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# Hypothalamic-pituitary-adrenal axis activation and immune regulation in heat-stressed sheep after supplementation with polyunsaturated fatty acids

**M. Caroprese,*[1] M. G. Ciliberti,* G. Annicchiarico,† M. Albenzio,* A. Muscio,* and A. Sevi***
*Department of the Sciences of Agriculture, Food and Environment (SAFE), University of Foggia, Via Napoli, 25, 71122 Foggia, Italy
†Council for Research and Experimentation in Agriculture, 71020 Foggia, Italy

## ABSTRACT

The aim of this study was to assess the effects of supplementation with polyunsaturated fatty acids from different sources on immune regulation and hypothalamic-pituitary-adrenal (HPA) axis activation in heat-stressed sheep. The experiment was carried out during the summer 2012. Thirty-two Comisana ewes were divided into 4 groups (8 sheep/group): (1) supplemented with whole flaxseed (FS); (2) supplemented with *Ascophyllum nodosum* (AG); (3) supplemented with a combination of flaxseed and *A. nodosum* (FS+AG); and (4) control (C; no supplementation). On d 22 of the experiment, cortisol concentrations in sheep blood were measured after an injection of ACTH. Cellular immune response was evaluated by intradermic injection of phytohemagglutinin (PHA) at 0, 15, and 30 d of the trial. Humoral response to ovalbumin (OVA) was measured at 0, 15, and 30 d. At 0, 15, and 30 d of the experiment, blood samples were collected from each ewe to determine production of T-helper (Th)1 cytokines (IL-12 and IFN-γ), and Th2 cytokines (IL-10, IL-4, IL-13), and concentrations of heat shock proteins (HSP) 70 and 90. Ewes supplemented with flaxseed alone had greater cortisol concentrations and a longer-lasting cell-mediated immune response compared with ewes in the control and other groups. Anti-OVA IgG concentrations increased in all groups throughout the trial, even though ewes in the FS+AG group had the lowest anti-OVA IgG concentrations at 15 d. The level of IL-10 increased in all groups throughout the experiment; the FS+AG group had the lowest IL-13 concentration at 15 and 30 d. The concentration of HSP 70 increased in AG ewes at the end of the experiment and decreased in FS ewes, whereas that of HSP 90 increased in FS ewes compared with FS+AG ewes. Flaxseed supplementation was found to influence in vivo HPA activation in heat-stressed sheep, resulting in increased cortisol concentrations, probably to meet increased energy demand for thermoregulation. Flaxseed supplementation also supported Th1 response via a complex cross-talk between IL-10, IL-12, and IFN-γ production.
**Key words:** cytokine, sheep, polyunsaturated fatty acids, heat stress, immune regulation

## INTRODUCTION

Stressors of different natures and intensity can induce production of extracellular and intracellular mediators to modulate cell responses. Heat stress stimulates signal transduction pathways to alter gene expression of immune cell mediators (i.e., cytokines) and can activate heat shock response. Heat shock response evokes large changes in the secretion and activity of heat shock proteins (**HSP**), which are strictly connected to the endocrine and immune system (Collier et al., 2008). The HSP are classified into different families according to their molecular weights; those of approximately 90, 70, and 27 kDa are referred to as HSP 90, HSP 70, and HSP 27, respectively. Cultured sheep lymphocytes have been demonstrated to produce HSP 90 and HSP 70 in response to thermal stress (Guerriero and Raynes, 1990). The engagement of extracellular HSP also includes the promotion of cytokine activity (Martin et al., 2009). Distinct cytokine patterns are responsible for the effector functions and development of T-helper 1 (**Th1**) and T-helper 2 (**Th2**) cell responses. The Th1 cells activate cellular immunity and inflammatory responses, whereas the Th2 cells control humoral immunity and promote antiinflammatory responses. The ability of animals to produce selectively Th1 cytokines (IFN-γ and IL-12) and Th2 cytokines (IL-10, IL-4, IL-13), and thereby to regulate the Th1:Th2 cytokine balance, is highly temperature dependent (Park et al., 2005). Hyperthermia can downregulate Th1 cytokines in favor of the secretion of Th2 cytokines, thus suppressing cell-mediated immunity (Murzenok et al., 1997; Elenkov and Chrousos, 1999; Webster et al., 2002). Ewes subjected to heat stress showed impairment of their cellular immune

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[1]Corresponding author: mariangela.caroprese@unifg.it

response after an intradermal injection of mitogens and had increased cortisol concentrations (Sevi et al., 2002; Caroprese et al., 2012). It has been demonstrated that corticosteroids, which can increase under stress conditions, bind to DNA and inhibit the expression of genes involved in T-cell activation and cytokine production (Sgorlon et al., 2012). Antiinflammatory properties of corticosteroids lead to decreased phagocytic cell activity, which in turn alters lymphocyte function (Ronsen at al., 2001). Several ruminant diseases, including Johne's disease, require a strong cell-mediated immune response to protect against pathogenic bacteria (Begg et al., 2005). Therefore, the maintenance of the proper Th1:Th2 balance could be a critical factor in facing immunological challenges, particularly if they occur during the summer season.

