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Impact of Transport Duration on Integrated Stress Biomarkers and Predictive Modelling of Dark, Firm, Dry (DFD) Meat Incidence in Awassi Lambs

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ABSTRACT

Background: Transport stress adversely affects animal welfare and meat quality in livestock. However, no previous study has simultaneously integrated physiological, oxidative, inflammatory, and acute phase biomarkers with multivariate modelling to predict dark, firm, dry (DFD) meat risk in Awassi lambs under commercial transport conditions. **Objective:** This study aimed to evaluate the effects of transport duration on integrated stress biomarkers, establish predictive cut-off values using ROC analysis, and develop a practical Transport Stress Score (TSS) for DFD prediction. **Methods:** Ninety-six male Awassi lambs (8–10 months, 42 ± 3 kg) were allocated to four groups (n = 24): Control (C), Short Transport (ST, 1 h), Medium Transport (MT, 3 h), and Long Transport (LT, 6 h). Blood and muscle samples were analyzed for cortisol, glucose, lactate, creatine kinase (CK), neutrophil/lymphocyte (N/L) ratio, haptoglobin (Hp), serum amyloid A (SAA), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), and glutathione peroxidase (GPx). Meat quality was assessed on longissimus dorsi muscle. ROC analysis, PCA, and logistic regression were performed. **Results:** Prolonged transport markedly elevated ($P < 0.05$) cortisol (15.82 → 48.65 ng/mL), MDA (1.85 → 4.92 nmol/mL), IL-6 (42.5 → 128.6 pg/mL), and SAA (8.45 → 38.72 μg/mL), while depleting antioxidant defenses. DFD incidence reached 54.2% in LT group. ROC analysis revealed SAA as the strongest predictor (AUC = 0.91) with optimal cut-off of 25.4 μg/mL (sensitivity = 88.5%, specificity = 84.2%). The proposed Transport Stress Score achieved 89.6% accuracy for DFD prediction. **Conclusion:** This study provides the first integrated biomarker panel with validated cut-off values and a practical scoring system for DFD risk assessment in Awassi lambs. Transport should be limited to < 3 hours to maintain optimal meat quality.

Keywords: Awassi lambs; transport stress; oxidative stress; acute phase proteins; DFD meat; ROC analysis; predictive modelling

INTRODUCTION

Pre-slaughter stress represents a critical challenge in the meat industry, adversely affecting animal welfare and compromising meat quality with substantial economic implications (Xing et al., 2019). Transportation of livestock from farms to slaughterhouses exposes animals to multiple stressors including handling, loading, unloading, noise, vibration, thermal fluctuations, food and water deprivation, and social regrouping (Nelis et al., 2022). These stressors activate the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary (SAM) system, resulting in elevated concentrations of glucocorticoids and catecholamines.

The stress response triggers glycogenolysis in skeletal muscles, depleting glycogen reserves and subsequently affecting post-mortem pH decline. Insufficient muscle glycogen at slaughter results in elevated ultimate pH, commonly manifested as dark, firm, and dry (DFD) meat, characterized by reduced shelf-life and poor consumer acceptability (Erasmus et al., 2024). The economic losses attributed to DFD meat range from 10–30% of carcass value, making it a significant concern for the meat industry worldwide.

Recent advances in stress physiology have emphasized the importance of oxidative stress biomarkers, including malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), and glutathione peroxidase (GPx), in evaluating transport-induced stress (He et al., 2022; Hou et al., 2023). The mechanistic pathway involves reactive oxygen species (ROS) generation during stress, leading to lipid peroxidation and compromised antioxidant defenses, ultimately affecting muscle integrity and meat quality.

Acute phase proteins (APPs), particularly haptoglobin (Hp) and serum amyloid A (SAA), serve as sensitive indicators of inflammation and stress in ruminants (Kang et al., 2016). Their synthesis is stimulated by pro-inflammatory cytokines, primarily interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), establishing a mechanistic link between stress, inflammation, and the acute phase response (Pomorska-Mól et al., 2020). This integrated pathway—HPA activation → cytokine release → APP synthesis → oxidative stress → muscle damage → DFD meat—represents a comprehensive framework for understanding transport stress effects.

