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USE OF PUPILLOMETRY IN THE DIAGNOSIS OF STRESS IN PIGLETS TRANSPORTED IN A TROPICAL CLIMATE

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KEYWORDS

animal transport, image analysis, animal welfare, precision livestock farming.

ABSTRACT

The objective of this study was to evaluate the reactivity of the pupillary area (PA) as a non-invasive method for diagnosing stress in piglets submitted to transport. A total of 10 journeys were monitored and grouped according to the transport distance (15 and 75 km). Were sampled 360 piglets (25±3.8 kg) to measure the PA, body temperature (BT), and serum concentrations of cortisol and creatine kinase (CK). The micrometeorological characterization of the load was performed using enthalpy comfort index (ECI). It was possible to identify an increase of 19.32% (+18.91 mm²; P<0.001) in PA of piglets transported in shorter journeys (15 km), as well as the highest mean values of BT (+ 1.24 °C; P<0.001), cortisol (+ 2.37 ng/mL; P<0.001) and CK (+ 379 U/L; P=0.025). In this study, high correlations of PA were observed with r = 0.922, 0.900, 0.842 and 0.829 (P<0.05) for cortisol and CK, BT and ECI of trailer, respectively. In conclusion, we state that PA reactivity is a physiological response for the diagnosis of stress of piglets transported. Further research will refine this technique in order to make this non-invasive acquisition method more practical and adaptable to different transport conditions.

INTRODUCTION

Transport is considered one of the most critical operations for animal welfare (Pinheiro et al., 2021). During transport, pigs are subjected to various stressors, such as mixing lots, deprivation of food and water, changes in the environment, vibrations and noise (Mota-Rojas et al., 2014; Benincasa et al., 2020). All with the potential to cause physiological and psychological stress (Faucitano, 2018; Tasse & Molento 2019).

Although it is a routine practice among farms with multiple production sites (Rioja-Lang et al., 2019), the transport of piglets is a complex operation regarding animal welfare because it causes severe negative impacts through stress, pain, and suffering during the journey (Rocha et al., 2016), which leads to serious economic losses by reducing feed consumption at finishing stage (Mota-Rojas et al. 2014), dehydration and deaths on arrival (DOA) (Johnson et al. 2018).

Nowadays, stress monitoring techniques are often invasive and require animal restriction or specific training, like using iButton sensors (Pereira et al., 2018), radio-telemetry (De Jong et al., 1998), and salivary cortisol (Dalla-Costa et al., 2009). The use of a non-invasive method would facilitate routine in animal production systems and could provide better welfare conditions for animals during transport.

In this context, pupillometry presents itself as a useful tool for practical application, especially because the regulation of the pupillary area (PA) is controlled by the autonomous nervous system. Previous studies show that PA has shown to expand (mydriasis) as a consequence of mental effort and exposure to stressors in humans (Lempert et al., 2015) and in goats subjected to heat stress (Marques et al., 2018).

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Pupillometry can be an important tool for precision livestock farming as it is a non-invasive method with the potential to monitor chronic stress in breeding systems. This technology can assist producers and transporters in decision making and transport planning in order to optimize production losses by monitoring the welfare state of animals.

In animal science, the use of pupillometry to evaluate the stress of animals is still incipient as far as the knowledge reaches, requiring further investigations. Therefore, the objective of this study was to evaluate the reactivity of the pupillary area as a non-invasive method for diagnosing stress in piglets submitted to transport.

MATERIAL AND METHODS

The experimental procedures in animals were performed according to the guidelines of the Animal Ethics Committee of the Federal University of Ceará and were approved by this committee with protocol number 9871250719/2019.

Location and Logistics

The journeys were performed under commercial conditions at a farm located in the municipality of Maracanaú - CE (3°54'46 "S 38°39'19 "W at 43m altitude), between the period from 16 to 28 July 2019. A total of 10 journeys were monitored, 5 to the municipality of Maranguape - CE (3°51'50 "S 38°40'05 "W at 68m altitude) and 5 to the municipality of Caridade - CE (4°09'19 "S 39°05'30 "W at 144m altitude), corresponding to distances of 15 km and 75 km, respectively.

