Effects of different rest-stop durations at control posts during a long journey on the welfare of sheep

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Animal welfare,
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Rest,
Sheep,
Stop duration,
Transport.

Summary
All livestock transport within the European Union must comply with the EC Regulation 1/2005. For sheep, this law prescribes a maximum journey of 29 hours after which animals must rest in control posts (CP) for 24 hours before further transportation. However, there is no scientific evidence defining the effects of different stop duration on sheep recovery during long journeys. The aim of this study was to assess if a shorter stop could be provided without impairing ewes' welfare. Ninety-six adult ewes were divided into 4 homogenous groups. One group stayed at the farm (control) and the other 3 were transported for 29 hours (long-transport, LT), stopped at CP for different times (8 hours (S8 group); 16 hours (S16 group); 24 hours (S24 group)) and were re-transported for 6 hours (short-transport, ST). Blood and saliva were collected to assess dehydration, muscular damage, and adrenocortical stress before departure, after LT, after the stop, and after ST. The LT affected the hydration of all transported groups (*i.e.* higher BUN/creatinine levels than controls, $p < 0.001$), but basal values were restored after the ST, regardless of the stop duration. After the ST, S8 group had higher muscular damage than the other groups ($p < 0.05$). No differences in stress level were observed. These results suggest that, in this trial, ewe's welfare was not impaired by a stop reduction from 24 hours to 16 hours.

Effetto dei diversi tempi di sosta al posto di controllo durante il lungo viaggio sul benessere delle pecore

Parole chiave
Benessere animale,
Pecore,
Regolamento (CE) 1/2005,
Riposo,
Tempi di sosta,
Trasporto.

Riassunto
I trasporti commerciali di animali vivi all'interno dell'Unione Europea devono soddisfare i requisiti e le prescrizioni previste dal Regolamento CE 1/2005. Tale norma prevede, per gli ovini adulti trasportati per più di 29 ore (h) (14 h di viaggio, 1 h di sosta intermedia e 14 h di viaggio), una sosta obbligatoria di 24h in un posto di controllo (CP). Ciononostante, non esiste a oggi evidenza scientifica degli effetti di soste di durata differente sulle condizioni di benessere di ovini adulti durante i lunghi viaggi. Scopo di questo studio preliminare è valutare se la durata di tale sosta possa essere ridotta senza impattare negativamente sul benessere degli animali trasportati. Novantasei pecore adulte sono state divise in 4 gruppi omogenei: un gruppo (controllo, C) è rimasto nell'allevamento di origine per l'intera durata dell'esperimento mentre gli altri tre sono stati trasportati per 29 h (lungo viaggio, LV), scaricati in CP ove hanno sostato per tempi diversi (8 h, S8; 16 h, S16; 24 h, S24) ed infine ritrasportate per un viaggio di 6 h (viaggio breve, VB). Campioni di sangue e saliva sono stati prelevati per valutare lo stato di disidratazione, danno muscolare e stress adrenocorticale alla partenza, all'arrivo al CP, dopo la sosta e alla fine dello studio. Dopo il LV, tutti i gruppi trasportati presentavano valori più elevati di BUN/creatinina rispetto al gruppo di controllo ($p < 0,001$) ma nessuna differenza significativa è emersa dopo VB, indipendentemente dalla durata della sosta. Il gruppo S8 ha mostrato valori di creatin chinasi più elevati di tutti gli altri gruppi ($p < 0,05$) dopo VB. Nessuna differenza significativa tra gruppi è emersa dall'analisi del cortisolo salivare. Alla luce di questi risultati preliminari si può supporre che, nell'esperimento in oggetto, una riduzione dei tempi di sosta da 24 a 16 ore non avrebbe impatto negativo sul benessere degli ovini trasportati.

