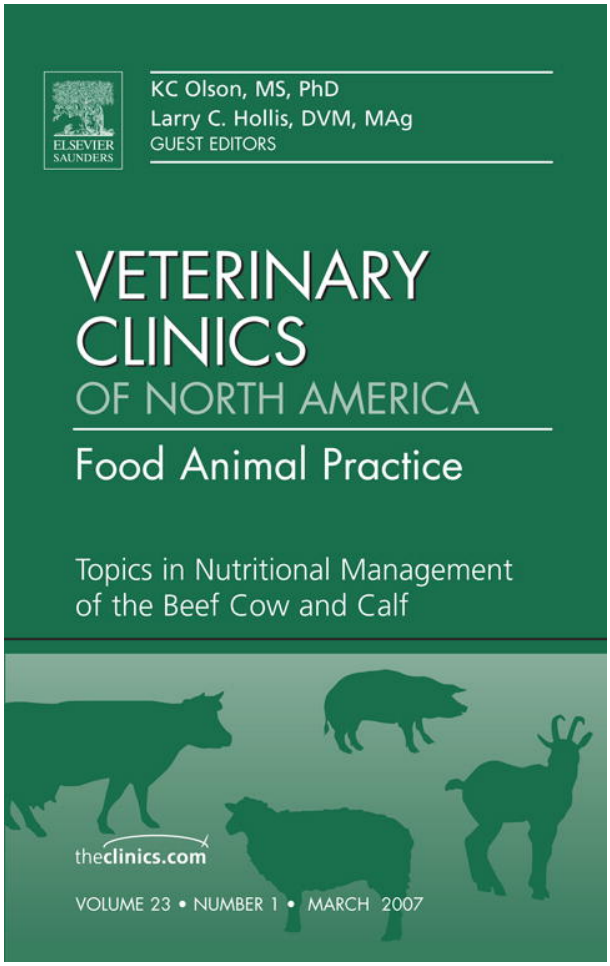

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Influence of Stress and Nutrition on Cattle Immunity

Jeffery A. Carroll, PhD[a,*], Neil E. Forsberg, PhD[b,c]

[a]*USDA-ARS, Livestock Issues Research Unit, 1604 East FM 1294, Lubbock, TX 79403, USA*
[b]*Department of Animal Sciences, Oregon State University, 112 Withycombe Hall, Corvallis, OR 97331-6702, USA*
[c]*OmniGen Research LLC, 2001 NW Monroe, Suite 203, Corvallis, OR 97330, USA*

General concepts of stress regulation

The debate among animal scientists concerning the definition and quantification of stress as it relates to animal productivity and well-being is ongoing. However, an increased appreciation and understanding of the effects of stress on livestock production has emerged throughout the scientific community and with livestock producers. Although the physiologic consequences of stress on the body have been of scientific interest for many years, scientists have yet to elucidate fully all the endocrine, neuroendocrine, and immunologic pathways that are altered as a result of stress. In the 1930s, Hans Selye was the first scientist to introduce the term "stress" into the medical community, at which time he proposed that, regardless of the stimuli, the body would respond in the same physiologic manner in an effort to maintain homeostasis. More than 6 decades later, the term stress, as it relates to bodily functions, continues to be defined in a similar manner as the sum of all biologic reactions to physical, emotional, or mental stimuli that disturb an individual's homeostasis [1]. These stimuli, known as stressors, elicit coordinated, physiologic responses within the body in an attempt to reestablish homeostasis, primarily through activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system [2].

Hypothalamic regulation

In response to both interoceptive and exteroceptive stimuli, corticotropin-releasing hormone (CRH) and vasopressin (VP) are secreted within

* Corresponding author.
E-mail address: jacarroll@lbk.ars.usda.gov (J.A. Carroll).

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the hypothalamus by their respective neurons. Although CRH and VP can mediate glucocorticoid secretion independently at the level of the pituitary and adrenal glands, they may also work in concert with one another to control the magnitude and duration of the glucocorticoid response. Both CRH and VP are potent stimulators of adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary gland. The relative potency of these neurohormones has been reported to be species-specific. Previous studies have demonstrated that in cultured bovine and porcine corticotrophs, CRH is the more potent stimulator of ACTH secretion [3–5]; however, in cultured ovine corticotrophs, VP has been reported to be the more potent stimulator of ACTH secretion [6,7]. An intriguing aspect of ACTH regulation is the ability of VP to potentiate CRH-induced ACTH secretion, which has been reported in several species, including rats, humans, pigs, and cattle [8–11]. What remains to be elucidated fully is the biologic need for multiple stimulators of ACTH secretion within the body, which highlights the relative importance of this system in the maintenance of homeostasis.

Plotsky and coworkers [12] demonstrated that specific stressors elicit specific ACTH secretagogues. For instance, CRH, VP, oxytocin, and catecholamines (all known stimulators of ACTH secretion) are released during hemorrhaging; however, during hypotension, CRH is the only secretagogue released [12,13]. Other studies have provided additional evidence that supports the concept that activation of the HPA axis is stressor-specific by demonstrating that specific, stressful stimuli elicit specific patterns of neurohormonal activation [14,15]. Given that CRH and VP are synthesized in approximately one half of the neurons of the paraventricular nucleus within the hypothalamus, and are co-localized in secretory granules of the median eminence [16–18], it appears that the brain has the ability to distinguish among stressful stimuli and release CRH, with or without VP, depending on the physiologic response needed to cope with the current stressor. Thus, contrary to Hans Selye's hypothesis, the body does not mount an all-or-none activation of the HPA axis, but rather elicits a specific physiologic response of the magnitude necessary to maintain or return to homeostasis.

The anterior pituitary gland

The pituitary gland (also referred to as the hypophysis), is an endocrine gland located in a small, bony cavity called the sella turcica of the sphenoid bone, at the base of the brain. It is composed of three separate endocrine organs: (1) the anterior lobe, (2) the posterior lobe, and (3) the intermediate lobe. The role of the intermediate lobe is species-specific. In fish, the intermediate lobe is thought to control physiologic color changes. In humans, and in a small number of other mammals, the intermediate lobe is considered rudimentary. The posterior lobe is an extension of the floor of the third ventricle that maintains physical contact with the ventricle by way of the

infundibulum (also referred to as the pituitary stalk). It acts primarily as a storage site for oxytocin and VP, both of which are synthesized in specialized neurons in the hypothalamus. The synthesis and release of VP as it relates to the stress response were described previously. The anterior pituitary, derived from the oral ectoderm, is composed of five phenotypically distinct cell types (somatotrophs, mammotrophs or lactotrophs, thyrotrophs, gonadotrophs, and corticotrophs) that can be classified histologically into three groups (chromophobes, acidophils, and basophils), based on their staining characteristics. Chromophobes are cells that do not stain and their biologic function has not been elucidated fully. Acidophils, as their name implies, readily stain with acidic dyes. Acidophils are primarily somatotrophs, which secrete growth hormone, and mammotrophs, which secrete prolactin. Mammosomatotrophs, a dual functioning acidophilic cell type, secrete both growth hormone and prolactin. The third histologic group is the basophils, which stain with a basic dye and synthesize and secrete trophic hormones. Basophils include thyrotrophs, which produce thyroid-stimulating hormone, gonadotrophs, which produce luteinizing hormone and follicle-stimulating hormone, and corticotrophs, which produce ACTH and related peptides. The reminder of this section focuses on neurohormonal (CRH and VP) stimulation of corticotrophs and the subsequent release of ACTH from the anterior pituitary gland. For a comprehensive overview of the effects of stress on growth hormone, prolactin, thyroid-stimulating hormone, and gonadotropins, readers are referred to a review by Matteri and colleagues [19].

Corticotrophs of the anterior pituitary synthesis ACTH [20] are part of a larger 256–amino acid polyprotein that serves as a precursor prohormone, known as proopiomelanocortin, which is cleaved enzymatically to form three melanocyte-stimulating hormones (α-, β-, and γ-MSH), three endorphins (α-, β-, and γ-endorphin), and ACTH. In pigs, cattle, sheep, and humans, ACTH is a single-chain polypeptide that consists of 39 amino acids [21]. The N-terminal amino acids 1–24 are identical in each of these species and are the portion of the protein that is responsible for the full biologic activity of the complete molecule. Species specificity of the ACTH molecule resides primarily in amino acids 25–33 of the C-terminus. The primary action of ACTH is to stimulate the synthesis and release of steroids from the adrenal gland, and to promote the uptake of cholesterol and its conversion to pregnenolone.

CRH and VP bind to their distinct membrane-bound receptors in the anterior pituitary gland [22,23]. On binding to their respective receptors, CRH activates protein kinase A, which is coupled to adenylate cyclase to produce 3',5' cyclic AMP (cAMP), whereas VP has been shown to activate protein kinase C, but does not stimulate the production of cAMP directly [24]. However, VP is capable of augmenting the CRH-induced production of cAMP in corticotrophs. Although the CRH and VP receptors vary in their second messenger signaling pathways, they do share similar properties, such

as desensitization and down-regulation in response to chronic stimulation [25,26]. Anther common characteristic is that stimulation of corticotrophs with either CRH or VP results in an increase in intracellular calcium, albeit by way of different mechanisms. Stimulation of corticotrophs with CRH causes a calcium influx from cytosolic origin, whereas stimulation of corticotrophs with VP causes mobilization of calcium from intracellular stores. Given that both CRH and VP stimulation of corticotrophs results in an overall increase in intracellular calcium, it has been speculated that the release of intracellular pools of calcium may be the main regulatory signal that activates ACTH release, irrespective of the secretagogue [27].

