Cortisol levels in sheep

Does litter size influence cortisol levels in hair in ewes or their lambs?

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Introduction

Animal welfare has become increasingly important in the breeding, housing and treating of farm animals. Although Lawrence et al. (2008) suggested that a growing acceptance that animals are sentient (which means that animals are capable of having feelings, like humans are) may be an possible and partial explanation for this increase in attention to welfare, Lawrence and colleagues also explain that no single reason can explain these developments.
Because of this increased importance, recently more research has been done into animal welfare. Nevertheless, different conceptions of animal welfare make it a difficult field of research (Fraser 2008, 1).

One method to determine whether animal welfare is affected is to measure physiological stress responses of animals. Although the use of physiological measures does not reveal whether an animal is psychologically affected or not, they are commonly used to detect particular perturbations of animal welfare (Brydges and Braithwaite 2008). Different endocrine, behavioural and immunological variables are used to measure physiological stress. None of these are proven to be sufficient in all situations to assess whether animal welfare is at stake (Mench and Moberg 2000, 3). However, physiological indicators of stress are valuable tools in a research field as animal welfare (Möstl 2002, 67). Measuring cortisol levels is described as a reliable physiological indicator of stress (Mench and Moberg 2000, 3) and is therefore often used to determine whether an animal experiences stress or not. Cortisol has been described in cattle (Christison 1972, 1005), goats (Aoyama 2008, 116), horses (Visser 2008, 521), pigs (Turner 2005, 398) and sheep (Smith 2002, 75) to be a useable and reliable measure of the stress response.

There are several ways to measure cortisol. Measuring cortisol in plasma is one possibility (Ingram and Matthews 2000, 123). Cortisol levels in plasma may increase for many reasons, including pleasurable experiences. However, a doubling of cortisol plasma concentrates is considered to be suggestive for a reduced welfare (Reece and Erickson 2004). Salivary cortisol is another useful way to determine cortisol levels, as long as the researcher is aware of the possible causes of variance (Hellhammer 2009, 163). The measurement of cortisol in both plasma and saliva is likely to be influenced by the method of sampling. Taking such samples from an animal requires usually handling of the animal and can cause an acute stress response. As a result the reliability of the results will decrease.
Measuring cortisol in urine is possible as well (Möstl 2002, 67). An important advantage of this method is that it can be noninvasive when the urine is spontaneously obtained. Despite using urine samples to measure cortisol levels has proven to be effective and reliable, urinary glucocorticoids and catecholamines sum up only over several hours (Hay 1998, 119). Therefore using urine samples to determine the extent to which an animal is experiencing stress over a longer period is difficult.
Another option is measuring cortisol in feces (Palme 1996, 43). As with urine samples, taking samples of feces for analysis is a noninvasive method. However, with this method it is difficult to follow the animal for a longer period as well.

More recently cortisol measuring in hair samples has gained interest. It has been proven as a reliable method to measure cortisol levels in dogs (Bennett 2010, 171), pigs (Bacci 2014, 218), cattle (Comin 2013, 36), sheep (Stubsjøen et al. 2015, 25-31) and human (Russell 2012, 589; Kalra 2007, 103; Kirschbaum 2009, 32). Several advantages to using cortisol in hair as a biomarker of stress are described. Samples can be collected noninvasively, results are not influenced by sampling and the cortisol levels of the samples are representative for extended periods of time (Russell 2012, 589). Despite the fact that it is not possible to determine the exact moment the stress occurred, it is an useful method to measure average stress responses of longer periods of time.

