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Sheep as an Experimental Model: Individual Housing Allowing Visual, Auditory, Olfactory and Tactile Contact is not an Obstacle for Studies Involving Hormonal Interrelationships

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Summary

Thirty-two ewes were used to determine whether individual housing, allowing contact with neighbours, induces a stress response. Ewes were housed in individual pens designed to allow the ewes to see, hear, smell and touch adjacent animals, and were distributed into four groups (n=8/group): ewes with subcutaneous implants containing melatonin and oestradiol (M+E), melatonin (M), oestradiol (E) and non-implanted control ewes (C). Heart rate, stress indicators (plasma cortisol, glucose, lactate and creatine kinase (CK) concentrations) and luteinizing hormone (LH) concentrations were measured hourly and compared with the resting values (before and after pen housing). Heart rate increased significantly during the introduction into the pen (P<0.001) in all groups, in comparison with the resting values. No significant differences between groups were observed for cortisol concentrations, with the exception of the M group, which showed the highest response (P<0.001) when ewes were introduced into the pens. Lactate, CK and glucose changes in comparison with the resting values were similar between groups. LH concentrations during pen housing decreased significantly in all groups in comparison with resting values. In conclusion, individual confinement of sheep allowing visual, auditory, olfactory and tactile contact with their neighbouring animals was not an obstacle for investigating particular hormonal interrelationships with multiple sampling procedures. However further investigations are required to determine if this conclusion applies to other hormone systems in sheep.

Introduction

The use of large animals as experimental models has allowed progress in the understanding of some human physiological and pathological mechanisms. They can provide larger volumes of sampling material (blood, urine, faeces), and with greater frequency, than small mammals (*Arney, 2009a*). Large animals have a much longer lifespan than small mammals, which may be of interest for long term studies. In particular, sheep (*Ovis aries*) are attractive animals for medical, veterinary and fundamental biological research: they are docile, rarely show aggression and are gregarious (*Arney, 2009b*). The biomedical applications of sheep as models for human diseases have been reviewed by Scheerlinck *et al.* (*2008*). Experimental protocols involving animal models usually include procedures that may have the potential to cause pain or distress to the animals. The response to stress depends on several factors but one of the most important is the nature of the stressful stimuli (*Parrot et al., 1994*). In commercial sheep management practices, transport, manipulation, shearing or health management can induce the stress response of the

animals (*Barnet & Hemsworth, 1990*). These practices can also affect reproductive performance of the ewes (*Dobson et al., 2012*). The nature of the stressor to which an animal is exposed should be considered when studying the endocrine response to adverse stimuli (*Parrot et al., 1994*). A stressful environment elevates cortisol concentrations and this could affect the pulsatility of luteinizing hormone (LH) release with the consequent reduction of oestradiol secreted by dominant follicles, preventing or delaying the pre-ovulatory surge (*Breen & Karsch, 2004*). Elevated plasma ACTH/corticosteroids concentrations have been shown to reduce significantly the concentration of follicular LH receptors, cause unusual pathological changes in follicles and corpora lutea, and inhibit ovulation in ewes (*López-Diaz & Bosu, 1997*). This situation could negatively affect the development and functionality of the oocyte, and the viability of the future offspring (*Dobson et al., 2012*).

Individuals from gregarious domestic species can become highly stressed if they are isolated from the social group. In sheep, confinement and isolation cause an elevation in the cortisol concentration which is much higher than with restraint (*Parrto et al., 1994*). The usual handling associated with frequent blood sampling for hormone analysis includes spatial isolation in a pen, jugular venous catheterization and close human contact. Some studies on sheep have found that social isolation induced pronounced physiological stress responses, including acceleration of the heart rate and increase in plasma cortisol concentrations. Increased heart rate has been recorded in relation to visual isolation of ewes (*Baldock & Sibly, 1990*) and restraint of the animal (*Palestrini et al., 1988*). Moreover, it has been demonstrated that if one ewe is prevented from seeing and smelling her flock mates, it causes a rise of the cortisol concentration, which can be maintained for at least six hours (*Dobson et al., 2012*).