Introduction

The establishment of the common market in the European Union (EU) has increased the overall trade in livestock and led to significant cross-border transportation and mutual dependencies between producers and consumers across these borders (Hop *et al.* 2013). Moreover, the drop in number of small abattoirs and the establishment of big slaughter plants collecting animals from large areas has resulted in increased travel durations for animals (Dalmau *et al.* 2014). Transport involves several potential stress factors that could negatively affect animal welfare (Fazio and Ferlazzo 2003); these concern the experience and condition of the animals, the nature of the journey, and the duration of transport (Broom 2003). Long journeys have been suggested as being especially likely to impact upon the general status of the animals (Gavinelli *et al.* 2008). In order to protect animal welfare during transport, the Regulation 1/2005 was issued by the European Council[1] and applies to all transport of live animals occurring within the EU. This regulation allows for a maximum journey time (29 hours for adult sheep, being 14 hours travel plus 1 hour rest on the truck plus 14 hour travel), after which animals must be unloaded, fed, and watered in dedicated staging points, the control posts (CP), before continuing their journey. The stop at CP shall last at least 24 hours. While the duration of the resting time in CP provided by the Regulation is the same for all species, the tolerance of animals to transport is influenced by several factors, including the species itself (Nielsen *et al.* 2011). Sheep, for example, appear to withstand transport well (Fisher *et al.* 2010, Knowles 1998) compared to other livestock species (*e.g.* better tolerating feed and water deprivation and having lower mortality rates). Therefore, it could be hypothesised that their need for resting time might also differ from that of other species. Previous results have highlighted that, when conditions are optimal, healthy and fit animals could withstand long journeys without experiencing welfare impairment (Nielsen *et al.* 2011). These findings highlight that additional research aiming to define resting requirements per species is still needed. Transport can affect different aspects of the animal's body and biological functions, having a range of potentially negative effects: dehydration, muscular damage, risk of injury, thermal and physical discomfort (Cockram *et al.* 1996). Thus, the assessment of transport impact will need to include the sampling of a number of different parameters (Fazio and Ferlazzo 2003).

[1] European Council (EC) 2004. Council Regulation (EC) 1/2005 on the protection of animals during transport and related operations and amending and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No 1255/97. *Off J*, **L 3**, 05/01/2005, 1-43.

Previous work evaluating the welfare of transported sheep and the effect of resting time during the journey (Cockram *et al.* 1997, Knowles *et al.* 1996, Parrott *et al.* 1998) has shown that after long transport, 1 hour rest was insufficient to restore the physiological conditions of adult sheep (Parrot *et al.* 1998), and that a 12 hours stop was preferable to a 3 hours one (Cockram *et al.* 1997). Studies conducted to assess the impact of long journeys on lambs showed that after a 24 hours travel, an 8 hours stop was beneficial, although the full recovery needed more than 24 hours rest (Cockram *et al.* 1997, Cockram *et al.* 1996).

The aim of the present work is to investigate the effect of different stopover durations (24 hours, 16 hours, and 8 hours) after a long journey (29 hours) on adult sheep. Ewe's condition was assessed using a large set of physiological parameters in order to evaluate whether sheep's welfare would be impaired by a reduction of the 24 hours stop provided by the Regulation.

Materials and methods

Animals

Ninety-six adult dry ewes of Comisana breed were used in this trial. All animals came from the same dairy farm, located in the Apulia region, in Southern of Italy (41°7′0″N, 16°29′0″E). Lambing season for dairy ewes in this area is in late Autumn. Lactation lasts about 28 weeks (until the mid-Summer), with sheep being milked twice a day, and ewes are dried-off in late summer. Sheep were normally grazed during the hot season and were housed in outside pens during night and Winter. Since transport was found to have a greater impact on the behaviour of sheep moved directly from pasture than on those transported from an outside pen (Cockram *et al.* 2000), all the animals taking part in the experiment were housed in a unique pen 5 days before transport began. Sheep had *ad libitum* access to water and hay (clover and oats) and received 400 g/day of concentrate feed (corn, peas, and barley) during the whole trial, with the exception of the time spent on the truck.