For a long time, welfare of sheep was not an big issue in the public concern. Probably this was due to the 'natural' circumstances under which the animals were kept (Lawrence 2008). However, over the last decades welfare of sheep has become more important (Lawrence 2008; Phillips and Dwyer 2008). Due to pressure on economic efficiency farmers tend to seek maximize returns. Therefore it is very likely they try to maximize results regarding the litter sizes of their sheep (Lawrence 2008).
Breeders often strive for litter sizes of exactly two, at least as often as possible (SanCristobal-Gaudy et al. 2001, 249-272). However, due to losses during parturition and raising the lambs, farmers sometimes will strive for bigger litter size to achieve this goal (Reinard Everts, personal communication, may 23, 2017).
However, Schoenian & Burfening (1990) found in their research average litter sizes of Rambouillet ewes between 1.13 and 1.45 lambs per ewe. More recently, higher average litter sizes were reported: 1.38 in Texel sheep, 1.36 in Shropshire sheep, 1.55 in Oxford Down sheep and 1.48 in Suffolk sheep (Maxa et al. 2007, 312-317). In this article there has been no distinction between primiparous and multiparous, although it has been proven that multiparous ewes have bigger litter sizes than primiparous ewes (Owens et al. 1985, 359-372; Cloete 1993, 38-38; Smith 1977, 745-753; Dwyer and Lawrence 2000, 1391-1413; Sidwell and Miller 1971, 1084-1089). A publication of Wageningen University reports litter sizes of 1.3 lambs in primiparous Texel sheep, 1.8 in multiparous Texel sheep (Anonymous 2002). According to this publication Suffolk ewes give birth to 1.7 lambs per ewe, but it is unclear what the differences are between primi- and multiparous ewes. In addition to the influence of parity on litter size, differences in breeds also result in different litter sizes (Freetly and Leymaster 2004, 612-618). In table 1 the most recent data from The Netherlands regarding litter sizes in Texel sheep is showed, collected by the NSFO (Dutch sheep and goat breeding organization) (Reinard Everts, personal communication, may 23, 2017).

Year	Average litter size
2012	1,81
2013	1,79
2014	1,84
2015	2,01
2016	1,78

Table 1. Average litter sizes in Texel sheep in the Netherlands between 2012 and 2016

Over the past decades litter sizes of pigs have grown steadily and the concerns about the effects of these bigger litter sizes grew with it. (Rutherford et al. 2013, 199-218; Rutherford et al. 2011) Rutherford et al. (2011) showed that larger litters have negative effects (such as increased neonatal mortality, reduced piglet viability) on piglets. Emotionality, learning and memory in piglets during growing up may be affected by litter size as well, but that has not been proven yet (10). Although this is more uncertain, larger litters may have an impact on sow welfare as well (Rutherford et al. 2013, 199-218; Rutherford et al. 2011). These results suggests that bigger litter sizes are appealing for farmers, but have negative consequences for farm animals. Bigger litter sizes could be a possible stressor in sheep as well. Therefore the aim of this study is to determine whether bigger litter sizes (>1) induce a bigger long term physiological stress response (higher cortisol levels in hair) than smaller litter sizes (=1) in Swifter ovine ewes and their lambs.

Materials & Methods

This explorative study was conducted to investigate whether cortisol levels in wool could be used as an indicator for animal welfare. For this study we used hair of ewes and their lambs, which was collected in a non-invasive, pain free manner. Consequently, this study did not need approval by an ethics committee, according to Dutch law ("Wet op de dierproeven", the Dutch Experiments on Animals Act from 18 Dec. 2014, §1, article 1, 1b, 13a).

Animals and housing

For this research 126 sheep (*Ovis aries*) and 261 lambs of the swifter breed were used. This research was conducted at the Tolakker, the farm of the Faculty of Veterinary Science of Utrecht University. The flock of sheep is mainly kept for meat production, besides that the farm is also used for education and research purposes of the Faculty of Veterinary Science of Utrecht University.

The sheep were kept on grasslands during the year but were housed in stables during the lambing season. The herd was divided based on their expected lambing date into four successive batches. For this study the last three batches were used. The second batch was expected to give birth in the beginning of February, the third one in the beginning of March and the last one around half April, with three weeks between each group. Approximately four weeks before lambing all ewes were shaved. During the first part of the housing period the sheep were kept in a herd of approximately 50 individuals in a deep litter straw pen with a size of approximately 85m^2. After lambing they were kept in individual pens for approximately two days. After two days, the lambs were strong enough to re-join the other ewes and lambs in another pen.

Water and grass silage were available ad libitum. In addition, sheep were fed concentrate pellets two times a day. They also had access to a mineral lick stone. Each ewe had a collar number for identification.

Experimental design

For cortisol measurement the ewes and lambs were categorised by parity, as shown in table 2.

