

# Chapter 1

## Impact of Sex Steroids on the Stress Response and Welfare in Female Farm Ruminants



Aline Freitas-de-Melo and Rodolfo Ungerfeld

**Abstract** The concentrations of sex steroids modify the sensitivity to stressors in mammals: progesterone, reduce the stress response, while progesterone withdrawal increases it. Estrogens appear to increase the sensitivity to stressors, influencing the hypothalamic-pituitary-adrenal axis response. As the concentration of progesterone and estrogens differ according to the physiological status, such as pregnancy, anestrus or the phases of the estrous cycle, it may also modify the sensitivity to stressors. Therefore, it would be important to consider this information for various practices in farm animals as they are frequently subjected to stressful situations, including artificial weaning, shearing, isolation from the group, and transport. Moreover, pharmacological treatments with these hormones are commonly applied to cows, ewes, does, or buffaloes for using different biotechnologies, such as estrous synchronization, estrous induction, or synchronization of the ovulations. Accordingly, it is important to consider that those treatments might modify the sensitivity of females' farm ruminants to human handling.

The chapter summarizes how sexual steroid profiles might change female ruminants' stress response and welfare. The major points included are: a brief description of the natural variation in sex steroid profiles in female ruminants; the main hormones and administration pathways used in the application of reproductive biotechnologies; the different physiological indicators of stress and welfare considered in farm animals; and the main knowledge available on the effects of sex steroids on the stress responses of female ruminants to different farm practices. Lastly, we propose directions in which research is needed to understand better if stress, behavior, and welfare of female farm animals could be affected by the application of reproductive biotechnologies.

**Keywords** Progesterone · Progestagens · Estrogen · Progesterone withdrawal · Oestrous cycle · Hypothalamic-pituitary-adrenal axis · Glucocorticoids · Sheep · Cattle · Goat

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A. Freitas-de-Melo (✉) · R. Ungerfeld  
Departamento de Biociencias Veterinarias, Facultad de Veterinaria,  
Universidad de la República, Montevideo, Uruguay

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## Abbreviation

GABA<sub>A</sub> aminobutyric acid type A

### 1.1 Introduction

During the last years, hormonal treatments for reproductive biotechnologies in farm animals increased widely throughout the world. The development of fixed-timed artificial insemination treatments in cattle has a significant impact on the economy and employment of farm animal production (Mapletoft et al. 2018). Most of these treatments include administering sexual steroids, mainly progesterone or progestogens, and different estradiol formulas. Sex steroids are primarily related to reproductive functions, but these hormones also affect metabolism (Ashley et al. 2000; Kalkhoff 1982), immunological status (Bouman et al. 2005), functions in the central nervous system (Genazzani et al. 2002), and can also modulate the stress response (Sze and Brunton 2019). The effects of sex steroids on the stress response are of particular interest in farm animals as they might directly influence their welfare. However, these issues have been mainly studied in laboratory animals and humans, with a paucity of information on farm animals. In ewes, it was reported that the administration of progesterone reduces the stress response at weaning (Freitas-de-Melo et al. 2013), while progesterone withdrawal increases the secretion of cortisol after social isolation (Freitas-de-Melo et al. 2016). Ewes treated with estradiol benzoate respond to an ACTH challenge with greater cortisol concentrations than non-treated ewes (Van Lier et al. 2014).

The concentration of progesterone and estrogens differs according to the physiological status (pregnant, anestrus, or cycling females); thus, it might modify the sensitivity to stressors. Pregnant ewes have slighter behavioral responses than non-pregnant ewes to social isolation or a surprise effect (Viérin and Bouissou 2001) and shearing (Ungerfeld and Freitas-de-Melo 2019). Furthermore, the sensitivity to stressors is greater during the follicular than during the luteal phase (Pinto-Santini and Ungerfeld 2019; Freitas-de-Melo et al., 2022). The reactivity of cows also varies according to the reproductive status, with decreased reactions of pregnant cows to human handling (Freitas-de-Melo et al. 2019).