The transportation was performed in a Ford® cargo 1519 truck, with a double-deck (two-story Triel® - HT model), containing six compartments (2.92 m length × 2.4 m width × 1 m height) per floor, totaling 12 compartments and a load capacity of 13 tons (Figure 1). The vehicle was not equipped with an air conditioning system, and the compartments did not contain any type of bedding or water supply system. The vehicle was not equipped with an air conditioning system, and the compartments did not contain a water supply system. Feeding was suspended only when the animals started to load and the density of the load during transport was 136 kg/m².

FIGURE 1. Trailer used to transport piglets.

The loading of piglets submitted to 75 km journeys started at 13:30±8 min. the animals were handled with flags during the loading procedure. Pre-molded loading ramps with a 30° slope was used for the lower deck, and a metal ramp with 3 meters and 58° slope for the upper deck. After loaded, the piglets were wetted randomly by a farm employee, using a hose as described by Pinheiro et al. (2020) and the average water used (± SE) was 2,852±210 liters per load.

The truck loading finished at 14: 20±10 min, the of animal's journeys of 15 km began at 15:00±6 min as it is a standard farm management practice where the study was conducted. The transportation time was 120±14 min and 80±12 min, respectively, for 75 km and 15 km journeys. The journeys were made on asphalt roads.

Thermal parameters

Twelve data loggers (Onset®, model U23-001 HOBO® Pro v2, with TA ± 0.2 ° C and RH ± 2.5% accuracy) were setted to measure temperature (TA) and relative air humidity (RH) every 10 min. The data loggers were distributed in the twelve truck trailer compartments, at the height of the animals, according to Machado et al. (2021a). The micrometeorological characterization of the load was performed with Enthalpy Comfort Index (ECI, kJ/kg dry air) proposed by Rodrigues et al. (2011), estimated by [eq. (1)].

$$ECI = 1.006 \text{ x } TA + \frac{RH}{Pb} .10^{7.5 \text{ x } TA (237.3 + TA)^{-1}} \text{ x } (71.28 + 0.052 \text{ x } TA) \quad (1)$$

Where:

TA: temperature (°C);

RH: relative air humidity (%);

Pb: local barometric pressure (mm Hg).

Animal’s data acquisition

A total of 2160 crossbred piglets (Large White x Landrace x Duroc) at 7 weeks of age and average with an average body weight of 25±4.5 kg were transported. From these, a sub-sample of 360 animals (72 per journey) with an average body weight of 25±3.8 kg was randomly selected and identified with markings on the dorsal and auricular region in the farm pen 80±15 min before loading.

The piglets were housed in all trailer compartments (3 per compartments). The protocol to register physiological variables was applied about 30±8 min after transport. Initially, the reactivity of the pupillary area (PA) of the piglets at the time of unloading, during the weighing of the piglets, that is, before being housed in the pens of the finishing farm, was evaluated.

Body temperature was collected prior to the piglet’s containment, without physical interference. Then, the animals were held lightly and briefly (maximum 2 min) in a supine position for blood collection to analyze the serum concentration of cortisol and creatine kinase, as previously described by Machado et al. (2020a). All samples were taken from each animal in 3 ± 1 min.

The measurement of PA was performed after the unloading of the piglet's, before the collection of the physiological variables. The animals were taken to an individual platform scale (Baltec Master® ACB, Araçatuba, SP, Brazil) containing a device for automatic image acquisition according to Lopes Neto et al., (2018).

The PA image acquiring equipment consists of a camera with a 16-megapixel resolution (Nikon® CoolPix B500 16MP Full HD, São Paulo, SP, Brazil), an Arduino® UNO system, a 5 V module and an ultrasonic sensor HC-SR04. The ultrasonic sensors triggered the image capturing as they detected the presence of the animal emitting an electric pulse to the Arduino® microcontroller. The controller then sends a signal to the camera's USB input, triggering the image capture (Figure 2A).