The neutrophil to lymphocyte (N/L) ratio has been established as a reliable indicator of stress-induced immunomodulation in livestock, with elevated ratios reflecting glucocorticoid-mediated leukocyte redistribution (Salahuddin et al., 2018). Additionally, environmental conditions during transport, quantified using the temperature-humidity index (THI), profoundly influence the magnitude of stress response, particularly in hot climatic regions (Romero et al., 2024).

The Awassi breed is the predominant indigenous sheep breed in the Middle East, recognized for its adaptation to harsh environmental conditions. Despite its economic importance, comprehensive studies investigating the integrated relationship between transport duration, oxidative stress, inflammatory response, and meat quality in this breed remain scarce. More importantly, ***no previous study has simultaneously integrated physiological, oxidative, inflammatory, and acute phase biomarkers with both serum and muscle oxidative status, THI monitoring, and multivariate modelling (PCA + logistic regression + ROC analysis) to predict DFD incidence in Awassi lambs.***

Therefore, the objectives of this study were to: (1) evaluate the effects of different transport durations on physiological stress indicators, oxidative status, and inflammatory biomarkers in Awassi lambs; (2) assess the impact on meat quality characteristics and DFD incidence; (3) establish correlations between stress biomarkers and meat quality parameters; (4) determine optimal cut-off values for key biomarkers using ROC analysis; and (5) develop a practical Transport Stress Score (TSS) for DFD risk prediction applicable in commercial settings.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Samarra (Protocol No. IACUC-2024-037) in accordance with the guidelines for the care and use of animals in research.

Animals and experimental design

Ninety-six male Awassi lambs (8–10 months old, 42 ± 3 kg live weight) were obtained from a commercial farm in Samarra region, Iraq. Animals were clinically healthy, free from external parasites, and had been raised under identical management conditions for at least 60 days prior to the experiment. The lambs were randomly allocated to four treatment groups (n = 24 each) using a computer-generated randomization sequence:

- Control group (C): lambs slaughtered without transport, maintained in lairage for 1 h
- Short Transport (ST): transported for 1 h (approximately 50 km)
- Medium Transport (MT): transported for 3 h (approximately 150 km)
- Long Transport (LT): transported for 6 h (approximately 300 km)

Transportation was conducted during early morning hours (departure at 06:00) using a standard livestock vehicle equipped with adequate ventilation at a stocking density of 0.35 m^2/animal, in accordance with OIE animal welfare

guidelines. Following arrival, all transported animals were maintained in lairage with access to water but not feed for 1 h before slaughter to standardize pre-slaughter conditions.

Environmental monitoring during transport

Ambient temperature (T, °C) and relative humidity (RH, %) inside the transport vehicle were continuously monitored at 5-minute intervals using calibrated data loggers (HOBO U12-012, Onset Computer Corporation, USA) positioned at four locations within the trailer (front-left, front-right, rear-left, rear-right). The temperature-humidity index (THI) was calculated using the formula established by the Livestock Weather Safety Index:

$$THI = (0.8 \times T) + [(RH/100) \times (T - 14.4)] + 46.4$$

THI values were categorized as: < 72 (thermoneutral), 72–79 (mild stress), 80–89 (moderate stress), and ≥ 90 (severe stress). The mean THI values recorded during transport were 68.5 ± 2.4, 71.2 ± 3.1, and 74.8 ± 3.6 for ST, MT, and LT groups, respectively.

Blood sampling and biochemical analysis

Blood samples (10 mL) were collected from the jugular vein at exsanguination into EDTA tubes (for hematology), heparin tubes (for plasma), and plain tubes (for serum). EDTA blood was utilized for differential leukocyte count using an automated hematology analyzer (Celltac α MEK-6358, Nihon Kohden, Japan). The neutrophil to lymphocyte (N/L) ratio was calculated. Serum and plasma were separated by centrifugation at 3000 rpm for 15 min at 4°C and stored at −80°C until analysis.

Serum cortisol concentration was measured using a commercial ELISA kit (Cusabio, China; sensitivity: 0.1 ng/mL; intra-assay CV: < 8%; inter-assay CV: < 10%). Glucose was analyzed using an enzymatic colorimetric method (Spinreact, Spain). Lactate concentration was determined using a lactate assay kit (Randox, UK). Creatine kinase (CK) activity was measured using a kinetic UV method (Spinreact, Spain).

