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Heart rate variability in Dorper sheep in the fetal and neonatal periods until 120 days of age: Use of the technique in the field

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ABSTRACT. The evaluation of the autonomic cardiac functions may be performed through the analysis of the heart rate variability. Heart rate variability is defined as the fluctuations in the heart rhythm or rate, and represents a useful tool in the evaluation of the autonomic nervous system through the sympathetic and parasympathetic components, as well as its balance and its reflexes on the cardiorespiratory control system. Fetal electrocardiography provides important information regarding the well-being of the fetus since, in human fetuses, there are changes in the behavior of the fetal heart rate during the second and third trimesters of pregnancy due to an increase in parasympathetic activity. Therefore, considering the importance of evaluating fetal viability, this study aims at evaluating the behavior of fetal heart rate and heart rate variability in Dorper sheep, as well as the activity of the autonomic nervous system during fetal life and in newborn lambs. The species is often used in experimental studies and autonomic nervous system activity is a prognostic index, therefore, the diagnosis of modifications in the sympathovagal balance may represent an early index for fetal viability and well-being in lambs. The analyses were performed in 10 Dorper sheep during pregnancy and in 10 lambs after birth until 120 days of age. There was a decrease in the fetal heart rate and heart rate variability indexes during the fifth month of pregnancy, but without statistical significance for the period evaluated. The heart rate of the lambs decreased gradually until they were 21 days old. The indexes SDNN (standard deviation of RR intervals) and RMSSD (square root of the mean of successive differences between adjacent RR intervals) diverged according to age, being high at day 60. Fetal viability is relevant in sheep fetuses to avoid losses during pregnancy and risks to the health of the mother. In the species, there seems to be a predominance of parasympathetic activity starting from the 21st day of age. Heart rate variability may be employed as a tool in the evaluation of the fetus and development of lambs, since changes in its behavior may represent an adverse effect to fetal and neonatal health.

KEY WORDS: autonomic nervous system, fetus, heart rate, pregnancy sheep

The monitoring of the FHR (fetal heart rate) has been used since the 1970 and has become a standard component when evaluating the fetal well-being [17]. One of the objectives of monitoring the FHR is to avoid fetal asphyxia and acidosis, as well as any long-term adverse effects on the neurological development [41]. In human fetuses, there are changes in the behavior of the FHR during the second and third trimesters of pregnancy, with a decrease from 175 beats per minute (bpm) to 140 bpm from the ninth week until the end of the pregnancy [42]. It is believed that this reduction is mediated by an increase in parasympathetic

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activity that voids the influence of the sympathetic tone. The alterations in vagal function may, in addition, lead to an increase in heart rate variability (HRV) [3].

Several physiological modifications happen during pregnancy to facilitate the supply of maternal oxygen and nutrients to the growing fetus while maintain a reserve large enough for the mother [8]. During labor, the fetus may be exposed to acute hypoxia during some uterine contractions or intrapartum insults [13] and has compensatory cardiovascular defense mechanisms to ideally prevent hypoxic-ischemic encephalopathy. Reduced variability or lack of variability in the FHR with deceleration is the most consistent predictor for neonatal acidemia with unfavorable outcomes [8, 9]. The degree of FHR deceleration is usually proportional to the force of the associated uterine contraction. It is believed that the initial decelerations compress the head of the fetus, altering the blood flow to the brain and leading to a decrease in FHR due to the vagal reflex [25].

The use of instrumented fetal sheep models at the end of pregnancy provided important information regarding fetal physiology [32] that are otherwise limited in human fetuses, allowing a detailed analysis of the fetal cardiovascular function over long periods of time [33]. Using these models, studies have shown that acute hypoxia activates both branches of the Autonomic Nervous System (ANS) that control the FHR with a vagal dominance [27], and that the activation of the sympathetic nervous system (SNS) is key to rapidly provide support and increase the peripheral vascular resistance of the fetus and stabilize the arterial blood pressure in spite of the decrease in FHR. In addition, the progression of the pregnancy and prenatal therapy with glucocorticoids interferes in the magnitude and pattern of the FHR response to hypoxia and in the endocrine and metabolic compensation of the fetus, both of which contribute towards alterations in the variability of FHR [35].

In premature human newborns, the ANS is immature and is unable to deal with demands of the cardiorespiratory transition upon birth [43]. About 25% of all premature babies develop a brain injury that may compromise even further the maturation and function of the ANS [19]. The function of the ANS may be measure non-invasively through the behavior of the heart rate (HR) [15], respiratory rate (RR) and arterial blood pressure (ABP). The heart rate variability or the time fluctuation between successive beats (RR intervals) [1, 16], provides a measure of sympathetic and parasympathetic function and, therefore, of the maturation of the ANS. As the ANS matures, there should be an increase in parasympathetic function [44].

The cardiovascular system is widely controlled by the ANS, with the sympathetic and parasympathetic nervous systems responsible for increases and decreases in heart rate and arterial blood pressure [29, 30]. The autonomic control of the cardiovascular system is developed during the fetal life, but the sympathetic and parasympathetic branches develop differently [39]. The sympathetic branch seems to develop quickly during the first trimester of pregnancy in humans, continuing to develop slowly after that. On the other hand, the parasympathetic or vagal control becomes dominant later during the development of the fetus, around the 25th or 30th week of pregnancy. The ANS control over the cardiovascular parameters involves a complex interaction between the two branches, and the result of this interaction reflects the sympathovagal balance [6, 15].

Therefore, considering the importance of evaluating fetal and neonatal viability, this study aims at assessing the behavior of fetal HR and HRV in Dorper sheep fetuses, as well as evaluate ANS activity during fetal and neonatal life, considering that the specie is used in experimental studies and that ANS activity represents a prognostic index. Therefore, the diagnosis of modifications in the sympathovagal balance may represent an early index for fetal viability, fetal well-being and the physiological condition in lambs, which may contribute towards translational research and reduce the neonatal mortality rates.

MATERIALS AND METHODS

Study location

This study was conducted according to the animal well-being guidelines and approved by the Ethics Commission on Animal Use (CEUA, *Comissão de Ética no Uso de Animais*) of the School of Veterinary Medicine and Animal Science at *Universidade Estadual Paulista "Júlio de Mesquita Filho"*, Botucatu Campus, under protocol CEUA-0174/2016. The owners of the animals consented to the experimental plan and to the procedures performed.