Experimental procedure

Three days before the beginning of the trial, sheep were divided into 4 groups of 24 subjects each, balanced for weight (group mean ± SD: 42.2 ± 11.4 kg). Each group was housed in a separate pen of 58 m^2 (2.43 m^2 per animal). Animals were marked with spray colour on their back in order to be identified both per group (different colours) and individually (individual numbers),

and weighed to allow for making the live weight uniform among groups. None of the animals had previous experience of truck transport nor were they familiar with the vehicle, loading or unloading procedures. All transported ewes were positively evaluated as fit for transport and loading operations were carried out under veterinary supervision. At 8:00 a.m. of the third day after group formation, all the animals, with the exception of those assigned to the control group (C) (these stayed on farm for the whole trial) were loaded in 3 pens on the same deck of 1 truck. Overall, the loading procedure took less than 10 minutes. The truck was a semi-trailer vehicle with 3 movable decks and an automatic ventilation system, authorised for long journeys under EC Regulation 1/2005. The deck used in this trail was divided in 3 compartments of 6.51 m^2 in mean area (0.27 m^2 per animal, in compliance with EC Regulation 1/2005 provisions). The floor of the vehicle was covered with straw. Nipple drinkers were available in every compartment (8 per compartment, 1 nipple per 3 animals) at adequate height to be used by the animals, and water was available for the whole journey. The animals were transported for 29 hours (long transport, hereafter LT), with an intermediate 1 hours stop in compliance with the Regulation, until reaching on day 4 at 1 p.m. the CP located in Bitritto, Bari (authorisation CE 07/PS; 41°2′27″N, 16°50′9″E). The total travelled distance was 1,599 km. All animals were unloaded on arrival. The same group composition as during transport was kept and the animals were housed in 3 resting pens (mean area 45.7 m^2, 1.9 m^2/ animal), separated physically but not visually by partitions. Feed and water were made available immediately after arrival through similar systems to the ones used on the farm of origin. Each group stopped at the CP for a different period of time: 8 hours (group S8), 16 hours (S16) and 24 hours (S24). In order to allow the different stop durations, a derogation for experimental purposes to the provided minimum stop of 24 hours in CP was issued by the Italian Ministry of Health (Ministerial Decree no. 166/2011-B[2]), following positive evaluation by the Ethical Committee of the Istituto Superiore della Sanità. After the resting time, each group was reloaded on to the truck and transported for a further 6 hours journey (short transport, hereafter ST) until the final unloading back on to the farm of origin (360 km drive). Each of the loading/ unloading operation took on average less than 10 minutes. The truck travelled mainly on national highways. The experiment was carried out in early October 2011. The weather conditions were good, with no rain or strong winds during the whole trial and the external temperature ranged from 32.5°C to 12.5°C. The mean internal temperature in the truck was 21.5°C, ranging between 13°C and 32.5°C.

[2] The Decree authorised the derogation, for experimental purposes, to the provided minimum stop of 24 hours, allowing the stop duration to be reduced up to 8 hours. In addition, this same decree allowed the performing of the experimental procedures foreseen without submitting the animals to anaesthesia..

Physiological measurements

Saliva and then blood (both for whole blood and serum) samples were collected in 4 phases: (t0) when groups were formed, (t1) immediately after the LT, (t2) at the end of the resting time at the CP before departure, and (t3) after the ST at arrival to the farm of origin. Three teams of 2 people, at least 1 being a veterinarian, conducted the sampling procedures (1 at the farm and 2 at the CP). The teams followed the same sampling procedure and timing was coordinated in such a way as to ensure the operations were as simultaneous as possible. One member of the team gently restrained the animal while the other carried out sampling (starting with saliva and then blood). In order to avoid sampling group C more often than the other groups, sampling of this group was performed only at the same time as for group S8. The overall experimental and sampling design is reported in Figure 1. Salivary samples were collected using the 'Salivette®' kit (Salivette® cortisol system, Starstedt, Verona, Italy) according to manufacturer instructions. Each ewe was allowed to chew the swab for 1-2 minutes, to collect as much saliva as possible. The swab was then returned into the Salivette tube and centrifuged it at 6000 rpm for 10 minutes. The saliva samples obtained were stored at -20°C until further analysis.

