

Effects of Physical Isolation on Serum and Salivary Cortisol and Components of Complete Blood Counts in Yearling Ewes¹

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Summary

Isolation is often stressful to herd animals. The objective of this study was to compare changes in serum and salivary cortisol concentrations and complete blood count (CBC) components in physically isolated sheep. Twelve Suffolk x Hampshire yearling ewes (64 kg \pm 1.2 kg) were held indoors in either a common pen or individual pens for 10 consecutive days. Individual pens did not allow physical contact, but did not obstruct vision or sound of adjacent sheep. Serum and whole blood samples (venipuncture) and salivary samples (oral swab) were collected at 0700 (AM) and 1300 (PM) on each day with the exception of day 1 (no AM sample). Additionally, intensive samples were taken in 15-min intervals over a 2-h period on days 1, 5, and 10 for correlation purposes. Serum and salivary cortisol concentrations (RIA) and specific blood components (CBC) were determined. Serum cortisol concentrations did not differ between treatments in AM ($P = 0.452$) or PM samples ($P = 0.827$). Similarly, salivary cortisol concentrations did not differ between treatments for either period ($P > 0.768$). Serum and salivary cortisol concentrations were closely correlated among all samples ($r = 0.83$, $P < 0.001$). White blood cells were reduced ($P < 0.022$) by isolation on days 1, 2, 4, 5, and 6 in PM samples, but AM samples were not affected ($P = 0.594$) by

treatment. Isolation also reduced neutrophils ($P < 0.037$) and increased lymphocytes ($P < 0.049$) on days 1, 2, and 5 in PM samples only. Mean corpuscular volume was reduced ($P < 0.001$) by isolation on all days in both AM and PM samples. Conversely, mean corpuscular hemoglobin concentrations were increased ($P < 0.009$) in all samples. Hematocrit was reduced in isolated sheep from day 2 to day 6 in AM samples ($P < 0.037$) and on all but day 10 in PM samples ($P < 0.050$). Physical isolation did not appear to influence other CBC components, including red blood cells and hemoglobin concentration. In general, stress components were greater during the first two days of isolation, regardless of treatment. This was likely due to the unfamiliar environment. Data from this study indicate physical isolation of yearling ewes for 10 days without visual and auditory isolation did not elicit noticeable changes in cortisol concentrations, while alterations in immune components of CBC were generally mild and inconsistent. Although certain non-immune components were substantially affected by physical isolation, causes or physiological significance of these changes are unclear.

Key Words: Complete Blood Counts, Isolation Stress, Salivary Cortisol, Serum Cortisol

Introduction

Although sheep have evolved as herd animals, many non-traditional production conditions, such as club-lambing, research settings, and maintenance of superior breeding stock require individual penning of sheep for extended periods of time. However, ramifications of physiological stress associated with unfamiliar and unnatural conditions have been documented (Morrison, 1983). Currently, reports of physiological stress levels in sheep facing seclusion are varied. Stackpole et al. (2003) and Tilbrook et al. (2008) reported no increase in serum concentrations of the stress hormone, cortisol, in sheep isolated for brief periods of time, while others have described consistent increases in blood cortisol concentrations (Apple et al., 1995; Degabriele and Fell, 2001; Wagenmaker et al., 2009), especially when isolation includes visual and auditory obstruction from other sheep. In addition to increased cortisol production, some have reported changes in immune components in stressed sheep, including increased numbers of white blood cells and neutrophils and decreased numbers of lymphocytes (Minton and Blecha, 1990; Minton et al., 1992). Less understood are changes in non-immune blood components as a response to stress in sheep, including reductions in hematocrit (Ali et al. 2001) and increased hemoglobin (Al-Qarawi and Ali, 2005). The objective of the present study was to evaluate changes in traditional stress markers, including serum cortisol concentration, salivary cortisol concentration, and components of complete blood counts in sheep exposed to physical, but not visual or auditory, isolation for 10 days and to quantify physiological stress levels in these isolated sheep.