Litter size (lambs)	Primiparous ewes		Multiparous ewes		Total
	Ewes	Lambs	**Ewes**	Lambs	
1	**12**	12	**13**	13	50
2	**24**	48	**20**	40	132
3	**8**	24	**32**	96	160
4	**--**	--	**7**	28	35
Total	**44**	84	**89**	177	377

Table 2. Number of ewes which delivered lambs, and number of delivered lambs.

The lambs and ewes were shaved within 48 hours after giving birth. Disposable Prep Razor (Kai medical) were used to shave both ewes and lambs at their caudo-medial flank as close to the skin as possible (see fig 1 for location), resulting in hairs of approximately 1cm long. Approximately 4cm^2 was shaved, this surface provided sufficient wool for lab analysis. The wool of white and black lambs was used. The shaven wool was stored in the dark to prevent damage to the cuticle structure by UV-light, which leads to major cortisol loss. (Li 2012, 434) That is why it was packed in thick aluminium foil and the samples of all littermates and the mother ewe were kept together in a plastic re-sealable bag together with a completed form containing information about the ewes and labs (e.g. littersize, date of lambing, date of collecting and collar number of the ewe).

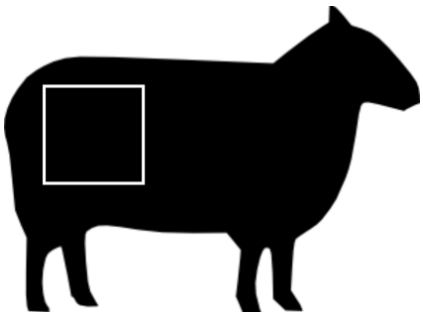

Figure 1. Approximate area from which a small sample of white or black wool will be taken.

In order to get rid of exogenous cortisol, samples were put into tubes. Then 20mL distilled water was added and the tubes were incubated on the rollerbank (Stuart) for three minutes at 30Hz. Next the water was drained from the tubes and 20mL of 80% Isopropanol (EMSURE) was added, after which it was again incubated on the rollerbank for three minutes at 30Hz. After cleaning, the wool samples were dried for seven days at

34°C in a stove and stored at room temperature in the dark until further analyses. The wool was cut into small pieces after which 35mg was used for further analysis. Three metal beads of 3.2mm (QIAGEN) were added to the sample, which was beadbeated in a TissueLyser II (QIAGEN) for 15 minutes at 30Hz. Afterwards the samples were centrifuged (VWR) at 17000G for five minutes. These two steps were repeated until all the wool in the tubes was turned into dust. In general, repeating these steps twice was sufficient. 1mL of Methanol (EMSURE) was added to the sample, the beads were removed and the sample was sonificated (Sonicor) for 30 minutes, followed by 24 hours on the rollerbank to extract the cortisol. After 24 hours the tubes were placed into the centrifuge for five minutes at 17000G after which 0,6mL of the supernatant placed into the Speedvac (Savant) for approximately three hours. The Methanol evaporates in the Speedvac and as a result only the cortisol remains. Afterwards 200µL of phosphate-buffered saline (PBS) from the ELISA kit was added, and the samples placed on the rollerbank for another 24 hours in order for the cortisol to dissolve. Next the ELISA was preformed according to the Protocol of the Salimetrics High Sensitivity Salivary Cortisol ELISA kit. The cortisol standard that was provided by the kit was used to calculate the cortisol values of our samples.

Retesting data

Because the first attempt to collect and interpret the cortisol values of these wool samples did not produce reliable and repeatable results, a new method to wash the samples was tried. In total, 108 samples (39 ewes and 69 lambs) were tried again with the new washing step. The other samples did not contain enough wool to repeat the procedure. The samples with enough wool were already processed like described before and were processed again with a new washing method.
For the new washing method, 20g Biotex Green (Unilever) was dissolved in 200mL distilled water. Biotex Green contains enzymes that break down stains consisting of fat, proteins, or starch. Then, the sample was placed in a water bath of 60ºC for one hour. Afterwards, 20mL distilled water was added and the sample was shaken with the hand for 5 seconds. Then, the water solution was thrown away and 20mL of distilled water was added and shaken with the hand for 5 seconds. This step was repeated 6 times. Then 20mL 100% n-hexane (J.T. Baker, VWR) was added and the samples were put on the Roller Bank for three minutes at 30RPM. In the end the same ELISA was performed as described before.