The study was conducted in a property located in the city of Botucatu, State of São Paulo, Brazil, in the Rubião Júnior District, latitude S-22.902107 and longitude W-48.516534, from July 2017 to April 2018.

Animals

Ten Dorper Sheep (*Ovis aries*) were evaluated during pregnancy (152 days, or approximately 5 months). The sheep underwent a general clinical examination and animals with any abnormalities in the results or in the body condition score were excluded from the study. Vaccination and deworming protocols were performed by the veterinarian responsible for the location.

The sheep were kept in semi-confined and received animal feed twice a day and water *ad libitum*. In addition, they received a mineral mixture composed of 65% ground corn, 10% corn bran, 20% soy bran and 5% NC (nucleus) for sheep. The basic composition of the NC was: calcium (minimum) 120.0 g/kg; calcium (maximum) 220 g/kg; phosphorus (minimum) 18.0 g/kg; sodium (minimum) 78.0 g/kg; magnesium (minimum) 7,700.0 mg/kg; potassium (minimum) 10.0 g/kg; iron (minimum) 250.0 mg/kg; zinc (minimum) 1,870.0 mg/kg; manganese (minimum) 650.0 mg/kg; iodine (minimum) 30 mg/kg; selenium (minimum) 8.00 mg/kg; cobalt (minimum) 20.00 mg/kg; monensin 600.00 mg/kg.

Fetal heart rate variability

The fetal ECG records were compiled using a Televet 100 system (Kruuse®, version 4.1.3, Marslev, Denmark) [22]. This device uses an electrocardiogram (ECG) filter that enables the visualization and analysis of the maternal and fetal HR, both together and separately, by amplifying the fetal signal. The filter used to obtain the fetal RR intervals in Televet was 50 Hz, as described and proposed by the manufacturer. For the correction of artifacts (noise) we also use the artifact correction tool of the software itself to obtain the results, where the Smooyhn priors filter was used for further analysis.

The fetal ECG records were obtained by placing the adhesive hydrogel electrodes on the pregnant sheep, as recommended by Nagel *et al.* [21]: the green electrode was placed on the right side of the neck; the yellow electrode was placed on the left flank, approximately at the height of the hips; the black electrode (neutral) was placed on the left lumbar region of the sheep (Fig. 1); and the red electrode was placed on the right side of the abdomen, at the height of the knees (Fig. 2).

Neonatal heart rate variability

The same system was used to record the neonatal ECG, but the positions of the electrodes was changed as follows: the green electrode was placed three centimeters from the sternum; the yellow and black electrodes were placed 20 and 30 cm below the withers on the left side of the thorax; and the red electrode was placed similarly to the yellow one, but on the right side of the thorax [12] (Fig. 3). The data was registered during eight min and transferred to a computer via Bluetooth technology.

Heart rate variability analysis

The analysis of the heart rate variability was performed using the software Kubios (Biomedical Signal Analysis Group, Applied

Fig. 1. Position of the electrodes (left side) for registering the Fetal heart rate variability in Dorper sheep.

Fig. 2. Position of the electrodes (right side) for registering the Fetal heart rate variability in Dorper sheep.

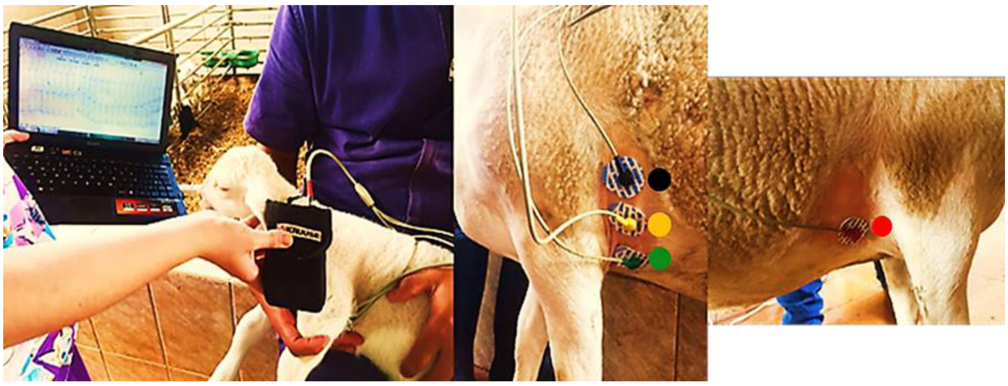

Fig. 3. Recommended position of the electrodes for conventional heart rate variability record in Dorper lambs.

Physics Department, University of Kuopio, Finland). Before being input in the software, the data was manually corrected in an Excel spreadsheet due to the possibility of there being artefacts that could compromise the analysis, as explained by Jonckheer-Sheehy *et al.* (2012) [14].

The HR and the HRV parameters were obtained from all ECG records taken for an individual sheep and the fetal records were obtained. Based on the registered RR intervals, the HRV indexes SDNN (Standard Deviation of RR Intervals) and RMSSD (square root of the mean of successive differences between adjacent RR intervals) [38] were calculated. The time-domain index PNN50% (proportion of differences between successive RR intervals exceeding 50 msec) was also recorded.

In the frequency domain, spectral analysis was conducted through the Fast Fourier Transform (FFT) algorithm and the following indexes were compiled and expressed in normalized units (n.u.): the high frequency component (HF) varying between 0.15 and 0.4 Hz; and the low frequency component (LF), varying between 0.04 and 0.15 Hz.

Statistical analysis

The results are shown as the mean, standard deviation, and minimum/maximum values. The test employed to verify the normality of the data was the Kolmogorov-Smirnov test, while the test employed to compare the proposed moments was Friedmann's test, which was later employed to compare specific moments (24 hr after birth, and at 7, 14 and 21 days of age). All discussions considered a significance level of 5%.