An intriguing aspect associated with CRH- and VP-stimulated ACTH release is that, whereas they regulate corticotroph activity differentially by way of different second messenger pathways (cAMP versus protein kinase C, respectively), a synergistic effect of these two neurohormones on ACTH secretion exists that can be linked to VP potentiation of cAMP accumulation in anterior pituitary corticotrophs [28]. Another interesting aspect is that not all corticotrophs equally express receptors for both CRH and VP. Some corticotrophs express receptors for only CRH or VP, whereas others express receptors for both [29,30]. The activation of corticotrophs and the subsequent release of ACTH become even more complex, considering that previous work has demonstrated that activation of one corticotroph appears to influence the responsiveness of another corticotroph to other secretagogues [31].

Ultimately, an increase in plasma concentration of ACTH stimulates the release of glucocorticoids from the adrenal cortex. Maintaining appropriate concentrations of glucocorticoids within the body is essential for the continuance of homeostasis and for overall survival.

The adrenal gland: glucocorticoid production

Regulation of the stress response at the level of the adrenal glands is no less complex than that of CRH and VP within the hypothalamus or ACTH within the anterior pituitary gland. As with the pituitary gland, the adrenal glands can be divided into two endocrine organs: (1) the adrenal cortex and (2) the adrenal medulla.

The adrenal cortex is composed of three distinct zones: (1) the zona glomerulosa, (2) the zona fasciculata, and (3) the zona reticularis. It is responsible for the synthesis and release of three major classes of adrenocortical steroid hormones (ie, mineralocorticoids, glucocorticoids, and androgens). Glucocorticoids and androgenic steroids are the major products of the zona fasciculata and zona reticularis, whereas mineralocorticoids (predominately aldosterone), are synthesized in the zona glomerulosa. Glucocorticoids and mineralocorticoids are essential for survival; however, adrenal androgens are thought to have a limited role in reproductive performance. Mineralocorticoids maintain sodium balance and extracellular fluid volume within the body, functions critical to sustaining life. During acclimation to

hot environments, aldosterone simulates the kidneys to conserve sodium and release potassium, while decreasing sodium content in sweat.

Glucocorticoids elicit a plethora of biologic effects on the body, including the metabolism of carbohydrates and protein, alterations in the growth and reproductive axes, regulation of the stress response, and influence on overall immune function. In rodents, corticosterone is the primary glucocorticoid, but in humans and most mammals, cortisol is the primary glucocorticoid produced in the adrenal cortex. Glucocorticoids play an important role in gluconeogenesis, the generation of glucose from other organic molecules like pyruvate, lactate, glycerol, and amino acids, during the flight or fight response. Glucocorticoids increase blood glucose concentrations by stimulating the liver to convert fat and protein to these intermediate metabolites that are ultimately converted to glucose for energy. Glucocorticoids also support the primary defense response by enhancing the synthesis and secretion of catecholamines, which control physiologic processes, such as heart rate, pupil dilation, vasoconstriction in the skin and gut, vasodilation in leg muscles, and increased glucose production by the liver, all of which are essential processes during the flight or fight response. Often considered the second line of defense is the suppression of the inflammatory and immune systems by glucocorticoids. Suppression of these systems prevents excessive and chronic stimulation which could prove deadly to the organism [32]. Specifically, glucocorticoids suppress the release of various cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-12, which can cause systemic disease [33]. Chronic exposure to high concentrations of glucocorticoids can cause severe physiologic and psychologic problems, such as excessive protein catabolism, hyperglycemia, immunosuppression, and depression. In domestic livestock, excessive concentrations of glucocorticoids have been linked to reduced rates of reproduction, suboptimal growth, suppressed milk production, and suppression of the immune function that could increase susceptibility to disease [34,35]. Equally important is the case of an inadequate supply of glucocorticoids, which can cause hypoactivity of the stress axis, leading to clinical conditions such as posttraumatic stress disorder [36] and chronic fatigue [37]. In the total absence of glucocorticoids, pathologic symptoms of myopathy, fatigue, hypotension, and susceptibility to autoimmune disorders and inflammation can develop, [38] and in adrenalectomized animals exposed to bacteria, death occurs at several logs lower than that of normal animals [39].

In addition to ACTH-stimulated glucocorticoid secretion, CRH and VP may play integral roles in the regulation of glucocorticoid production and secretion by way of paracrine actions within the adrenal glands. Previous studies have demonstrated the localization of CRH protein and mRNA in the adrenal gland [40–42], and CRH has been reported to directly stimulate glucocorticosteroid secretion from the adrenal gland in several species, including humans [43,44], rats [45], and cattle [46,47]. Similarly, VP may be involved in sustaining glucocorticoid and catecholamine secretion by acting

directly through V1a receptors in the adrenal cortex, or indirectly, by way of VP receptors in the adrenal medulla that stimulate local ACTH secretion [48–50]. In fact, VP has been reported to stimulate in vitro secretion of cortisol from cultured bovine adrenocortical cells [47].

Because ACTH has yet to be recognized as the primary physiologic regulator of cortisol production, researchers continue to investigate the relative roles and physiologic relevance of other adrenal steroid regulators, such as angiotensin II, cytokines, and various growth factors. Accordingly, as novel approaches are employed to elucidate the complex and integrative aspects of glucocorticoid synthesis and secretion further, the conventional view of HPA activation and regulation of the stress response is undergoing continual modification.

The adrenal gland: catecholamine production

Discussing the involvement of the adrenal glands in the activation of the HPA axis would be incomplete without at least elaborating briefly on the symbiotic relationship between the adrenal cortex and adrenal medulla. The adrenal medulla, which is part of the sympathetic division of the autonomic nervous system, is located in the center of the adrenal gland and is composed primarily of chromaffin cells. It originates during embryonic development from the neural crest and becomes a specialized sympathetic ganglion that is the principal site of the conversion of the amino acid tyrosine into the catecholamines. In response to psychologic or physical stress, catecholamines are secreted directly into the circulatory system, thereby functioning more as an endocrine and paracrine organ. The primary catecholamines secreted by the adrenal medulla are epinephrine (E), also referred to as adrenaline, norepinephrine (NE), also referred to as noradrenaline, and dopamine. The most prominent effects of E and NE include increased heart rate, blood vessel constriction, bronchiole dilation, and increased metabolism. Specifically, E is associated with increased heart rate and force of heart contractions, increased blood flow to the muscles and brain, relaxation of smooth muscles, and conversion of glycogen to glucose in the liver. Conversely, NE has minimal effect on smooth muscle activity, metabolic processes, and cardiac output. However, NE elicits strong vasoconstrictive effects that increase blood pressure. Although E and NE are both associated with increased alertness and awareness, E secretion is associated more with anxiety and fear. In humans, E accounts for most of the catecholamine (80%–85%) output from the adrenal vein, whereas in other species, NE is the primary catecholamine secreted. In addition to the aforementioned biologic actions, catecholamines have a substantial influence on the HPA axis and the overall stress response by way of regulation of hypothalamic neurohormone release, release of ACTH from the anterior pituitary gland, and stimulation of cortisol from the adrenal cortex [51–53].

As with the activation of the HPA axis and the associated stress response, the brain appears to be capable of mediating the magnitude of the sympathetic response in a manner that is customized for each individual, depending on the stressor and the individual's current physiologic status. Therefore, it appears to be this central interpretative regulation in the brain, coupled with the intercommunication between the sympathetic nervous system and the HPA, which work in unison to maintain homeostasis within the body.

General concepts of immunology

Generally, the immune system can be separated into three broad components: natural immunity, innate immunity, and acquired immunity, all of which must be developed fully and functioning properly to provide adequate immunologic protection. Typically, natural and innate immunity are grouped together under the category, innate immunity. Therefore, for the purposes of this article, the immune system is presented as two distinct arms that work in tandem to prevent infections: the innate immune system and the acquired immune system [54].

Innate immunity

The innate immune system is an evolutionarily ancient mechanism for fighting disease and represents the antigen-nonspecific defense mechanisms of the immune system, which are elicited immediately, or up to several hours (0 to 4 hours) after, exposure to an antigen. Innate immunity, which includes physical barriers such as the skin, mucosal secretions, tears, urine, and stomach acid, as well as complement and antigen-nonspecific cellular components, is considered to be the first line of defense against pathogens, whether bacterial, viral, protozoal, or fungal. Beneficial microorganisms in the intestine and respiratory tract that compete against invading pathogens are also an important part of innate immunity. By providing this front-line barrier, the innate system provides the time required by the acquired immune system to develop an antibody response against a specific pathogen, usually several days to several weeks. Although the term ''innate immunity'' implies that it is stable or unwavering, this is not the case. Innate immunity, although always present to some degree, is regulated and may be strengthened or weakened by a number of factors, including wounds, dehydration, nutritional status, genetics, and even stress. When the innate immune system is functioning properly, most of the pathogenic organisms encountered by an animal on a daily basis do not cause disease because either their invasion is blocked from entering the body by the aforementioned physical barriers, or they are detected readily and eliminated once they enter the body.

The cellular component of the innate immune system consists of phagocytic cells (eg, neutrophils, monocytes, macrophages, and dendritic cells), natural killer (NK) cells, and cells that release inflammatory mediators (basophils, mast cells, and eosinophils). Phagocytic cells are activated at sites of infection where they attack and kill pathogens before the pathogens have the opportunity to proliferate and cause a significant infection. Unlike adaptive immunity, phagocytic cells of the innate immune system do not recognize every possible antigen. Instead, they recognize a few highly conserved structures present in many different microorganisms. The structures recognized, called pathogen-associated molecular patterns (PAMPs) [54], interact with receptors on the surface of the immune cells [55]. Recognition is mediated through a structurally diverse set of pattern-recognition receptors that belong to several different protein families [56]. Generally, the pattern-recognition receptors can be divided into three functional groups: (1) circulating humoral proteins, such as the endotoxin receptor CD14 and complement proteins, (2) endocytic receptors that are expressed on the cell surface and mediate endocytosis, and (3) signaling receptors, such as toll-like receptors that are expressed on the surface of the cell [57]. Binding of PAMPs to toll-like receptors initiates killing mechanisms by the neutrophils and macrophages. Specifically, pathogens contain molecules not found typically in mammalian cells and, through this strategy, phagocytic cells of the innate system are able to recognize invading pathogens. Examples of PAMP molecules associated with pathogens that are recognized by innate cells include lipopolysaccharide from the gram-negative cell wall, peptidoglycan, lipoteichoic acids from the gram-positive cell wall, the sugar mannose (common in microbial glycolipids and glycoproteins, but rare in humans), bacterial DNA, N-formylmethionine found in bacterial proteins, double-stranded RNA from viruses, and glucans from fungal cell walls. Most body defense cells have pattern-recognition receptors for these common PAMPS, so the invading microorganism evokes an immediate response. PAMPs can be recognized also by a series of soluble, pattern-recognition receptors in the blood that function as opsonins and initiate the complement pathways.