MATERIALS AND METHODS

Animal care and facilities

The New Mexico State University Institutional Animal Care and Use Committee approved all procedures. Before use, ewes were weighed ($64 \text{ kg} \pm 1.2 \text{ kg}$) and examined for health. Animals were held indoors under artificial cooling ($25^\circ \text{ C} \pm 4^\circ \text{ C}$) and light

(approximately 12 h of light and 12 h of dark each day) for the duration of the experimental period. All ewes had free access to water and were fed chopped alfalfa hay (approximately 1.25 kg/ewe) once daily at 0600. Approximately 7 cm of wood shavings were used as bedding over concrete floors. Bedding was changed every 72 h. Experimental procedures were conducted at New Mexico State University, Las Cruces, N.M. ($32^\circ 19' 11'' \text{ N}$, $106^\circ 45' 55'' \text{ W}$; elevation 1,219 m) beginning on March 5, 2008. All ewes were of mixed Suffolk x Hampshire breeding and were produced at New Mexico State University. Ewes ranged from 10 mo to 12 mo of age and averaged from moderate to good body condition. Following the experimental period, all ewes received a cautionary dose of liqumysin (LA-200, 5 mL, s.c.).

Isolation model

Six ewes were randomly assigned to a common 6-m x 5-m indoor control pen with no divisions, while six ewes were penned in individual 2-m x 5-m indoor pens. All pens were constructed of galvanized-mesh, cattle panels (6 cm x 12 cm openings), thus, ewes in individual pens were physically isolated but not visually or audibly obstructed from other ewes. Individual pens were spatially separated by no less than 2 m to avoid across-fence

physical contact. Ewes were placed in assigned pens for 10 d, beginning at 1200 on d 1. Blood and salivary samples were collected at 0700 and 1300 each day with the exception of d 1, on which no morning sample was collected. On d 1, d 5, and d 10, samples were collected intensively at 15-min intervals for 2 h, beginning at 1300 for use in correlation comparisons.

Sample collection and analysis

Blood samples were collected via jugular venipuncture into 10 mL vacuum tubes (Corvac serum-separator, Kendall Health Care, St. Louis, Mo.). Blood samples were kept at room temperature for 30 min to 60 min and were centrifuged ($1,500 \times g$ at 4° C for 15 min). After centrifugation, serum was stored in plastic vials at -80° C until assayed. For each blood sample, a simultaneous saliva sample was collected via 30 to 45 second swab of the mouth with a 1 by 2 cm cotton strip held with surgical forceps as previously described (Yates et al., 2010). Saliva samples were placed in salivette tubes (Sarstedt AG and Co., Numbrecht, Germany) and cooled in ice immediately after collection, then centrifuged ($1,500 \times g$ at 4° C for 15 min) and stored at -80° C until assayed. Serum cortisol was quantified by solid phase RIA using components of a com-

Figure 1. Serum cortisol concentrations in samples collected at 0700 from young ewes housed in a common pen (control) or those maintained in individual pens (isolated) for 10 days. Housing effect, $P = 0.452$, day effect, $P < 0.001$, isolation x day, $P = 0.110$.