Individual housing of sheep has been a frequent practice in our studies, especially when control of individual food consumption is required, or a frequent bleeding regime to measure pulsatile hormones, particularly LH, is necessary (*Lozano et al., 1998; Abecia et al., 1996, 2002; Forcada et al., 1997, 2002, 2003, 2007; Sosa et al., 2009*). It is important to note that although we kept animals isolated from their flock mates, the design of the pens used in these experiments allowed full visual, auditory, olfactory and tactile contact with adjacent sheep. Under these constraints, it is logical to raise the question as to whether or not this practice could affect the results of these studies. Treatment with melatonin has been part of the experimental procedures of our studies, in order to determine the effect of this hormone on LH release under different nutritional treatments or social environments. Some authors have proposed the hypothesis that some of the positive effects of melatonin could be affected by a more efficient stress response of the animals treated with this hormone (*Chuang et al., 1993*), or diminishing the endocrine and behavioural impact of social isolation in ewes (*Guesdon et al., 2013*).

The aim of this study was to determine whether or not individual housing which allows visual, auditory, olfactory and tactile contact with flock mates produces a stress response, and if this response could be affected by exogenous hormones. This is of particular importance when hormones under study are able to modulate the physiological adaptive syndrome *per se*.

Material and Methods

The study was conducted at the experimental farm of the University of Zaragoza, Spain (41°N). All procedures were approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza (Institutional Review Board/Independent Ethics Committee number IRB00006869; Office for Human Research Protections number OHRP IORG0005699). The care and use of animals were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Animals and experimental procedures

Thirty two sexually mature Rasa Aragonesa ewes were used, with a mean (± SD) weight of 59.2±7.6 kg and a mean (± SD) body condition (score from 0 to 5; *Russel et al., 1969*) of 3.10±0.47. These animals had not been used previously for experimental purposes. Animals were ovariectomized in the first week of August under deep anaesthesia at the Veterinary Hospital of the University of Zaragoza. Ewes were housed in an uncovered communal pen without supplementary light, and always in total absence of males. In mid-October, 16 ewes (8 from the melatonin treated group and 8 non-treated with melatonin ewes) received a subcutaneous silastic implant (length: 1.5 cm; internal diameter: 3.3 mm; external diameter: 4.6 mm) (*Karsch et al., 1973*) containing crystalline oestradiol (Sigma-Aldrich Química S.A., Madrid, Spain). To prevent an initial peak of ster-

oid release, implants were pre-soaked in water. One week after oestradiol implantation, 16 ewes received a single subcutaneous implant containing 18 mg melatonin (Melovine®, CEVA Salud Animal, Barcelona, Spain) (eight of them had been previously implanted with oestradiol). These implants were designed to maintain high plasma melatonin concentrations for at least 90 days. Thus, animals were distributed into four groups: ewes implanted with melatonin and oestradiol (group M+E, n=8), ewes implanted with melatonin (group M, n=8), ewes implanted with oestradiol (group E, n=8) and non-implanted control ewes (group C, n=8).