RESULTS

The fetal heart rate variability indexes were recorded starting at the second month of pregnancy, but only from the fourth month onwards it was possible to obtain them without a considerable number of artefacts. The data is represented in Table 1. Figure 4 shows the fetal electrocardiographic tracing obtained through the Televet 100® system on the fifth month of pregnancy. There were no statistically significant differences for fetal heart rate and HRV indexes between the fourth and fifth months of pregnancy, but we observed that the mean and maximum fetal heart rate decreased on the fifth month in comparison with the fourth. In addition, the HRV indexes PNN50 (proportion of differences between successive RR intervals exceeding 50 msec) and high frequency (n.u.) were higher during this period.

Table 2 shows the HRV indexes on lambs staring 24 hr after birth until 120 days of age. The heart rate decreased gradually as

Table 1. Fetal heart rate variability indexes (mean, standard deviation, minimum, maximum) on the fourth and fifth months of pregnancy in Dorper sheep

Fetal HRV	4th month	5th month	*P*
HR Min	153.00 ± 11.20 (136;173)	153.00 ± 13.49 (136;183)	0.941
Mean	195.00 ± 12.00 (158;208)	191.00 ± 16.43 (159;220)	0.404
Max	258.00 ± 22.45 (169;270)	248.00 ± 23.44 (182;267)	0.184
RR (msec)	308.00 ± 21.18 (288;379)	315.00 ± 28.23 (272;376)	0.369
SDNN (msec)	6.00 ± 4.81 (2.3;23.3)	5.70 ± 2.68 (2.5;12.0)	0.843
RMSSD (msec)	4.26 ± 4.25 (1.2;18.6)	3.71 ± 2.30 (1.6;8.9)	0.643
PNN50 (%)	0.37 ± 1.63 (0;7.14)	0.60 ± 0.25 (0;1)	0.455
LF (n.u.)	59.17 ± 25.27 (13.22;93.15)	48.78 ± 29.71 (1.77;88.42)	0.271
HF (n.u.)	40.68 ± 25.18 (6.81;86.56)	50.97 ± 29.51 (11.57;98.08)	0.273
LF/HF	3.18 ± 3.66 (0.15;13.68)	1.96 ± 2.19 (0.01;7.64)	0.252

Comparison between moments: Friedmann's Test; *significance $P<0.05$; n.u.=normalized units. HR: heart rate; Min: minimum; Max: maximum; RR: RR intervals; SDNN: standard deviation of all RR intervals; RMSSD: square root of the mean of successive differences between adjacent RR intervals; PNN50: proportion of differences between successive RR intervals exceeding 50 msec; HF: high frequency; LF: low frequency.

Fig. 4. Fetal electrocardiographic tracing obtained through the Televet 100® system on the fifth month of pregnancy on Dorper sheep.

Table 2. Heart rate variability in Dorper lambs (mean, standard deviation, minimum, maximum) starting at 24 hr after birth until 120 days of age

HRV	24 hr	7 days	14 days	21 days	30 days	60 days	90 days	120 days	P
Min HR	178 ± 39.43^{a} (120;246)	165 ± 35.70^{ab} (110;221)	164 ± 26.93^{bc} (136;219)	137 ± 39.77 ^{bc} (82;227)	134 ± 25 (100;197)	130 ± 21.88 (86;173)	140 ± 13.48 (107;157)	131 ± 18.18 (105;158)	*0.000
Mean HR	199 ± 38.71^{a} (134;255)	199 ± 37.74^{ab} (148;247)	179 ± 27.45^{bc} (144;230)	159 ± 40.57^{bc} (102;253)	149 ± 28.09 (107;222)	162 ± 25.03 (106;198)	154 ± 17.33 (110;175)	149 ± 17.53 (123;175)	*0.000
Max HR	239 ± 34.30^{a} (164;270)	245 ± 32.25^{ab} (190;270)	213 ± 38.70^{bc} (151;270)	205 ± 47.14^{bc} (122;261)	171 ± 31 (120;254)	217 ± 27.98 (166;251)	176 ± 22.07 (118;200)	199 ± 38.76 (139;259)	*0.000
RR (msec)	311 ± 62.64^{a} (236;449)	311 ± 61.30^{ab} (243;407)	340 ± 50.44^{bc} (261;417)	398 ± 98.18^{c} (237;589)	414 ± 70.85 (270;559)	380 ± 71.23 (304;568)	393 ± 53.38 (343;543)	405 ± 48.59 (343;487)	*0.000
SDNN (msec)	1.77 ± 1.39^{ac} (0.40;4.10)	3.19 ± 2.90^{ab} (0.60;9.30)	1.17 ± 1.75^{b} (0.50;6.70)	3.53 ± 4.15^{c} (0.70; 15.20)	0.69 ± 0.14 (0.60;1.10)	3.83 ± 2.98 (0.90;11.30)	0.71 ± 0.09 (0.60;0.90)	3.14 ± 2.52 (0.90;7.60)	*0.000
RMSSD (msec)	0.66 ± 0.44^{a} (0.20;1.40)	1.38 ± 1.22^{bc} (0.30;4)	0.56 ± 0.59^{a} (0.20;2.40)	1.45 ± 1.45^{c} (0.40;5.50)	0.52 ± 0.14 (0.30;0.80)	1.70 ± 1.07 (0.60;3.50)	0.48 ± 0.08 (0.40;0.70)	1.30 ± 0.84 (0.50;3)	*0.000
PNN50 (%)	0 ± 0 (0;0)	0.01 ± 0.04 (0;0.15)	0.01 ± 0.03 (0;0.1)	0 ± 0 (0;0)	0 ± 0 (0;0)	0.03 ± 0.07 (0;0.19)	0 ± 0 (0;0)	0.01 ± 0.04 (0;0.15)	0.474
LF (n.u.)	75.45 ± 8.20^{ab} (57.35;90.96)	75.19 ± 16.44^{a} (40.91;95.33)	72.81 ± 9.48^{a} (56.20;83.09)	65.08 ± 15.68^{b} (43.99;92.4)	60.58 ± 16.47 (27.93;77.7)	53.73 ± 17.90 (29.50;79.5)	66.77 ± 6.94 (54.32;76.7)	62.86 ± 11.03 (40.83;77)	*0.008
HF (n.u.)	24.51 ± 8.19^{ab} (9.01;42.61)	24.75 ± 16.40^{a} (4.66;58.91)	27.14 ± 9.45^{a} (16.87;43.77)	34.81 ± 15.63^{b} (7.47;55.65)	39.35 ± 16.49 (25.68;49.4)	46.17 ± 17.90 (20.44;70.4)	34.50 ± 9.83 (23.19;58.4)	37.04 ± 10.98 (22.93;58.8)	*0.005
LF/HF	3.65 ± 2.20^{ab} (1.35;10.10)	5.12 ± 5.16^{a} (0.69;20.44)	3.10 ± 1.35^{a} (1.28;4.93)	3.07 ± 3.38^{b} (0.79;12.37)	1.93 ± 1.08 (0.39;3.50)	1.59 ± 1.26 (0.42;3.89)	5.49 ± 11.39 (1.21;41.59)	1.90 ± 0.78 (0.69;3.36)	*0.008