Statistical analysis

Statistical analysis were carried out with SPSS (version statistics 24). Statistical analysis were separately performed for ewes and lambs. Based on the Shapiro-Wilk test and visual analysis of Q-Q plots, the data was verified whether it was normal distributed or not.

One-way ANOVA in ewes

Ewes from several groups were compared in both an unpaired T-Test and a one-way ANOVA test. Cortisol values from ewes with one lamb were compared to cortisol values from ewes with multiple lambs in an unpaired T-test. Besides this test, cortisol values of

ewes with one lamb were also compared to cortisol values from ewes with two lambs and ewes with more than two lambs separately in an one-way ANOVA test.

One-way ANOVA in lambs

Due to the fact that the cortisol values of lambs were not independent, but lambs from the same litter had the common influence of the mother, a correction was performed before a one-way ANOVA test was carried out. First, the cortisol values of the ewes were subtracted from the cortisol values of the lambs of the litters of these ewes. We assumed that the influence of the ewes on the cortisol values of the lambs was eliminated this way. Then these differences were checked whether they are normally distributed or not, by the Shapiro-Wilk test and visual analysis of Q-Q plots. Finally, the lambs of litter sizes of one lamb were compared to the lambs of litter sizes of two and more than two lambs with an ANOVA test.

Results

The data of 108 samples was categorised in 6 categories: ewes with singletons (n=8), singleton lambs (n=7), ewes with twins (n=18), twin lambs (n=30), ewes with triplets and quadruplets (n=13) and lambs from litters of three and four (n=32). Because the ewes were compared to each other and the lambs to each other as well, statistical analysis for normality was done for ewes and lambs separately. According to the Shapiro-Wilk test the data was normal distributed for both the ewes (p=0,521) and lambs (p=0,082). This is also visual confirmed with the Q-Q plots (figure 2).

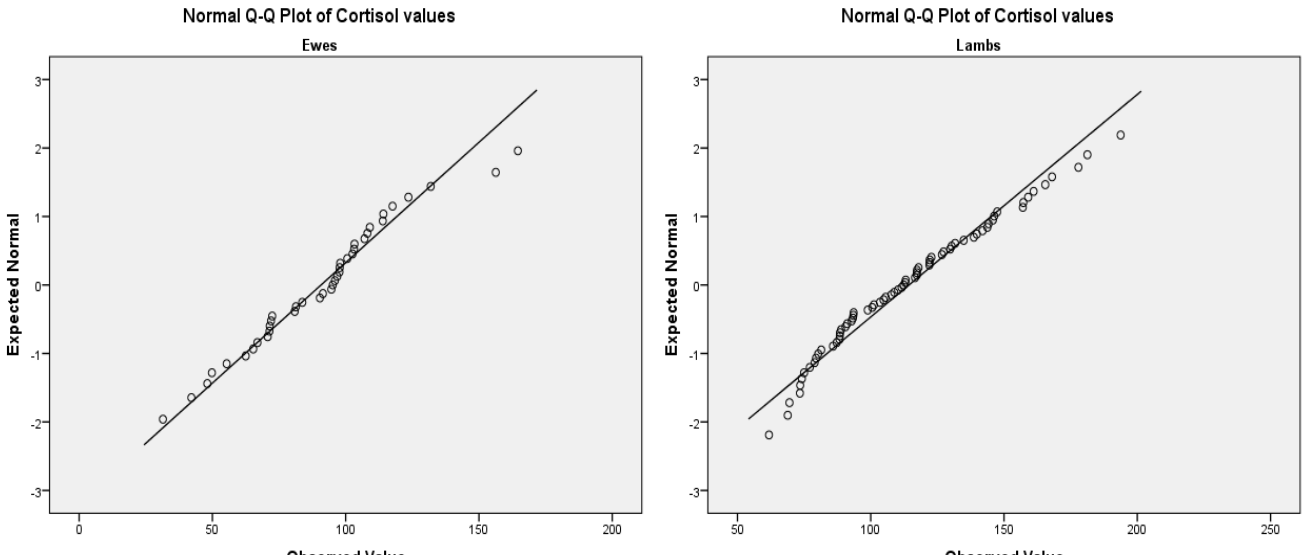

Figure 2. Normal Q-Q plots of cortisol values of ewes and lambs

The means of ewes and lambs, and their standard mean of error are shown in table 3 and figure 3.