Unlike phagocytic cells, NK cells are distinctive in that they do not attack the pathogens themselves, but attach and kill cells that have been infected by pathogens [58]. They are termed NK cells because they do not need to be activated by a specific antigen before taking action. NK cells target abnormal cells, such as tumor cells, and protect against various infectious microbes. On contact and binding to target cells, NK cells release a lethal burst of chemicals that produce holes in the cellular membrane of the target cell, which allows fluids to penetrate and burst the target cell. NK cells also contribute to activation of other immunologic processes by secreting high levels of cytokines. The release of the proinflammatory cytokines IL-1, IL-6, and IL-12, TNF-α, and interferon-γ (IFN-γ) by the innate immune system, in response to infection, initiates the acute phase response

(APR) and the adaptive immune response [59–61]. The initial release of proinflammatory cytokines is augmented by their paracrine actions, which cause further release of these cytokines and eventually results in the systemic release of cytokines.

Ultimately, the primary function of the innate immune response to a pathogen is to provide the first line of defense by either eliminating the pathogen from the host by way of its antimicrobial responses, or by controlling it until an acquired immune response can be generated to assist with the pathogen. Subsequent to activation of the innate immune system, the acquired immune response is initiated through a series of cellular events such as antigen presentation or cytokine signaling pathways.

Innate immunity: the acute phase response

The APR is a component of the innate body defense, which is characterized by varied reactions of the body to infection, inflammation, disease, or trauma. Characteristic reactions include fever; shifts in liver synthesis from normal products to acute phase proteins; alterations in plasma iron (Fe), zinc (Zn), and copper (Cu); increases in circulating white blood cells; and changes in behavior, such as lethargy (increased sleep), anorexia (decreased food and water intake), decreased social and sexual behavior, decreased aggressive behavior, and hyperalgesia (increased pain reactivity). Additionally, the stress response is enhanced with increased release of pituitary-adrenal and sympathetic hormones. The APR is stimulated by the release of proinflammatory cytokines (IL-1, IL-6, and TNF-α) from macrophages and monocytes at the site of inflammation or infection (Fig. 1). The initial release of proinflammatory cytokines is augmented by their paracrine actions, which cause further release of these cytokines and eventually results in a systemic release of cytokines. This increase in circulation of the proinflammatory cytokines stimulates the release of acute phase proteins from the liver.

Basically, two physiologic responses are regarded as being associated with acute inflammation: the febrile response and alterations in liver metabolism and gene regulation. The febrile response involves alteration of the temperature set point in the hypothalamus. The proinflammatory cytokines, IL-1, IL-6, and TNF-α, are considered to regulate the fever response through the induction of prostaglandin E2. Additionally, IL-1 and IL-6 act on the pituitary to increase ACTH secretion and, subsequently, cortisol from the adrenal cortex, which provides a negative feedback loop because glucocorticoids inhibit cytokine gene expression. The febrile response is a phylogenetically very old and ubiquitous defensive response that is associated with an acceleration of enzymatic processes involved in killing pathogens and preventing the formation of bacterial protective coats. Fever itself is sufficient to destroy many bacteria and microorganisms within the host and accelerates the cellular proliferation of many immune cell types. It is also important for the alterations in plasma metals that affect pathogen

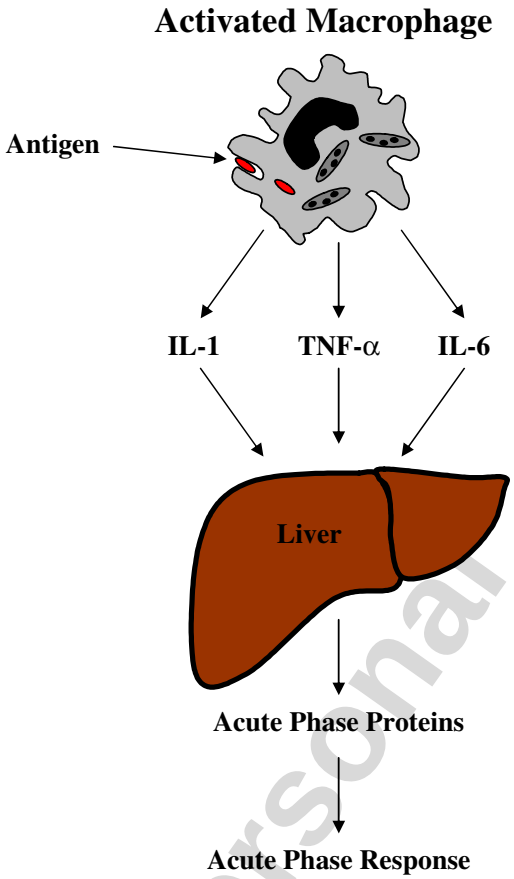

Fig. 1. Activation of the APR by proinflammatory cytokines IL-1, IL-6, and TNF-α, which are released by activated macrophages and monocytes at the site of inflammation or infection. The profile of acute phase proteins released from hepatocytes within the liver varies, depending on the physiologic circumstances.

growth. Fever is highly adaptive, and reducing fever has been shown to reduce the survival rates of the infected host.

The second physiologic reaction associated with the APR is alterations in liver metabolism and gene regulation. Under normal circumstances, hepatocytes in the liver synthesize a characteristic range of proteins, referred to as acute phase proteins, at a relatively steady state, that have various biologic functions, such as proteinase inhibitors, enzymes, coagulation proteins, metal-binding proteins, and transport proteins. However, during an inflammatory response, proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, mediate the hepatocyte production and secretion of these proteins. When the production of these proteins is either increased (positive acute phase proteins) or decreased (negative acute phase proteins) because of inflammation, bacterial infection, endotoxin exposure, neoplasia, or physical injury, many of these proteins become important mediators of immunologic functions and play an active role in tissue repair and remodeling. Each hepatocyte possesses the capacity to produce the entire spectrum of acute phase proteins. Following stimulation of a single hepatocyte within individual lobules, one observes a stimulation of further hepatocytes, and this process continues until almost all the hepatocytes produce the acute phase proteins

and release them into the circulation. The various acute phase proteins differ markedly in their rise or decline in the plasma and also in their final plasma concentrations. APRs generate a characteristic serum protein profile that is species-specific. The levels of elevated expression can differ widely from species to species, and some proteins that function as an acute phase protein in one species may not be an acute phase protein in another species. Some acute phase proteins have more of a direct role in the immune responses, such as activation of macrophages, and tissue repair and remodeling, whereas other acute phase proteins have more of an indirect role, acting as transport proteins for products generated during the inflammatory process. Broadly, acute phase proteins can be classified as positive or negative acute phase proteins. Positive acute phase proteins represent those proteins whose plasma concentrations dramatically increase following infection and the subsequent proinflammatory cytokine stimulation of liver hepatocytes. In man and domestic animals, the positive acute phase proteins can be divided into three major groups: (1) those whose plasma concentration increases by 50% (ie, ceruloplasmin and complement factor-3); (2) those which demonstrate a twofold to threefold increase in plasma concentration (ie, haptoglobin, fibrinogen, lipopolysaccharide-binding protein); and (3) those whose plasma concentration increases rapidly up to 5 to 1000 fold (ie, C-reactive protein and serum amyloid A). (See review by Gruys and colleagues Ref. [62]). In cattle, a number of proteins have been proposed as acute phase proteins, including haptoglobin, serum amyloid A, α1-acid glycoprotein, ceruloplasmin, α1-antitrypsin, α1-antichymotrypsin, α2-macroglobulin, and fetuin [63] Although haptoglobin is probably the most widely studied acute phase protein in cattle [64], numerous studies have investigated plasma profiles of α1-acid glycoprotein, fibrinogen, and serum amyloid A as potential indicators of bovine acute and chronic inflammation and disease. For a more comprehensive view of acute phase proteins as they relate to domestic livestock, the reader is referred to the review by Petersen and colleagues [65].

