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**Extending the interval between second vaccination and slaughter: I. Effects on growth, scrotal size and stress responses of immunocastrated ram lambs.**

T. Needham1,2, H. Lambrechts1 and L.C. Hoffman1,3

1Department of Animal Sciences, University of Stellenbosch, Private Bag X1, Matieland, Stellenbosch, 7602, South Africa.

2Department of Animal Science and Food Processing, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, Kamýcká 961/129, Prague 165 00, Czech Republic.

3Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation (QAAFI). The University of Queensland, Health and Food Sciences Precinct, 39 Kessels Rd, Coopers Plains 4108. Australia.

Corresponding author: Louw Hoffman. louwrens.hoffman@uq.edu.au

Growth of immunocastrated and castrated rams

**Table S1** *Analysed nutrient composition of supplementary commercial sheep complete finisher diet fed to immunocastrated, Burdizzo-castrated and intact Dohne Merino ram lambs for 52 days at 500g per sheep per day.*

|  |  |
| --- | --- |
| Analysed Nutrient Composition, As-is basis | Value |
| Gross Energy (MJ/kg) | 16.36 |
| Neutral detergent fibre (g/kg) | 247.1 |
| Acid detergent fibre (g/kg) | 145.1 |
| Crude protein1 (g/kg) | 139.6 |
| Crude fat (g/kg) | 23.3 |
| Calcium (g/kg) | 10 |
| Phosphorus (g/kg) | 2 |
| 123.9 % derived from urea |  |

**Supplementary Material S1** *Serum cortisol extraction protocol and analysis*

Cortisol was extracted using a liquid-liquid extraction (Quanson *et al.*, 2016). Firstly, 50 µL of deionized water containing the internal standard of 15 ng cortisol-9, 11, 12, 12-d4 (Cambridge Isotope Laboratories, Andover, USA) was added to 500 µL of the collected serum samples. Subsequently 1.5 mL of UHPLC-grade tert-Methyl Butyl Ether (Sigma-Aldrich, Steinheim, Germany) was added to the serum and vortexed at 1000 RPM for 10 minutes. The samples were then frozen at -80 °C for 60 minutes after which the non-frozen, non-polar phase was transferred and evaporated under nitrogen gas at 55 °C. Samples were reconstituted using 50 µL of 50 % methanol (ROMIL, Cambridge, England), vortexed and transferred into vials to be stored at -20 °C until analysis.

 Standard curves were established using cortisol-9, 11, 12, 12-d4 in 50 % methanol. Cortisol concentration was quantified using ultra-performance convergence chromatography tandem mass spectrometry (UPC2-MS/MS) according to Quanson *et al.* (2016). The Acquity UPC2 system was fitted with an Acquity UPC2 BEH 2-EP column (3 mm x 100 mm; 1.7 µm particle size; Waters Corporation, USA) with a mobile phase comprising of carbon dioxide modified with methanol. A four-minute linear gradient from 2 to 9.5 % methanol with a constant flow rate of 2.0 mL/minute was used to separate the C19 steroids within the prepared samples at an injection volume of 2 µL. Column temperature was 60°C and automated back pressure regulator was 2000 psi. Quantification of cortisol was performed using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA) attached to a make-up pump supplying 1% formic acid in methanol at a flow rate of 0.2 mL/minute. Sample were analysed using Multiple Reaction Monitoring mode with an electrospray probe in positive ionization mode under 3.8 kV capillary voltage, 120 °C source temperature, 500 °C desolvation temperature, 1000 L/h desolvation gas and 150 L/h cone gas. Analysis of raw data was performed using MassLynxTM software (Waters Corporation, USA).

**Figure S1** The injection site surface skin temperature (top), scrotal surface temperature (middle) and rectal temperature (bottom) of Dohne Merino ram lambs immunocastrated at either six (ICS6; n = 10) or four (ICS4; n = 10) weeks before slaughter (Day 57), Burdizzo-castrated on D2 (B; n = 10) and intact rams (R; n = 10). Vaccinations are indicated by arrows and vertical bars denote SEM.