How female farm animals cope with stressful situations directly affects its' productive, reproductive, and welfare outcomes; thus, the reproductive status and the concentration of different sex steroids should be considered when different managements are applied in these animals. For example, it has been widely known that stress affects cow fertility (see review: Dobson and Smith 2000). However, it is not well studied if the application of hormonal treatments for reproductive biotechnologies might modify the easiness of animal handling, productive efficiency, or animals' welfare.

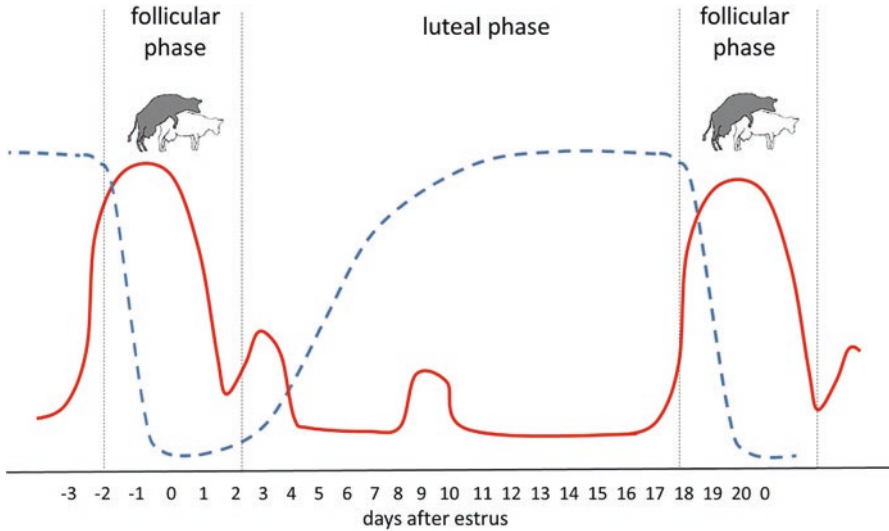
This chapter aimed to present a brief reminder on how profiles of sexual steroids vary according to the physiological status in female ruminants; the main hormones and administration pathways used in the application of reproductive biotechnologies; the main physiological indicators of stress and welfare; and the knowledge available on the effects of sex steroids during stressful situations in female ruminants. Lastly, we propose directions in which research is needed to understand better if the application of reproductive biotechnologies can affect the sensitivity to stressors, the behavioral responses, and the welfare of female farm animals.

## 1.2 Natural Variation in Sex Steroid Profiles in Female Ruminants

### 1.2.1 *Estrous Cycle*

Sexual steroid concentrations vary naturally throughout the reproductive lives of female ruminants. Before puberty, progesterone concentrations remain at basal levels, and estrogen concentrations may have some oscillations due to the physiological reproductive changes that determine puberty. However, estrogen concentration does not reach concentrations similar to those observed during regular estrous cycles. Since puberty, the estrous cycle is a set of reproductive events that repeats successively. While in sheep, it lasts an average of 17 days, in other ruminants, such as cow, goat, or buffalo, its length is approximately 21 days. The estrous cycle can be divided into a luteal phase, which in ewes extends from day 2–3 (heat = day 0) of the cycle, to approximately day 13–14; and a follicular phase that goes from luteolysis that occurs on day 13–14 until day 2. In species with estrous cycles of 21 days, the general pattern is the same, maintaining the proportion of the duration of these two phases (Fig. 1.1).

The follicular phase extends from the regression of the corpus luteum to ovulation. During the follicular phase, the growth of the ovulatory follicle ends with the luteinizing hormone surge, ovulation, and the beginning of the follicle luteinization. The ovulatory follicle(s) secrete substantial concentrations of estrogens, which are responsible for triggering the increase in luteinizing hormone secretion, reaching the luteinizing hormone peak during the second half of heat. In turn, these estrogens are responsible for determining the receptive behavior in the female. In summary, estrogen concentrations are high during the follicular phase, increasing from the beginning of this phase until ovulation, when a rather abrupt decrease in concentration begins. The preovulatory luteinizing hormone peak triggers the ovulation of the preovulatory follicle(s), and luteinization of the remaining structure(s), with the subsequent formation of the corpus luteum. As the corpus luteum develops, the amounts of progesterone secreted by it increase. After ovulation, the weight of the corpus luteum increases associated with an important angiogenesis process and an increase in the size of the luteal cells (Smith et al. 1993). The newly formed corpus