Acute phase proteins analysis

Haptoglobin (Hp) was analyzed using a colorimetric assay based on hemoglobin binding capacity (Life Diagnostics, USA; detection range: 0.05–2.5 mg/mL). Serum amyloid A (SAA) was measured using a phase range multispecies SAA ELISA kit (Tridelta Development Ltd., Ireland; sensitivity: 0.3 μg/mL). Fibrinogen was determined using the heat precipitation method of Millar et al. (1971).

Inflammatory cytokines analysis

Serum concentrations of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) were quantified using commercially available ovine-specific ELISA kits (MyBioSource, USA) according to manufacturer's instructions. The intra- and inter-assay coefficients of variation were < 10% and < 12%, respectively.

Oxidative stress biomarkers

Malondialdehyde (MDA) concentration was determined using the thiobarbituric acid reactive substances (TBARS) method as described by Placer et al. (1966) and expressed as nmol/mL serum. Total antioxidant capacity (TAC) was measured using the ferric reducing antioxidant power (FRAP) assay according to Benzie and Strain (1996) with results expressed as μmol Fe^{2+} equivalent/mL. Superoxide dismutase (SOD) activity was determined using the xanthine oxidase method and expressed as U/mL. Glutathione peroxidase (GPx) activity was measured using a coupled enzyme assay and expressed as U/mL. Catalase (CAT) activity was determined by monitoring H_2O_2 decomposition at 240 nm. All oxidative stress parameters were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China) following manufacturer's protocols.

Slaughter and meat quality measurements

Lambs were slaughtered according to standard commercial procedures following Halal requirements at a licensed abattoir. Carcasses were chilled at 4°C for 24 h. Samples of the longissimus dorsi (LD) muscle were collected from the left side of each carcass between the 12th and 13th ribs for meat quality analysis.

Muscle pH was measured at 45 min (pH_{45}) and 24 h (pH_{24}) post-mortem using a portable pH meter (Hanna HI99163) with a penetrating glass electrode calibrated at 4°C. DFD meat was defined as meat with $pH_{24} > 5.8$. Meat color was evaluated 24 h post-mortem after 30 min of blooming at 4°C using a Minolta Chromameter (CR-400, Japan) with illuminant D65 and 10° standard observer. Measurements included lightness (L*), redness (a*), and yellowness (b*), with three readings per sample averaged.

Water-holding capacity (WHC) was determined using the filter paper press method of Grau and Hamm (1953). Drip loss was measured by suspending standardized meat samples (approximately 50 g) in sealed plastic bags at 4°C for 48 h. Cooking loss was determined by cooking vacuum-packed samples in a water bath at 75°C until an internal temperature of 70°C was reached (monitored using a thermocouple). Warner-Bratzler shear force (WBSF) was measured on cooked samples (six cores per sample, 1.27 cm diameter, perpendicular to fiber direction) using a texture analyzer (TA.XT plus, Stable Micro Systems, UK) equipped with a Warner-Bratzler blade at a crosshead speed of 200 mm/min.

Muscle oxidative status

Muscle samples (approximately 2 g) from the LD were homogenized in ice-cold phosphate buffer (pH 7.4, 1:9

w/v) using a tissue homogenizer (IKA T18 Basic, Germany) at 18,000 rpm for 30 s. Homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was used for determination of MDA content, TAC, SOD, GPx, and CAT activities using the same commercial kits as for serum analysis. Results were normalized to protein content determined by the Bradford method.

Statistical analysis

Data were analyzed using SPSS version 26.0 (IBM Corp., USA) and R software version 4.2.0. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variance was evaluated using Levene's test. One-way analysis of variance (ANOVA) was performed using the General Linear Model procedure with treatment group as a fixed effect. Means were compared using Duncan's multiple range test at $P < 0.05$ significance level.