Acquired (adaptive) immunity

The second arm of the immune system is termed the adaptive system. It represents that part of the immune system that adapts and builds a specific immune response for each antigen it encounters. The adaptive immune system is characterized by the production of antibodies that are directed against specific antigens and possess immunologic memory, which allows subsequent exposures to the same pathogens to elicit faster and stronger immune responses than those associated with the original pathogen exposure [54]. The use of vaccines to protect animals from various pathogens is an example of adaptive immunity. Adaptive immunity can be classified as either cell mediated or humoral immunity. Cell-mediated immunity represents the immunologic response associated with immune cells that act directly against

pathogen-infected cells. Humoral immunity, on the other hand, involves the generation of specific antibodies that are directed against the invading pathogens. Adaptive immunity requires the involvement of specialized white blood cells known as lymphocytes. The lymphocyte population consists of subsets of both B and T lymphocytes (also referred to as B and T cells, respectively), which are critical in the adaptive immune response. The B cells produce antibodies that recognize and attach to a specific antigen in its native form. Following recognition and antigen attachment, the B cell ingests the antigen and processes it for presentation of T cells. The T cells, which develop in the thymus, provide cell-mediated immunity, and are divided into two groups, the T-helper (T_H) cells (T_{H1} and T_{H2}) and cytotoxic T-lymphocyte (CTL). The T_H cells produce cytokines to help the other T and B cells grow and divide, and grow and divide themselves to produce more cells to fight future infections. Although antigen-presenting cells of the innate immune system are important for activation of the T_H cells that form the adaptive immune response [66], it is the cytokine profile associated with the innate immune system that directs the initial adaptive immune response into primarily cellular (T_{H1}) or humoral (T_{H2}), by dictating T_H cell proliferation/differentiation and cytokine production [61,67]. For example, secretion of the proinflammatory cytokine IL-12 from activated macrophages stimulates the release of IFN-γ from NK cells, which supports the differentiation of T_{H1} cells. The T_{H1} cells then produce a proinflammatory cytokine profile, which supports cellular immunity [68]. If differentiation favors the T_{H2} cells, an anti-inflammatory cytokine profile (IL-4, IL-10, and IL-13) prevails that promotes humoral immunity. Alternatively, the T_H cell, in response to IL-2, develops into a CTL, which is responsible for destroying pathogen-infected cells. Pathogens can be phagocytosed and digested by antigen-presenting cells (eg, macrophages, B lymphocytes, and dendritic cells) and the digested pieces of pathogens presented on the surface of the antigen-presenting cell to T_H cells. The T_H cells may then stimulate clonal expansion of a B-cell lineage, which then secretes antibodies. CTLs express antibodies tethered to their cell surface and can mediate destruction of cells infected with a pathogen. Antibodies produced by CTL take on various forms and are referred to as immunoglobulins (Ig). The most common immunoglobulin isotopes include IgM and IgG. The IgM isotopes are the first antibodies to be produced by the immune system in response to an infection. Although they arrive on the scene as the first antibody type following an infection, they possess relatively low affinity against antigen. The more specific IgG isotypes (IgG1, IgG2, IgG3, and IgG4) require additional time for their development.

Relationship of the innate and acquired immune systems

Prior separate discussion of the innate and acquired arms of the immune system implies that these systems function independently. However, the two arms communicate with one another and, to some extent, rely on similar

communication molecules. In the past 10 years, for example, it has been learned that up-regulation of the innate system provides an important feed-forward system for antibody production. For example, activation of neutrophils by an invading pathogen causes the neutrophils to release IL-1β, which, in turn, stimulates the acquired system. Maintaining an intricate balance between proinflammatory and anti-inflammatory cytokine profiles, and cellular versus humoral immunity, is critical to the overall success of the immune system.

Stress and immune function

Scientists have known for decades that stress can have detrimental effects on the immune system. However, what had not been distinguished until recently are the divergent effects of acute stress compared with long-term or chronic stress. As scientists expanded their scope of exploration beyond traditionally defined pathways of neuroendocrinology, endocrinology, and immunology, multidisciplinary efforts emerged that have elucidated cross-communication among these systems and led to a better understanding of homeostatic regulation within the animal. No longer is the stress response considered an all-or-nothing biologic activity strictly associated with the fight or flight behavior, nor is stress considered strictly immunosuppressive. Indeed, stress may elicit bidirectional effects on immune function, such that acute stress may be immunoenhancing, whereas chronic stress may be immunosuppressive.

Today, our knowledge base has expanded, and a greater appreciation and understanding has emerged regarding the plethora of immune system activities that are influenced by glucocorticoids, such as stimulation of cytokine expression and secretion, stimulation of immune cell proliferation and differentiation, and regulation of effector cell function [69–71]. In addition to these stimulatory actions, glucocorticoids are known to inhibit aspects of immune function. In rodents, social stress has been reported to have negative effects on both innate and adaptive immune responses such as changes in leukocyte subset populations, decreased mitogen-induced proliferation, decreased cytokine production, and decreased antibody production [72–74]. Ultimately, within the animal, the immune system response to stress depends on the type of stress encountered (ie, acute versus chronic). In some instances of acute stress, such as that resulting from bites, punctures, scrapes, or other challenges to the integrity of the body, stress hormones are associated with priming the immune system in a manner that prepares for potentially invading pathogens and subsequent infection. However, when an animal experiences prolonged or chronic stress, the effect of stress hormones on the immune system shifts from a preparatory event to a series of suppressive events, first at the cellular level and then, eventually, across the entire immune system spectrum.

A prime example of the detrimental effect of chronic stress on the immune system is associated with continual glucocorticoid stimulation of immune system effector cells. Initially, glucocorticoids stimulate effector cells to prepare for pathogen invasion, but under chronic stress, these cells are stimulated constantly and prepared for a large-scale immune response. Activated immune cells secrete proinflammatory cytokines, which stimulate the further release of glucocorticoids [75], thus providing a feed-forward pathway. At this heightened alert stage, effector cells potentially could lose the ability to recognize self and attack body tissues. Therefore, as a built-in safety precaution, the cells down-regulate under continual glucocorticoid stimulation, resulting in effector cells that potentially could become tolerant to future stimulation by glucocorticoids and lack the ability to respond. Unfortunately, developing a tolerance to elevated concentrations of glucocorticoids that prevents the negative-feedback response could also lead to uncontrolled inflammation [76]. In a biologic effort to prevent total immunologic suppression by glucocorticoids, a negative-feedback system is also present in the HPA axis that is responsive to elevated concentrations of glucocorticoids. Glucocorticoids themselves inhibit subsequent production of CRH, VP, and ACTH at the level of the hypothalamus and pituitary gland [77].

Glucocorticoids are also known to inhibit gene expression by way of activation of the glucocorticoid receptor within immune cells. Given that glucocorticoids are lipophilic molecules, they pass readily through the plasma membrane of all cells in the body and bind to their respective receptors. The two receptors for glucocorticoid hormones are the GR and the mineralocorticoid receptor, with the mineralocorticoid receptor having a higher affinity for cortisol than the GR [78]. Therefore, at low-circulating concentrations, glucocorticoid hormones preferentially bind to the MR. However, at high circulating concentrations, such as those observed during a stress response, the GR becomes occupied [79]. Immune cells such as lymphocytes, macrophages, and granulocytes, which posses the GR, are therefore more responsive to elevated concentrations of glucocorticoid hormones during periods of stress [80]. Activation of the GR in immune cells has been reported to invoke several cellular activities, including proliferation, cytokine secretion, antibody production, and cytolytic activity [81], by interfering with the primary transcription factor, nuclear factor kappa B (NF-κB) [82,83]. Within eukaryotic cells, activated NF-κB regulates cell proliferation and cell survival, and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, bacteria, and viruses. The activated GR has been reported to bind directly to NF-κB, which prevents transmigration to the nucleus of the cell, thereby interfering with the production of various cytokines from both macrophages and T_H cells [84,85]. In inflammatory diseases, such as inflammatory bowel disease, arthritis, and sepsis, NF-κB is chronically active [86–88].

In addition to the aforementioned desensitization or down-regulation of the GR on effector cells, and the effects of glucocorticoids on NF-κB,

glucocorticoids can influence overall immunity through their actions on the thymus, which is essential to the development and proper functioning of the immune system. Within the thymus, developing thymocytes and stromal cells drive hematopoietic progenitor cells to become mature T lymphocytes. Glucocorticoids are known to influence the survival, proliferation, and differentiation of these developing thymocytes [89]. Under normal conditions, endogenous glucocorticoids regulate the pool of T cells and the $CD4^+$/$CD8^+$ T cells ratio [90]. However, during chronic elevations of glucocorticoids, pre-B and pre-T cells experience accelerated programmed cell death (eg, apoptosis), which reduces the formation of lymphocytes, and causes atrophy of the thymus gland [91]. In mice, exogenous glucocorticoids decreased the cellular content and size of the thymus within 24 hours of injection [92], and induced apoptosis in normal immature thymocytes [93]. Similar results have also been reported in calves exposed to low doses of a synthetic glucocorticoid (dexamethasone) for extended periods of time, ranging from 14 to 25 days [94]. The investigators reported severe thymus atrophy, increased apoptosis within the thymus, and an overall decrease in the lymphocyte proliferative response of calves injected with dexamethasone.

Although the discussion pertaining to the influences of stress on the immune system has been limited primarily to the actions of glucocorticoids to this point, one cannot discount the involvement of the catecholamines E and NE as modulators of the immune system. Indeed, the HPA axis and the sympathetic nervous system are the two major pathways able to alter the immune system. Similar to the glucocorticoid hormones, E and NE bind to multiple receptors classified as α or β adrenergic receptors. The β_2 adrenergic receptor that binds both E and NE is a seven-membrane–spanning, serpentine receptor embedded in the plasma membranes of many cell types, and has been identified previously on all immune cell types, with the exception of the T_{H2} cells [95]. An increase in circulating concentrations of catecholamines following a stressful event has been reported previously to modulate immune cell activities, such as proliferation, cytokine and antibody production, cytolytic activity, and cell migration [96]. As with glucocorticoids, the effects of E and NE on the immune system can be stimulatory and inhibitory. In vivo and in vitro studies have reported potential immunoenhancing roles for E and NE. Alaniz and colleagues [97] noted that transgenic mice lacking the enzymes necessary to synthesize NE and E had an impaired immune system reactivity to infectious agents, thus indicating an immunoenhancing role for these catecholamines. In cell cultures, the addition of E or NE at the initiation of culture increased cytolytic activity, indicating an enhancement in the generation of CTLs [95]. Treatment of peripheral blood leukocytes with catecholamines in vitro has been shown to suppress IL-12 synthesis, and increase IL-10 production, thus potentially favoring a primarily cellular (T_{H1}) versus humoral (T_{H2}) differentiation of T_H cells [98]. Catecholamines have also been reported to reduce phagocytosis

and inhibit lymphocyte proliferation, antibody secretion, and production of proinflammatory cytokines.

Ultimately, the combined immunologic effects of glucocorticoid hormones and catecholamines result in a well-orchestrated event designed to prevent overstimulation of innate immunity and the T_{H1} cytokines, while simultaneously priming the humoral immune response through stimulation of the T_{H2} cells. Therefore, the final type of immune response that prevails within an animal depends on the overall effect of stress hormones on the production of the T_{H1} and T_{H2} cytokines [61]. For a more detailed review of the influence of glucocorticoids and catecholamines on immune function, the reader is referred to the review by Padgett and Glaser [99].