Normality test: Kolmogorov-Smirnov; * significance $P<0.05$; n.u.=normalized units. HR: heart rate; Min: minimum; Max: maximum; RR: RR intervals; SDNN: standard deviation of all RR intervals; RMSSD: square root of the mean of successive differences between adjacent RR intervals; PNN50: proportion of differences between successive RR intervals exceeding 50 msec; HF: high frequency; LF: low frequency; ^{ab} different lower case letters superscripted on the same line indicate very significant differences ($P<0.001$) between moments of the neonatal period (24 hr after birth until 21 days of age).

the animals developed, decreasing more considerably starting at day 21. Minimum, mean and maximum HR diverged significantly between the moments, also decreasing starting at day 21. There were also differences in the RR intervals, which presented increased durations starting at day 21.

The indexes SDNN (standard deviation of RR intervals) and RMSSD (square root of the mean of successive differences between adjacent RR intervals) diverged significantly between the moments, peaking at day 60. There were also statistically significant differences between the moments for the indexes low frequency (n.u.), which decreased starting at day 21; high frequency (n.u.), which increased starting at day 21; and also, in the LF/HF ratio.

Friedmann's statistical test was the applied to evaluate possible significant differences between specific moments for the HRV indexes, starting 24 hr after birth until 21 days of age, which was when the start of the decrease in the heart rate was observed. There were very significant differences for the RR interval when comparing 24 hr after birth with 14 and 21 days, and between 7 and 21 days. The same was observed in the aforementioned periods for minimum, mean and maximum heart rate. The index SDNN diverged significantly when comparing the following moments: 24 hr and 14 days; 7 and 21 days; 14 and 21 days.

The index RMSSD presented significant differences when comparing 24 hr after birth with 7 and 21 days, and between 7 days and 14 days, and 14 days and 21 days. The indexes LF and HF (n.u.), as well as the LF/HF ratio, presented significant differences between 7 days and 21 days, and between 14 days and 21 days, as shown in Table 2.

Figure 5 shows the electrocardiographic tracings obtained through the Televet 100® method in Dorper lambs 24 hr after birth, and Fig. 6 shows the electrocardiographic tracings obtained through the Televet 100® method in Dorper lambs at 21 days of age.

Friedmann's test was also applied to compared the HRV indexes between the fetal and neonatal periods. The fetal RR interval during the fourth month of pregnancy presented significant differences with 30 days, 90 days and 120 days of age. Mean HR during the fourth month of pregnancy diverged with the mean HR at 30 days of age. Maximum HR, SDNN and RMSSD presented differences between the fourth month of pregnancy and 14 days of age, fourth month and 30 days of age, and fourth month and 90 days of age. The FHR was lower during the fourth month of pregnancy than 24 hr after birth and at 7 and 14 days of age. This is in line with the fetal heart rate behavior observed through the HRV indexes that indicate parasympathetic activity (PNN50 and RMSSD in the time domain, and HF in the frequency domain), which were high during this period. The same happened with the fetal heart rate during the fifth month of pregnancy. Table 3 shows the comparisons between the fetal period during the fourth month of pregnancy and neonatal period.

The fetal RR interval at the fifth month of pregnancy presented significant differences with 30, 90 and 120 days of age. Mean and maximum HR presented differences between the fifth month of pregnancy and 30 days of age. The time-domain index SDNN presented differences between the fifth month of pregnancy and 14, 30 and 90 days of age, while the index RMSSD presented

Fig. 5. Neonatal electrocardiographic tracing obtained through the Televet 100® method in Dorper lambs 24 hr after birth.

Fig. 6. Neonatal electrocardiographic tracing obtained through the Televet 100® method in Dorper lambs at 21 days of age.

Table 3. Comparison between the heart rate variability indexes during the fetal period (4th month) and neonatal period in Dorper sheep

