Physiology and miRNA expression in confined sows with different pupillary light reflex

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ABSTRACT

Long-term physical restriction may lead to affective and physiological disorders in sows; pupillary light reflex (PLR) characteristics might be a good indicator to diagnose these symptoms. Accordingly, the physiological and psychological states of sows with different PLR characteristics were investigated in this study. Gestating sows of three parities (parity 0, 2, and 5) were divided into strong reflex (SR) and weak reflex (WR) groups, according to a PLR test. In each group, miRNA expression and serum physiological indices were detected. Compared with the SR group, WR group showed lower 5-hydroxytryptamine levels and higher cortisol, interleukin-6, and beta-endorphin levels \((p<.05)\) in all parities; higher immunoglobulin A and tumour necrosis factor-\(\alpha\) levels in the parity 0 and 2 sows \((p<.05)\); and higher miR-335, miR-16, and miR-1202 expression \((p<.05)\) in higher miR-132, miR-504, miR-34a, and miR-30e expression in the parity 2 and 5 sows. Compared with the parity 0 sows, parity 5 sows showed higher cortisol, interleukin-6, tumour necrosis factor-\(\alpha\), and beta-endorphin levels; higher miR-504 and miR-34a expression \((p<.05)\); and lower miR-335, miR-16, miR-134, and miR-1202 expression in both groups. Thus, psychophysiological state differs among sows with different PLR characteristics, and, that of sows with weak PLR characteristics, is consistent with depression disorder, and as parity increases, these psychophysiological differences widen.

HIGHLIGHTS

- PLR characteristics could reflect different physiological states including neurophysiological and immunological states in sows.
- The miRNA molecular markers and serum physiological indices of sows with weak pupillary light responses were consistent with depression disorder.
- Long-term physical restriction might worsen physiological and psychological disorders in sows.

Introduction

With a growing interest in better animal welfare, crates have been gradually eliminated in modern pig farming. However, many sows (especially lactating sows) are still confined in crates to improve space utilisation in intensive pig farms. The narrow and barren environment of crates may cause psychological frustration (Fraser et al. 2013), depressed psychological responses (Yin et al. 2019), and even physiological disorders in sows. These psychophysiological abnormalities of animals are often evaluated by monitoring their behaviour (such as stereotypical behaviour) or physiological discomfort. However, these indicators cannot directly and accurately reflect the psychological state of sows. Pupillary light reflex (PLR) characteristics are considered sensitive markers of psychiatric status (Bao et al. 2013) and might be a better way to monitor psychological status of animals.

PLR is controlled by the sympathetic and parasympathetic nerves of the autonomic nervous system (Bar et al. 2004). Parasympathetic nerves play an inhibitory role in regulating PLR, while sympathetic nerves play an excitatory role. Certain diseases, drugs, emotions, and other factors affect the activity of the autonomic nervous system, affecting the heart rate, blood pressure and PLR indices (Laeng et al. 2012). Abnormal
PLR characteristics indicate a disorder of the autonomic nervous system, which might be caused by an individual psychological or neurological disorder. Patients with neurological disorders, including traumatic neurological disorders, anxiety disorders (Bakes et al. 1990), specific phobias (Kojima et al. 2004), and depression (Laeng et al. 2012; Sokolski et al. 2000; Siegle et al. 2001), exhibit specific PLR characteristics. Bao et al. found that stall-housed sows showed more prolonged PLR latencies and longer PLR durations than group-housed sows (Bar et al. 2004). These symptoms indicated the poor psychological state of sows, and the chronic stress caused by their restricted activities played an important role in these results.