The images were zoomed in, focusing on the orbital region (Figure 2B), and PA measurement was performed in the imageJ® software. Five images of each animal were selected and the eye perimeter was manually outlined to obtain the PA, as shown in Figure 2C. The software calibration was performed using a method adapted from Marques et al. (2018), using a reference scale of 5 mm in the cage of the individual platform scale.

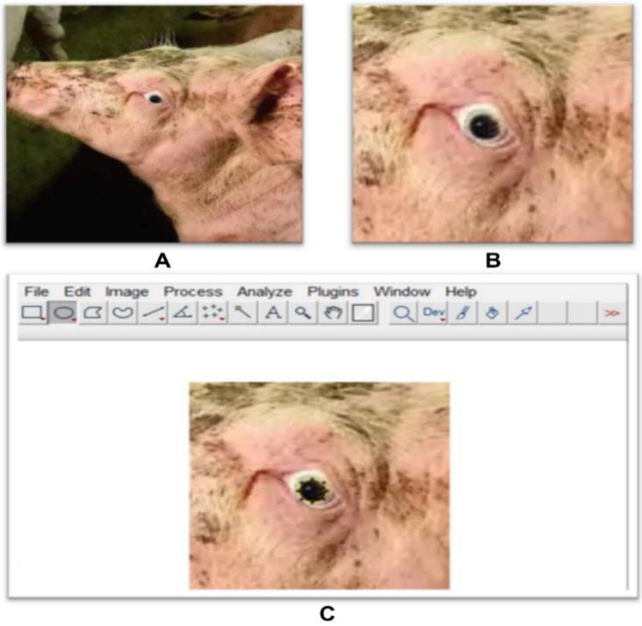

FIGURE 2. Image (A), zoom in the orbital region (B) and ImageJ interface, delimiting the perimeter of the pupil area.

Body temperature was obtained by thermal imaging from a thermographic camera (TiS10, Fluke, 4,800 pixels resolution), by adjusting the environmental temperature at the time of image recording, and emissivity of biological tissues ε = 0.98 (Machado et al., 2021b). Thermal images were obtained with a fixed distance of 0.50 m between the animal and the observer (Soerensen et al., 2014), analyzed by SmartView 4.3 software. All thermal images were analyzed in the SmartView 4.3 software.

Blood samples of 10 mL were collected in the animals' jugular vein using hypodermic needles of 18 x 38 mm by a veterinarian, after the animals were restrained for further analysis of cortisol and creatine kinase (CK). This procedure was followed by hemostasis, to avoid any type of sequelae. Then the samples were stored in tubes (BD Vacutainers, Kasvi K50204S).

Cortisol determination was performed with an Elisa commercial kit (Neogen Corp. Lexington, KY, USA) with a microplate reader (BioTek In®, ELx808™, Winooski, VT, USA). CK was determined using the commercial kit (Creatine Kinase-SL, Sukisui Diagnostics) and CK serum concentrations were obtained by spectrophotometer (Konica Minolta®, CM-3600A).

Statistical Analyses

The data obtained were analyzed by ANOVA using SAS® PROC GLIM (SAS Inst. Inc., Cary, NC) followed by comparisons of means with Tukey test (P < 0.05). The degree of relation between the studied variables (P < 0.05) was obtained based on Pearson's linear correlation coefficient (r) using the PROC CORR.

RESULTS AND DISCUSSION

The piglets that were transported for 15 km showed higher pupillary area (P<0.001) compared to animals transported for 75 km, with an increase of 19.32%. These animals also showed the highest serum concentrations of cortisol (+ 2.37 ng/mL; P<0.001) and CK (+ 379 U/L; P=0.025) and the highest body temperature (+ 1.24 °C; P<0.001) when compared to piglets transported for 75 km. It is important to note that we found a significant reduction in the heat contained in the trailer (-5.24 kJ / kg of dry air) for a 75 km journey when compared to 15 km journey (Table 1).

TABLE 1. Mean (±SE) of the pupillary area, body temperature, serum concentrations of cortisol and creatine kinase of piglet's and enthalpy comfort index of trailer during transport.