HRV	4th month	24 hr	7 days	14 days	21 days	30 days	60 days	90 days	120 days
Min HR	153 ± 11.20^{a}	178 ± 39.43^{a}	165 ± 35.70^{a}	164 ± 26.93^{a}	137 ± 39.77^{a}	134 ± 25^{a}	130 ± 21.88^{a}	140 ± 13.48^{a}	131 ± 18.18^{a}
Mean HR	195 ± 12.00^{a}	199 ± 38.71^{ac}	199 ± 37.74^{ac}	179 ± 27.45^{ac}	159 ± 40.57^{ac}	149 ± 28.09^{c}	162 ± 25.03^{ac}	154 ± 17.33^{ac}	149 ± 17.53^{ac}
Max HR	258 ± 22.45^{a}	239 ± 34.30^{abcd}	245 ± 32.25^{abcd}	213 ± 38.70^{bcd}	205 ± 47.14^{ab}	171 ± 31^{bcd}	217 ± 27.98^{abcd}	176 ± 22.07^{bcd}	199 ± 38.76^{abcd}
RR (msec)	308 ± 21.18^{a}	311 ± 62.64^{abcd}	311 ± 61.30^{abcd}	340 ± 50.44^{abcd}	398 ± 98.18^{abcd}	414 ± 70.85^{bcd}	380 ± 71.23^{abcd}	393 ± 53.38 ^{bcd}	405 ± 48.59 ^{bcd}
SDNN (msec)	6 ± 4.81^{a}	1.77 ± 1.39^{abcd}	3.19 ± 2.90^{abcd}	1.17 ± 1.75^{bcd}	3.53 ± 4.15^{ab}	0.69 ± 0.14^{bcd}	3.83 ± 2.98^{abcd}	0.71 ± 0.09^{bcd}	3.14 ± 2.52^{abcd}
RMSSD (msec)	4.26 ± 4.25^{a}	0.66 ± 0.44^{abcd}	1.38 ± 1.22^{abcd}	0.56 ± 0.59^{bcd}	1.45 ± 1.45^{ab}	0.52 ± 0.14^{bcd}	1.70 ± 1.07^{abcd}	0.48 ± 0.08^{bcd}	1.30 ± 0.84^{abcd}
PNN50 (%)	0.37 ± 1.63^{a}	0 ± 0^{a}	0.01 ± 0.04^{a}	0.01 ± 0.03^{a}	0 ± 0^{a}	0 ± 0^{a}	0.03 ± 0.07^{a}	0 ± 0^{a}	0.01 ± 0.04^{a}
LF (n.u.)	59.17 ± 25.27^{a}	75.45 ± 8.20^{a}	75.19 ± 16.44^{a}	72.81 ± 9.48^{a}	65.08 ± 15.68^{a}	60.58 ± 16.47^{a}	53.73 ± 17.90^{a}	66.77 ± 6.94^{a}	62.86 ± 11.03^{a}
HF (n.u.)	40.68 ± 25.18^{a}	24.51 ± 8.19^{a}	24.75 ± 16.40^{a}	27.14 ± 9.45^{a}	34.81 ± 15.63^{a}	39.35 ± 16.49^{a}	46.17 ± 17.90^{a}	34.50 ± 9.83^{a}	37.04 ± 10.98^{a}
LF/HF	40.68 ± 25.18^{a}	3.65 ± 2.20^{a}	5.12 ± 5.16^{a}	3.10 ± 1.35^{a}	3.07 ± 3.38^{a}	1.93 ± 1.08^{a}	1.59 ± 1.26^{a}	5.49 ± 11.39^{a}	1.90 ± 0.78^{a}

Comparison between moments: Friedmann's test; n.u.=normalized units. HR: heart rate; Min: minimum; Max: maximum; RR: RR intervals; SDNN: standard deviation of all RR intervals; RMSSD: square root of the mean of successive differences between adjacent RR intervals; PNN50: proportion of differences between successive RR intervals exceeding 50 msec; HF: high frequency; LF: low frequency.

Table 4. Comparison between the heart rate variability indexes during the fetal period (5th month) and neonatal period in Dorper sheep

HRV	5th month	24 hr	7 days	14 days	21 days	30 days	60 days	90 days	120 days
Min HR	153 ± 13.49^{a}	178 ± 39.43^{a}	165 ± 35.70^{a}	164 ± 26.93^{a}	137 ± 39.77^{a}	134 ± 25^{a}	130 ± 21.88^{a}	140 ± 13.48^{a}	131 ± 18.18^{a}
Mean HR	191 ± 16.43^{a}	199 ± 38.71^{ab}	199 ± 37.74^{ab}	179 ± 27.45^{ab}	159 ± 40.57^{ab}	149 ± 28.09^{b}	162 ± 25.03^{ab}	154 ± 17.33^{ab}	149 ± 17.53^{ab}
Max HR	248 ± 23.44^{a}	239 ± 34.30^{ab}	245 ± 32.25^{ab}	213 ± 38.70^{ab}	205 ± 47.14^{ab}	171 ± 31^{b}	217 ± 27.98^{ab}	176 ± 22.07^{ab}	199 ± 38.76^{ab}
RR (msec)	315 ± 28.23^{a}	311 ± 62.64^{abcd}	311 ± 61.30^{abcd}	340 ± 50.44^{abcd}	398 ± 98.18^{abcd}	414 ± 70.85^{bcd}	380 ± 71.23^{abcd}	393 ± 53.38^{bcd}	405 ± 48.59^{bcd}
SDNN (msec)	5.7 ± 2.68^{a}	1.77 ± 1.39^{abcd}	3.19 ± 2.90^{abcd}	1.17 ± 1.75^{bcd}	3.53 ± 4.15^{abcd}	0.69 ± 0.14^{bcd}	3.83 ± 2.98^{abcd}	0.71 ± 0.09^{bcd}	3.14 ± 2.52^{bcd}
RMSSD (msec)	3.71 ± 2.30^{a}	0.66 ± 0.44^{b}	1.38 ± 1.22^{b}	0.56 ± 0.59^{b}	1.45 ± 1.45^{a}	0.52 ± 0.14^{b}	1.70 ± 1.07^{a}	0.48 ± 0.08^{b}	1.30 ± 0.84^{a}
PNN50 (%)	0.6 ± 0.25	0 ± 0	0.01 ± 0.04	0.01 ± 0.03	0 ± 0	0 ± 0	0.03 ± 0.07	0 ± 0	0.01 ± 0.04
LF (n.u.)	48.78 ± 29.71	75.45 ± 8.20	75.19 ± 16.44	72.81 ± 9.48	65.08 ± 15.68	60.58 ± 16.47	53.73 ± 17.90	66.77 ± 6.94	62.86 ± 11.03
HF (n.u.)	50.97 ± 29.51	24.51 ± 8.19	24.75 ± 16.40	27.14 ± 9.45	34.81 ± 15.63	39.35 ± 16.49	46.17 ± 17.90	34.50 ± 9.83	37.04 ± 10.98
LF/HF	50.97 ± 29.51	3.65 ± 2.20	5.12 ± 5.16	3.10 ± 1.35	3.07 ± 3.38	1.93 ± 1.08	1.59 ± 1.26	5.49 ± 11.39	1.90 ± 0.78

Comparison between moments: Friedmann's test; n.u.=normalized units. HR: heart rate; Min: minimum; Max: maximum; RR: RR intervals; SDNN: standard deviation of all RR intervals; RMSSD: square root of the mean of successive differences between adjacent RR intervals; PNN50: proportion of differences between successive RR intervals exceeding 50 msec; HF: high frequency; LF: low frequency.

differences between the fetal period and most moments evaluated, except for 21, 60 and 120 days of age. The comparisons between the fifth month of pregnancy and the neonatal period are shown in Table 4.