After saliva sampling, 2 blood samples were taken from the jugular vein of each animal, 1 in a tube with anticoagulant for whole blood (Venosafe + EDTA 5 ml, Terumo Italia Srl, Rome, Italy) and another in a dry tube for serum (Venosafe 9 ml secca, Terumo Italia Srl, Rome, Italy). The EDTA added samples were kept at 4°C until further processing, while the tubes for serum were left in incubation at room temperature until clot formation and then centrifuged at 3500 rpm for 5 minutes. The serum collected was stored at -20°C until further analysis.

The frozen serum samples were used to assess the degree of muscular stress [measuring creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH)] and dehydration, measuring total proteins (TP), and BUN/creatinine ratio (BUN/ Crea). These samples were analysed as described by Dalmau and colleagues (Dalmau *et al.* 2014).

Haematocrit was assessed from the refrigerated whole blood samples using a micro-haematocrit centrifuge (Ematomed, GIMA S.p.A, Milan, Italy) following the manufacturer instructions. The assessment of salivary cortisol was performed with an immune-enzymatic assay using 'Salivary Cortisol'

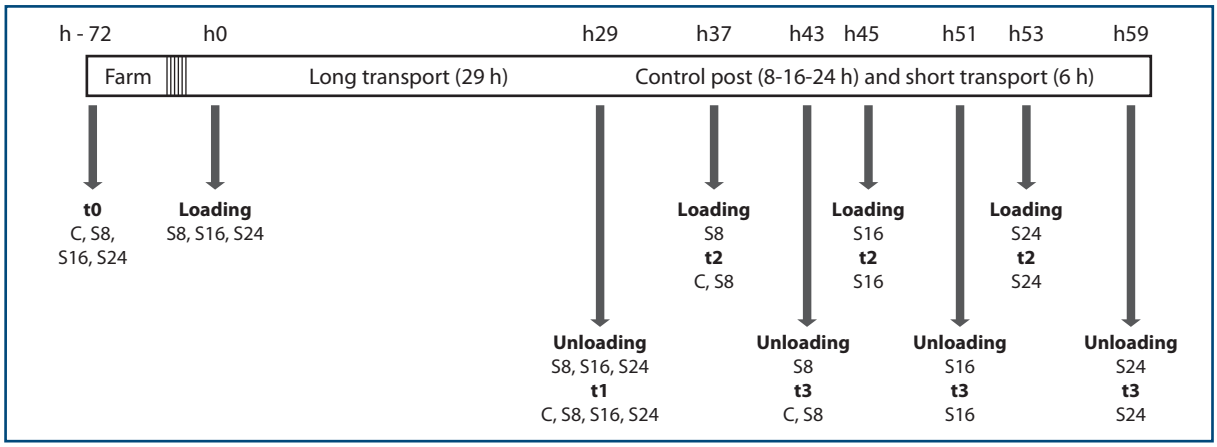

Figure 1. *Experimental design.* At the top of the bar the total count of the hours is reported from the t0 sampling (h - 72) to the final unloading of the last group to the farm (h59). The arrows indicate the time where experimental procedures [in bold, (un)-loading and sampling] were carried out. The groups undergoing each procedure are reported under the corresponding procedure.

kit (Salimetrics, LLC, State College, PA, USA). All above analyses were carried out at the laboratories of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (Teramo, Italy).

Statistical analysis

Data were analysed using the SAS statistical package (SAS Inst. Inc., Cary, NC, USA). The effects of treatment (C, S8, S16, and S24) and time of sampling (t0, t1, t2, t3) were evaluated using PROC GENMOD with repeated measures for each physiological value between the 4 groups of animals. Analysis of maximum likelihood was used as a method of estimation of the mean standard error (s.e.) and the differences between means and the least square means of fixed effects were used when the analysis of variance indicated differences ($p < 0.05$).