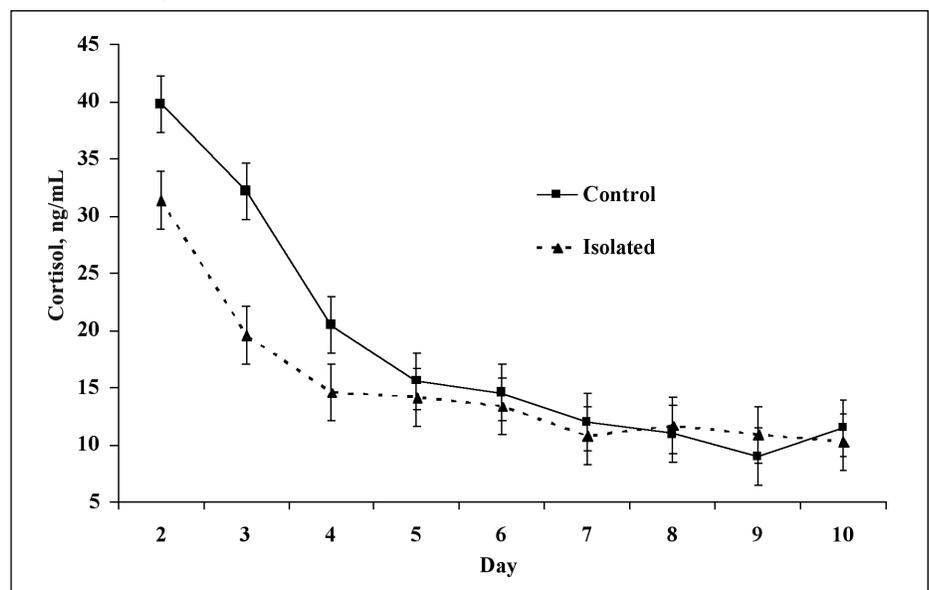


Figure 2. Serum cortisol concentrations in samples collected at 1300 from young ewes housed in a common pen (control) or those maintained in individual pens (isolated) for 10 days. Housing effect, $P = 0.827$, day effect, $P < 0.001$, isolation x day, $P = 0.657$.

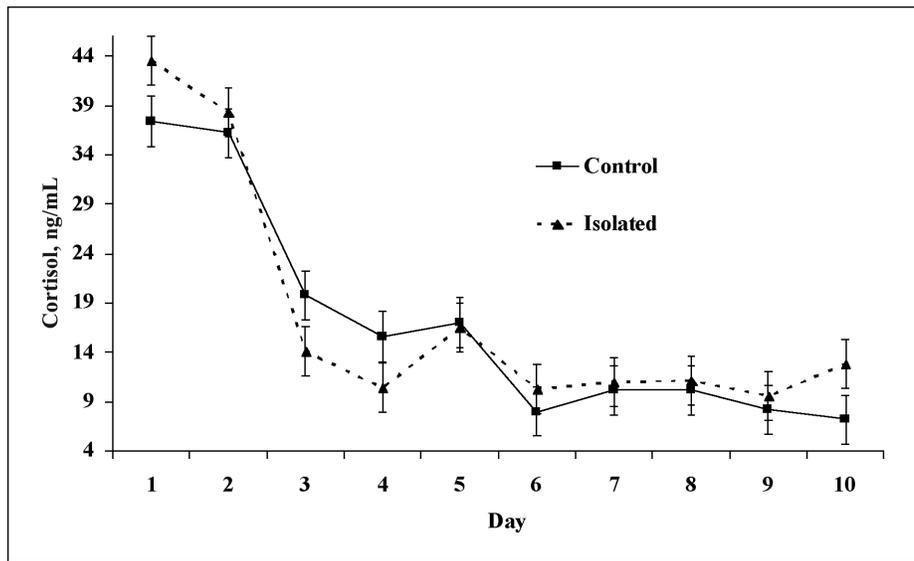
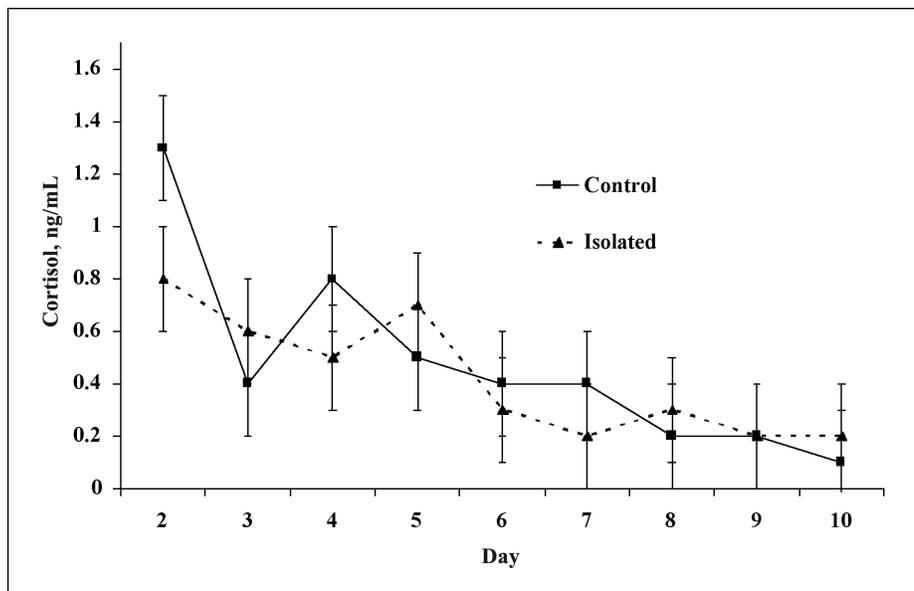