	Mean cortisol values of ewes	SEM of mean cortisol values of ewes	Mean cortisol values of lambs	SEM of mean cortisol values of lambs
Singletons	77,24	10,12	99,51	10,53
Twins	96,26	7,93	113,76	5,52
Triplets and quadruplets	91,42	4,89	118,21	5,61

Table 3. Mean cortisol values (pg/µg) with the standard error of the means

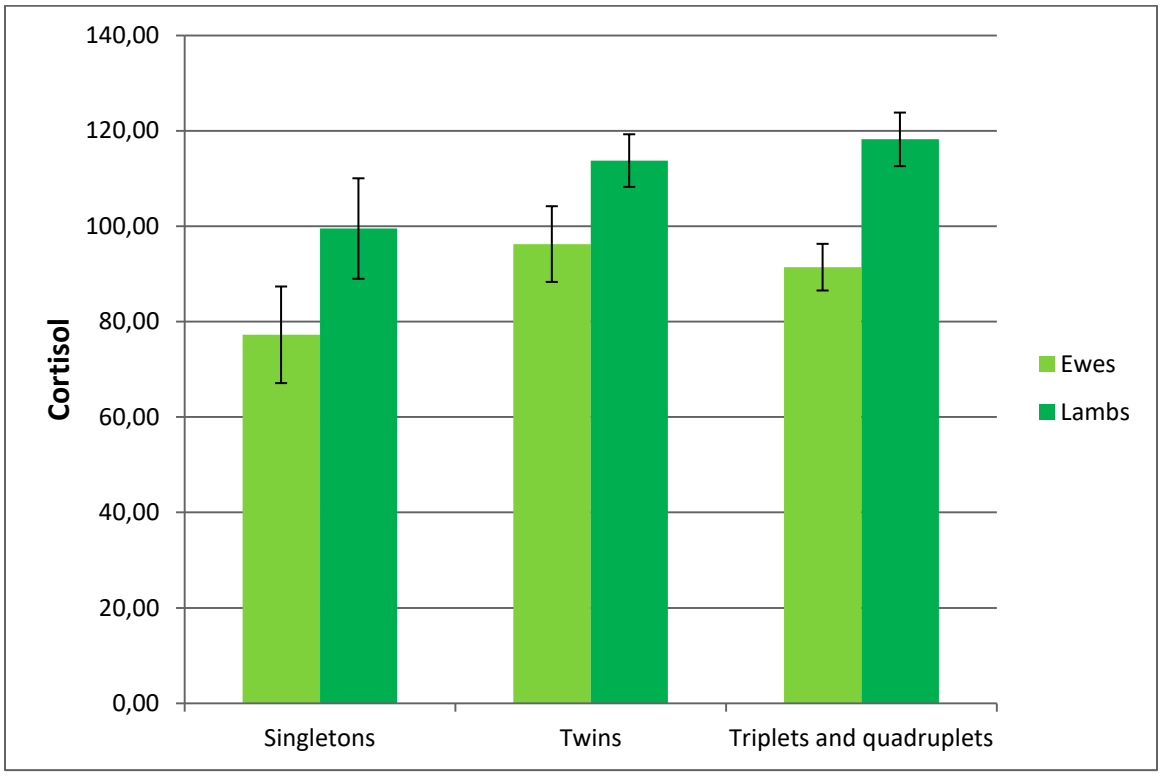

Figure 3. Mean cortisol values with the standard error of the means

Ewes

A one-way ANOVA was conducted to compare the effect of litter size on cortisol values in ewes. Three groups were compared: ewes with one lamb (n=8), ewes with two lambs (n=18) and ewes with more than two lambs (n=13). The differences in the mean cortisol values between the groups ewes and the corresponding standard errors of means are shown in table 4. However, according to the one-way ANOVA, these differences were not significant [$F_{(2, 36)} = 1,26$; $p = 0,29$].