Animals in a poor psychological state have also been found to exhibit other physiological disorders, such as immune response disorders (Laeng et al. 2012; Singh and Chaudhuri 2014); inflammatory response disorders (Bergink et al. 2014; Muller 2018); and the abnormal release of endogenous opioid peptides (Lutz and Kieffer 2013), neurotransmitters 5-hydroxytryptamine (5-HT), dopamine (DA), and stress hormones (Keay et al. 2006; Joels et al. 2007; Kim and Haller 2007). These symptoms resulted from long-term confinement in a narrow and barren environment, and could also predict the psychological distress of the animal. Studies have shown that immune inflammatory responses can induce schizophrenia and bipolar disorder (Lutz and Kieffer 2013; Muller 2018). In addition, increased levels of proinflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), have been found in patients with schizophrenia (Bergink et al. 2014; Fillman et al. 2014) and depression (Hussein et al. 2015; Filho et al. 2018; Mao et al. 2018). The immunoglobulin A (IgA) concentration was significantly higher in the serum of patients with depression than in that of other people (Gold et al. 2012; Maes et al. 2012). Major depressive disorder (MDD) has also been found to be associated with changes in endogenous opioid peptides (Keay et al. 2006). Emotional abnormalities caused by environmental stress, such as depression and anxiety, have been related to abnormal levels of neurotransmitters such as DA and 5-HT (Xiao et al. 2017; Li et al. 2020). 5-HT levels were also reduced in a model of depression induced by social isolation stress in adult mice (Wang et al. 2019). These studies provide a basis for revealing the physiological value of different PLR characteristics in sows.

Specific PLR characteristics can be observed in sows following psychological distress and changes of physiological state in sows reflect various psychological disorders. It is therefore necessary to accurately identify these states in sows. MiRNA is a short, non-coding RNA that regulates brain development and dendritic spine morphology. They play a role in the pathogenesis of many psychiatric disorders, including schizophrenia (Jian et al. 2017; Xu et al. 2019) and depression (Zurawek et al. 2016; Tavakolizadeh et al. 2018); thus, they have been used as an important clinical diagnostic biomarker (Smigielski et al. 2020). MiR-335 (Li et al. 2015), miR-1202 (Fiori et al. 2017; Lopez et al. 2017), and miR-16 (Song et al. 2015) are down-regulated in patients with depression. Brain-derived neurotrophic factor (BDNF) expression and synaptic plasticity are regulated by miR-134 and miR-132 (Shen et al. 2019), and the former is involved in nervous system function and functional plasticity during depression (Castren and Rantamaki 2010). In patients with schizophrenia, plasma miR-30e (Sun et al. 2015a) and brain tissue miR-34a expression were significantly increased and miR-30e expression was significantly reduced after treatment with antipsychotic drugs (Sun et al. 2015b). Rats under chronic stress were found to have high miR-504 expression in their nucleus accumbens, and their miR-504 levels were positively correlated with the severity of depression-like behaviour after stress (Zhang et al. 2013). In summary, miRNA may be a good biomarker for the diagnosis and treatment of multiple affective disorders. Therefore, detecting the expression levels of miRNA markers in sows with different PLR characteristics could identify the psychological state of sows.

In this study, we focus on the variations in the PLR characteristics of sows (same parity) continuously confined for similar periods and use this variation as the basis for distinguishing their affective states. This could help create a sow psychological states evaluation system from a new perspective. The affective disorders of confined sows with different PLR characteristics were diagnosed by determining the levels of immunoregulatory factors, endogenous peptides, neurophysiological indicators, and the relative expression of some serum miRNAs to verify if the psychological state of sows can be determined by PLR characteristics. This study could elucidate whether there is differentiation among the affective states of sows affected by confining environments, and promote sow welfare research from qualitative to quantitative methods.

Materials and methods

**Animals, treatments, management, and feeding**

This experiment was approved by the Animal Ethics Committee of the College of Animal Science and
Veterinary Medicine, Heilongjiang Bayi Agricultural University and conducted on a commercial pig farm (Heilongjiang Damuren Animal Husbandry Co, Ltd.) located in Heilongjiang Province in northeast China. A total of 48 Yorkshire/C2 Landrace sows with different parity (parity 0, 2, and 5; 16 sows each) were selected. All sows had been pregnant for 42–95 d and were housed in identical gestation crates (2.15 × 0.65 × 0.96 m, length × width × height). The sows were selected from a large group through strict healthy cheques and had received standard immunisation procedures. The physiological status of each sow was as consistent as possible. The housing temperature and humidity were maintained at 18–20 °C and 60–62%. All sows were fed at 06:00 am (3.0 kg each) and were provided ad libitum access to water. The nutritional standards of the formula feed for pregnant sows are shown in Table 1. The gestation crates were cleaned at 06:30 am; other management practices were conducted following the uniform standards for commercial pig farms.

