ratio between placental cortisone and cortisol concentration (rPL). The graph **(2)** shows the relationship between sow's variables – at the level of individuals, and sow dietary treatments – high fiber diet (HF) and low fiber diet (LF).

Sows groups	Day	Time	N	Mean (pg/50µl)	s.d.	Mean ratio
G1	75	06:00	7	0.414	0.285	2.076
		18:00	6	0.243	0.124	

	90	06:00 18:00	7	0.510 0.213	0.312 0.089	2.871
G2	75	06:00 18:00	9	0.331 0.190	0.255 0.102	2.826
	90	06:00 18:00	10	0.472 0.204	0.226 0.055	2.452
G3	75	06:00 18:00	4 5	0.240 0.204	0.102 0.072	1.362
	90	06:00 18:00	4 5	0.554 0.654	0.248 1.078	2.381

Supplementary Table S1: Descriptive measures from saliva cortisol collected twice daily (at 06:00 and 18:00) from sows at 75 and 90 days of pregnancy. Number of animals is abbreviated as N. Standard deviation is abbreviate as s.d. Average of each variable is presented as *mean*.

Sows groups	Placental hormone	N	Mean	s.d.	p-value of t-test
G1	Cortisol Cortisone	6	3.537 22.954	0.684 14.842	0.024
G2	Cortisol Cortisone	8	3.900 26.537	0.570 15.183	0.004
G3	Cortisol Cortisone	5	3.983 16.489	1.489 8.785	0.032

Supplementary Table S2: Descriptive measures of placental cortisol/cortisone concentrations and results of intraspecific comparisons in the sows groups G1, G2 and G3.

Measure	**Sow group G1 (N=7)**		**Sow group G2 (N=10)**		**Sow group G3 (N=5)**	
	Mean	s.d.	Mean	s.d.	Mean	s.d.
Average daily litter weight gain (Kg)	2.295	0.248	2.402	0.501	2.647	0.538
Average daily weight gain per animal (Kg)	0.224	0.047	0.238	0.043	0.242	0.036
Gestation length (days)	115	0.577	114	1.886	114.2	1.095
Number of crushed piglets	0.571	0.787	1.3	1.059	0.6	0.548
Total number of piglets born	11.428	2.878	12.8	2.300	12.8	2.168
Total number of piglets born alive at farrowing	11.143	2.609	11.9	2.331	12	1.871
Total litter weight at 21 days of age (Kg)	65.6	9.958	65.24	16.010	67.76	7.516
Total litter weight at 27 days of age (Kg)	83.514	8.206	87.86	16.461	90.56	17.032
Total litter weight at farrowing (Kg)	17.06	2.459	18.827	2.690	19.35	2.505

Supplementary Table S3: Descriptive measures of performance data from the sow groups G1, G2 and G3.

Measure	Age (days)	Piglets group G1			Piglets group G2			Piglets group G3		
		N	Mean	s.d.	N	Mean	s.d.	N	Mean	s.d.
Weight (Kg)	0	74	1.61	0.24	113	1.67	0.36	57	1.70	0.25
	21		6.21	1.28	105	6.21	1.14		5.94	1.12
	27	73	8.00	1.54	104	8.45	1.86		7.95	1.68
Number of skin lesions	28	52	2.69	2.55	66	5.08	4.65	38	2.95	3.59
	29		24.79	18.03		32.49	16.10		30.58	19.96
	30		22.42	13.02		23.45	11.84		23.24	14.05

Supplementary Table S4: Descriptive measures of weight at birth at 21, and 27 days of age; and skin lesions at 28, 29 and 30 days of age in piglets from groups G1, G2 and G3.

Measure	Test	Piglets group G1 (N=47)		Piglets group G2 (N=62)		Piglets group G3 (N=33)	
		Mean	s.d.	Mean	s.d.	Mean	s.d.
Latency●	Open field	6.53	10.380	5.658	6.993	5.484	6.050
Central Quadrants●		29.979	10.109	29.871	12.609	26.909	7.341
Lateral Quadrants●		56.915	24.437	60.193	30.040	57.515	18.762
Activity●		87.319	30.029	89.097	38.451	84.667	24.729
Vocalizations▲		219.126	98.028	170.790	102.573	183	99.936
Latency●	Novel object*	83.614	108.047	71.659	84.043	60.473	66.511
Exploration●		17.191	14.979	14.164	9.510	18.667	18.261
Near to the object●		41.532	28.993	31.836	21.143	37.818	27.617
Vocalizations▲		221.510	97.801	160.541	87.347	177.848	81.221

Supplementary Table S5: Descriptive measures from the variables analyzed during open field and novel object test. ● The unit of these measures is seconds. ▲ The unit of these measures is in frequency; it means the number of all types of vocalizations realized during 300 seconds for each test. *The number of individuals in the group G2 were 61.