Figure 3. Salivary cortisol concentrations in samples collected at 0700 from young ewes housed in a common pen (control) or those maintained in individual pens (isolated) for 10 days. Housing effect, $P = 0.722$, day effect, $P = 0.002$, isolation x day, $P = 0.590$.



mercial kit (Coat-A-Count, Siemens Medical Solutions Diagnostics, Los Angeles, Calif.) with modifications described by Kiyama et al. (2004). Within-assay and between-assays CV were 2.5 percent and 1.4 percent, respectively, for serum determinations. Salivary cortisol concentrations were determined using the same commercial kit with modifications described by Yates et

al. (2010). Within-assay and between-assays CV were 3.6 percent and 16.8 percent, respectively, for salivary determinations. Whole-blood samples were obtained by jugular venipuncture (EDTA-containing, whole-blood vacuum tubes, Kendall Health Care, St. Louis, Mo.). Immediately after sampling, whole-blood samples were cooled on ice and shipped (refrigerated) overnight to

the New Mexico Department of Agriculture Veterinary Diagnostics Services, Albuquerque, N.M., for analysis of specific immune and non-immune components of complete blood counts.

Statistical analysis

Ewe was the experimental unit, and data were grouped for analysis into AM (0700) or PM (1300) samples. Experimental design was a split plot with isolation treatment in the main plot and day and treatment x day interaction in the sub plot. When treatment x day interactions were observed, data were examined for treatment effects within each day. Split-plot data were analyzed using the mixed procedure of SAS with the repeated measures function (SAS Inst. Inc., Cary, N.C.). Correlation coefficients were determined using the correlation procedure of SAS. For correlation determination, AM, PM, and intensively collected samples were included.

Results and Discussion

Serum and salivary cortisol concentrations

Serum cortisol concentrations did not differ ($P > 0.452$) between treatments in AM samples (Figure 1) or PM samples (Figure 2). Additionally, salivary cortisol concentrations did not differ ($P > 0.722$) between treatments for the AM (Figure 3) or PM period (Figure 4). The lack of induced change in serum or salivary cortisol concentrations in the present study indicates that physical isolation for 10 d without visual or auditory isolation did not elicit a typical stress response, as the hypothalamic-pituitary-adrenal axis did not appear more active in isolated sheep. This finding complements previous work in which isolation did not result in increased serum cortisol concentration (Tilbrook et al., 2008). Stackpole et al. (2003) also reported no difference in cortisol concentrations in sheep that were both isolated and restrained. However, inclusion of restraint with isolation in other studies increased circulating cortisol (Apple et al., 1995; Minton et al., 1995; Rivalland et al., 2007), even when stress was applied for short durations only. Additionally, Wagenmaker et al. (2009) reported increased serum cortisol con-

Figure 4. Salivary cortisol concentrations in samples collected at 1300 from young ewes housed in a common pen (control) or those maintained in individual pens (isolated) for 10 days. Housing effect, $P = 0.449$, day effect, $P < 0.001$, isolation x day, $P = 0.092$.