**Pupillary light reflex test**

PLR characteristics in each group were assessed using a hand-held pupillometer Neur Optics PLR-200 (NeurOptics, Laguna Hills, CA). The PLR test was conducted from 08:00 to 09:00 am each day under an illuminance intensity of <250 lx. Each eye of every sow was measured three times for the following parameters at an interval of >5 min: maximum pupil diameter, minimum pupil diameter, contraction rate of pupil, latency of the pupillary response, average contraction velocity, maximum contraction velocity, average dilation velocity, and time for 75% recovery of the initial pupil diameter. All indicators of PLR are defined in Table 2.

**Sample collection**

The blood of all sows was sampled from the anterior vena cava before feeding. The sampling time of each sow did not exceed 5 min. All blood sample were collected in 5-mL anticoagulant or 5-mL procoagulant tubes. The samples in procoagulant tubes were centrifuged (1200 g; 10 min; Cence, Changsha HN, China) after allowing them to stand for 30 min. The serum was collected in enzyme-free EP tubes. All blood samples were stored in a liquid nitrogen container until the assay.

**Serum indicator measurements**

The concentrations of cortisol (COR), 5-HT, DA, IgA, TNF-α, and beta-endorphin (β-EP) were determined using commercial enzyme-linked immunosorbent assay kits (MLBIO Biotechnology, Shanghai, China).

**miRNA expression analysis**

The expression of miR-335, miR-1202, miR-16, miR-134, miR-132, miR-504, miR-30e, and miR-34a in each group was detected by quantitative real-time polymerase chain reaction (qPCR). Total RNA was extracted from whole blood using a TRizol kit and dissolved in diethyl pyro carbonate (Sigma, St. Louis, MO). The concentration and purity of the RNA were determined using an ultra-micro spectrophotometer K5600 (KAIAO Technology, Beijing, China). Reverse transcription (RT)-PCR was performed according to the manufacturer instructions for the Prime Script RT Reagent Kit with gDNA Eraser (Takara Bio, Dalian, LN, China). RT-PCR was conducted in a PCR machine, the Cycler™ II (Select Bioproducts, New York City, NY); the program was set as follows: 37 °C, 60 min → 85 °C, 5 min → 4 °C, ∞. After the reaction was completed, the

| Table 1. Nutritional standards of formula feed for pregnant sows (%). |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| CP | CF | CA | Ca | TP | NaCl | Lys |
| --- | --- | --- | --- | --- | --- | --- |
| ≥15.0% | ≤10.0% | ≤10.0% | 0.5–1.3 | ≥0.4 | 0.3–1.0 | ≥0.5 |

Diet-nutrient concentration was calculated from the nutrient composition of raw materials in the NRC (2012) (National Research Council, 2012). CP: crude protein; CF: crude fat; CA: crude ash; Ca: calcium; TP: total phosphorus; NaCl: sodium chloride; Lys: lysine.

| Table 2. Definitions of pupillary light reflex indicators. |
|-----------------------------|-------------|
| Indicator | Definition |
| Maximum pupil diameter | The diameter of the pupil before light stimulation |
| Minimum pupil diameter | The smallest pupil diameter when light stimulated |
| Contraction rate of pupil | (Maximum pupil diameter − Minimum pupil diameter)/Maximum pupil diameter |
| Latency of pupillary response | The response time of the pupil’s reaction to light stimulation |
| Average contraction velocity | The average contraction velocity of the pupil after light stimulation |
| Maximum contraction velocity | The maximum contraction velocity of the pupil after light stimulation |
| Average dilation velocity | The average velocity of the pupil from minimum pupil diameter to initial diameter |
| Time for 75% recovery of the initial pupil diameter | The duration of recovery from minimum pupil diameter to 75% of the initial diameter |
obtained cDNA solution was stored at −20°C. For conducting qPCR, three replicate wells per sample were prepared. The internal reference for the primers (Sciencia Biotech, Harbin, HL, China) (Table 3) was U6. qPCR was carried out using a Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA), according to the following reaction conditions: 95°C/C14, 60°C, 30 s → 95°C, 5 s → 4°C, ∞, repeated for 40 cycles. The Ct value of each sample was recorded, and the relative miRNA expression was calculated using the 2^−ΔΔCt method.

**Statistical analysis**

IBM SPSS statistics 19.0 (IBM, Armonk, NY, USA) was used to analyse the data. The PLR data from the left and right eye of each sow were analysed by Fisher’s exact test. The data are expressed as means ± standard deviations. As shown in Table 4, PLR data from the left and right eyes of the sows showed no significant differences (p > 0.05, Table 4). Therefore, the PLR data from left and right eyes were regarded as repeated data.