(A) Litter size	(B) Litter size	Mean Difference (A - B)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-19,02	12,00	,122	-43,37	5,32
	>2	-14,18	12,69	,271	-39,92	11,57
2	1	19,02	12,00	,122	-5,32	43,37
	>2	4,85	10,28	,640	-16,01	25,70
>2	1	14,18	12,69	,271	-11,57	39,92
	2	-4,85	10,28	,640	-25,70	16,01

Table 4. Comparisons of mean cortisol values (pg/µg) between ewes

Lambs

Because the data of the lambs was dependent on the ewes, a correction was performed. The cortisol values of the ewes were subtracted from the cortisol values of the lambs of the litters of these ewes. Thereby, the differences in cortisol values of ewes and lambs are found. We assume that therefore the influence of the ewe on the her litter is eliminated. These differences are according to the Shapiro-Wilk test normal distributed (p=0,763). This is also visual confirmed with a Q-Q plot (figure 4).

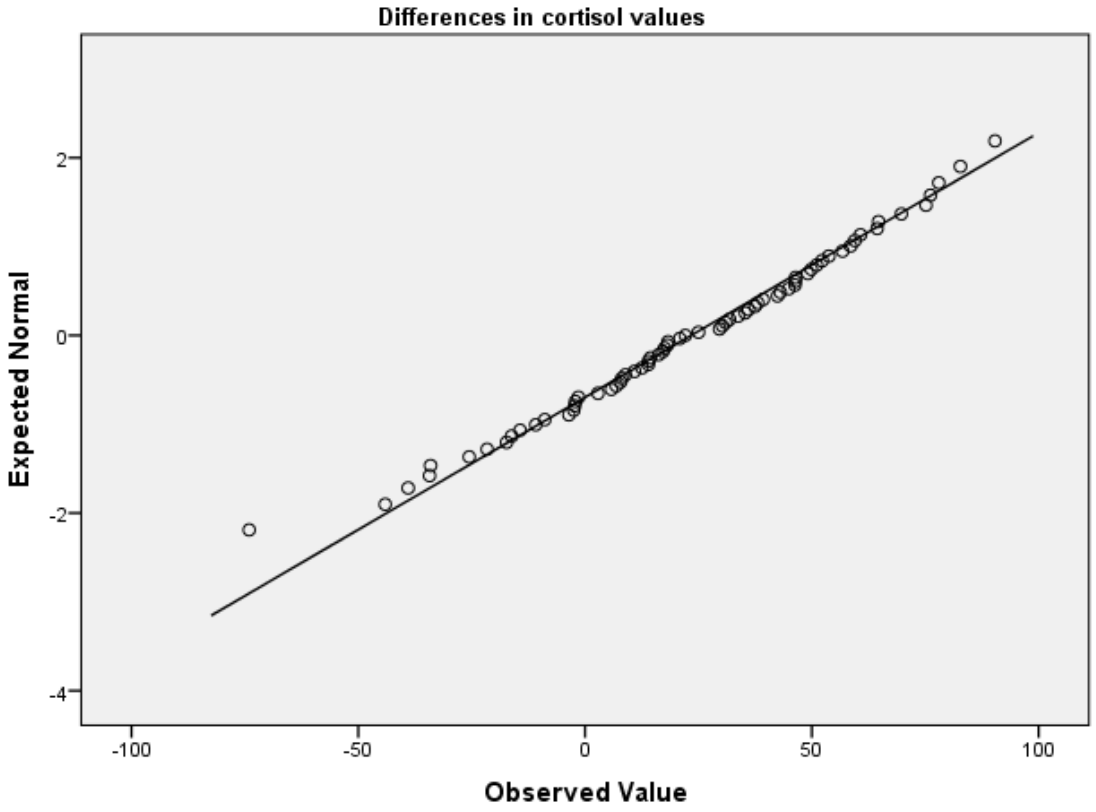

Figure 4. Normal Q-Q plots of the difference between cortisol values of lambs and cortisol values of their mothers

The mean differences and their standard error of mean are shown in table 5 and figure 5.