Stress in cattle

Cattle experience numerous environmental, managerial, and nutritional stressors throughout the production cycle that potentially could inhibit overall productivity and well-being because of neuroendocrine disruption and stress-induced immunosuppression. Generally, in the case of livestock, stressors can be grouped into the following three broad categories: (1) psychologic stress; (2) physiologic stress; and (3) physical stress. Typically, stress associated with fear, such as that experienced during commingling or social mixing, exposure to novel environments and loud or unusual noises, and restraint, is considered psychologic stress. Physiologic stress can result from deviations in normal endocrine or neuroendocrine function caused by various conditions, such as nutrient restriction or deficiencies, glandular disorders, and other endocrine disruptors. Physical stress represents stress associated with injury, thermal stress, hunger and thirst, fatigue, and disease [100]. Some stressors can be prevented or overcome through alternative management practices and various nutritional strategies; however, some stressors, such as thermal stress (heat and cold stress), are often difficult to prevent, and impose significant economic burdens on the cattle industry.

Because of elevated climatic temperatures, cattle may experience periods of heat stress, resulting in decreased performance, increased discomfort, and even death [101–103]. The physiologic consequences associated with acute heat stress include increased respiratory rates, decreased feed intake, increased water intake, and imbalances in blood gases and plasma electrolytes [104,105]. In the summer months, finishing feedlot cattle often experience heat stress because of hot climatic conditions resulting from above-normal ambient temperatures, high relative humidity, high solar radiation, and low wind speed [106–108]. As a coping strategy, cattle attempt to reduce core body temperature by decreasing metabolic heat production through reduced dry matter intake, which ultimately reduces overall performance [109–111]. To appreciate the economic significance of heat stress on the

cattle industry, one need only consider the severe heat stress events that occurred during 1995, 1997, and 1999 [112]. During these years, cattle producers in the Northern Plains and Cornbelt lost more than 100,000 head, and the total economic impact on the cattle industry exceeded $20 million per heat episode [108,112,113]. In addition to the severe heat episodes in the mid-1990s, cattle producers experienced significant losses associated with early snowstorms and severe cold during this time. In 1992 and 1997, more than 30,000 head of feedlot cattle were lost each year because of early snowstorms in the Southern Plains [112]. During the winter of 1996–1997, above-average snowfall and greater than normal winds caused producers in the Northern Plains to lose more than 100,000 head of cattle [112]. In the winter of 2000–2001, it was reported that efficiencies of gain and daily gain in feedlot cattle decreased by approximately 5% and 10%, respectively, compared with previous years, because of late-autumn and early-winter moisture combined with prolonged cold stress conditions [114–116]. Implementing various management practices during periods of heat stress, including environmental modifications within the feedlot such as shading, sprinkling, misting, and fogging, or reducing metabolic energy intake through feed restriction programs, could reduce the overall heat load, enhance feed conversion, and improve overall productivity [117–119]. Employing environmental and nutritional strategies to intervene during periods of severe cold stress may prove to be feasible economically in the feedlot, in cow-calf production, and in stocker production [112,120].

In addition, castration, weaning, transportation, disease, social mixing, inadequate diets, and dietary changes can impose significant stresses on cattle, resulting in reduced performance, increased morbidity, and death. Incoming feedlot cattle typically experience multiple stressors within hours of exposure to the transportation stress associated with shipping to the feedlot. Typically, cattle are exposed to various stressors shortly after arrival at the feedlot, such as physical restraint, vaccination, horn tipping, social mixing, and dietary changes. For intact bulls, the processing event becomes even more stressful because of castration. The stress stemming from castration can affect numerous factors, including behavior, endocrine and immune profiles, and physiologic responses, all of which ultimately influence overall well-being and growth performance. Castration on arrival at the feedlot has been reported to reduce feeding and watering bouts, decrease average daily gain, and increase morbidity and mortality, compared with bulls castrated before arrival [121]. In this particular study, the investigators reported the results of three, 21-day receiving trials composed of 448 newly received Brahman crossbred male calves that were shipped for approximately 27 hours from Texas sale barns to a commercial feedlot in Arizona during the months of August to October (trial 1, August 19 to September 9, n = 150; trial 2, September 9 to September 30, n = 150; and trial 3, October 1 to October 22, n = 148). The investigators reported that final body weight and average daily gain were reduced by 7 kg and 0.32 kg/day, respectively,

in bull calves that were castrated on arrival at the feedlot, compared with bull calves castrated before arrival at the feedlot. Additionally, the investigators reported that bull calves castrated at the feedlot experienced a 92.5% greater incidence of morbidity (35.8% versus 18.6%) and increased mortality (3.5% versus 0%), compared with the bull calves castrated before arrival. An alternative management practice may be to castrate bull calves at a younger age to eliminate some of the performance losses. In fact, recent studies have demonstrated that as the bull calf ages, the weight loss associated with castration increases. In a recent review, Bretschneider [122] reported that weight loss for the 30-day period following castration is reduced when the procedure is done closer to birth. Lents and colleagues [123] reported that body weight gain is decreased for at least 30 days when bull calves are castrated beyond 6 months of age. These investigators also reported that leaving bulls intact until 6 to 7 months of age did not result in heavier weaning weights compared with bull calves banded at birth or banded at birth and implanted with an estrogenic implant [123]. Similar results have been reported in other domestic species. In pigs, for example, several studies have demonstrated that when castration occurs earlier in life, the animals exhibit fewer negative behavioral and physiologic responses [124–126]. Although the effect of castration on weight gain following castration in pigs has been less than decisive in the literature [127–130], it is clear that the act of castration does elicit a stress response, regardless of age [126].

Within the beef cattle production system, transportation stress is one of the most widely recognized stressors, impacting the overall productivity and health of cattle, and has been associated with increased glucocorticoid concentrations for nearly 3 decades [131]. In live cattle being shipped to feedlots and international markets, immunosuppression caused by transportation stress is a significant concern because of the reported increase in incidences of shipping fever [100,132]. Previous studies have reported that stress-induced glucocorticoid concentrations in response to transportation stress are linked to immunosuppression, which may predispose cattle to infectious diseases [100,133–135]. Several studies have reported alterations in various aspects of the immune system following transportation stress, including increased blood neutrophil numbers, decreased lymphocyte responsiveness and proliferation to myogenic stimulation, and altered acute phase protein profiles [136–138]. Recent work by Arthington and colleagues [137] has focused on the use of acute phase proteins profiles in transported calves, as opposed to serum cortisol, as a more stable indicator of stress following transportation. Specifically, the investigators reported higher mean serum concentrations of amyloid-A, fibrinogen, and ceruloplasmin, and lower haptoglobin concentrations in transported calves, compared with nontransported calves. Unfortunately, the results have been somewhat variable in subsequent studies and the investigators acknowledge that further research is needed before the use of acute phase protein profiles can be endorsed as reliable

indicators of stress in cattle. For additional information regarding the effects of transportation on cattle, the reader is referred to a recent review by Fike and Spire [139].

Routinely, the stress associated with weaning and transportation is imposed on domestic livestock simultaneously, often making in difficult to separate out the immunosuppressive effects of these events independently. In pigs, the nutritional, psychologic, or environmental stressors associated with weaning have been linked to disruption of the somatotrophic axis [19] and reduced growth rates [140]. Previous studies have indicated that the endocrine profile observed in the recently weaned pig is similar to that observed in undernourished animals [141–143]. Various growth-related hormones, such as growth hormone, insulin-like growth factors 1 and 2, thyroid hormones, and glucocorticoids, which are influenced strongly by nutritional status, can also have a significant impact on the immunocompetence of the animal. In cattle, several studies have demonstrated successfully in experimental models that abrupt weaning and maternal separation can have significant effects on behavioral responses [144], acute phase protein profiles [145], and the immune system of calves [146]. Using cortisol as a stress index, Zavy and colleagues [147] reported that weaning elicited a stress response similar to that of transportation in both *Bos indicus* and *Bos taurus* calves. In addition to increased concentrations of cortisol, weaning has been associated with increased concentrations of catecholamines [146,148]. As with transportation stress, the stress associated with weaning has been reported to have negative effects on the cell-mediated and humoral immunity of calves [134,136,146,149]. With regard to cell-mediated immunity, Hickey and colleagues [146] reported that even in calves that had been habituated to handling, abrupt weaning that included maternal separation and disruption of established social grouping decreased in vitro secretion of IFN-γ following stimulation with both Con A and keyhole limpet hemocyanin (KLH) for up to 7 days postweaning. Likewise, Pollock and colleagues [150] reported that the antibody responses to KLH given near weaning were altered, compared with the antibody responses to KLH given at least 2 weeks before weaning. Mackenzie and colleagues [134] also reported that transportation and weaning resulted in a decreased humoral immunity that lasted for several weeks in calves.

As noted by Hutcheson and Cole [151], the management of feeder cattle through normal production systems, including weaning, castration, and transportation, results in stress that could alter the nutritional requirements of calves, thus requiring dietary changes that promote or support immunocompetence. For example, previous work has demonstrated that during the peak morbidity stage of disease in cattle infected with infectious bovine rhinotracheitis virus (IBRV), urinary Cu and Zn excretion is increased [152], thereby potentially causing these cattle to enter a phase of mineral deficiency that could result in immunosuppression.