Results

Table I shows the means ± s.e. of physiological parameters for sheep in the control group (C) and transported groups stopping for different times at control post (8 hours, S8; 16 hours, S16; 24 hours, S24) at t0, after long transport (t1), different stop at control post (t2) and short transport (t3).

Dehydration

The analysis on the BUN/Crea parameter highlighted significant group*time effects as follows. Group C presented higher BUN/Crea values at t0 ($p < 0.01$) compared to all other groups. Comparing variations in time, BUN/Crea values of group C remained stable, while values of transported groups all increased significantly at t1 compared to t0 ($p < 0.0001$ for all comparisons). At t1, all transported groups had BUN/Crea values significantly higher than the control ($p < 0.0001$) and groups S16 and S24 had also higher values than S8 ($pS16 = 0.0008$; $pS24 = 0.02$). At t2, all groups showed a significant decrease in BUN/crea values compared to t1 ($p < 0.05$ for all comparisons). At this time, all groups differed significantly ($p \leq 0.01$), with S8 being the group with the highest mean values (see Table I). At t3, after the short transport, BUN/crea values remained stable for group S24 (compared to values at t2); while for both S8 and S16 values decreased significantly ($p < 0.05$). Inter-group comparisons at t3 showed no significant differences.

No significant differences were observed for total proteins.

Haematocrit concentration was similar among groups at t0, except for S16 that had lower mean values than group C ($p = 0.04$). After the LT, at t1, values did not vary significantly from t0 for all groups, although, when comparing groups within t1, S24 had a significantly higher concentration than groups C and S16 ($p < 0.05$). At t2 and t3, haematocrit concentration did not differ significantly among groups. Groups C and S16 had significantly increased mean values between t1 and t2; all transported groups showed significantly higher values at t2 than at t0 ($p_{S8} = 0.008$; $p_{S16} < 0.001$; $p_{S24} = 0.009$). Within-group values also remained stable for all groups, when comparing t2 to t3. No differences were observed from basal values (t0) and values at the end of the trial for all groups, with the exception of S16, which showed increased values in t3 ($p = 0.03$) (Table I).

Muscular damage

The analysis on the CK serum concentration highlighted the following group*time effects: at t0,

Table I. *Mean ± s.e. values of physiological parameters for sheep of control group (C) and transported groups stopping for different times at control post (8 h, S8; 16 h, S16; 24 h, S24).* Basal values before transport (t0), after long transport (t1), at the end of stop at control post (t2) and short transport (t3). Physiological parameters investigated are BUN/creatinine ratio (BUN/Crea), creatine kinase (CK), total proteins (TP), haematocrit, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and salivary cortisol.