All PLR indicators were measured using the Kaiser-Meyer-Olkin test. The results of the Bartlett’s test showed that the PLR data could be used for factor analysis. The principal component load matrix (U) was calculated using the formula \( U_i = A_i / \sqrt{\lambda_i} \) (U: principal component load matrix; A: component matrix; \( \lambda_i \): eigenvalue). The Analyse—Descriptive Statistics—Descriptives procedure was followed to standardise the variables, and the comprehensive evaluation score (Y) was calculated using the Transform—Compute Variables procedure. The results showed that the variance contribution rates of the principal components, \( Y_1 \) and \( Y_2 \), were 66.413% and 17.527% (\( Y_1 = 0.243X_1 + 0.380X_2 + 0.413X_3 + 0.374X_4 + 0.407X_5 + 0.386X_6 \)

\[ \begin{align*}
Y_1 &= 0.243X_1 + 0.380X_2 + 0.413X_3 + 0.374X_4 + 0.407X_5 + 0.386X_6 \\
Y_2 &= 0.653X_1 + 0.381X_2 + 0.050X_3 + 0.068X_4 - 0.177X_5 - 0.332X_6 + 0.529X_7 + 0.023X_8
\end{align*} \]

where \( W_1 \) and \( W_2 \) correspond to the cumulative variance contribution rates. According to the results of the Kolmogorov–Smirnov test, data on physiological and miRNA expression were found to be normally distributed and were therefore analysed using the independent samples t-test and Fisher’s exact test. The results were expressed as means ± standard deviations.

**Results**

**Neurophysiological indicators among confined sows with different parities and different PLR**

Compared with the serum COR and 5-HT concentrations in SR group sows of all parity, the COR concentration was significantly higher in the WR group (\( p < 0.05 \), Figure 1(A1)), and the serum 5-HT concentration was significantly lower (\( p < 0.05 \), Figure 1(B1)). Compared with the serum DA concentrations in the SR group of gilts (parity 0), the concentration in the WR group was significantly lower (\( p < 0.05 \), Figure 1(C1)).

When comparing the serum concentrations of the neurophysiological indicators between sows of different parities with the same PLR characteristics, the serum 5-HT and DA concentrations were significantly lower in in parity 2 or 5 than in parity 0 both the WR and SR groups (\( p < 0.05 \), Figure 1(B2), C2), whereas the serum COR concentration was significantly higher in

| Table 3. Primer sequence. |
|---------------------------|
| **Primer name** | **Sequence** | **Size** |
| miR-335-Pig-F | CGGCCTAAGGACATTAACGAAAATG | 26 |
| miR-1202-Human-F | GTCAGCGTGGACGTTG | 16 |
| miR-16-Pig-F | AATACGAGCATTGGAATGCGG | 23 |
| miR-134-Human-F | TTGCGACTCTGAGGAGCAGG | 20 |
| miR-132-Pig-F | TAACAGTGCTACGATGGTACGG | 22 |
| miR-504-Human-3P | CGACAGCCGGGTGCTGCACTTATCTC | 23 |
| miR-504-Human-5P | TATATACGAGGTGCGAGCCAGGG | 23 |
| miR-304-Human-3P | CGGCTTTTGCCGATGGGTTTACGCTC | 25 |
| miR-304-Human-5P | CGCCGTGAACATATCCGTGACTGGAAG | 26 |
| miR-334-Human-SP | CGCCATGCTGTGTTTACGCTTCTC | 23 |
| miR-34a-Human-3P | CGGAATCGCAATGATATAGCG | 24 |

| Table 4. PLR indicators in left and right eyes of sows. |
|---------------------------|
| **PLR indicators** | **Left eye** | **Right eye** | **p-value** |
| Maximum pupil diameter (mm) | 9.82 ± 0.036 | 9.78 ± 0.038 | .44 |
| Minimum pupil diameter (mm) | 8.83 ± 0.056 | 8.85 ± 0.061 | .81 |
| Contraction rate of pupil (%) | −0.1 ± 0.004 | −0.1 ± 0.005 | .47 |
| Latency of pupillary response (s) | 0.54 ± 0.018 | 0.58 ± 0.024 | .12 |
| Average contraction velocity (mm/s) | −1.41 ± 0.068 | −1.44 ± 0.068 | .76 |
| Maximum contraction velocity (mm/s) | −3.22 ± 0.111 | −3.05 ± 0.104 | .27 |
| Average dilation velocity (mm/s) | 0.41 ± 0.03 | 0.53 ± 0.045 | .06 |
| Time for 75% recovery of the initial pupil diameter (s) | 1.51 ± 0.153 | 1.46 ± 0.132 | .81 |