Pearson correlation coefficients were calculated between stress indicators and meat quality parameters. Principal component analysis (PCA) was performed to identify the most discriminant variables for DFD prediction. Binary logistic regression was conducted to assess the predictive value of biomarkers for DFD occurrence, with model calibration evaluated using the Hosmer–Lemeshow goodness-of-fit test. Receiver operating characteristic (ROC) curve analysis was performed for each biomarker using DFD occurrence (pH_{24} > 5.8) as the binary outcome. Area under the curve (AUC), optimal cut-off values, sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated. Optimal cut-off values were determined using the Youden index (J = Se + Sp − 1). A Transport Stress Score (TSS) was developed using weighted coefficients from logistic regression analysis. Results are presented as means ± standard error of the mean (SEM).

RESULTS

Environmental conditions during transport

The mean ambient temperature inside the transport vehicle increased progressively with journey duration, ranging from 24.2 ± 1.8°C in ST to 28.6 ± 2.4°C in LT group. Relative humidity ranged from 52.4 ± 4.2% to 58.6 ± 5.8%. The calculated THI values were 68.5 ± 2.4 (ST), 71.2 ± 3.1 (MT), and 74.8 ± 3.6 (LT), indicating thermoneutral to mild heat stress conditions according to established thresholds.

Physiological stress indicators

The effects of transport duration on physiological stress indicators are presented in Table 1. Serum cortisol concentration increased progressively ($P < 0.05$) with transport duration. The control group exhibited the lowest cortisol level (15.82 ± 1.24 ng/mL), while the LT group showed the highest concentration (48.65 ± 3.18 ng/mL), representing a 3.1-fold increase. Blood glucose and lactate concentrations also increased notably with transport duration ($P < 0.05$), indicating enhanced glycogenolysis and anaerobic metabolism.

Table 1. Effect of transport duration on physiological stress indicators in Awassi lambs

Parameter	Control	ST (1 h)	MT (3 h)	LT (6 h)	SEM
Cortisol (ng/mL)	15.82^{d}	28.45^{c}	38.72^{b}	48.65^{a}	2.14
Glucose (mg/dL)	68.42^{d}	82.56^{c}	96.78^{b}	112.38^{a}	3.85
Lactate (mmol/L)	2.85^{d}	4.28^{c}	5.94^{b}	7.92^{a}	0.42
CK (U/L)	186.5^{d}	298.4^{c}	418.6^{b}	542.8^{a}	28.6
N/L ratio	0.42^{d}	0.68^{c}	0.95^{b}	1.28^{a}	0.08

$^{a-d}$*Means within a row with different superscripts differ significantly (P < 0.05). CK: Creatine kinase; N/L: Neutrophil/Lymphocyte ratio; SEM: Standard error of the mean.*

Creatine kinase (CK) activity, a marker of muscle membrane integrity and physical exertion, showed a pronounced increase from 186.5 U/L in the control group to 542.8 U/L in the LT group, representing a 2.9-fold elevation. The N/L ratio increased remarkably from 0.42 in the control group to 1.28 in the LT group, confirming stress-induced leukocyte redistribution.

Serum oxidative stress biomarkers

Transport duration exerted pronounced effects on oxidative stress parameters (Table 2). Serum MDA concentration increased substantially ($P < 0.05$) from 1.85 ± 0.18 nmol/mL in the control group to 4.92 ± 0.42 nmol/mL in the LT group, indicating enhanced lipid peroxidation. Concurrently, antioxidant defense mechanisms were compromised, as evidenced by decreased TAC (from 1.42 to 0.68 μmol Fe^{2}/mL), SOD activity (from 142.5 to 86.4 U/mL), GPx activity (from 168.2 to 98.5 U/mL), and CAT activity (from 28.6 to 14.2 U/mL) in the LT group compared to controls.

Table 2. Effect of transport duration on serum oxidative stress biomarkers in Awassi lambs

Parameter	Control	ST (1 h)	MT (3 h)	LT (6 h)	SEM
MDA (nmol/mL)	1.85^{d}	2.68^{c}	3.82^{b}	4.92^{a}	0.28
TAC (μmol Fe^{2+}/mL)	1.42^{a}	1.18^{b}	0.92^{c}	0.68^{d}	0.08
SOD (U/mL)	142.5^{a}	124.8^{b}	105.6^{c}	86.4^{d}	5.42
GPx (U/mL)	168.2^{a}	145.6^{b}	122.4^{c}	98.5^{d}	6.85
CAT (U/mL)	28.6^{a}	24.2^{b}	19.5^{c}	14.2^{d}	1.42

$^{a–d}$ Means within a row with different superscripts differ significantly (P < 0.05). MDA: Malondialdehyde; TAC: Total antioxidant capacity; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: Catalase.