Nutrition and immunity

As livestock production systems intensified and herds of animals were maintained within confined areas, producers began relying on preventative medicines and chemotherapeutic drugs to ensure the health of their livestock. For more than a half a century, livestock producers have used antimicrobials to enhance growth and to prevent disease in their herds [153]. In recent years, however, livestock producers have been under considerable pressure from consumer groups to reduce or eliminate the use of antimicrobials in livestock production because of a growing concern that this common production practice may lead to an increase in the number of antibiotic-resistant human pathogens [154,155]. Because of these rising concerns, several European countries already have restricted the use of antimicrobials in livestock production systems [156]. And, if the current trend continues, United States producers could be forced to eliminate the use of antimicrobial agents in livestock production in the future. If antimicrobials are removed from livestock production, it will be imperative that United States producers have proven nonantimicrobial alternatives readily available to them, if the livestock industry is to remain viable. Therefore, enhancing our knowledge now, with regard to how nutritional supplements may be used to support or enhance immune function, will aid in our ability to improve the overall well-being and performance of the livestock, and will provide nonantimicrobial alternative management practices for the United States producer.

All physiologic processes within the body, including the immune system, are influenced by nutrient availability, or lack thereof. For more than 3 decades, scientists have investigated the use of various nutritional strategies to enhance the immune system of livestock throughout various stages of production. In addition to vitamin and mineral supplementation, lipid, protein, and amino acid forms and concentrations within livestock diets have been evaluated extensively as to their potential impact on the immune system. Countless studies have documented the immunomodulatory properties of various vitamins (A, B_6 and B_{12}, C, D, and E), minerals (Zn, Cu, Fe, magnesium, and selenium [Se]), animal products (spray-dried plasma, fish oil, and fish meal), yeast products, and plants (echinacea, yucca, and quillaja) within domestic livestock diets. The reminder of this section focuses on the more commonly used supplements within livestock diets. For an overview of the effects of dietary energy and dietary protein on the productivity and overall health of feedlot cattle, the reader is referred to the recent review by Duff and Galyean [157].

For many years, numerous dietary factors, including protein concentration [158], potassium concentration [151,159], and energy concentration [160], have been known to influence the performance and health of cattle. To provide a comprehensive overview of all nutrition–immune system interactions is well beyond the scope of a single article; therefore, the reader will

be referred periodically to various reviews for more detailed information. In 2002, Calder and Kew [161] published a review on nutrients with known effects on immunity. In nonruminants, essential amino acids, linoleic acid, vitamin A, folic acid, vitamin B_6, vitamin B_{12}, vitamin C, vitamin E, Zn, Cu, Fe, and Se have been reported to affect one or more indexes of immunity [161]. According to Calder and Kew, vitamin E and Zn have received the most attention as immunostimulatory nutrients. A review of the potential roles of magnesium in support of both innate and acquired immunity in humans and animal models has also been published recently [162]. Unfortunately, less is known about the nutritional regulation of immunity in ruminant livestock. But it may be assumed safely that nutrients, at the tissue level, will have similar effects on immunity in ruminants as in nonruminants.

Vitamins

Vitamin deficiencies have been linked to immune system disorders and diseases for hundreds of years. The proper development and function of virtually every aspect of the immune system can be linked to an adequate level of one particular vitamin or another. Vitamins exert essential roles in hematopoiesis, maintenance and function of lymphocytes, NK cells, and neutrophils, and even antibody production. Antioxidant vitamins also play important roles in inactivating harmful reactive oxidative species (ROS) such as oxygen ions, free radicals, and peroxides produced through normal cellular activity, such as oxygen metabolism, that can destroy cellular membranes, cellular proteins, and nucleic acids. Lymphocytes, for example, are highly active cells that, as part of their normal cellular activity, generate ROS continuously. In a recent review [163], the investigators suggest that immune cells are particularly susceptible to oxidative damage for two reasons: (1) one mechanism by which cells of the immune system provide protection is by phagocytizing and killing pathogens through an oxidative bactericidal mechanism termed the ''respiratory burst,'' which generates large amounts of ROS; and (2) immune cells have a high percentage of polyunsaturated fatty acids in their plasma membranes, which makes them more sensitive to oxidative stress [164]. As a result, immune cells are dependent particularly on high levels of antioxidants to protect them from ROS-mediated cell damage and membrane damage. Although the body produces a number of endogenous antioxidants as a defense mechanism against ROS, under conditions of high oxidative stress their ability to eliminate ROS can be exceeded. In these instances, dietary sources of antioxidants such as vitamin E, vitamin C, carotenoids, Zn, and Se can be very beneficial in helping to eliminate damaging ROS and in maintaining normal cellular function and health [163].

The available literature pertaining to the relationship between vitamin deficiencies/supplementation and the immune system is overwhelming. Thus,

the intent of this section is to provide only a brief overview of some of the more commonly used vitamins in livestock production. Although the immunostimulatory properties of the B vitamins (B_6, B_{12}, folic acid) and vitamin C will be covered, dietary sources of these vitamins may be less important in ruminants because these are either synthesized endogenously (ie, vitamin C) or provided by a healthy microbial population (ie, essential amino acids, B vitamins).

Vitamin A

Vitamin A in the form of retinol or beta (β)-carotene has long been recognized as an important immune system nutrient. More than 40 years ago, researchers demonstrated that vitamin A could have significant effects on the pig's ability to generate antibodies in response to antigen stimulation [165]. Continued research over the past 20 years has provided additional evidence that vitamin A has important roles in providing immunologic protection against viral, bacterial, and protozoan infections [166]. In numerous in vivo and in vitro studies, vitamin A and its retinoids (a class of chemical compounds that are related chemically to vitamin A) have been reported to have significant effects on various aspects of immunity, including lymphopoiesis, apoptosis, cytokine expression, and antibody production. Vitamin A has also been associated with the inhibition of Type 1 lymphocyte cytokine production [167] and the proliferation of white blood cells [168]. The function of virtually all immune cells, including neutrophils, NK cells, macrophages, T lymphocytes, and B lymphocytes are affected by vitamin A [166].

Our current understanding of the role or roles vitamin A plays in the maintenance of the immune function is not clear-cut because its effect on immune modulation during infections may depend on the pathogen or pathogens involved, and the vitamin A status of the animal itself. For instance, one major role attributed to vitamin A is associated with the maintenance of the protective mucous membranes of the respiratory and gastrointestinal tracts. It is thought that vitamin A deficiency causes damage to these membranes, which allows bacteria and viruses an opportunity for invasion [169]. In fact, in humans and rodents, a vitamin A deficiency has been associated with an increased severity of infections and an increase in mortality [170]. However, in human trials, the beneficial effect of vitamin A supplementation has been variable. In patients exhibiting lower respiratory tract infections, vitamin A supplementation did not improve the clinical signs of illness significantly [166]. Overall, it appears that a vitamin A deficiency, rather than vitamin A excess through supplementation, has the most profound and significant effects on immune function. Duff and Galyean [157] have suggested recently that, in the case of bovine respiratory disease (BRD), a vitamin A injection to calves known to be deficient or marginal in vitamin A status would benefit by rapidly increasing body stores of vitamin A. But the

investigators also note that in the absence of a deficiency, it is unlikely that vitamin A supplementation would be beneficial.

Carotenoids are a class of natural, fat-soluble pigments and antioxidants found primarily in plants, algae, and photosynthetic bacteria. Carotenoids are defined by their chemical structure, and include β-carotene, lutein, canthaxanthin, lycopene, and astaxanthin [163]. Carotenoids (ie, β-carotene) have been viewed traditionally as a source of vitamin A by way of the cleavage of β-carotene precursor into active forms of vitamin A. However, studies in the past 2 decades have shown that carotenoids have immunostimulatory properties independent of their roles as precursors of vitamin A. Rodent studies conducted by Bendich and Shapiro [171] were the first to document the immunostimulatory properties of carotenoids. Specifically, they reported that canthaxanthin, a carotenoid that cannot be converted to vitamin A, increased mitogen-stimulated lymphocyte proliferation in rats. Since then, numerous studies have documented mechanisms by which carotenoids benefit immunity, independent of their provitamin A activity. In vitro studies have demonstrated that β-carotene increased lymphocyte blastogenesis [172] and increased neutrophil killing activity [173,174]. In humans and animals, β-carotene and other carotenoids have been reported to possess immunomodulatory activities, including enhanced lymphocyte blastogenesis, increased lymphocyte cytotoxic activity, and stimulation of cytokine secretion [175]. In ruminants, it has been suggested that the immunostimulatory actions of carotenoids may improve overall health, including mammary and reproductive health [175]. In fact, in dairy cows, supplementation with β-carotene at dry-off was reported to reduce mammary gland infections [176].

Vitamin C

Vitamin C, also known as ascorbic acid, is a water-soluble, antioxidant vitamin that is important in the formation of collagen, which is an important protein that gives structure to bones, tendons, ligaments, muscle, and blood vessels. Vitamin C also plays an important role in the synthesis of the NE, a neurotransmitter produced in the adrenal medulla that is critical to brain function and the body's response to stress. The secretion of NE and its roles in immunity were discussed previously. For centuries, vitamin C was known primarily for its ability to prevent scurvy; however, because of its highly effective antioxidant properties, vitamin C is recognized now as an important protective agent against free radicals in the body. Vitamin C accumulates in tissues like the adrenal gland and the cells of the immune system [177]. Because of the highly effective nature of vitamin C, only small amounts are necessary to protect proteins, lipids, carbohydrates, DNA, and RNA from damage caused by free radicals and reactive oxygen species, which are generated in the body because of metabolic process and immune cell activity. An equally important property of vitamin C may be its ability

to regenerate other antioxidants, such as vitamin E [178]. Vitamin C has been reported to stimulate innate and acquired immunity in several species, including mice, guinea pigs, humans, and rabbits [179]. In pigs, in vitro studies have demonstrated that vitamin C alters the proliferative response of lymphocytes to mitogens and induces transient changes in the populations of T and B cells [180]. These investigators also demonstrated the effects of vitamin C on interleukin gene regulation and metabolism of IL-2, thus indicating a role for vitamin C in the maintenance of immune homeostasis. Vitamin C has also been reported to increase in vitro phagocytosis, chemotaxis, and cell adherence of macrophages [181]. Although evidence exists supporting an in vitro a role for vitamin C in maintaining immune homeostasis, the in vivo evidence is inconclusive. Despite numerous attempts over the years to elucidate a potential role for vitamin C on growth performance, stress responses, and in vivo immune status, the results have been inconsistent [182–186]. In cattle, few studies have evaluated the potential immunoenhancing properties of vitamin C, and those available have provided inconsistent data as well [179,187]. Recent work in cattle has suggested that the beneficial role of vitamin C may be attributed to its interaction with other antioxidants, such as vitamin E [187].