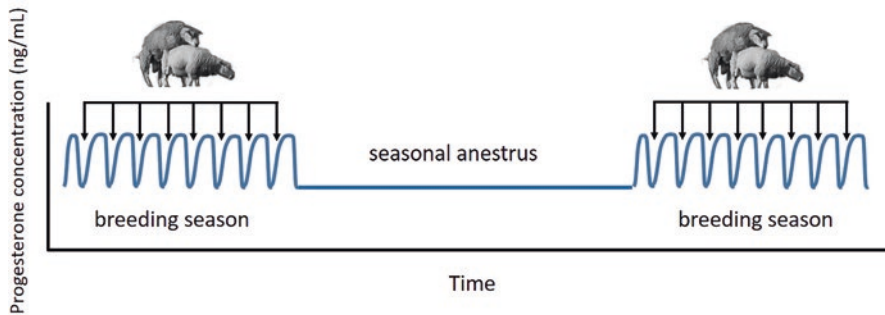


**Fig. 1.1** An illustration with the follicular phase on the right and left and the luteal phase in the middle. The follicular phase has an image of cows mating. Progesterone (blue line) and estrogen (red line) profiles during an estrous cycle in cows. The luteal phase is characterized by high concentrations of progesterone and low concentration of estrogens, and during the follicular phase, the opposite hormonal pattern is observed. The line for progesterone is at its peak in the luteal phase and descends in the follicular phase and the one for estrogen is at its peak during the follicular phase and lowest during the luteal phase

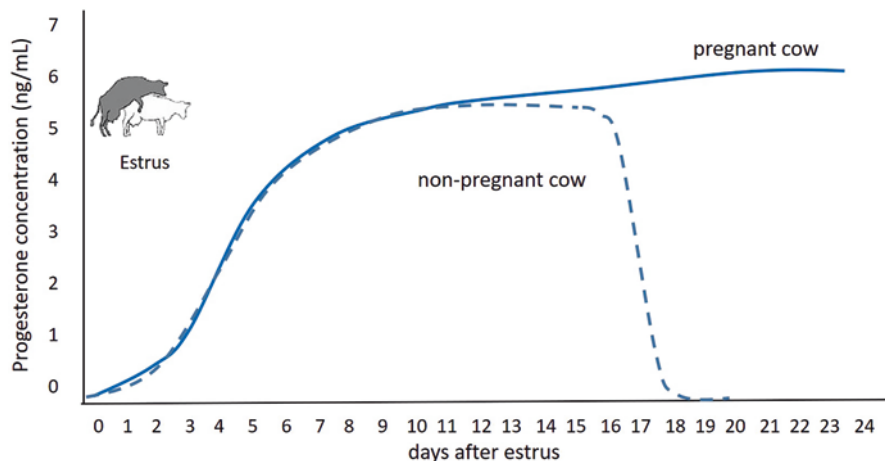
luteum receives substantial amounts of blood flow in relation to its size. The production of progesterone increases more than ten times due to the increase of the enzymes that control the precursors of this steroidogenic pathway. In contrast, the production of androgens and estrogens decreases due to the enzymes' loss involved in their synthesis.

### 1.2.2 Seasonality

According to the species, the estrous cycles are repeated successively in the non-pregnant animal throughout the year in non-seasonal polyestric species, like cows, or only during part of the year, in seasonal polyestric species, like sheep and goat (Fig. 1.2). Although there are variations according to the breed and the latitude where sheep and goats live, the breeding season comprises successive estrous cycles occurring during summer and autumn. The period of the year in which there are no estrous cycles or ovulations is the seasonal anestrus, when progesterone concentration remains at baseline, at subluteal concentrations. During this period, there are small oscillations in the estrogen concentration associated with follicular development, but without reaching the level of a follicular phase.