Journeys	Pupillary area (mm²)	Cortisol (ng/mL)	Creatine kinase[1] (U/L)	Body temperature (°C)	ECI[2] (kJ/kg of dry air)
15 km	97.51±1.69[a]	34.82±2.64[a]	4367±86.15[a]	40.34±0.78[a]	83.50±1.28[a]
75 km	78.60±2.08[b]	32.45±2.14[b]	3988±54.47[b]	39.10±0.83[b]	78.26±1.80[b]
CV (%)	7.96	8.04	16.47	4.02	6.60
P value	<0.001	<0.001	0.025	<0.001	0.015

Means followed by different letters (lines) are different from each other by the Tukey test at 5% significance. CV = coefficient of variation; [1]Data transformed to log10 and [2] Enthalpy Comfort Index.

The results show that the adverse effects of stress on piglets is reduced for higher journey. We attribute this to the fact that the longer transport duration allows a “rest period” with effect similar to a waiting time at the slaughterhouse (Haley et al., 2008). Thus, the piglets adapt to transport conditions (social and physical environment), reducing the impacts caused by loading (Goumon & Faucitano, 2017).

This hypothesis is also supported by reduced heat in the trailer on longer journeys (75 km). According to Silva-Miranda et al., (2012) environments with ECI values above 80 kJ/kg of dry air are favorable for the thermal stress of piglets. This suggests that the micrometeorological profile of longer trips was, on average, more favorable, possibly due to the lower need for heat and water vapor dissipation into the environment by the animals due to their greater thermal balance.

In a study carried out in Spain, Perez et al., (2002) concluded that pigs submitted to shorter trips (15 minutes) showed a more intense stress response (increases of 19.02 mg/mL and 29.46 ng/mL of lactate and cortisol in the blood, respectively) and worse meat quality (reduction of 0.09 and 0.17 of the pH of *Longissimus Thoracis* and *Semimembranosus* and discoloration), in comparison with pigs submitted to longer journeys (3 hours), when they were immediately slaughtered on arrival at the slaughterhouse.

In this study, the pupillary area showed significant high positive correlations with other physiological responses and with the heat in the load (ECI). The linear correlation coefficients obtained for the serum concentrations of cortisol and creatine kinase, body temperature, and ECI were r = 0.92, 0.90, 0.84, and 0.82, respectively (Table 2 and Figure 3). These findings show the potential use of pupillometry in precision livestock farming, specifically the reactivity of pupillary area as a diagnosis of stress, including commercial animal transport conditions.

TABLE 2. Correlations of the pupillary area with the enthalpy comfort index, body temperature, serum concentrations of cortisol and creatine kinase.

Item	Linear Models			P-value
	a	b	r	
Enthalpy Comfort Index	0.824	15.160	0.829	0.031
Body temperature	0.086	31.192	0.842	0.018
Cortisol	0.243	12.346	0.900	<0.001
Creatine kinase[1]	17.812	2519.000	0.922	<0.001