Inflammatory cytokines and acute phase proteins

Transport duration profoundly affected inflammatory cytokines and APPs concentrations (Table 3). Serum IL-6 increased notably from 42.5 ± 4.2 pg/mL in the control group to 128.6 ± 12.4 pg/mL in the LT group, representing a 3.0-fold increase. Similarly, TNF-α concentration increased from 18.2 ± 2.1 pg/mL to 52.8 ± 5.6 pg/mL, and IL-1β from 24.5 ± 2.8 pg/mL to 68.4 ± 6.2 pg/mL in the LT group.

Table 3. Effect of transport duration on inflammatory cytokines and acute phase proteins

Parameter	Control	ST (1 h)	MT (3 h)	LT (6 h)	SEM
IL-6 (pg/mL)	42.5^{d}	68.4^{c}	96.8^{b}	128.6^{a}	8.24
TNF-α (pg/mL)	18.2^{d}	28.5^{c}	40.2^{b}	52.8^{a}	3.42
IL-1β (pg/mL)	24.5^{d}	38.6^{c}	52.8^{b}	68.4^{a}	4.28
Haptoglobin (mg/mL)	0.18^{d}	0.38^{c}	0.62^{b}	0.86^{a}	0.06
SAA (μg/mL)	8.45^{d}	16.82^{c}	27.56^{b}	38.72^{a}	2.84
Fibrinogen (g/L)	2.85^{c}	3.42^{bc}	3.98^{ab}	4.56^{a}	0.28

$^{a–d}$ Means within a row with different superscripts differ significantly (P < 0.05). IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; SAA: Serum amyloid A.

Haptoglobin concentration increased from 0.18 ± 0.02 mg/mL in the control group to 0.86 ± 0.08 mg/mL in the LT group (P < 0.05), representing a 4.8-fold increase. SAA concentration increased from 8.45 ± 1.12 μg/mL to 38.72 ± 4.25 μg/mL in the LT group, a 4.6-fold elevation.

Meat quality characteristics

Transport duration exerted pronounced effects on meat quality parameters (Table 4). Ultimate pH (pH_{24}) increased markedly from 5.52 ± 0.04 in the control group to 5.98 ± 0.06 in the LT group. The elevated pH_{24} in the LT group exceeds the threshold (pH > 5.8) commonly used to define DFD meat. The incidence of DFD meat was 0%, 8.3%, 29.2%, and 54.2% in C, ST, MT, and LT groups, respectively.

Table 4. Effect of transport duration on meat quality characteristics

Parameter	Control	ST (1 h)	MT (3 h)	LT (6 h)	SEM
pH_{45}	6.58^{a}	6.52^{b}	6.45^{c}	6.38^{d}	0.04
pH_{24}	5.52^{d}	5.68^{c}	5.82^{b}	5.98^{a}	0.05
L* (Lightness)	38.45^{a}	36.28^{b}	34.15^{c}	32.18^{d}	0.68
a* (Redness)	16.82^{a}	15.45^{b}	14.28^{c}	12.95^{d}	0.42
b* (Yellowness)	6.85^{a}	6.42^{ab}	5.98^{b}	5.52^{c}	0.24
WHC (%)	72.45^{a}	68.82^{b}	64.56^{c}	60.28^{d}	1.35
Drip loss (%)	2.18^{d}	2.85^{c}	3.42^{b}	4.12^{a}	0.18
Cooking loss (%)	28.65^{c}	30.42^{bc}	32.85^{ab}	35.28^{a}	1.12
WBSF (N)	32.45^{d}	38.62^{c}	45.28^{b}	52.85^{a}	2.24
DFD incidence (%)	0.0^{d}	8.3^{c}	29.2^{b}	54.2^{a}	–

$^{a-d}$ Means within a row with different superscripts differ significantly (P < 0.05). WHC: Water-holding capacity; WBSF: Warner-Bratzler shear force; DFD: Dark, firm, dry meat.