**Fig. 1.2** Model of the seasonality of reproduction in sheep. Blue lines show progesterone concentrations, with increases in each estrous cycle. The black arrows indicate when heat occurs, and the estrogen concentration is more remarkable. A graph with time on the x-axis and progesterone concentration on the y-axis. At the beginning and end are a series of continuous peaks for the breeding season with an image of ewes mating, and in between these two series of peaks is a flat line for seasonal anestrus. Arrows are pointing to the dips in the peaks



**Fig. 1.3** Changes in progesterone concentrations during the estrous cycle (dotted line) or early gestation in cows (continuous line). A graph with days after estrus on the x-axis and progesterone concentration on the y-axis. An image of a cow mating with estrus written below is presented at beginning of the graph. The line for the pregnant cow has an ascending curve that later flatlines and the line for non-pregnant cow has the same ascending curve but dips completely at 16

### 1.2.3 Gestation and Postpartum Anestrus

Progesterone is the main hormone responsible for the maintenance of pregnancy, so during this period, luteolysis does not occur, maintaining active the corpus luteum (Fig. 1.3). After conception, the embryo produces chemical signals that prevent luteolysis, which in ruminants is mainly interferon tau. The progesterone produced

by the corpus luteum is essential to maintain pregnancy during the first half of it in all species. In some species, such as sheep, the placenta also produces progesterone in amounts enough to maintain pregnancy from the mid-gestation until its end. In other species, e.g., cow and goat, the placenta also produces progesterone, but the amount produced is below the threshold necessary to maintain pregnancy *per se*, so progesterone produced by the corpus luteum is essential to maintain the pregnancy. In any case, progesterone concentrations remain elevated throughout pregnancy.

### 1.3 Exogenous Hormonal Control of Reproduction in Female Ruminants

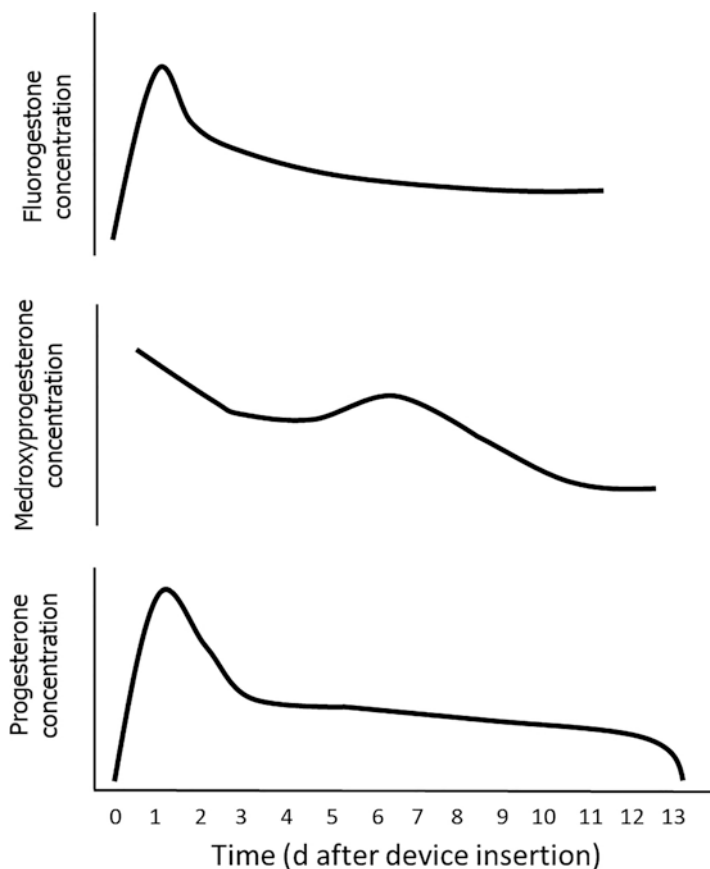
The control of reproduction for the application of reproductive biotechnologies requires the exogenous administration of sexual steroids. Treatments for applying different biotechnologies, such as estrous synchronization, estrous induction, synchronization of the ovulations, or follicular development for superovulation, require controlling the estrous cycle and/or follicular development. There are several reviews on these topics (Bó et al. 2016; Bó and Baruselli 2014), so this section summarizes the different hormones and routes of administration used in farm ruminants. Table 1.1 summarizes information on the progestogens more frequently used in reproductive management in farm ruminants.