Pupil contraction is opposite to the direction of dilation, “−” indicate the direction of contraction.
parity 2 or 5 than in parity 0 ($p < .05$, Figure 1(B2)). Parity 5 sows in the WR group also had higher COR serum concentrations than parity 2 sows ($p < .05$, Figure 1(B2)).

**Immunoregulatory factors among confined sows with different parities and PLR characteristics**

Compared with the serum IgA, TNF-α, and IL-6 concentrations in SR-group sows of the same parity, those
concentrations in the WR group were significantly higher in the parity 0 and 5 ($p < .05$, Figure 2(A1–C1)). And the serum IL-6 c concentrations of the WR group sows were also significantly higher in the parity 2 ($p < .05$, Figure 2(B1)).

When comparing the serum concentrations of immunoregulatory factors among sows of different parities with the same PLR characteristics, these concentrations generally increased with parity. The serum IL-6 and TNF-α concentrations in parity 5 sows were significantly higher than those in gilts in both the WR and SR groups ($p < .05$, Figure 2(B2, C2)). The serum TNF-α concentrations in parity 5 was significantly higher than those in parity 2 sows in the SR groups ($p < .05$, Figure 2(B2)). However, IgA concentration was not significantly different among different-parity sows ($p > .05$, Figure 2(A2)).

**miRNA expression among confined sows with different parities and different PLR characteristics**

MiR-335, miR-16, miR-1202, miR-134 were high expression in the SR group of all parity, while miR-132, miR-504, miR-30e, miR-34a were high expression in the WR group of all parity. And, the relative miRNA expression differences between the WR and SR groups became larger with the increase of parity. However, the differences between the WR and the SR group were significant only in parity 2 or 5 sows (Figure 4).

In parity 2 sows, the relative expression of miR-335, miR-16, and miR-1202 was significantly lower in the WR group than in the SR group ($p < .05$, Figure 4(A,B,D)). However, the relative expression of miR-132, miR-504, miR-30e ($p < .05$, Figure 4(E,F,H)), and miR-34a ($p < .01$, Figure 4(G)) was higher in the WR group.

In parity 5 sows, the relative expression of miR-335, miR-16, miR-134, and miR-1202 was extremely significantly lower in the WR group than in the SR group ($p < .01$, Figure 4(A–D)), whereas the relative expression of miR-132, miR-504, miR-30e ($p < .01$, Figure 4(E,F,G)), and miR-34a ($p < .05$, Figure 4(H)) was higher in the WR group.

When comparing the relative miRNA expression among sows with different parities but the same PLR characteristics, the relative expression of miR-335, miR-16, miR-134, and miR-1202 of gilts were significantly higher than those of parity 2 or 5 sows in both the WR and SR groups ($p < .05$, Figure 5(A–D)), whereas the relative expression of miR-504 and miR-34a of gilts was lower than those of parity 2 or 5 sows in the WR groups ($p < .05$, Figure 5(F,H)).

**β-EP among confined sows with different parities and different PLR characteristics**

Serum β-EP concentrations in WR sows were significantly higher than those in SR sows of the same parity, for every parity ($p < .05$, Figure 3(A1)).

When comparing β-EP concentrations among sows of different parities with the same PLR characteristics, the serum β-EP in parity 5 sows was significantly higher than those in parity 0 and 2 sows in both the WR and SR groups ($p < .05$, Figure 1(G)), whereas the serum β-EP in parity 2 sows was significantly higher than that of gilts in both the WR and SR groups ($p < .05$, Figure 3(A2)).

