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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.**

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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.