Figures 7–9 show, respectively, a schematic representation of the behavior of minimum, mean and maximum HR during the fetal and neonatal periods in Dorper lambs, while Figs. 10 and 11 show, respectively, the behavior of frequency-domain HRV indexes LF (n.u.) and HF (n.u.) during the fetal and neonatal periods.

The behavior of the HR and the HRV indexes during the fetal and neonatal periods are shown in Supplementary Figs. 1– 3. Maximum FHR was higher than the maximum HR observed in neonates. Mean HR remained similar in the fetal and neonatal phases, but at day 21 the mean HR was lower than the values observed during the fetal phase. The LF index was higher in developing lambs than in fetuses. The HF index was lower in neonates than in fetuses, but increased as the animal developed.

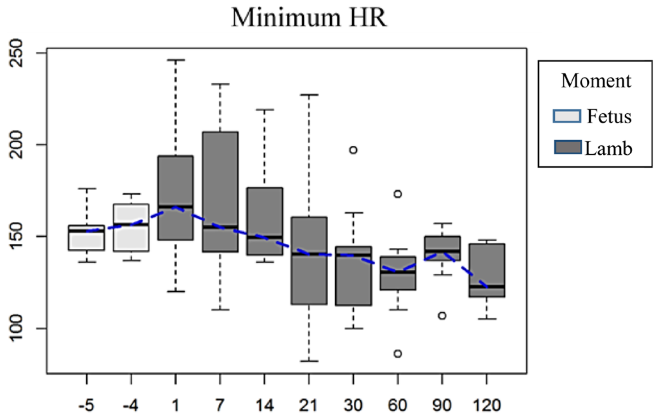

Fig. 7. Schematic representation of minimum heart rate during the fetal and neonatal period in Dorper lambs (−5: 5th month of pregnancy; −4: 4th month of pregnancy; 1: 24 hr old; 7, 14, 21, 30, 60, 90 and 120 days old).

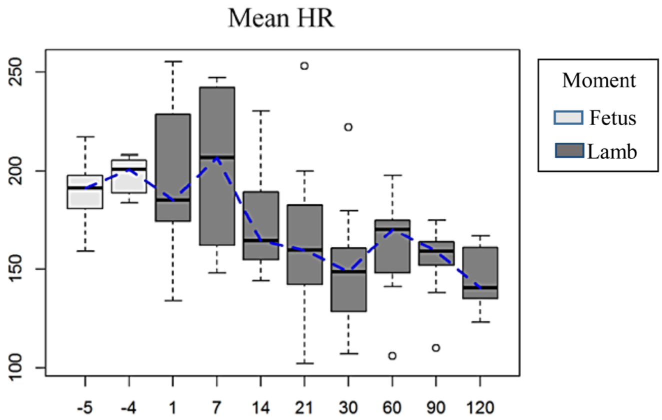

Fig. 8. Schematic representation of mean heart rate during the fetal and neonatal period in Dorper lambs (−5: 5th month of pregnancy; −4: 4th month of pregnancy; 1: 24 hr old; 7, 14, 21, 30, 60, 90 and 120 days old).

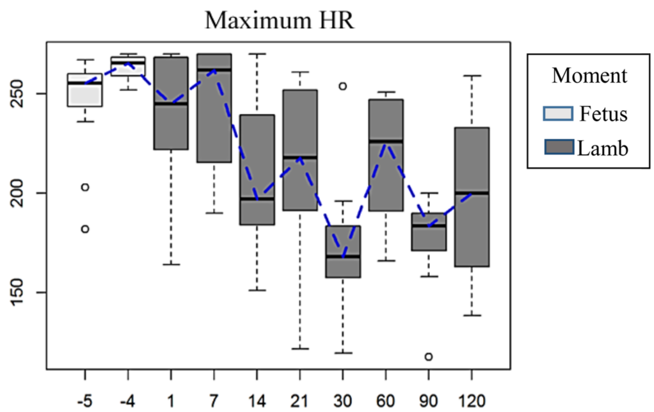

Fig. 9. Schematic representation of maximum heart rate during the fetal and neonatal period in Dorper lambs (−5: 5th month of pregnancy; −4: 4th month of pregnancy; 1: 24 hr old; 7, 14, 21, 30, 60, 90 and 120 days old).

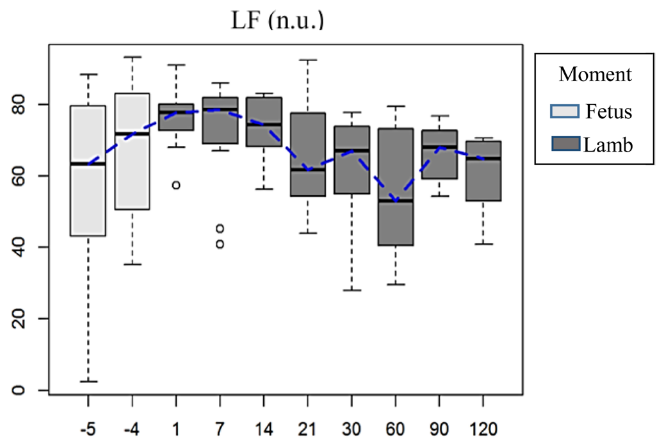

Fig. 10. Schematic representation of frequency-domain heart rate variability index LF (n.u.) during the fetal and neonatal period in Dorper lambs (−5: 5th month of pregnancy; −4: 4th month of pregnancy; 1: 24 hr old; 7, 14, 21, 30, 60, 90 and 120 days old).

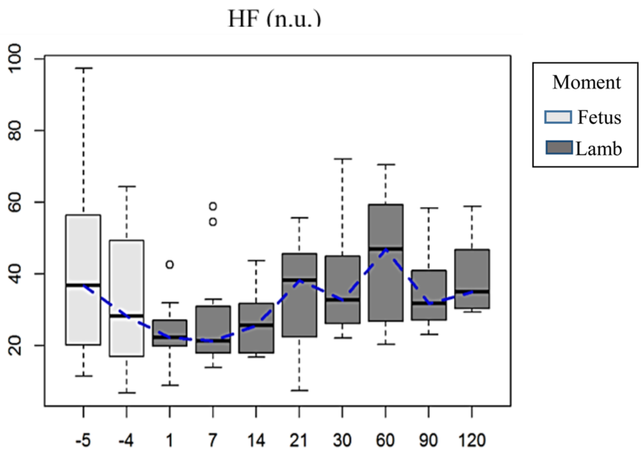

Fig. 11. Schematic representation of frequency-domain heart rate variability index LF (n.u.) during the fetal and neonatal period in Dorper lambs (−5: 5th month of pregnancy; −4: 4th month of pregnancy; 1: 24 hr old; 7, 14, 21, 30, 60, 90 and 120 days old).