On the 10th December, we housed the ewes in individual pens (2 x 2 m). Wall pens (height 1.5 m) were made with 5 iron bars (length 2 m), so that ewes could see, hear, smell and touch their adjacent sheep (between 3 and 5 depending on cage's position). Pens were elevated on a slatted floor with automatic cleaning of manure. They were provided with individual food and water bowls. Heart rate, stress indicators (plasma cortisol, glucose, lactate and creatine kinase (CK) concentrations) and plasma LH concentrations were measured at seven occasions through the experimental period: 1) in the communal pen, resting before uploading animals to the pens (Rest Before), 2) just when they were introduced into the pens (Ascent), 3) after 1 hour in the pen (Pen 1 h), 4) after 2 hours in the pen (Pen 2 h), 5) after 3 hours in the pen (Pen 3 h), 6) after 4 hours in the pen (Pen 4 h), and 7) resting 1 hour after returning the ewes to the communal pen (Rest After). Blood samples were obtained by jugular venous catheters, which were inserted the day before sampling by the same trained team. Catheters were provided with a 3-way stopcock with one male luer-lock port and two female luer-lock ports, so that heparinized saline prevented coagulation of the catheters. Local anaesthesia was used for the catheterization procedures. Ewes were uploaded one by one to their pens, and immediately, the first blood sample was collected from the first ewe. After that, the second ewe was uploaded and sampled, followed by the other animals in the same order as before. This stratified procedure was followed throughout the whole sampling period. Identically, at the end of the penned period, the first ewe was downloaded to the group pen, and sampled, then the second ewe and so on until the last animal. Plasma was separated by centrifugation and stored at –20° C until analysis.

Heart rate monitoring

Heart rate (beats per minute) was recorded using a Polar Sport Tester monitor (Polar S610 tm, Polar Electro Oy, Finland), which was placed onto each animal the day before monitoring. The transmitter was attached to a girth belt supplied by the manufacturer for use in humans (model S-160) and adapted to sheep with a neoprene strip. One electrode was placed behind the scapula and the other electrode was situated on the ventral abdomen. The receiver (codified for each ewe) was attached to the belt on the back of the animal. To improve the reception of the signal, the electrodes were impregnated with ultrasound gel. The heart rate signal was telemetrically transmitted within a range of 1 m to the receiver. The monitor calculated heart rate based on a pulse to pulse time-averaging algorithm at 5, 15 or 60 sec intervals (*Seaward et al., 1990*). In this particular study the signal was recorded every 5 sec. Data were downloaded to a computer at the end of the study.

Hormonal and metabolite assays

Plasma glucose (mmol/L) and CK (IU/L) concentrations were determined with a Multichannel Technicon Analyser (RA-500), using reagents for RA Technicon systems (Bayer Diagnostics, Spain) (glucose, Ref. T01-1492-56; CK Ref. T01-1885-01). Plasma cortisol concentrations (nmol/L) were determined in duplicate by a single enzyme immunoassay (EIA) (*Chacon-Perez et al., 2004*). The concentration of lactate (mmol/L) was determined in fluoride oxalate plasma using a Sigma Diagnostic Kit (Lactate 735-10) and a spectrophotometer (Lamda 5, Perkin Elmer). Plasma LH concentrations (IU/L) were measured using a simple sandwich EIA on 96-well polyvinyl microtiter plates (Valares et al., 2007). The intra-assay coefficients of variation were 10, 8, 14, 5 and 7% for glucose, CK, cortisol, lactate and LH, respectively.

Statistical analysis

Data were analysed using least square techniques to determine the influence of the fixed effects included in the model. The general representation of the model used was: $y = Xb + e$, where y was an N×1 vector of records, b denoted the fixed effect in the model with the association matrix X and e was the vector of residual effects. Data were presented as least square means ± standard error (SE). The main effects were the treatment, with four concentrations (M, E, M+E and C) and the seven sampling times. After testing that the interaction effects were not significant, they were removed from the full model. The analysis was

performed using the PROC MIXED procedure of SAS statistical software package.

Results

The highest mean heart rate values throughout the experiment were observed in the E group, being significantly different to that obtained by the other groups at most time points (P<0.001) (Figure 1). Heart rate increased significantly during the introduction to the individual pen (P<0.001) in all the groups, in comparison with the resting values, both before uploading to the pens and at the end of the