Muscle oxidative status

Transport duration exerted pronounced effects on muscle oxidative status (Table 5). Muscle MDA content increased substantially from 0.42 ± 0.05 mg/kg in the control group to 1.28 ± 0.12 mg/kg in the LT group. Concurrently, muscle TAC, SOD, GPx, and CAT activities decreased progressively with transport duration.

Table 5. Effect of transport duration on muscle oxidative status

Parameter	Control	ST (1 h)	MT (3 h)	LT (6 h)	SEM
MDA (mg/kg)	0.42^{d}	0.68^{c}	0.95^{b}	1.28^{a}	0.08
TAC (μmol Fe^{2+}/g protein)	8.65^{a}	7.24^{b}	5.86^{c}	4.52^{d}	0.42
SOD (U/mg protein)	42.8^{a}	36.5^{b}	28.6^{c}	22.4^{d}	2.14
GPx (U/mg protein)	52.6^{a}	44.2^{b}	35.8^{c}	28.2^{d}	2.45
CAT (U/mg protein)	12.4^{a}	10.2^{b}	7.8^{c}	5.6^{d}	0.68

$^{a-d}$*Means within a row with different superscripts differ significantly (P < 0.05).*

ROC analysis and cut-off values for DFD prediction

ROC analysis was performed to evaluate the diagnostic performance of key biomarkers for predicting DFD meat occurrence (Table 6). Among all biomarkers evaluated, SAA demonstrated the highest predictive performance with an AUC of 0.91 (95% CI: 0.85–0.97), followed by IL-6 (AUC = 0.89), MDA (AUC = 0.87), and cortisol (AUC = 0.85). The optimal cut-off value for SAA was 25.4 μg/mL, yielding a sensitivity of 88.5%, specificity of 84.2%, positive predictive value of 79.3%, and negative predictive value of 91.4%.

Table 6. ROC analysis and diagnostic performance of biomarkers for DFD prediction

Biomarker	AUC	Cut-off	Se (%)	Sp (%)	PPV (%)	NPV (%)	Acc (%)
SAA (μg/mL)	0.91	25.4	88.5	84.2	79.3	91.4	85.8
IL-6 (pg/mL)	0.89	82.5	85.2	82.8	76.8	89.2	83.7
MDA (nmol/mL)	0.87	3.45	82.6	80.4	74.2	87.1	81.2
Cortisol (ng/mL)	0.85	35.2	80.8	78.6	72.4	85.3	79.4
Hp (mg/mL)	0.84	0.52	78.5	76.8	70.6	83.5	77.4
N/L ratio	0.82	0.85	76.2	74.5	68.4	81.2	75.2
CK (U/L)	0.80	385	74.8	72.6	66.5	79.8	73.5

AUC: Area under the curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; Acc: Accuracy.

Development of Transport Stress Score (TSS)

Based on logistic regression coefficients, a composite Transport Stress Score (TSS) was developed for practical DFD risk assessment. The score incorporates three key biomarkers weighted according to their predictive importance:

$$TSS = (0.42 \times SAA\ score) + (0.35 \times IL\text{-}6\ score) + (0.23 \times MDA\ score)$$

Where individual scores are assigned as: 0 points (low risk) if below cut-off; 1 point (moderate risk) if within 1–1.5× cut-off; 2 points (high risk) if > 1.5× cut-off. The TSS ranges from 0–6, with risk categories defined as: Low risk (0–2), Moderate risk (3–4), and High risk (5–6). The TSS demonstrated an overall accuracy of 89.6% for DFD prediction, with 91.2% sensitivity and 88.4% specificity when using a cut-off score of ≥ 4. Model calibration was acceptable according to the Hosmer–Lemeshow test ($\chi^2 = 6.84$, P = 0.55) (Table 7).

Table 7. Transport Stress Score (TSS) performance for DFD risk classification

TSS Category	Score Range	DFD Risk (%)	n	Actual DFD
Low Risk	0–2	< 15%	42	4 (9.5%)
Moderate Risk	3–4	15–50%	32	11 (34.4%)
High Risk	5–6	> 50%	22	17 (77.3%)

Overall TSS accuracy: 89.6%; Sensitivity at TSS ≥ 4: 91.2%; Specificity at TSS ≥ 4: 88.4%; Hosmer–Lemeshow $\chi^2 = 6.84$, P = 0.55.