**Figure 3.** β-EP indicator levels in confined sows with different parity and different pupillary light reflex (PLR) characteristics, including β-EP Among Confined Sows with Different PLR (A1), β-EP Among Confined Sows with Different Parities (A2). The parities include parity 0 (P0), parity 2 (P2), and parity 5 (P5). The PLR characteristics include weak reflex (WR) and strong reflex (SR). Differences in the (A1, A2) serum beta-endorphin (β-EP) level among different parity and different PLR groups in confined sows. The standard deviation is expressed by error bars, *$p < .05$. 


The relative expression of miR-134, miR-504, and miR-34a showed no significant differences between parity 2 and 5 sows with the same PLR characteristics, in either the WR or SR groups \( (p > .05, \text{Figure 5(C,F,H)}) \). The relative expression of miR-16, miR-1202, and miR-30e of the WR group was significantly lower in parity 5 sows than in parity 2 sows \( (p < .05, \text{Figure 5(B,D,G)}) \). Moreover, the relative expression of miR-1202 in parity 5 sows was significantly lower than that of parity 2 sows in the SR group \( (p < .05, \text{Figure 5(D)}) \). The relative expression of miR-132 and miR-30e of parity 5 sows was significantly higher than that of parity 0 or 2 sows in the SR group \( (p < .05, \text{Figure 5(E,G)}) \).

**Figure 4.** Relative miRNA expression in sows with different PLR characteristics but the same parity. (A) The relative expression of miR-335; (B) miR-16; (C) miR-134; (D) miR-1202; (E) miR-132; (F) miR-504; (G) miR-30e; and (H) miR-34a. The standard deviation is expressed by error bars, \(* p < .05; ** p < .01.\)

**Discussion**

**Neurophysiological indicators of confined sows with different PLR characteristics**

There were differences in serum 5-HT, COR, and DA concentrations in confined sows with different PLR characteristics, indicating that these sows had different neurophysiological states. Compared with the SR group, the WR group had lower 5-HT and DA serum concentrations and higher COR serum concentrations. As a suppressive neurotransmitter, 5-HT can regulate mood. Therefore, patients with depression usually report a gloomy and dejected mood, accompanied by
low levels of 5-HT. In a rat model of depression, blood 5-HT concentration was effectively relieved after treatment with antidepressants (Jang et al. 2019). The serum concentrations of DA were found to have the same trends as those of 5-HT in this study. Zhu et al. demonstrated that both maternal deprivation and chronic mild stress could induce depression-like behaviour in rats via the dopaminergic system (Zhu et al. 2011). Li et al. (2020) found that oxytocin exerts antidepressant-like effects by improving DA levels in the medial prefrontal cortex. This work shows that the low level of 5-HT is consistent with depression in the WR group sows. In addition, Maeda et al. (2019) found that patients with depression and Alzheimer’s disease show higher COR levels, suggesting that neurodegenerative lesions, mainly causing cognitive dysfunction, may occur under stress. Collectively, this work has confirmed that low levels of 5-HT and DA in the blood are typical symptoms of depression and that chronic stress owing to a restrictive environment is the main reason for depression in sows. The results of this study indicate that sows with different PLR characteristics ...
suffer from different mood disorders. In addition, the sows in the WR group exhibited the typical neurophysiological symptoms of depression. For sows with different parities, the serum concentrations of 5-HT and DA reduced with increasing parity and those of COR showed an increasing trend. These indicators in parity 0 sows were significantly different from those in parity 2 and 5 sows. The narrow and barren environment in the crates restricted the normal behaviour and normal psychological expression of the sows, which is a source of anxiety in captive animals. This leads to increased activity of the hypothalamic–pituitary–adrenal axis and the release of glucocorticoids (Morris et al. 2012). In this study, serum COR concentration increased with restriction duration, implying that long-term high levels of COR in sows resulting from chronic stress could affect the body’s emotional state (Joels et al. 2007). Studies have shown that environmental stress leads to an increased risk of depression and anxiety (Pace et al. 2006), and animals reared in a stressful environment are more likely to show depression-like behaviour in adulthood. In this study, parity 2 and 5 sows were restricted for a longer time, and their 5-HT and DA serum concentrations were significantly lower than those of gilts. These sows could be considered to have depression. In summary, sows in the WR group had higher levels of COR than those in the SR group, which might indicate the dysfunction of hypothalamic–pituitary–adrenal (HPA) axes. The changes of 5-HT and DA levels might be the manifestation of their psychological disorder. In addition, as parity increases, these symptoms worsen.