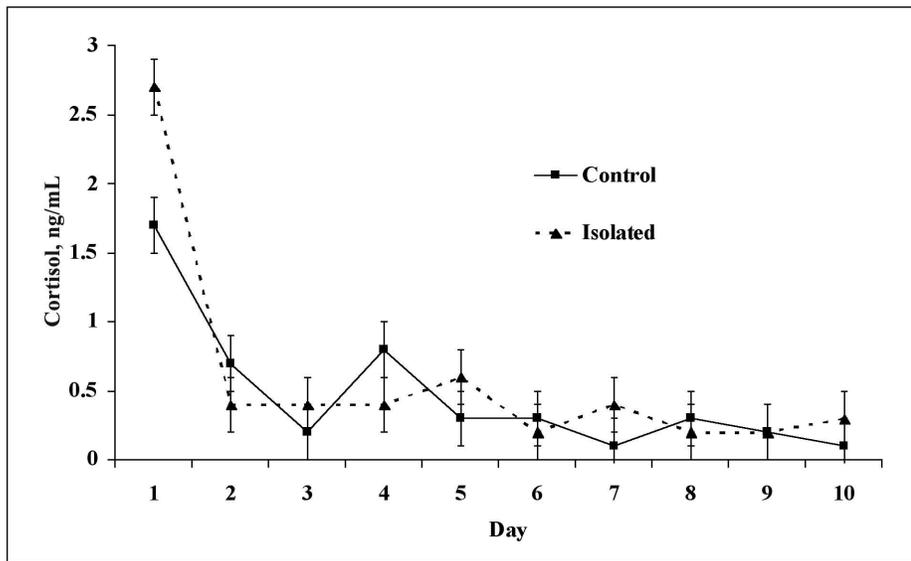
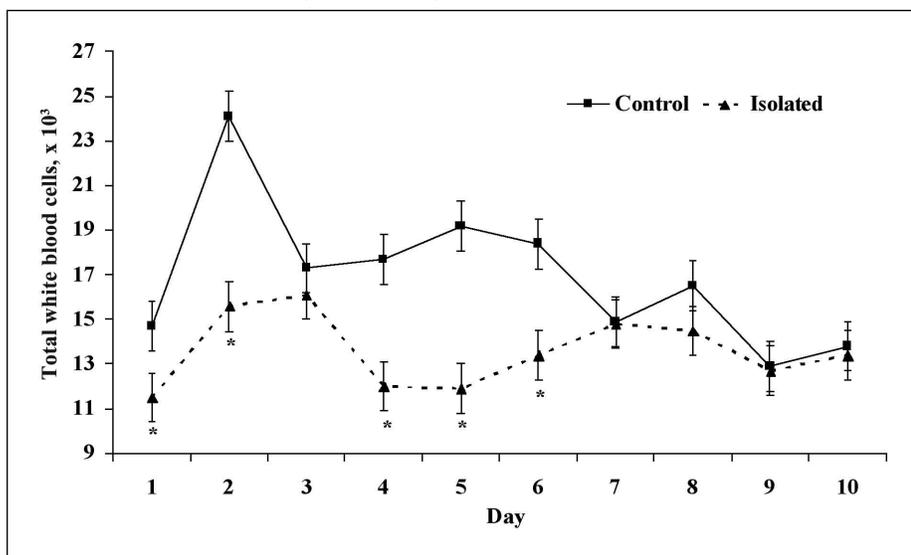


Figure 5. Total white blood cells in samples collected at 1300 from young ewes housed in a common pen (control) or those maintained in individual pens (isolated) for 10 days. Isolation x day, $P < 0.001$. Treatment differences ($P < 0.05$) within each day denoted by '*'.



concentrations when isolation was coupled with blindfolding and predator cues. In the present study, serum and salivary cortisol concentrations differed among days ($P < 0.001$). In both biological fluids, cortisol concentrations were elevated on d 1 and 2, but fell and stabilized thereafter, suggesting acclimation to the new surroundings. Importantly, salivary cortisol concentrations were correlated with contemporary serum

cortisol concentrations ($r = 0.83$, $P < 0.001$), which supports previous findings indicating that salivary cortisol concentration is a suitable non-invasive indicator of serum cortisol concentration (Yates et al., 2010).