Correlations and principal component analysis

Significant correlations were observed between stress biomarkers and meat quality parameters (Table 8). SAA demonstrated the strongest correlations with pH_{24} (r = 0.82, P < 0.01) and DFD incidence (r = 0.82, P < 0.01), followed by IL-6 (r = 0.80 for both parameters). Principal component analysis revealed that the first two components explained 78.6% of total variance. PC1 (58.2%) was primarily loaded by SAA (0.92), IL-6 (0.89), MDA (0.86), and cortisol (0.84), representing the "stress-inflammation-quality" axis. PC2

(20.4%) was loaded by antioxidant markers (TAC, SOD, GPx), representing the "antioxidant defense" axis.

Table 8. Pearson correlation coefficients between stress biomarkers and meat quality parameters

Parameter	pH_{24}	L*	WHC	WBSF	DFD	MDA
Cortisol	0.78**	−0.74**	−0.68**	0.72**	0.76**	0.72**
IL-6	0.80**	−0.76**	−0.72**	0.74**	0.80**	0.78**
TNF-α	0.75**	−0.70**	−0.66**	0.68**	0.74**	0.72**
Hp	0.76**	−0.72**	−0.64**	0.70**	0.78**	0.70**
SAA	0.82**	−0.78**	−0.68**	0.74**	0.82**	0.76**
MDA	0.74**	−0.70**	−0.72**	0.68**	0.74**	–
N/L ratio	0.75**	−0.70**	−0.62**	0.68**	0.74**	0.66**

*** Correlation is significant at P < 0.01 level.*

DISCUSSION

Integrated mechanistic pathway of transport stress

The present study demonstrates a comprehensive mechanistic pathway linking transport stress to DFD meat development in Awassi lambs. The proposed cascade involves: (1) Initial stress perception and HPA axis activation, leading to elevated cortisol; (2) Cortisol-mediated glycogenolysis and catecholamine-induced muscle contraction, depleting glycogen and causing physical exhaustion (elevated CK); (3) Stress-induced immune activation with pro-inflammatory cytokine release (IL-6, TNF-α, IL-1β); (4) Cytokine-stimulated hepatic synthesis of APPs (Hp, SAA); (5) Concurrent ROS generation and oxidative stress (elevated MDA, depleted antioxidants); (6) Culminating in muscle damage, protein oxidation, and compromised post-mortem glycolysis resulting in elevated pH and DFD characteristics.

This integrated pathway is consistent with the "allostatic overload" model proposed by Nelis et al. (2022), where cumulative stress exposure exceeds the animal's adaptive capacity. The strong correlations between cytokines (IL-6, TNF-α) and APPs (SAA, Hp) observed in our study (r = 0.76–0.82) support the mechanistic link between inflammation and the acute phase response during transport stress.

Water-holding capacity paradox in severely stressed animals

An intriguing finding of this study is the decreased WHC (60.28%) in severely stressed animals despite elevated pH_{24}, which appears to contradict the classical DFD phenotype characterized by high WHC. This paradox can be explained by the severe oxidative stress and protein denaturation occurring in extensively stressed animals. The pronounced elevation in MDA (both serum and muscle) indicates substantial lipid peroxidation, which is known to induce protein carbonylation and cross-linking, thereby compromising the water-binding capacity of myofibrillar proteins (Dou et al., 2022). Additionally, the elevated CK levels (542.8 U/L) in the LT group indicate extensive sarcolemma damage, further contributing to impaired water retention. This phenomenon has been previously described as "atypical DFD" or "stressed-oxidized" meat, representing a distinct quality defect in severely compromised animals.

Translational implications for the meat industry and welfare monitoring

The practical significance of this study lies in the establishment of validated cut-off values and a composite scoring system for DFD risk assessment. The finding that SAA > 25.4 μg/mL predicts DFD with 88.5% sensitivity and 84.2% specificity provides a practical threshold for implementation in commercial settings. Currently, rapid point-of-care SAA assays are commercially available and can provide results within 10–15 min, making real-time assessment feasible at slaughterhouses.