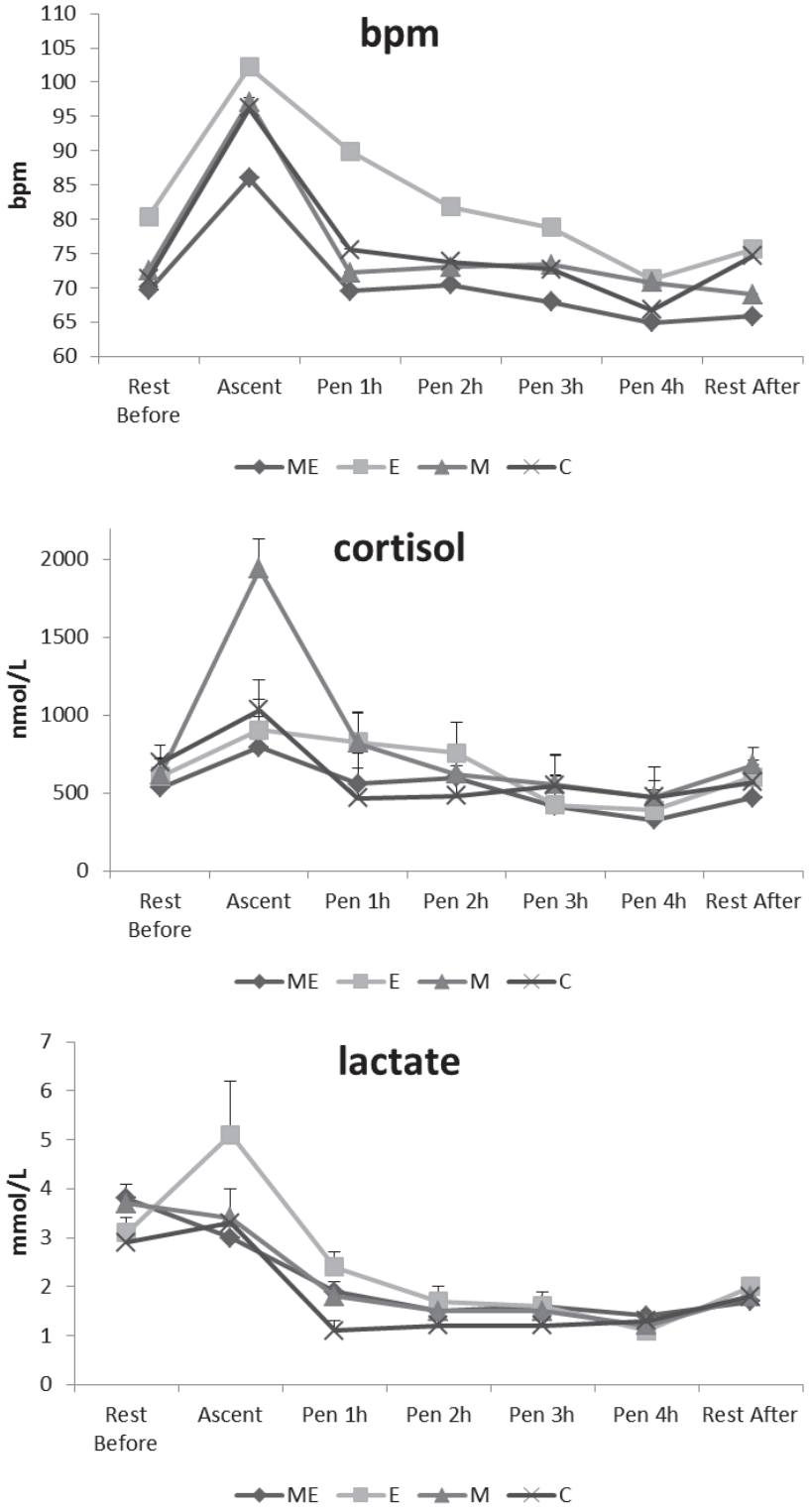

Figure 1. Least square means (±SE) of heart rate (bpm), cortisol (nmol/L) and lactate (mmol/L) in the communal pen, resting before uploading animals to the pens (Rest Before), just when they were introduced into the pens (Ascent), after 2, 3 or 4 h in the pen (Pen 1 h, 2h, 3h, 4h) and resting 1 hour after returning the ewes to the communal pen (Rest After), of ovariectomized Rasa Aragonesa ewes treated with melatonin (M) and/or oestradiol implant (ME, E) or not treated (C).