DISCUSSION

We believe that, in this study, both the stage of the pregnancy and the development of the autonomic nervous system contributed towards acquiring higher quality data during the last two months of pregnancy. The maturity of the autonomic nervous system is a factor that should be considered when collecting fetal data in sheep, since the fetal heart rate was lower during the fifth month than in the fourth. The cardiac activity of the fetus depends on the stage of the pregnancy and, therefore, on the maturation of the autonomic nervous system. During the pregnancy, the regulation of the cardiac activity of the fetus by the sympathetic and parasympathetic nervous systems becomes progressively more complex [23], and the development of the autonomic ability to adapt to the variable offers and demands in the organism is one of the key needs during the maturation of the fetus [37].

According to the behavior of the fetal heart rate and heart rate variability observed in this study, we believe that there is predominantly parasympathetic activity in sheep fetuses during the last month of pregnancy, but there were no significant differences between the periods studied, which could be a result of the relatively small sample size. Garabedian *et al.* [7] conducted a study aiming to evaluate the fetal heart rate variability indexes and whether the indexes high frequency and RMSSD, which are considered representative of parasympathetic activity, where reliable in this representativeness. For this purpose, the authors administered propranolol (sympathetic activity blocker) and atropine (parasympatholytic) to sheep fetuses. They observed that the spectral component high frequency and the index RMSSD decreased after atropine was administered, confirming that these indexes are representative for activity of the parasympathetic nervous system.

The LF/HF ratio did not present statistically significant differences between the fourth and fifth month of pregnancy in this study, but we observed that it decreased slightly in the last month. Since the high frequency index represents parasympathetic activity, we believe that this decrease in the last month is a consequence of an increase in parasympathetic activity. The predominance of parasympathetic activity during the last month of pregnancy, as well as the lower fetal heart rate values observed in the fifth month, may be explained by the proximity of birth. There is a negative correlation between the progress of the pregnancy and the fetal heart rate [21], which is interpreted as progressively more complex cardiac regulation by autonomic nervous system towards the end of the pregnancy [24].

In bovines, the release of cortisol by the fetus increases markedly during the last week of pregnancy [33]. This increase coincides with changes in the heart rate variability of the full-term fetuses, apparently reflecting the final maturation of the regulation of the cardiac function by the ANS. On the other hand, in equines, this marked increase in the release of cortisol happens only during the last two days before birth and is yet to be associated with any increases in fetal heart rate variability [18].

The evaluation of the fetal HRV during the second and third months of pregnancy was compromised in this study due to the presence of artefacts with encountered in the species' ECG, which may be explained by the body fat of the mother, since the Dorper breed is primarily a meat breed, and by the gastrointestinal tract of the species. The artefacts may also be caused by the method employed to evaluate the HRV (Televet 100®), since the system is designed primarily for equines. Further studies employing other methods to evaluate fetal HRV or other breeds (milk breeds, lower body weight) may reduce the interference of artefacts and facilitate data collection, increasing our understanding regarding ANS activity during the fetal period.

Trenk *et al.* [40], in a study with bovines, observed that only 50% of the recording time in cows provided a constant and reliable signal that allowed the evaluation of fetal HR and HRV, and that this time increased to 60% in heifers. The authors believe that this is probably caused by the smaller size of the mother and the improved access to the fetus, and that, although fetomaternal electrocardiography consistently allow the detection of cardiac signals in bovines towards the end of the pregnancy, the technique was less reliable than in equines. According to the authors, in humans, the fetal HRV increases markedly after the 30th week of pregnancy, reflecting the maturation of the ANS.

In this study, data collection was considerably better during the fourth and fifth months of pregnancy, and, while it is possible to apply the method to sheep, there are several limitations. In equines, transcutaneous fetal electrocardiography (ECG tc) may be used to assess fetal viability in pregnant mares starting at the 150th day of pregnancy, but the acquisition and interpretation of the cardiac signals are compromised by artefacts caused by the position of the fetus, by the more evident maternal ECG signal, which masks the fetal signal, and by electronic noise. The interpretation of the fetal electrocardiogram is also limited by the small amplitude of the fetal ECG tc signal and by marked differences in HR on each fetus [20].

For the time-domain HRV indexes analyzed in the lambs, the minimum, mean and maximum HR decreased starting at the 21st day, while the duration of the RR intervals started increasing at the same time, which may reflect a balance between the ANS branches starting at this point. However, on the 60th day, mean and maximum HR increased again. We believe that may happened due to handling, since, as the animals developed, the handling became more limited and they became more agitated, exemplifying the limitation of working in the field.

Chiacchio *et al*. [4], in a study aiming to assess the HRV in Ile de France lambs through the Holter exam during the neonatal period, observed that the heart rate decreased progressively over the course of the study (from birth until 35 days old), and that the index PNN50 increased during this period, highlighting the fact that the maturity of the parasympathetic nervous system progresses with the development of the animal. On the other hand, Koether *et al*. [10], in a study aiming to assess the electrocardiographic parameters in Bergamasca lambs during their development, also during the same period evaluated by Chiacchio *et al*., observed that the HR decreased and the RR interval increased from birth until the 35th day, showing a negative correlation between age and HR.

The HRV indexes SDNN (standard deviation of RR intervals) and RMSSD (square root of the mean of successive differences between adjacent RR intervals) presented oscillations during the development of the animals, increasing and decreasing upon each period evaluated. Since the index SDNN (standard deviation of RR intervals) reflects the activity of both branches of the ANS and the index RMSSD (square root of the mean of successive differences between adjacent RR intervals) reflects parasympathetic activity [18], we believe that these oscillations are caused by the activity of both branches at the same time, aiming to maintain the autonomic balance. We also observed that, during the same 60-day period during which the heart rate increases again, the index RMSSD (square root of the mean of successive differences between adjacent RR intervals) also increased, revealing an increased parasympathetic activity during the period, probably to counterbalance the sympathetic stimulus resulting from the agitation caused by handling.