**Immunoregulatory factors in confined sows with different PLR characteristics**

The body’s immune functioning is influenced by the environment. Meanwhile, immune function also affects mood and the body’s psychological condition (Herrmann-Lingen et al. 2019). Abnormal immune and inflammatory reactions could affect the brain, resulting in emotional dysfunction. These reactions are also involved in the development of various mental diseases, including schizophrenia and bipolar disorder (Lutz and Kieffer 2013; Muller 2018). Zhang et al. (2017) found that IL-6 mRNA was highly expressed in the hippocampus, frontal cortex, and hypothalamus of confined sows, and this was closely associated with the psychological stress of the sows. Therefore, chronic inflammation and immune disorders might be symptoms of psychological abnormalities in sows. Our results showed that the TNF-α, IgA, and IL-6 serum concentrations of the WR group were significantly higher in parity 0 and 5. The dysfunction of the immune system and HPA axis dysregulation are common in chronic stress-induced depression (McEwen 1999). Dysfunction of the 5-HT axis can also cause dysfunctions of the HPA axis, which in turn activates the cytokine interleukin (IL)-1β signalling pathway, while proinflammatory cytokines could further induce emotional disorders such as depression (Pineda et al. 2010; Leonard 2014). Studies using a mouse depression model have shown that the mice not only exhibit major pathological symptoms related to depression but also have elevated IL-6 and TNF-α levels in their blood (Ghosh et al. 2020; Alshammari et al. 2020). Simeonova et al. (2020) detected higher blood levels IgA in patients with MDD. Day et al. also found that German shepherds with depression had higher serum IgA levels (Day et al. 1985). These findings indicate that the changes in serum immunomodulatory factors in the WR group sows are consistent with HPA axis dysregulation, which might also contribute to the psychological abnormalities of sows.

In addition, the IL-6 and TNF-α concentrations of gilts were significantly lower than those of parity 5 sows in both the WR and SR groups. This might be the result of long-term chronic stress owing to restricted activities. Environmental stress causes the level of various proinflammatory markers to increase in sows. This was also found in patients with MDD and schizophrenia (Filho et al. 2018), and an increasing number of studies have confirmed that chronic psychological stress mediates a variety of affective disorders, including depression and anxiety in animals (Bao et al. 2013; Muller 2018; Maeda et al. 2019). Studies have found that long-term stress also increases the risk of MDD and the level of inflammatory markers (such as IL-6) (Tannous et al. 2020). Elevated levels of inflammatory cytokines could cause neuroinflammation or damage brain functioning, which is an important factor leading to various affective disorders (Maeng and Hong 2019).

**β-EP in confined sows with different PLR characteristics**

β-EP and endomorphin-2 function as both hormones and neuromodulators (Janecki et al. 2004). Emotional state is significantly influenced by β-EP (Smith et al. 1990), and research shows that serum β-EP levels in patients with depression are significantly increased (Millan et al. 1981). β-EP serum concentrations in the WR group were significantly higher than those in the
SR group in every parity and the β-EP levels in parity 2 and 5 sows were significantly higher than those of gilts in both the WR and SR groups. These results indicate that the sows in the WR group were depressed and that these symptoms will worsen with increasing restriction time.

miRNA expression in confined sows with different PLR characteristics

MiRNAs target and regulate the expression of various genes and are involved in the development, proliferation, and differentiation of neurons. MiRNAs have been shown to be related to the pathogenesis of many psychiatric disorders, including schizophrenia (Ambros 2004). Therefore, some miRNAs can be used as biomarkers for psychological obstacles. They were effective for evaluating psychological disorders in sows with different PLR characteristics in our study. The downregulation of miR-335 (Fiori et al. 2017), miR-1202 (Song et al. 2015), and miR-16 (Shen et al. 2019) might be related to the depression of the sows. Among these miRNAs, miR-335 was down-regulated in the prefrontal cortex (Smalheiser et al. 2012) and blood (Lopez et al. 2017) of patients with depression and was up-regulated after anti-depressant treatment. MiR-1202 is related to the pathophysiology of depression, which can be improved by the regulation of the target gene of this miRNA, GRMA. Based on this, previous studies have reported that miR-1202 is significantly reduced in the brain of patients with MDD (Lopez et al. 2014) and that it increases after treatment (Song et al. 2015; Lopez et al. 2017). MiR-16 might participate in the physio-pathological process of MDD by regulating the expression of serotonin transporter genes (Shen et al. 2019). Low levels of miR-16 have been observed in animal depression models of chronic mild stress, which may prevent the animals from resisting stress (Smigielski et al. 2020). There was a correlation found between miR-134 and effective mood stabilisers (Rong et al. 2011); downregulation of the miR-134 pathway was found in a chronic stress-induced depression model, which may be related to its effect of stabilising emotions (Castren and Rantanamaki 2010). The relative expressions of miR-335, miR-16, miR-134, and miR-1202 in the WR group were lower, indicating that the WR group might be in a depressed psychological state, and chronic stress seems to be the main reason for these results.