	Sampling time							
	t0		t1		t2		t3	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
BUN/crea								
C	21.4^{aA}	1.78	20.9^{cA}	0.74	11.2^{dC}	0.58	13.8^{B}	0.64
S8	17.0^{bC}	1.07	27.9^{bA}	0.89	24.3^{aB}	0.78	13.9^{D}	0.89
S16	16.4^{bB}	0.52	32.9^{aA}	0.77	16.6^{bB}	0.83	14.0^{C}	0.7
S24	17.4^{bB}	0.82	31.9^{aA}	1.12	14.2^{cC}	1.13	12.4^{C}	1.08
CK (IU/l)								
C	184.5^{abB}	29.87	162.4^{bB}	15.38	207.7^{aA}	33.1	139.3^{bB}	19.09
S8	171.3^{abB}	21.1	270.6^{aA}	47.74	300.8^{aA}	61.11	377.4^{aA}	76.41
S16	149.1^{b}	14.32	197.7^{ab}	26.64	154.3^{b}	13.12	178.6^{b}	21.43
S24	231.8^{a}	39.39	229.4^{ab}	49.8	219.7^{a}	56.15	176.1^{b}	37.81
TP (g/dl)								
C	7.1	0.12	7.4	0.1	7.3	0.11	7.0	0.1
S8	7.1	0.14	7.7	0.16	7.4	0.16	7.3	0.15
S16	7.3	0.16	7.7	0.18	7.2	0.16	7.3	0.17
S24	7.0	0.12	7.6	0.13	7.0	0.12	7.0	0.11
Haematocrit (%)								
C	31.8^{aAB}	0.8	30.6 ^{bB}	0.7	33.0^{A}	0.71	32.5^{AB}	0.8
S8	30.6^{abB}	0.62	31.7^{abAB}	0.82	33.5^{A}	0.71	32.1^{AB}	0.8
S16	29.9^{bC}	0.45	31.4^{bBC}	0.62	34.4^{A}	0.95	32.5^{AB}	0.68
S24	31.4^{abB}	0.66	33.5^{aAB}	0.76	34.4^{A}	0.94	32.9^{AB}	0.53
LDH (IU/l)								
C	1,110.7	39.48	1,051.3^{b}	35.63	1,094.5^{ba}	39.17	997.9^{b}	34.62
S8	1,058.4^{B}	50.56	1,225.1^{aA}	65.64	1,202.2^{aA}	66.67	1,249.4^{aA}	78.48
S16	1,013.1	36.08	1,135.8^{ba}	48.4	1,023.2^{b}	41.87	1,079.1^{b}	43.78
S24	1,055.7	38.57	1,022.5^{b}	40.48	1,005.4^{b}	56.83	1,032.7^{b}	59.05
AST (IU/l)								
C	100.7	3.86	100.9	5.67	101.8	5.69	95.3	5.36
S8	94.8	5.03	110.4	7.01	112.8	7.69	117.0	8.73
S16	95.9	2.62	107.2	4.37	99.7	3.79	102.2	4.18
S24	102.8	3.41	102.7	4.34	101.6	7.93	102.8	8.79
Salivary cortisol (ng/ml)								
C	0.2	0.05	0.1	0.01	0.2	0.01	0.2	0.04
S8	0.1	0.01	0.1	0.01	0.1	0.01	0.2	0.01
S16	0.2	0.03	0.1	0.01	0.2	0.09	0.1	0.02
S24	0.2	0.02	0.3	0.03	0.3	0.06	0.3	0.05

$^{a, b, c, d}$ Different lower case letters at the same columns for one parameter mean significant difference within treatments at $p < 0.05$.
$^{A, B, C, D}$ Capital letters at the same row means differences among times within a group at $p < 0.05$.

S24 had a significantly higher CK concentration than S16 ($p = 0.01$). After the long transport, CK values did not show significant changes, except for S8, which showed a significant increase in CK levels when comparing t0 and t1 ($p = 0.01$). Comparing groups at t1, S8 had a CK level that was comparable to the other transported groups, but was significantly higher than the control group ($p = 0.007$). After rest (t2), values of the transported groups did not vary significantly compared to t1, although S16 had a significantly lower CK concentration when compared to the other groups ($p < 0.05$ for all comparisons, Table I). Group C had a peak at t2, when CK level was significantly higher than at both t1 and at t3 ($p < 0.01$). Finally, the short transport did not significantly affect CK level within transported groups of sheep, although S8 had significantly higher CK levels at t3 compared to the other groups ($p < 0.0001$ for all comparisons).

Analysis of LDH highlighted a significant mean increase for S8 after the LT ($p = 0.01$). At t1, LDH level for S8 was significantly higher than S24 and group C ($p \leq 0.01$). After rest (t2) and after ST (t3), S8 maintained significantly higher LDH values than the other groups ($p < 0.01$ for all comparisons (Table I). No significant differences emerged from the AST analysis.

Stress evaluation

The assessment of transport stress was performed using salivary cortisol, however no significant effects of time, group or group*time interaction were detected.