**Table 1.1** Progestogens frequently used in reproductive management in farm ruminants

Species	Administration	Hormone	Treatment length
Cattle			
	Intravaginal device	Progesterone	5–7 days
	Solution	Progesterone	Single administration
	Subcutaneous implant	Norgestomet	5–7 days
Small ruminants			
	Intravaginal sponges	Medroxyprogesterone acetate	Short treatments: 5–6 days Traditional treatments: 12–14 days in ewes, 15–16 days in does
		Flurogestone acetate	Short treatments: 5–6 days Traditional treatments: 12–14 days in ewes, 15–16 days in does
	Intravaginal device	Progesterone	Short treatments: 5–6 days Traditional treatments: 12–14 days in ewes, 15–16 days in does
	Solution	Progesterone	Single administration

### 1.3.1 Progestagens and Progesterone Treatments

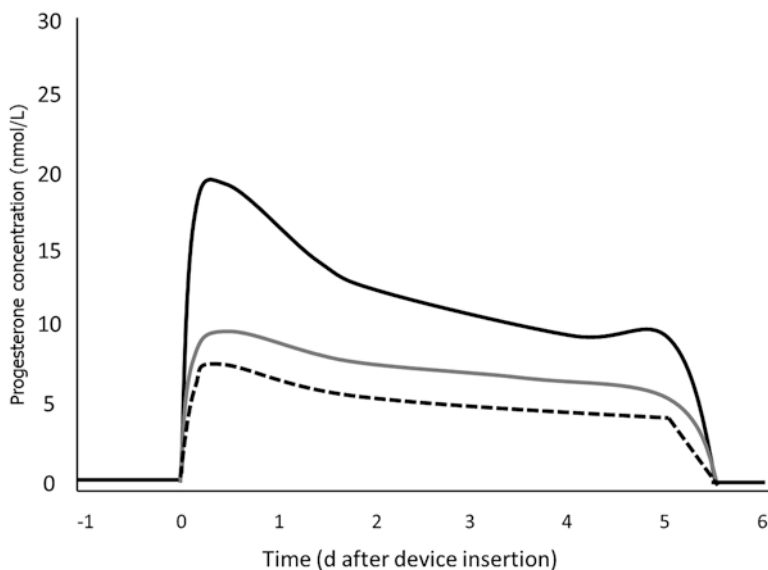
The use of treatments based on mimicking a luteal phase requires the administration of sustained amounts of progesterone or progestagens. Progestagens are synthetic molecules with similar reproductive effects to progesterone. In cattle, sheep, and goats progesterone is administered by intravaginal silicone devices impregnated with synthetic progesterone, identical to that natural molecule produced by the corpus luteum. These devices are inserted and remain *in situ* for 5–7 days, depending on the protocol used. After the insertion of these devices, progesterone concentration achieved in the blood increases sharply, remaining at luteal concentrations throughout the treatment (Fig. 1.4). These devices are frequently used more than



**Fig. 1.4** Patterns of fluorogestone, medroxyprogesterone, and progesterone blood concentrations in small ruminants after the insertion of commercial devices. The pattern is based on Gaston-Parry et al. (1988), Greyling et al. (1994), and Rubianes et al. (1998). There are three line graphs with time on the x-axis and progestogen concentration on the y-axis. The graph on top has a steep increase and later dips a little and flatlines. The second graph begins from the top and descends with a small peak in between. The last one has a steep peak which dips a little and flatlines, and finally dips completely

once (Vilarinho et al. 2011, 2013; Oliveira et al. 2001). Some authors autoclaved these devices before a second application, increasing progesterone concentration shortly after being inserted, achieving values greater than with used non-autoclaved devices (cattle: Zuluaga and Williams 2008; sheep: Ungerfeld et al. 2013; goats: Alvarez et al. 2013). However, in general, concentrations achieved with used devices are lower than those achieved with new devices (Fig. 1.5).