The relative expression of miR-132, miR-504, miR-30e, and miR-34a in the WR group was higher than that in the SR group, which also indicates that the WR group might be in a depressed psychological state. The high levels of miR-30e and miR-34a were also consistent with schizophrenia in the WR group sows, but this has not been previously reported in sows. MiR-132 and BDNF have a mutually regulating effect that plays an important role in maintaining neuronal activity and regulating neuronal morphology. It is closely related to the occurrence of various mental diseases. MiR-132 expression was found to be up-regulated in patients with depression (Li et al. 2013). MiR-504 plays an important role in the development of the rat neurotransmission system, which also affects brain functioning and emotional state; high expression of miR-50 was observed in rat depression models (Huang and Li 2009). In addition, the miR-34 family (including miR-34a) is associated with environmental stress, and strongly induced by the TP53 gene, which is a key cell cycle control gene and can also regulate the expression of emotional molecules (Haramati et al. 2011). The expression characteristics of miR-30e is positively correlated with depression and the onset of symptoms. Studies have shown that the expression of miR-30e is increased in the peripheral blood samples and post-mortem brain tissue samples of patients with depression (Gorinski et al. 2019). These findings indicate that high expression of miR-30e is also an important factor in depression. The latest research shows that miR-34a is involved in the pathological process of Alzheimer’s disease, schizophrenia, depression, and other mental diseases (Cogswell et al. 2008).

Same-parity sows with different PLR characteristics showed different relative expression levels of miRNAs. Although relative miRNA expressions did not significantly differ between the WR and SR groups in gilts, the gap is widening noticeably with the increasing of parities. The relative expression of miR-335, miR-16, miR-134, and miR-1202 down-regulated with the increase of parity, and the relative expression of those miRNA in the parity 5 sows was significantly lower than that of gilts in both the WR and SR groups. While the relative expression of miR-132, miR-504, miR-30e, and miR-34a up-regulated with the increase of parity, and the relative expression of miR-504, miR-34a in the parity 5 sows was significantly higher than that of the gilts in both the WR and SR groups. These results indicate that short-term physical activity restrictions could cause physiological abnormalities in sows, including immune-related and neurophysiological abnormalities, while long-term confinement can induce psychological abnormality in sows over time. In addition, the relative expression of miRNA in the serum of the WR group sows were consistent with
depression and that this depression-like physiological state became more obvious as parity increased.

The evidence presented in this study indicates that individualised differences in the effects of adverse environments on emotions of sows can be reflected by PLR. However, there are limitations in the quantitative relationship between PLR characteristics and affective disorders, and the mechanisms that causes sows to manifest specific PLR characteristics are still unclear.

Conclusions

Differences in the levels of immunoregulatory factors, endogenous peptides, and neurophysiological indicators and the relative expression of some miRNAs among confined sows with different PLR characteristics show that there were different psychophysiological states among these sows. Short-term physical activity restriction could cause physiological abnormalities in sows, including immune-related and neurophysiological abnormalities. As parity increases, recurrent periods of restriction can induce the psychological abnormality in sows over time. The physiological and psychological states of sows in the WR group were consistent with depression disorder, and the chronic stress caused by restricted activities was an important factor that led to this condition. Our results could help create a sow welfare evaluation system and promote sow welfare research from qualitative to a quantitative process. Further, to improve the evaluation criteria of PLR, a correlation analysis experiment will be performed using a large amount of data in our next study.

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Ethical approval

This study was approved by the Animal Ethics Committee of the Animal Science and Veterinary College of Heilongjiang Bayi Agricultural University.

Disclosure statement

No potential conflicts of interest were reported by the author(s).

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