Discussion

Previous research evaluating the welfare of transported sheep and the effect of resting time during the journey has already been described in the relevant literature. However, these studies focused on shorter travelling time *i.e.* up to 42 hours of transport, including resting time (Cockram *et al.* 1997, Knowles *et al.* 1996, Parrott *et al.* 1998), and used the farm of origin, which was familiar to the animals, as a resting point (Cockram *et al.* 1997). In this trial, the whole procedure lasted between 43 hours and 59 hours, and it included the use of novel pens and new handlers as resting points, thus reflecting commercial conditions. It has been observed that sheep of different ages differ in their response to transportation, in both muscular and adrenocortical stress (Zhong *et al.* 2011). For instance, Zhong and colleagues (Zhong *et al.* 2011) found that adult sheep are more susceptible to muscular damage during transport than younger animals. Therefore, only adult sheep were used in this trial.

Since no replication of the trial was performed, care should be exercised in extrapolating the results to field conditions from our specific experimental conditions, and the breed of tested sheep used should be also taken into consideration. Nonetheless, we believe that the findings of this study may provide a valuable basis to support the development of further research on this topic.

The 29 hours journey affected the level of hydration of some transported animals, but all ewes restored their basal values after the second journey regardless of the stop duration. Although water deprivation is one of the major concerns when transporting animals for long periods (Nielsen *et al.* 2011), sheep are known to be particularly resilient to dehydration (Cockram *et al.* 1997, Knowles *et al.* 1995, Parrott *et al.* 1996). Previous studies have shown that healthy un-transported sheep can tolerate feed and water withdrawal for up to 3 days (Cole 1995); whilst if transported without feed availability, they remain in water balance for 2 days. However, some studies have indicated that the effects of feed and water deprivation are noticeable on long journeys (Knowles *et al.* 1995). In this trial, a set of parameters was used to measure the level of hydration of the animals: haematocrit, blood urea nitrogen (BUN)/creatinine ratio, and serum total proteins. Interestingly, although varying across the different phases of the trial, all values always remained within the normal expected physiological ranges (Merck Veterinary Manual 2010). This suggests that, although some effects on the hydration status of the animals could have existed, the experimental treatment did not cause severe dehydration in adult sheep. Nevertheless, after the stop at CP (t2), significantly different BUN/crea values were found between the groups, with increasing mean values as the resting time decreased. This finding seems to suggest that increasing resting time at CP improves the restoring of hydration level in adult sheep. It should also be noted that sampling occurred at different times of the day for the different groups. It may be argued that there could be an effect of the sampling time. Furthermore, group C and S8 were sampled at the same time and showed very different patterns, suggesting that any sampling time effect would have played a minor role, if any. Once the animals returned to the farm of origin, levels of BUN/crea did not differ among groups. A possible explanation for this might be that the ewes were not familiar with nipple drinkers, having had troughs on their farm and so they might have needed more time to get used to them. Similar behaviour has been observed in other ruminant species in similar situations (Werner *et al.* 2013). If this would be the case, this study would support the hypothesis that the drinkers available on the truck (1 per 3 ewes) were allowing an appropriate water intake to the animals during travel. This would be in line with results from previous studies (Bøe *et al.* 2012), which concluded that in farm conditions, 1 nipple drinker with an acceptable flow rate may serve up to 30 pregnant ewes without negatively affecting their water intake. It might be argued then that, even if in our study the available space to the animals was inferior to that being normally available on farm, the animals managed to access the drinker adequately. In addition, feed ingestion at CP might have played a role, with concentrate consumption increasing the BUN serum concentration and therefore the BUN/crea ratio, which took some hours to return to pre-departure values.