Vitamin E

Vitamin E is a lipid-soluble vitamin that exists in eight different forms and exhibits different degrees of biologic activity, depending on its form [188] Vitamin E is the generic name for all tocol and tocotrienol derivates. The form alpha-tocopherol (α-tocopherol) is the most active form of vitamin E in humans and is a powerful biologic antioxidant. As an antioxidant, vitamin E protects cells against free radicals, which are potentially damaging by-products of energy metabolism and activation of the immune system. In addition to its antioxidant properties, vitamin E has a supportive role in maintenance of the immune system, in DNA repair, and in other metabolic processes [189,190]. Vitamin E is an important constituent of all the membranes found in cells (plasma, mitochondrial, and nuclear) and is the major antioxidant in body tissue. Although vitamin E is found throughout all cells of the body, it is more concentrated in the immune cells, where it may provide protection against the destructive free radicals used by white blood cells to destroy pathogenic organisms. In humans, vitamin E supplementation has been reported to have beneficial immune-enhancing properties in both sick and healthy individuals [191,192]. In sheep [193] and chickens [194,195], vitamin E has been used in vaccines, as an adjuvant to increase antibody titer and disease resistance. In swine, supplementing the sow's diet with vitamin E was reported to improve both the sow's and pig's immune response [196]. Additionally, supplementing the weaned pig's diet with vitamin E has been suggested to increase the humoral immune response because improved antibody titers to sheep red blood cells have been

reported [197]. In contrast to these previously mentioned studies, Bonnette and colleagues [198] reported no significant effects of vitamin E supplementation (11, 110, 220 and 550 IU/kg) on either cell-mediate or humoral immunity in weaned pigs, suggesting that the beneficial effects of vitamin E are influenced by other factors, such as genetics, immune status, age, environment, or nutritional status.

In ruminants, a large portion of the benefits of vitamin E is related to its function as an antioxidant. In feedlot cattle, Carter and colleagues [199] demonstrated that vitamin E supplementation reduced medical treatment costs and serum concentrations of the acute phase proteins, serum amyloid A and α-1-acid glycoprotein. However, average daily gain and the acute phase proteins fibrinogen and haptoglobin were not affected by vitamin E supplementation. Similar results were reported in studies by Rivera and colleagues [200,201] in that vitamin E supplementation appears to reduce the severity and duration of an immune challenge in cattle, but does not enhance growth performance necessarily. In a recent review by Duff and Galyean [157] the investigators suggest that the available literature supports an overall protective role for vitamin E in the case of disease challenges such as BRD and IBRV, but that its effects on growth performance remain inconclusive.

Minerals

A number of minerals have been reported to be important for adequate functioning of the immune system in several species including humans, rodents, swine, and cattle. Minerals modulate immune responses primarily through their critical roles in enzyme activity, and a deficiency or an excess of minerals can alter immune system activities. Mineral deficiencies can have detrimental effects on proper immune system function though alterations in specific aspects of immunity, including antibody responses, cell-mediated immunity, and NK cell activity. What remains somewhat inconsistent in the literature is whether mineral supplementation beyond normal physiologic requirements enhances immune function in livestock. Contributing to these discrepancies are the influences of inorganic versus organic mineral sources, the true mineral status of the animals before mineral supplementation, the potential effect of mineral supplementation on the pathogen as opposed to the immune system of the host, and vitamin/mineral interactions that could occur. Studies evaluating the bioavailability, and, therefore, the nutritional value, of minerals from organic versus inorganic sources have been on-going for more than 20 years, and the data have been inconsistent [202–204]. Therefore, a comprehensive comparison of organic versus inorganic mineral sources is beyond the scope of this article and the reader is referred to the reviews by Galyean and colleagues [205], and Duff and Galyean [157] for more information and data sources on organic versus inorganic

mineral supplementation in cattle. This section focuses primarily on the potential immunologic benefits of dietary supplementation with Se, Cu, Zn, and chromium (Cr).

Selenium

Studies suggest that vitamin E and Se play overlapping and essential roles in support of the immune system in ruminant animals. Although vitamin E does act as an antioxidant, as discussed previously, the mechanism by which vitamin E and Se elicit their antioxidant properties differs significantly. Se plays an important role in removing hydrogen peroxide and organic hydroperoxides through its effects as a component of the antioxidant enzyme glutathione peroxidase, and a deficiency in Se can induce a state of oxidative stress in the host [206]. Oxidative stress brought about by the overproduction of free radicals and other oxidants causes damage to animal or plant cells by way of the actions of reactive oxygen species such as superoxide, singlet oxygen, peroxynitrite, and hydrogen peroxide. Oxidative stress can affect host cells in a number of ways, including damaging deoxyribonucleic acid (DNA), altering ion movement across cell membranes, and impairing cell membrane integrity, leading to the decrease or loss of cellular function [207,208]. In several species, Se deficiencies have been associated with lower resistance to infections, possibly caused by decreased antibody production and an impaired lymphocyte proliferative response [209–212]. Various studies have identified additional mechanisms by which Se supplementation enhances immune function, including neutrophil killing activity [213] and neutrophil adherence [214]. Spears speculated that altered neutrophil adherence could affect the ability of neutrophils to attack and sequester pathogens [215].

In ruminants, several studies over the years have reported various positive effects of Se on the immune system. For example, Smith and colleagues [216] reported that feeding elevated levels of Se to ruminants reduced the incidence of diseases and infections, and, in IBRV-challenged calves, reduced primary and secondary immune responses were associated with Se deficiency [210]. Subsequent studies have specifically demonstrated an enhanced antibody response associated with Se supplementation in cattle [217–219]. In 2001, Parnousis and colleagues [220] reported that injection of Se, either alone or in combination with vitamin E, improved the production of specific antibodies against *Escherichia coli*, and that the production of specific antibodies was greater after the administration of Se alone. More recently, Beck and colleagues [221] reported that in Se-deficient calves, Se supplementation enhanced macrophage phagocytosis, and tended to enhance the skin swelling response to a phytohemagglutinin (PHA) injection. However, the investigators reported no effect of Se supplementation on lymphocyte proliferation. Fry and colleagues [222] also reported no effect of Se supplementation, regardless of Se level or source (organic versus inorganic), on

lymphocyte proliferation or macrophage phagocytosis in 30 crossbred Angus steers supplemented with no Se (control), or with 1.7 mg/day of either an organic or inorganic Se source, during a 105-day trail.

Although it has been speculated that dietary Se is vital for virtually all components of the immune system [223], whether or not supplemental Se in excess of that necessary to prevent Se deficiency will further enhance immune function in ruminants remains unclear [157]. Interpreting the available literature associated with Se supplementation is virtually impossible without a priori knowledge of the Se status of the animals within the studies. Adding to the overall complexity is the issue of Se source, organic versus inorganic, another variable in the equation not yet resolved because of conflicting results [157,205] Beyond the effects Se may and may not elicit within the immune system of the host, one must also consider the potential effects of Se on the pathogen itself. For example, in mice, Beck and colleagues [224] reported that Se deficiencies invoked changes in the viral genome of the *Coxsackievirus* that allowed the avirulent strain of the virus to acquire virulence. Subsequent studies have demonstrated similar results with viral mutations occurring in Se-deficient mice when exposed to the influenza virus [225,226].

Copper

In humans and several other species, Cu is considered an essential nutrient and Cu deficiencies have been reported to be associated with numerous health concerns, including an impaired immune system. Prohaska and Failla [227] published a review based on experimental and clinical evidence that highlighted the effects of Cu on adaptive and innate immunity. This information has been updated subsequently with further research activity conducted in this area in a more recent review by Percival [228]. This recent review highlights results from previous rodent and human studies that have reported various effects of Cu deficiency on both adaptive and innate immunity, including reduced antibody production, reduced cytokine production, altered immune cell maturation, and altered immune cell function. Several studies have reported that Cu-deficient animals are more susceptible to parasitic, bacterial, and viral infection [229–231]. Although previous rodent studies have demonstrated that Cu deficiency decreases B-cell activity and decreases antibody responses to various antigens [232–238], research evaluating the effects of Cu on cell-mediated immunity has provided inconsistent results.

Although compelling evidence exists that Cu is essential for both adaptive and innate immunity in humans and rodents, research pertaining to Cu enhancement of the immune function in ruminant animals has provided equivocal results. Cu deficiencies associated with either low Cu intake or high intakes of molybdenum or Fe have been described previously as a widespread problem within the cattle industry [239,240], and have been associated with higher mortality rates in sheep [241]. In vitro work by Jones and Suttle [242]

was one of the first studies of a potential role for Cu in supporting the immune function in sheep and cattle. In these studies, the investigators reported that the ability of neutrophils to kill a common fungus, *Candida albicans*, was decreased in Cu-deficient animals, and repletion of Cu restored the neutrophil-killing activity. Subsequent in vitro studies have also reported that a Cu deficiency decreases the ability of bovine neutrophils to kill phagocytized microorganisms [243]. Cu deficiencies have also been reported to reduce antibody production and impair cytokine production (IFN and TNF) from mononuclear cells [215]. Wright and colleagues [244] reported that low Cu status in steers reduced mitogen-stimulated blastogenesis following weaning and IBRV challenge. Ward and Spears [245] reported a Cu-by-stress interaction associated with the humoral immune response in Angus steers to porcine red blood cells such that Cu decreased antibody titers to porcine red blood cells in unstressed steers, but increased antibody titers in stressed steers. However, these investigators concluded that Cu deficiency, even after a prolonged period, alters immune function in cattle only minimally. In a more recent study by Salyer and colleagues [246], the investigators reported that supplemental Cu did not affect performance or morbidity of lightweight, newly received heifers; however, the source of Cu affected the humoral immune response to ovalbumin immunization.