	Mean differences in cortisol values between lambs and ewe	SEM of mean differences in cortisol values between lambs and ewe
Singletons	18,35	11,18
Twins	20,00	7,21
Triplets and quadruplets	27,69	5,03

Table 5. Mean differences in cortisol value (pg/µg) between ewes and lambs with their standard errors of means

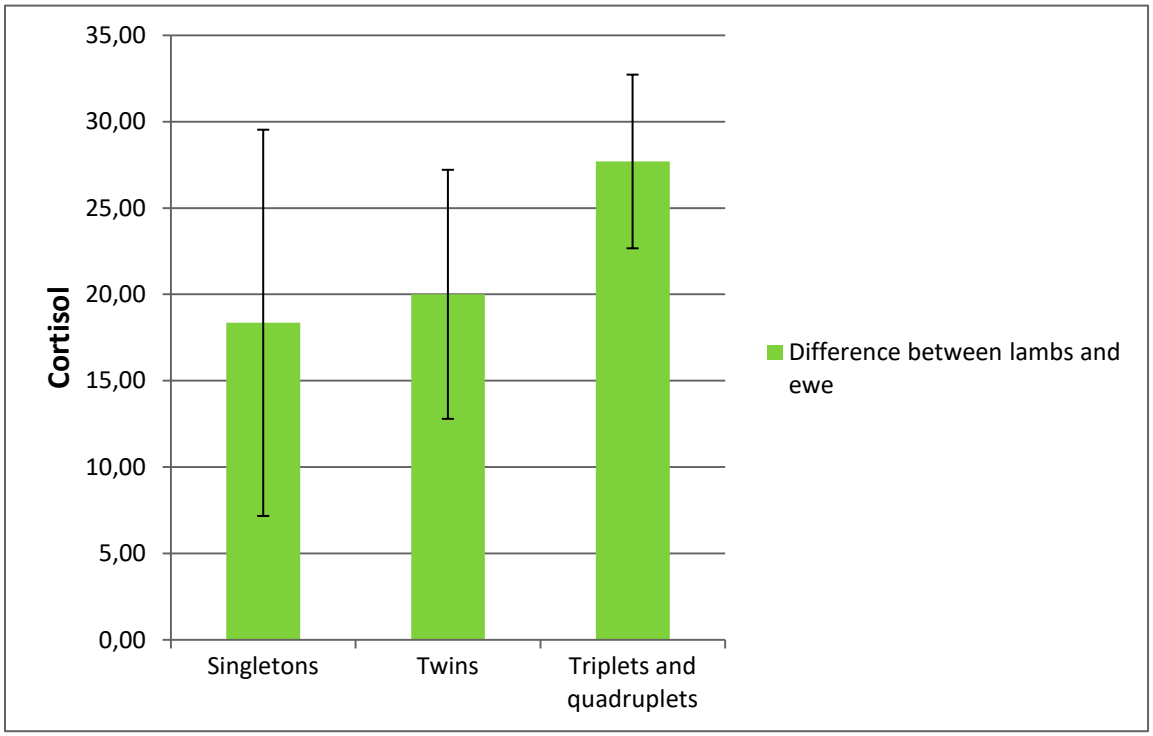

Figure 5. Mean differences in cortisol value between ewes and lambs with their standard errors of means

Because this data was normally distributed, a one-way ANOVA was conducted to compare the effect of litter size on the difference between cortisol values of lambs and cortisol values of their mothers. The means of these differences and their standard error of means are shown and table 6. However, according to the one-way ANOVA, these differences were not significant [$F_{(2, 66)} = 0,487$; $p = 0,62$].

					95% Confidence Interval	
(A) Litter size	(B) Litter size	Mean Difference (A-B)	Std. Error	Sig.	Lower Bound	Upper Bound
1	2	-1,65	14,20	,908	-29,99	26,69
	>2	-9,34	14,11	,510	-37,51	18,84
2	1	1,65	14,20	,908	-26,70	29,99
	>2	-7,68	8,60	,374	-24,85	9,47
>2	1	9,34	14,11	,510	-18,84	37,51
	2	7,69	8,60	,374	-9,47	24,85

Table 6. Comparisons of mean differences in cortisol values (pg/µg) between lambs

Discussion

In earlier research, an ELISA kit, designed for cortisol quantification in saliva, was used to determine the cortisol concentrations in hair of rhesus macaques. (Davenport et al. 2006, 255-261) The same ELISA kit was used in this study. However, in other studies in which cortisol concentrations in wool of sheep was investigated, different ELISA kits were used. (Stubsjøen et al. 2015, 25-31; Salaberger et al. 2016, 73-78) Possibly, the method we used in this study might not be the best available method to measure cortisol concentrations in wool.