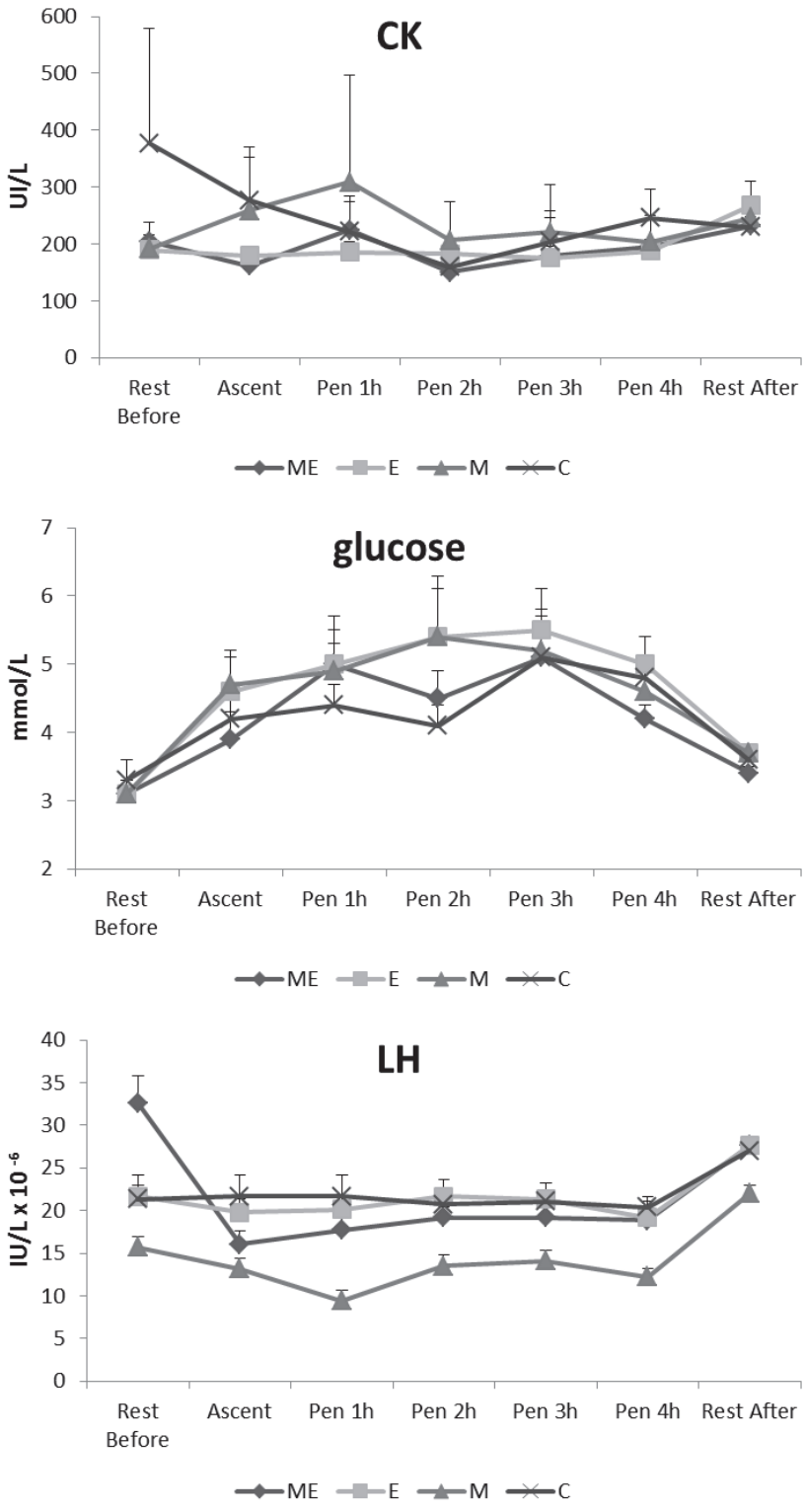

Figure 2. Least square means (±SE) of creatine kinase (CK) (UI/L), glucose (mmol/L) and LH (IU/L) in the communal pen, resting before uploading animals to the pens (Rest Before), just when they were introduced into the pens (Ascent), after 2, 3 or 4 h in the pen (Pen 1 h, 2h, 3h, 4h) and resting 1 hour after returning the ewes to the communal pen (Rest After), of ovariectomized Rasa Aragonesa ewes treated with melatonin (M) and/or oestradiol implant (ME, E) or not treated (C).

pen period. The relative increment of heart rate when ewes were uploaded to the pens was lower in the M+E and E groups and higher in M and C groups. These last two groups showed significant differences in Pen 1h and Pen 2h time and during the rest period post pen housing ($P<0.05$).

No significant differences between groups were observed for the cortisol concentrations throughout the experiment (Figure 1), with the exception

of the M group, which showed the highest response (P<0.001) when ewes were introduced into the pens. The variability between groups in the cortisol concentrations was higher during the introduction to pen (more than 1000 nmol/L) than during the end of this stressor action (213 nmol/L).

The lactate concentrations at the beginning of the stressor action were similar between groups, although slightly higher concentrations were observed in the E group (Figure 1). At the end of the stressor action the differences disappeared. Regarding CK values, no significant differences between groups were observed (Figure 2). Introduction to the pen did not provoke any increment of plasma CK concentrations. The glucose concentration profile during the experiment was similar in the four groups, values increasing up to 3 h after uploading the animals into the pens and decreasing at the end of the experiment (Figure 2).

At the beginning of the experiment, the M+E group presented the highest LH concentrations, being significantly different to the other groups (P<0.001). Except in the E and C groups, occupation of the individual pens had a reducing effect on LH concentrations, which significantly decreased after 1 h in the pen. This reduction was shown later, since no significant differences in the LH concentrations were observed between hours 2, 3 and 4 compared with the 1 h concentrations in groups, except in the M+E group. In the M group, LH release significantly increased during the rest post pen than at the beginning of the experiment, showing an increase of 39% in comparison with the initial concentration.

Discussion