Immune components of complete blood counts

Treatment x day interactions were observed ($P < 0.021$) in PM samples for

total white blood cells, neutrophils, and lymphocytes. For these variables, treatment effects were examined within each day. In PM samples taken from isolated sheep, total white blood cells were reduced ($P < 0.022$; Figure 5) on d 1, d 2, d 4, d 5, and d 6, neutrophils were reduced ($P < 0.037$; Figure 6) on d 1, d 2, and d 5, and lymphocytes were increased ($P < 0.049$; Figure 7) on d 1, d 2, and d 5. However, monocyte, eosinophil, and basophil concentrations did not differ ($P > 0.250$) between treatments in PM samples, and no differences were observed between treatments ($P > 0.629$) in AM samples for any immune component of CBC. In general, immune components were not greatly or consistently influenced by physical isolation in the present study. Increased lymphocyte numbers in the present study were in contrast to previous findings in which seclusion actually decreased total lymphocytes (Minton and Blecha, 1990; Minton et al., 1992; Al-Qarawi and Ali, 2005). However, Degabriele and Fell (2001) reported that increased or decreased lymphocyte numbers in response to three weeks of isolation were specific to lymphocyte type. Decreases in total white blood cells and neutrophils in the present study were also different from previously reported increases in these immune components in isolated and restrained sheep (Minton and Blecha, 1990), although other types of stress have reportedly decreased total white blood cells in birds (Voslárova et al., 2006). Basophils, monocytes, and eosinophils did not differ due to treatment in any samples in the present study, which is consistent with some previous findings (Minton and Blecha, 1990), but is contrary to others (Gupta and Flora, 2005).

Non-immune components of complete blood counts

Treatment x day interactions were observed in both AM and PM samples ($P < 0.020$) for hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin concentrations. For these variables, treatment effects were examined within each day. Mean corpuscular volume was reduced ($P < 0.001$) by isolation on all days in both AM and PM samples. Conversely, mean corpuscular hemoglobin concentrations were

Figure 6. Neutrophils in samples collected at 1300 from young ewes housed in a common pen (control) or those maintained in individual pens (isolated) for 10 days. Isolation x day, $P < 0.001$. Treatment differences ($P < 0.05$) within each day denoted by '*'.

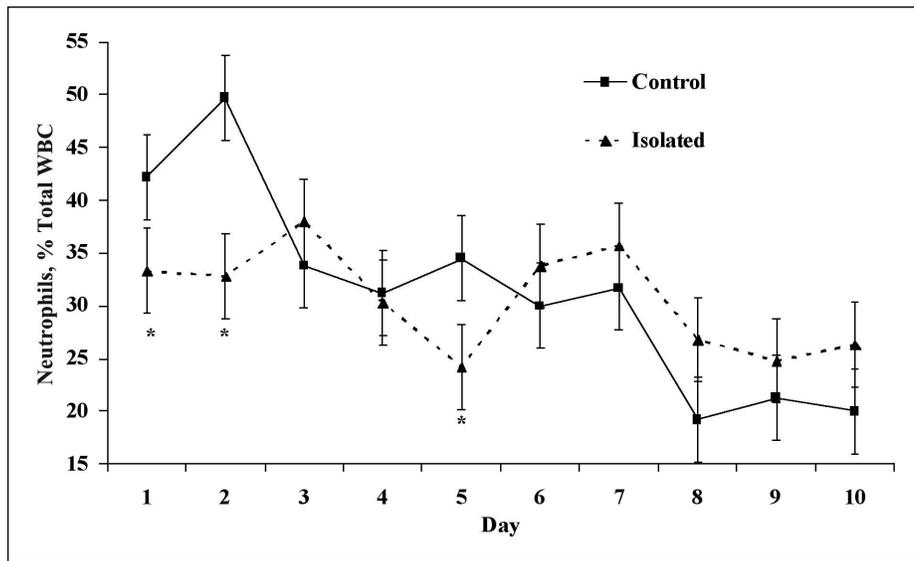
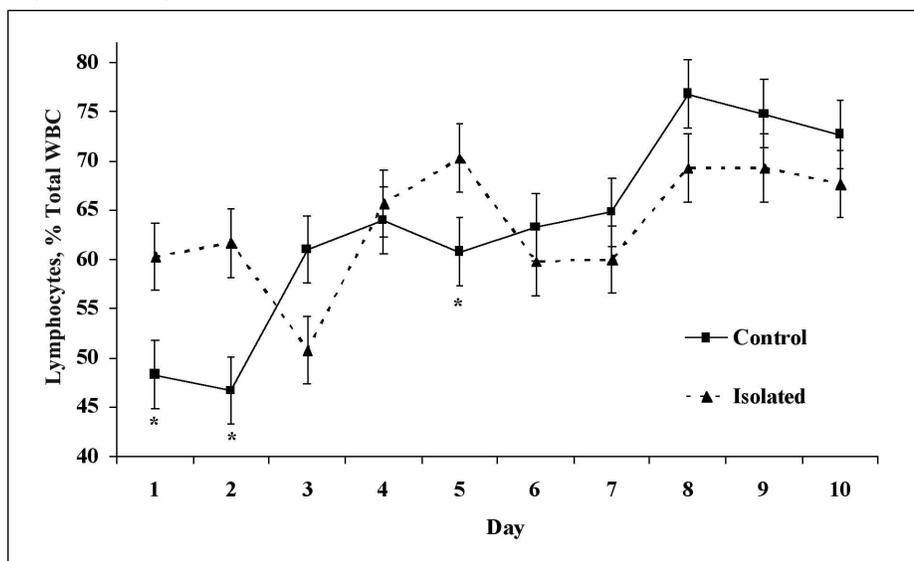