Muscular stress was assessed measuring serum CK, AST, and LDH. Creatine kinase is the enzyme of choice in sheep studies because of its sensitivity and rapid serum increase after muscular damage (Braun *et al.*, 2010), while AST has a longer half-life compared to CK (Boyd 1976). Lactate dehydrogenase (LDH) indicates intense muscular activity and also muscular

damage (Bórnez *et al.* 2009). Previous studies have indicated that CK activity is increased by transport (Earley and Murray 2010, Miranda-De la Lama *et al.* 2011) and duration of lairage (Braun *et al.* 2010). In this trial, no effect of the journey was observed on muscular damage for two of the transported groups (S16 and S24), while a significant increase in CK serum concentration was observed for group S8. At t3, when returning at the farm of origin, S8 still had a significantly higher CK level than all other groups. The progressive increase of CK level in S8 group might indicate an effect of repeated loading/unloading procedures. Although the half-life of this enzyme is quite short (about 4 hours, Russell and Roussel 2007), this did not allow the serum concentration to decrease to basal level. Previous studies have identified the loading/unloading procedures as the most stressful part of a journey for sheep (Cockram 2007, Knowles 1995, Trunkfield and Broom 1990). Subjecting sheep to repeated loading/unloading procedures in a short time (8 hours), without sufficient time to recover, can then be detrimental and appears to increase the risk of muscular damage for subsequent journeys.

However, it should be noted that CK levels also underwent some modification in the C group which, even when not moved from the farm, showed a peak at t2. Since both group C and S8 were sampled at the same time and with shorter intervals as compared to other groups, it might be argued that the catching and sampling procedures themselves might have played a relevant role in the muscular stress of the animals. However, as the sampling was performed by experienced veterinarians applying standard operational practices and took usually less than 2 minutes per animal, any such effect was kept to a minimum. Nevertheless, while CK values of the C group lowered between t2 and t3, the same did not happen for S8, indicating more likely that the whole transport process had a stronger influence on the muscular stress than the sampling procedure itself. The CK results were also confirmed by LDH analysis, highlighting higher mean values for S8 compared to the other groups at all time sampling periods. No significant differences were observed on AST levels during the whole trial possibly due to the lower sensitivity of the enzymes compared to CK (Boyd 1976).

Cortisol is an important indicator of stress and is commonly used in sheep to assess the effects of isolation, transport or different loading procedures (Broom *et al.* 1996, Cockram *et al.* 2000, Kent 1997, Krawczel *et al.* 2007, Parrott *et al.* 1994). Previous research has shown that the effect of transport on serum cortisol was most apparent at the start of the journey, decreasing throughout the journey (Broom *et al.* 1996, Cockram *et al.* 1996). Cockram and colleagues (Cockram *et al.* 1997) also found that serum cortisol concentration increased at the start of the journey and after 24 hours of continuous transport it was still greater than in control groups. We found no differences between control group and transported animals in salivary cortisol after a transport journey of 29 hours. Surprisingly, no differences in salivary cortisol in relation to control group were even found in S16 and S24 after they rested at CP, even though a circadian rhythm effect would have been expected, as they were obtained at different times of the day. A possible explanation is that, although cortisol levels were found to be rather low, the stress response produced on the animals by a person present in the pen during the sampling procedure was high enough to mask any other effect (transport or circadian rhythm). Therefore, it can be hypothesised that transport conditions in sheep produce a stress response on the animals on arrival no higher than that produced by the presence of unknown humans walking into the group. In fact, stress from human handling is likely to provoke cortisol increase (Hild *et al.* 2011). The lack of differences between the groups indicates that the duration of the stop in the control post and a second transport are also less stressful than unknown-human presence.

Conclusions

From these results, it appears that a 16-hour stop was sufficient for adult sheep to recover from the 29-hour journey and to undergo a further 6-hour travel without having detrimental consequences on their welfare when compared to 24-hour stop. In contrast, stop times of 8 hours seem to have detrimental effects on muscle indicators, probably due to the shorter times between unloading and loading procedures. It is suggested that, in order to facilitate a good watering of the animals after travel, the control post should have different types of drinkers available and adapt them to those provided on the farms of origin. In future research, other breeds and ages under different climatic conditions should be studied to confirm that resting periods from 16 hours to 24 hours can be enough for the recovery of sheep after a long transport journey. Number, type, and location of drinkers in the trucks under different scenarios should also be further investigated.

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