The initial rise of some the physiological stress-indicators, coincident with the introduction to the pen and during the first moments of isolation, demonstrates that the isolation conditions in the present experiment induced a certain degree of stress in the short term. The heart rate results show that the critical period was the upload to the individual pen in all groups although it was likely due to the exercise associated with being placed in the individual pens; unfortunately, no behavioural indicators of stress were recorded to confirm this observation. It has been proposed that the increased heart rate may be a reaction to handling rather than to separation per se (*Piccione et al., 2011*). Also, this could be explained by the fact that sheep, which are a gregarious and relatively defenceless species, show an innate and immediate response in a dangerous situation. In this case, the initial resistance of the animal to handling could constitute a situation of agitation corresponding to a notable increase in heart rate (*Baldock & Sibly, 1990*). In gregarious animals, behaviour among flock members is highly synchronized. When the amount of available space increases, sheep adjust the distance between themselves to maintain group cohesion (*Sibbad et al., 2000*). Extra activities are needed (i.e. locomotor) to maintain social aggregation and hierarchy in small spaces like stalls (*Piccione et al., 2011*).

The lower values of heart rate at the end of the stage in the individual pens (Pen 4 value) could be interpreted as a period of quietness of the animals due to a combination of the protection offered by a less variable environment (*Baldock & Sibly, 1990*), such as the visual, tactile and olfactory contact with social companions during the stage in the pen (*Gelez & Fabre-Nys, 2004*), and an habituation effect to the stressor stimulus (*Smith and Dobson, 2002)*. Thus, Hopster & Blockhuis (*1994*) showed that heart rate arousal reflects the locomotor activity more than a stress reaction *per se* when isolated cows are free to move. In relation to management and human presence, some studies have shown that sheep can habituate themselves more easily to the presence of people than to a particular situation. In addition, the animals used in this study had been housed on the experimental farm for at least six months before the experiment, and have had repeated tactile, visual and auditory contact with humans. In this context, it was reported (*Hargreaves & Hutson, 1990*) that this permanent human contact reduced the heart rate response of sheep in relation to an approaching human.