Few studies have focused on the effects of heat stress on nutrient utilization and circulating hormones to cope with immune challenges in sheep. Recently, Caroprese et al. (2012) found that in heat-stressed ewes, the supplementation of whole flaxseed, rich in the n-3 PUFA α-linolenic acid (C18:3, **ALA**), sustains humoral responses and induces an increase in cortisol secretion. The precise mechanisms underlying cellular immune and humoral functions in sheep under high temperatures, however, remain undefined, particularly with regard to cytokine profiles in vivo and to activation of the hypothalamic-pituitary-adrenal (**HPA**) axis.

To overcome heat stress in lambs, Saker et al. (2004) suggested supplementation with an extract from a brown seaweed, *Ascophyllum nodosum* (Tasco; Acadian Seaplants Ltd., Dartmouth, NS, Canada). The high antioxidant content of *A. nodosum* was found to be partly responsible for the enhanced immune function and antioxidant status in heat-stressed lambs. In addition, *A. nodosum* contains n-3 PUFA such as eicosapentaenoic acid (C20:5, **EPA**), which are considered health-promoting molecules, and fucoidan, which has antiinflammatory and antimicrobial activities.

No studies have evaluated the combined effect of flaxseed and algae as supplementation to heat-stressed sheep. We hypothesized that the administration of PUFA supplemented as whole flaxseed or algae, or in combination, to the diets of dairy sheep under heat stress could contribute to alter HPA activation and sustain their immune functions and Th1:Th2 balance.

The objective of the present experiment was therefore to determine the effects of supplementing the diet with PUFA from flaxseed or algae, or their combination, on immune regulation, in terms of cell-mediated and humoral immune responses and HPA activation in sheep under heat-stress conditions. We also investigated the balance between Th1 and Th2 cells by monitoring secretion of interleukins and production of HSP in vivo.

## MATERIALS AND METHODS

### *Animals and Experimental Design*

The experiment lasted 30 d and was conducted during the summer (June–July) 2012 at Segezia research station of the Council for Research and Experimentation in Agriculture (CRA-ZOE) in Apulia, Italy. Thirty-two late-lactation Comisana ewes (202.1 ± 5.3 d of lactation, mean ± SD) were divided into 4 groups of 8 balanced for milk yield, BW, and BCS. Groups were separately reared in external pens of 5 × 12 m bounded with a mesh fence; the trough and crib were located in the external areas. During the trial, ambient temperature and relative humidity in indoor and outdoor areas were monitored with thermo-hygrographs (I-20090, LSI, Settala Premenugo-Milano, Italy) placed at 1.5 m from the floor. The average temperature-humidity index (**THI**) was calculated using the formula of Kelly and Bond (1971).

All groups were individually fed twice daily and received 1.8 kg/ewe daily of oat hay. The control group also received 1 kg/ewe daily of pelleted concentrate (Mangimificio Molino Gallo, Potenza, Italy), whereas ewes in the experimental groups were supplemented with whole flaxseed (**FS**; Lin Tech, Tecnozoo srl, Torreselle di Piombino Dese, Italy), or *A. nodosum* (**AG**; Tasco), or their combination (**FS+AG**). Thus, ewes in the FS group received 750 g/ewe per day of pelleted concentrate, and 250 g/ewe per day of whole flaxseed; ewes in the AG group received 1 kg/ewe per day of pelleted concentrate in which 5% *A. nodosum* was incorporated; ewes in the FS+AG group were supplemented with both flaxseed (250 g/d) and pelleted concentrate (750 g/d) incorporating 5% *A. nodosum.* Dry matter intake was determined for each experimental group by weighing the refusals at 0800, 1200, 1600, and 2000 h. Water was available ad libitum for all groups from automatic drinking troughs at any time of day.

All procedures were conducted according to the guidelines of EU Directive 2010/63/EU (European Union, 2010) on the protection of animals used for experimental and other scientific purposes. Ewes were healthy and their conditions were carefully examined by veterinarians throughout the trial to exclude the presence of signs of diseases.

The chemical composition of diets was carried out by standard procedures (AOAC, 1990). Ingredients and chemical composition of diets are reported in Table 1. The determination of methyl esters of the diet ingredients was carried out according to O'Fallon et al. (2007). Briefly, 1 g of each sample was pipetted into a screw-cap (16 × 25 mm) reaction tube. Into each tube, 1.0 mL of the C13:0 internal standard (0.5 mg of C13:0/mL of methanol), 0.7 mL of KOH, and 5.3 mL

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