Starting at the 21st day, we observed that the index low frequency decreased while the index HF increased. This illustrates the fact that sympathetic activity remains dominant until this time, when the parasympathetic nervous system started being more active, resulting in an autonomic balance at this age group. We observed that the time-domain and frequency-domain indexes both presented significant differences between the 7th and 14th days, and between the 14th and 21st days, while allows us to infer that, in this age group, there is a transition towards parasympathetic predominance.

The more inadequate the function of the ANS, the more the organism tends to lose its chaotic behavior and default to linearity [11]. This also seems to be valid in newborns, particularly in those born before term, since the nervous system is not yet fully mature in this group. Age has a influence over this balance between the sympathetic and parasympathetic systems, and the higher the age, the lower the HRV. This relationship, however, inverts when considering young adults, probably due to the not fully developed ANS [34].

In this study, we did evaluate the HR and the HRV indexes regarding the circadian rhythm, but we believe this rhythm somehow influences the oscillations observed in the HRV and HR, as mentioned by Piccirillo *et al.* [28]. Kovács *et al.* [12], in a study assessing the behavior of the HRV indexes in bovines during the day and night periods, observed a predominance of sympathetic activity during active periods (day) and also during the summer, when both the HR and the spectral parameters presented a circadian rhythm.

In this study, the lambs were evaluated only during the summer and only during the day, so we believe that these factors, coupled with the high temperatures, may have influenced our results, except for those obtained 24 hr after birth since the animals were evaluated both during the day and during the night. In addition, as mentioned by Kovács *et al.* [12], environmental factors, diet and locomotor activity may have influenced the results. Further studies are need to evaluate the circadian rhythm in lambs during their development, as well as assess the behavior of the HRV in lambs and its relationship with the circadian rhythm.

For the interpretation of our results, we want to stress the importance of considering the stress factor involved, particularly in respect to the HRV indexes and the behavior of the HR. Vögeli *et al.* [41] studied the reactions of the sheep brain in the face positive and negative stimuli. They observed that, during the transition from negative to positive situations, there was a reduction in the number of movements of the sheep, in the rate of movements in the limbs, in the rate of asymmetric postures (reflecting behavioral changes), in the HR and RR (physiological changes), and that the activation of the frontal cortex was less evident, associated with a displacement towards the activation of the left side of the brain. The authors suggest that the emotional value should also be considered in farm animals, with this term meaning the emotional state attributed to a situation or stimulus varying between negativity and positivity.

The development of an artificial placenta has been the object of studies for over 50 years with limited success [33]. The main challenges faced were congestive heart failure due to the imbalance between the preload and afterload imposed on the heart of the fetus by the resistance of the oxygenator and by the circuits supported by the pump, as well as the use of open fluid incubators, resulting in contamination e fetal sepsis, and problems related to the umbilical vascular access, resulting in spasms [2].

Partridge *et al.* [26] have shown that premature-born lambs may be consistently supported by an extracorporeal device for up to four weeks without apparent physiological disorders or organ failure, and with adequate nutritional support the lambs presented normal somatic growth and pulmonary maturation, as well as adequate cerebral growth and myelination. Therefore, according to these results, translational research employing the species have gained prominence and resulting in advancements in the diagnosis and treatment of cardiovascular afflictions, as well as serving as a support for the artificial neonatal development, which results in improvements in the field of neonatology, promoting monitoring and early intervention for possible occurrences that could limit the development and the health of the fetus.

We have emphasized in this study the importance of performing the analysis of the fetal and neonatal HRV, since it has proven to be effective in the evaluation of the behavior of the ANS. It may also be employed to assess fetal viability and in cases involving afflictions that could limit the development of the fetus or neonate, considering the importance of providing assistance to the lambs during their development due to the series of physiological adaptations needed during the fetal-neonatal transition. Rivolta *et al.* [31] conducted a study aiming to assess the behavior of the fetal heart rate in cases of hypoxia and acidemia, observing that the higher the degree of acidemia, the more predominant the parasympathetic component of the autonomic nervous system became. Frasch *et al*. [5] reported that, in pregnant sheep, the index RMSSD (square root of the mean of successive differences between adjacent RR intervals) presented more marked alterations during acidemia, while Siira *et al*. [36] reported that, during cases of acute hypoxia without acidemia there is more sympathetic activity, while when there is acidemia, the vagal activity increases.

The activation of the ANS by initial and acute hypoxia probably constitutes a first-line adaptive response and is the result of a marked fetal heart rate modulation and increased cardiovascular response. In addition, the predominant involvement of the vagal tone has been associated to a more efficient modulation of the ANS [39]. In the presence of acute hypoxia, the reduction in the fetal heart rate, which is mediated by chemoreceptors through the parasympathetic branch, acts as a mechanism to protect the fetus, since it reduces the myocardial work and the consumption of oxygen. When the vagal regulation becomes inadequate, some adaptive mechanisms (such as circulatory adaptation mediated by chemoreceptors) may fail, leading to brain damage and ultimately death of the fetus [30].

Some inherent limitations were observed in this study. The small sample size may have contributed towards the non-significant results. The manual restraint used on lambs and mothers may have influenced the HRV indexes, since working on the field presents several limitations in data acquisition. Other methods of HRV analysis may also contribute towards illustrating the activity of the ANS during fetal and neonatal life.

In sheep, the maturation of the ANS appears to begin towards the end of the pregnancy, with a predominance of parasympathetic activity during this period. Despite the presence of parasympathetic activity during the last month of pregnancy, the complex interactions between both branches of the ANS still require further research. The HR decreases in lambs starting at the 21st day of life. These results show the importance of further researched regarding the activity of the ANS, since the detection of autonomic imbalances through the HRV may allow the investigation of probable system alterations and an early intervention fetuses and neonates, reducing the morbidity and mortality rates.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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