Earlier in this research project, similar analyzes have been made about the hypothesis that litter size may influence cortisol levels in wool of sheep. (Siemons 2016) In that study, another washing method was performed, followed by the same ELISA used in this study. Unfortunately, these results turned out to be not repeatable. In this study the samples were tested again, but with a different washing method, as described in Materials and Methods. However, this time the samples were not retested to verify if the data was repeatable. In a short trial to compare different washing methods, some samples were tested in the same way the samples were tested in this study. (Bentvelzen 2017) No statistical analysis have been made to compare these results to the results of this study. Therefore it is not sure if the results from this study are repeatable and thereby reliable.

As mentioned before, a different washing method was chosen after a trial to compare different washing methods. (Bentvelzen 2017) This trial showed that this washing method yielded the highest cortisol values of the tried and tested methods and was therefore selected for further analysis. It is clear that the reliability of this washing method must be confirmed. This has not been done yet, but is still ongoing. Until this is finished, the reliability of the results in this study are questionable.

Because this study was performed with the same samples as collected earlier in this research project, but with a different washing method, not all the samples collected in the first place could be used in this study. Due to some technical issues only 108 of 387 animals could be retested. (Bentvelzen 2017) That is the reason some groups became quite small. This study contained only one ewe with quadruplets. This ewe and her lambs were merged with the group ewes with triplets, to produce some reliable results. The group of singletons existed of eight ewes and seven lambs. This may have resulted in distorted results. A study with more animals in these groups is therefore advisable.

The differences in cortisol values found between groups in this study were not large and not significant. As mentioned before, that could be due to the methods used in this study. Other methods could give higher differences. However, these small differences could be relevant. In this study, sheep of the swifter breed were used. Although there is no scientific evidence, swifter sheep are possible more resistant to stress factors than other breeds. Among other things, good nursing qualities and a calm character are breeding goals for swifter ewes. (Bosgoed 2017) Structural selection for this goals could lead to more stress resistant animals. Similar studies in other breeds could give other results. In that case, small differences in swifter sheep could be clinically relevant.

However, it is possible the factors mentioned above, did not have such a large influence on the results of this research and therefore the results could be reliable. In that scenario the cortisol levels in wool of sheep are not influenced by litter size during gestation. Therefore, litter size does not seem to affect the stress levels in ewes, nor in lambs. However, that may not be entirely correct. Cortisol levels in wool are a parameter for long-term stress responses. As mentioned before, it shows in lambs the stress response from the latest period of the gestation to the time of shaving, and in ewes the stress response between shaving the first time and the second time. Therefore it does not say something about the individual moments in which stress responses may be higher in bigger litter sizes. Although it is very well possible ewes and/or lambs are sometimes exposed to a higher stress response, due to litter sizes, this research showed that these stress responses are not high enough to cause higher wool cortisol levels. Based on the results in this research it is safe to assume that litter size does not influence cortisol levels in hair in ewes or their lambs. However, for more certainty this research should be repeated and perfected.

Recommendations for further research

Approximately four weeks before parturition the ewes were brought inside. At that time the whole group was shaved. This exact timing was not the same in de different groups. Within 24 hours after parturition the wool samples were collected from both the ewe and the lambs. This was done by several different persons. Therefore the exact ways in which the samples were collected could differ from each other. A more standardized study design is therefore necessary in further research.

The data used in this study was retrieved from animals of one commercial farm. Stress factors may differ amongst different farms. Therefore it might be useful to compare sheep from different farms to each other.

Another important factor is to do some research to baselines of cortisol levels in sheep. Possibly, the baseline of cortisol levels in wool differs between sheep. That could influence study results, when not taken into account. When baseline cortisol levels in wool are established, it could be easier to interpret differences in cortisol values after pregnancy. Sheep with bigger litter sizes could have higher increased cortisol values in wool than sheep with smaller litter sizes.

As mentioned before, the type of breed could influence the effects of stress factors on the changes of cortisol values in wool. Therefore it is important to do more research to cortisol values in different breeds. It could support the results of this study if it turns out that swifter sheep are less likely to have increased cortisol values in wool.

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