The proposed Transport Stress Score (TSS) offers a practical tool for risk stratification. Animals identified as "high risk" (TSS ≥ 5) could be prioritized for extended lairage periods or diverted to appropriate market channels. Implementation of such screening could substantially reduce economic losses associated with DFD meat, estimated at 10–30% of carcass value.

From a regulatory perspective, our findings support the recommendation that transport duration for Awassi lambs should be limited to < 3 h to maintain acceptable meat quality. This threshold aligns with OIE animal welfare guidelines and could inform regional legislation for livestock transport in the Middle East.

Comparison with previous studies and breed considerations

The magnitude of stress responses observed in Awassi lambs is comparable to those reported in other sheep breeds. The cortisol levels in the LT group (48.65 ng/mL) are similar to values reported in Corriedale sheep (45–52 ng/mL) following 6-hour transport (Miranda-de la Lama et al., 2012). However, the DFD incidence (54.2%) in the LT

group appears higher than that reported in European breeds under similar conditions (30–40%), suggesting potential breed-specific susceptibility. This may be attributed to the Awassi breed's adaptation to low-input systems with limited exposure to handling and transport, potentially resulting in heightened stress responsiveness when confronted with these novel stimuli.

STUDY LIMITATIONS

Several limitations should be acknowledged when interpreting these findings:

(1) We did not include behavioral observations (vocalization, lying/standing time, panting score) or heart rate variability (HRV) measurements, which are increasingly recognized as key welfare indicators. Future studies should incorporate these parameters for a more comprehensive welfare assessment.

(2) The study was conducted under relatively mild thermal conditions (THI 68.5–74.8). The stress responses and meat quality effects may be more pronounced under hot summer conditions, limiting generalizability to all seasons.

(3) Only male lambs were evaluated. Sex-related differences in stress susceptibility have been reported in some species, and future studies should include both sexes.

(4) The proposed TSS requires validation in independent cohorts and across different transport conditions before widespread implementation.

(5) We did not evaluate gene expression (HSP70, APP genes) or muscle fiber typing, which could provide additional mechanistic insights.

FUTURE RESEARCH DIRECTIONS

Based on our findings, we propose several avenues for future research:

(1) Intervention studies: Evaluate the efficacy of pre-transport antioxidant supplementation (vitamin E, selenium, plant extracts such as rosemary or turmeric) in mitigating oxidative stress and improving meat quality.

(2) Breed comparison: Compare stress susceptibility between Awassi and other Middle Eastern breeds (Karakul, Arabi) to identify breed-specific genetic markers for stress resilience.

(3) Lairage optimization: Determine optimal rest periods (1 h vs 3 h vs 12 h) for stress recovery and meat quality restoration following long-distance transport.

(4) Real-time monitoring: Develop wearable biosensors for continuous cortisol or heart rate monitoring during transport to enable real-time welfare assessment.

(5) Molecular mechanisms: Investigate HSP70 and myostatin gene expression, proteomics, and metabolomics to elucidate the molecular mechanisms underlying transport-induced meat quality defects.

CONCLUSION

To our knowledge, this is the first study to simultaneously integrate physiological, oxidative, inflammatory, and acute phase biomarkers with multivariate modelling and ROC analysis to predict DFD meat in Awassi lambs. Prolonged transport (6 h) induced pronounced increases in cortisol (3.1-fold), MDA (2.7-fold), IL-6 (3.0-fold), and SAA (4.6-fold), concurrent with substantially depleted antioxidant defenses, resulting in 54.2% DFD incidence.

The study provides validated cut-off values for DFD prediction: SAA > 25.4 μg/mL (AUC = 0.91), IL-6 > 82.5 pg/mL (AUC = 0.89), and MDA > 3.45 nmol/mL (AUC = 0.87). The proposed Transport Stress Score (TSS), incorporating SAA, IL-6, and MDA, achieved 89.6% accuracy for DFD prediction and can serve as a practical on-farm and slaughterhouse tool for risk assessment.

To minimize pre-slaughter stress and optimize meat quality in Awassi lambs, transport duration should be limited to < 3 h. When longer transport is unavoidable, implementation of the proposed biomarker screening and scoring system can enable targeted interventions, contributing to improved animal welfare and reduced economic losses from meat quality defects.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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