The E group showed higher heart rates than the other groups throughout the experiment. An increase of 15-29% in heart rate in 17-ß oestradiol treated ovariectomized ewes compared to an untreated group has been reported (*Evans et al., 1988*). In the same way, a 1 µg/kg intravenous treatment of oestradiol has been associated with increased cardiac output and heart rate in ovariectomized ewes (*Magness & Rosenfeld, 1989*). The lower heart rate presented by the M+E group in comparison with the E group could indicate that melatonin mitigates the stressor effect of isolation on heart activity or the heart stimulation produced by oestradiol itself. In fact, it has been demonstrated that melatonin decreases heart rate (*Hussein et al., 2007*) and blood pressure (*Koziróg et al., 2011*) in men, and the administration of melatonin in rats produces a dose-related fall in mean arterial pressure and heart rate (*Chuang et al., 1993*).

No significant differences between groups were observed for the cortisol concentrations, with the exception of the M group, which showed the significantly highest response at introduction to the pen. An elevated adrenocortical response recorded in the serum corticosteroid concentrations 15 h after an intraperitoneal injection of 100 μg of melatonin in male rats has been reported (*Weidenfeld et al., 1993*), indicating that this hormone could directly affect plasma cortisol concentrations. This effect was not observed in the M+E group, indicating that the presence of exogenous oestradiol may neutralize the potential effect of melatonin on the adaptation process.

Tilbrook *et al.* (*2000*) observed a decrease in LH pulse frequency and amplitude during an isolation/restraint stressor in ovariectomized oestrogen implanted ewes during the breeding season. In our study, in ovariectomized ewes without oestrogen implants no differences in LH pulse concentrations were observed. However, the comparison of the current results to more frequent sampling periods, which are required to investigate LH pulsatility and amplitude, should be carefully considered. A decrease in the LH release (pulse frequency and amplitude), during 4 h of transport in ovariectomized ewes with or without prior steroid exposure at mid-breeding season has been reported (*Dobson et al., 1999*). Rivier & Rivest (*1991*) suggested that increased concentrations of circulating corticosteroids do not represent the sole modulator of stress-induced inhibition of LH secretion. On the other hand, cortisol suppresses pulsatile LH secretion by inhibiting pituitary responsiveness to GnRH, rather than by suppressing hypothalamic GnRH release in the ovariectomized ewe (*Breen & Karsch, 2004*). It is likely that the initial rise of cortisol concentrations observed at introduction to the pen could be responsible for the plasma LH decrease in the M group. However, the prolongation of stressor stimulus does not lead to continued suppression of LH release (*Smith et al., 2003*). This could explain the LH results in E, M and C groups, which had no significant differences in pen 2, pen 3 and pen 4 in comparison with the initial concentration. Rasmussen & Malven (*1983*) described that habituation to an acute confinement stress produced no change in average plasma LH concentrations in ovariectomized ewes. Thus, episodic secretion of LH was inhibited by the stress of initial confinement, but several days or hours (in this case) of habituation to the same periods of confinement minimized this inhibition and restored the episodic discharges of LH. Moreover, an increase of mean LH concentration after stressor stimulus (transport) has been observed, in comparison with the values following a stressor in ovariectomized ewes with no steroid treatment during the breeding season (*Dobson et al., 1999*). These results are in accordance with those obtained in the present study in the M and C groups.

In conclusion, individual confinement of sheep allowing visual, auditory, olfactory and tactile contact with their neighbouring animals was not an obstacle for investigating particular hormonal interrelationships with multiple sampling procedures. However further investigations are required to determine if this conclusion applies to other hormone systems in sheep.

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