Figure 7. Lymphocytes in samples collected at 1300 from young ewes housed in a common pen (control) or those maintained in individual pens (isolated) for 10 days. Isolation x day, $P = 0.021$. Treatment differences ($P < 0.05$) within each day denoted by '*'.



increased ($P < 0.009$) in all samples. Hematocrit was reduced in isolated sheep from d 2 to d 6 in AM samples ($P < 0.037$) and on all but d 10 in PM samples ($P < 0.050$). Total red blood cell numbers did not differ ($P > 0.442$) between treatments in AM or PM samples. Physical isolation in the present study appeared to elicit the greatest response from non-immune components

of CBC. Similar results for hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin concentrations have been found in stressed chickens (Bedánová et al., 2006) and stressed pheasants (Voslarova et al., 2006). Hematocrit reduction in isolated sheep also supports previous findings in sheep exposed to both isolation stress (Al-Qarawi and Ali, 2005) and transporta-

tion stress (Ali et al., 2001). Hemoglobin tended to be reduced in PM samples in isolated sheep but was unaltered in AM samples; a contrast to previous work that found increased hemoglobin in response to stress in sheep (Ali et al., 2001; Al-Qarawi and Ali, 2005) and other animals (Bedánová et al., 2006; Voslarova et al., 2006). Total red blood cell numbers did not differ, although stress has been shown to decrease erythrocyte counts in other species (Bedánová et al., 2006; Voslarova et al., 2006). Causes of altered non-immune components in this and other studies are unclear, although changes in both hematocrit and red blood cell numbers may be related more to dehydration associated with physical exertion (Averós et al., 2008) and, thus, might be more representative of changes elicited by physical activity than by actual physiological stress response.

Conclusions

Physical isolation without visual or auditory impairment for 10 d did not appear to stimulate the physiological stress response axis in young ewes, as neither serum nor salivary cortisol concentrations were affected by isolation treatment. Cortisol in both biological fluids was greatest on d 1 and d 2, but fell to stable levels by d 3, indicating acclimation to the unfamiliar indoor environment. Serum cortisol concentration was reflected in salivary cortisol concentration, indicating measurement of salivary cortisol might represent a suitable non-invasive alternative to measurement of cortisol in blood samples. Changes in immune components in response to the isolation model used in this study were largely inconsistent and contradicted previous reports in some cases. Although effects of isolation on non-immune blood components were more consistent and reflective of previous research, mechanisms for and physiological significance of these effects are unclear. Data from this study indicate that ewes were capable of coping with physical isolation for moderate periods of time, when allowed visual and auditory contact with other sheep.

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