Although natural Cu deficiencies have been associated with increased susceptibility of ruminant animals to disease [215], inducing a Cu deficiency by supplementing molybdenum, Fe, or S has often failed to increase the incidence of disease [245,247,248]. To date, specific mechanisms by which Cu supports the immune function in ruminants have not been described, and previous research on the effects of a Cu deficiency regarding specific immune responses in cattle have been inconsistent [215,231,249].

Zinc

Zn has been reported previously to be a cofactor in more than 300 enzymes that influence various physiologic processes within the body [250] Therefore, it should not be surprising that Zn deficiencies can have significant effects on multiple systems within the body, including the growth, nervous, reproductive, and immune systems. With regard to the immune system, Zn has been reported to influence several components of immunity, including cell-mediated immune functions, tissue regeneration, protein synthesis, and inflammatory responses [251], and is one of the most studied trace elements with regard to its effects on the immune system. Unlike other trace minerals, Zn has been reported to perform numerous unique functions within the immune system [252]. Zn supports humoral and cell-mediated immunity by facilitating proliferative reactions to stimulus by different mitogens by way of its action on immune cells as a cofactor for essential enzymes. Zn deficiency has been associated with decreased T-cell function and antibody responses [252]. Zn also plays an important role in transcriptional

control through its action as a Zn-finger motif and the ability of immune cells to proliferate. The immune response requires rapid proliferation of cells (eg, T- and B-lymphocytes) in response to specific antigens and, therefore, Zn deficiency prevents this aspect of immunity from developing. In humans, Zn deficiency has been noted to increase susceptibility to infectious disease [253], cause an imbalance between T_{H1} and T_{H2} functions, and decrease the production of IFN-γ, IL-2 and TNF-α [254,255]. Although Zn deficiencies are typically the culprit with regard to immune system disorders, it should also be mentioned that excessive levels of Zn have been reported to be immunosuppressive. Schlesinger and colleagues [256] previously reported that excessive Zn in human infants decreased the activities of polymorphonuclear leukocytes, decreased T-cell proliferation to mitogen, and decreased antibody production. Gross and colleagues [257] were the first to report that a diet-induced Zn deficiency in rodents resulted in a depressed immune system. Specifically, these investigators demonstrated that the mitogenic responses of the spleen, thymus, and peripheral blood lymphocytes to PHA and concanavalin A (ConA) were reduced significantly in Zn-deficient mice. Other early rodent studies demonstrated that a Zn deficiency was also associated with reduced thymus size and depleted macrophages and lymphocytes in the spleen [258–260]. The reader is referred to a recent review by Rink and Gabriel [250] for more detailed information on the known effects of Zn on immunity in nonruminants.

In contrast to studies with humans and laboratory animals, Spears [215] reported that marginal Zn deficiency has little effect on the immune function in ruminant animals, but Zn supplementation may be beneficial. However, earlier studies did report that Zn deficiencies decreased the immune response in ruminants, which increased their susceptibility to disease [241]. Engle and colleagues [261] reported that feed efficiency and cell-mediated skin swelling in response to a PHA challenge were reduced significantly in Zn-deficient calves. Subsequent work demonstrated that pharmacologic concentrations of Zn enhanced the recovery rate of IBRV-stressed cattle [262]. Galyean and colleagues [263] also reported that supplementing the diets of newly weaned steers with 70 mg of Zn from Zn oxide or Zn methionine reduced BRD-associated mortality by 52%. However, in a recent study by Salyer and colleagues [246], although the authors reported increased ovalbumin antibody response in heifers fed a bioavailable Zn supplement, they reported no effect of Zn supplementation on performance or morbidity. Thus, whereas Zn has been reported in a number of studies to have beneficial effects on immune function and performance in cattle, these beneficial effects may depend on the type of immune stimulus, the current mineral status of the animal, the concentration and bioavailability of the Zn supplemented, or the physiologic status of the animal itself. In fact, Galyean and colleagues [205] suggested that Zn supplementation may be beneficial only in stressed calves that are predisposed to succumbing to BRD. It becomes quite evident

in reviewing the literature associated with Zn supplementation in cattle that additional studies with Zn must consider the aforementioned variables to elucidate fully its potential roles in the maintenance of the immune system, overall health, and performance of cattle.

Chromium

Cr is thought of mainly as an essential mineral for the maintenance of normal glucose and insulin metabolism. Acting as a cofactor of insulin, Cr enhances glucose transport and promotes healthy blood sugar concentrations by facilitating the binding of insulin to its receptor on the cell surface, which results in enhanced insulin sensitivity and responsiveness in peripheral tissues [264–266]. In humans, Cr has long been recognized as an essential mineral that is required for the normal metabolism of carbohydrates, proteins, and lipids [267,268]. Cr deficiencies in humans are associated typically with metabolic and physical stresses brought on by events such as pregnancy, excessive or extreme exercise routines, physical trauma, disease, and excessive carbohydrate intake [269–272]. These stresses increase glucose metabolism, resulting in an acceleration of Cr mobilization and urinary loss, which eventually deplete Cr stores in the body [273].

In addition to its aforementioned metabolic effects on glucose and insulin, Cr has been reported to increase muscle growth, reduce fat deposition, and enhance immunity in swine and cattle, albeit the results have been somewhat inconsistent in the literature. For example, although some researchers have reported that supplemental Cr increases the longissimus muscle area and decreases the backfat thickness in swine [274,275], others have reported either variable or no effects of supplemental Cr on muscle growth or fat deposition [276,277]. With regard to immunity, supplementing Cr in the weaned pig's diet has been reported to increase antibody production to sheep red blood cells, decrease antibody production in response to ovalbumin, and enhance lymphocyte blastogenesis in response to pokeweed mitogen stimulation [278]. However, in a more recent study with weanling pigs, the investigators reported no significant effect of Cr supplementation on postweaning performance or immunocompetence [279].

With regard to beef cattle, studies evaluating supplemental Cr have reported improved growth performance, feed efficiency, morbidity rates, and immune responses. In vitro studies have demonstrated that dietary Cr supplementation in beef calves increases the proliferation of peripheral blood lymphocytes and that direct addition of Cr to the culture medium increases the in vitro proliferation of peripheral blood lymphocytes, with or without mitogen stimulation [280] In feeder calves subjected to transportation stress, supplemental Cr has been reported to increase total serum IgM concentrations [281], to enhance primary antibody response to human red blood cells, and to increase serum IgG concentrations [282]. Supplementing

stressed calves with high-Cr yeast has also been reported to increase antibody production in response to ovalbumin [283].

Several studies have indicated that supplementation of Cr to dairy cattle, in a biologically available form (eg, Cr-amino acid complex or Cr-yeast), benefits immunity. For example, Burton and colleagues [284] reported increased ConA-induced blastogenesis in Cr-supplemented periparturient cattle, and Chang and colleagues [280] reported increased blastogenesis in lymphocytes recovered from sick calves. However, this effect was not detected in lymphocytes taken from healthy calves. Additionally, Burton and colleagues [284] reported that Cr increased the development of titer to ovalbumin immunization and, in a subsequent study, they reported increased titer in Cr-supplemented cows following immunization with an IBRV antigen [285]. More recently, Faldyna and colleagues [286] reported that Cr, fed as a chelate, increased the IgG2 antibody response to tetanus toxoid.

In 2000, Spears reviewed studies on the value of adding Cr to livestock diets in relation to immunity and overall health [215]. A number of studies have indicated that Cr supplementation may improve cell-mediated and humoral immune response and resistance to respiratory infections in stressed cattle. With respiratory disease challenge models, Cr generally does not affect disease resistance [215]. Overall, individual studies have yielded conflicting results, which Spears attributed to variations in Cr status, supplementation protocol, and physiologic states of the animals. Although many studies have reported that Cr supplementation did not affect immune parameters (as cited in the review by Spears), a common theme among studies that have detected a benefit to Cr supplementation may be the presence of a stressor (shipping, parturition, weaning). In 1992, Mertz reported that stress and disease increase urinary excretion of Cr, thus depleting body stores of Cr [268]. It has been suggested that alleviating stress-induced immunosuppression may be one of the mechanisms by which Cr elicits its beneficial effects [284]. Consistent with this theory are reports that the immunomodulatory benefits of Cr supplementation are more pronounced during times of stress [273].

Energy usage and immune responses

Although activating and maintaining an immune response is essential for survival, there is an associated energy cost to the animal. Creating and maintaining a febrile response alone is very energy intensive. It has been estimated that metabolism is increased approximately 10% to 13% for every degree Celsius increase in body temperature associated with an immune response [287]. However, elevating the body temperature aids in reducing the survival and reproduction of most microbial organisms, and preventing the febrile response with antipyretic drugs can be detrimental [287]. For

instance, in goats infected with *Trypanosoma vivax*, it has been reported that administering antipyretic drugs actually increased the mortality from infection [288]. Above and beyond the energy required for the febrile response is the energy required for processes such as increased production of inflammatory cytokines, acute phase proteins, and antibody formation. To compensate for these increased energy needs, animals display various behavioral responses, such as increased sleep, decreased social activity, decreased sexual behavior, and decreased foraging, in an effort to conserve energy. Metabolic changes also occur, associated with increased glucocorticoid and NE secretion that liberate energy in response to illness. However, although these behavioral and metabolic responses aid in the animal's ability to conserve energy, they have an overall negative impact on productivity because the energy resources used to mount an adequate immunologic response limit the energy that could otherwise be used for other economically important biologic functions, such as growth, reproduction, and lactation. Nonetheless, activation of the immune system is essential for survival and to prevent disease within the animal. Without diverting these nutrients to support immunologic functions, significant economic losses would be associated with death loss, decreased feed efficiency, and reduced body weight gain.

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