[1]Data transformed to log10

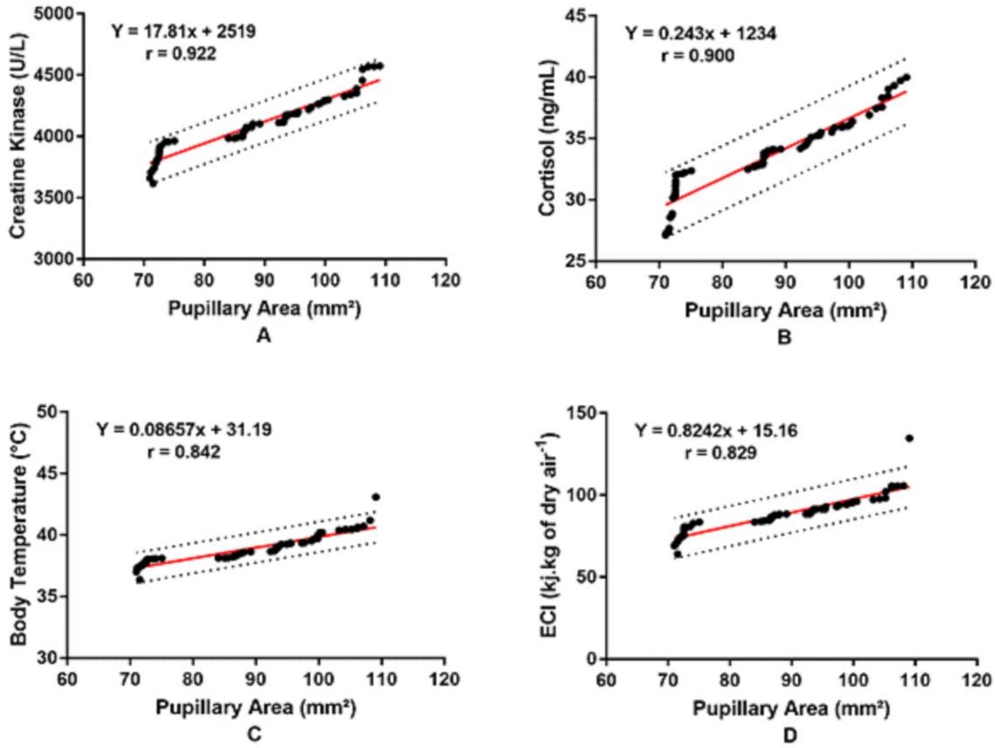

FIGURE 3. Diagrams of dispersion between pupillary area with serum concentration of CK[1] (A), cortisol (B), body temperature (C) and enthalpy comfort index (D). [1]Data transformed to log10

Pupillary area (PA) gives physiological response for the diagnosis of stress od piglets submitted to transportation. It was possible to detect that piglets subjected to short trips experienced more intense stress. These animals presented a pupil area dilation of 18.91 mm², about 19.32% greater than the PA of piglets subjected to longer journeys (Table 1).

These findings show the relationship between the PA the stress. The reason for this is that PA regulation is associated with immediate activation of the neuroaxis when facing stress, which leads to the activation of the sympathetic system of autonomic nervous system by the production and release of noradrenaline in the mediations of the locus coeruleus (Lempert et al., 2015).

The stress causes activation of the autonomic nervous system, increasing the serum levels of cortisol and CK in piglets subjected to shorter trips, also showing an increase in adrenocortical activity in these animals (Table 1). These parameters suggest greater physical stress (higher CK values), related to fatigue and muscle exhaustion, possibly due to the handling of piglets (Goumon & Faucitano, 2017).

Therefore, due to the stress during transport, the activation of the sympathetic system of the autonomic nervous system and the peripheral nervous system occurred, resulting in physiological changes (McDougall et al., 2015), which includes pupillary mydriasis (Laeng et al., 2012). Furthermore, factors related to human-animal interaction in the loading and unloading of pigs (Mota-Rojas et al., 2020).

In a bioclimatic chamber study, Marques et al., (2018) analyzed the pupillary dilation of crossbred goats and reported a significant PA increase of 37% with a 4°C rise in the temperature and a high correlation with linear correlation coefficient (r=0.99), indicating a direct relationship with the activation of sensitive mechanisms of heat loss to the maintenance of the animals' homeothermy.

In this study, we identified PA correlations from 0.82 to 0.92 with physiological stress indicators, especially with serum CK concentration (Table 2 and Figure 2). The CK is considered an important biochemical marker of muscle fatigue (Miles et al., 2008) used for measuring muscle exhaustion during pig transport (Correa et al., 2014). These results show that PA is a potentially tool in the description of the sympathetic system adaptations of the autonomic nervous system of piglets under stress conditions.

Although, the pupillary area can be used as a variable for measuring physiological responses in a non-invasive way and with a reasonable degree of accuracy in the studied conditions. Further research is needed to refine the technique in order to achieve a more practical and adaptable acquisition method to commercial transport conditions.

CONCLUSIONS

The reactivity of the pupil area is a useful physiological response for the diagnosis of stress in piglets during transport. However, further studies are needed to develop more refined image acquisition methods and image processing techniques to make the method more practical and adaptable to the farm and for animal transportation.

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