Similar devices are also used in sheep and goats, but traditional long treatments are frequently used, lasting 12–14 days in sheep and 14–16 days in goats. In this case, progesterone concentrations decrease to subluteal concentrations after 8–10 days of application (Fig. 1.4). During the last period of these treatments, progesterone concentrations are below to those observed during a normal late luteal phase. In these species, intravaginal sponges impregnated with synthetic progestagens, such as medroxyprogesterone and fluorogestone acetate, are more frequently used. There are commercial sponges with different quantities of progestagens, which in general exceed the amount needed for the desired reproductive effects (Ungerfeld et al. 2003). There is scarce information on how the concentrations of progestagens vary in blood after the insertion of the device. In all cases, progestagen concentration decreases throughout the treatment, but it is difficult to know its effects compared to progesterone, as progestogens have a longer half-life and potency. Concerning the main aim of this chapter, there is even less information



**Fig. 1.5** Variations in progesterone concentrations achieved in the blood of goats after the insertion of a new intravaginal silicone device impregnated with progesterone (continuous black line), a device used once before (grey line), or a device used twice before (black-spotted line) (Redrawn from Vilarinho et al. 2011). A line graph with the time of the x-axis and progesterone concentration on the y-axis. There are three lines in the graph, and all three of them have a steep peak that descends slowly and finally dips. The peak value of the first line is 20, and the lowest is around 7



about the possible impact of these hormones on the sensitivity to stressors in ewes and does.

Little is known on the effectiveness of injectable progesterone in oil-based formulas (cattle: Andrade et al. 2020; Morotti et al. 2018; Gimenes et al. 2009; goats: Alvarado-Espino et al. 2019a, b), which were developed to substitute the administration through devices. This strategy aims to reduce the hormonal residues in the environment, which can have deleterious effects on native fauna (Sauer et al. 2018; Liu et al. 2015). The effectiveness of injectable progesterone in oil-based is related to the formula used, determining when progesterone concentrations remain over luteal concentrations. The injectable progesterone might be impractical, as in most commercial products, the hormone should be administered several times. The scarce information available suggests that after a single administration of long-acting progesterone prepared in an oil solution decreases after 52 (Cavestany et al. 2008) to 96 h (Corr3a Rocha et al. 2011) in cows, or 12–16 h in ewes (Ungerfeld and Freitas-de-Melo, unpublished data).

### 1.3.2 Estrogens

Although in some countries, the use of estrogens is not allowed, these hormones are widely used, mainly in treatments to control the follicular dynamics in cows. Estrogens are used in treatments to synchronize the ovulation associated with fixed-timed artificial insemination in cows. Although originally follicular wave was synchronized with estradiol-17 $\beta$  (B3 et al. 1994), currently the hormones more commonly used are estradiol benzoate and estradiol cypionate (Monteiro et al. 2015; Sales et al. 2012; Mart3nez et al. 2000). As it has low solubility in water, estradiol cypionate has a longer half-life than estradiol benzoate or estradiol-17 $\beta$  (Vynchier et al. 1990). In sheep and goats, there are scarce studies using estradiol esters. In some studies, estradiol-17 $\beta$  was initially used to determine if it might synchronize the follicular wave (Ungerfeld et al. 2004), as occurs in cattle. Later, it was used associated with progesterone treatments to synchronize the follicular dynamics for estrous induction (Ungerfeld 2008, 2009) or associated with superovulatory treatments (Souza-Fabjan et al. 2017). More recently, it has been used to synchronize the ovulation for timed artificial insemination in ewes and does (Cosentino et al. 2019). Estradiol benzoate has also been used to promote cervical relaxation in ewes before insemination or embryo collection (Hauschildt Dias et al. 2020).

## 1.4 Stress Response and Welfare

According to Broom (1986), the welfare of an animal is its state regarding its attempts to cope with its environment. Welfare includes the coordination of different responses, including physiological, behavioral, and immunological responses, and

therefore, cannot be evaluated without including several aspects. In livestock cattle, sheep, and goats, females are repeatedly subjected to different challenges, which include human handlings, feeding management, social influences, and the occurrence of pathogens (Broom and Fraser 2015). All these challenges generate physiological, behavioral and immunological responses (Dobson and Smith 2000). As stress implies the modification of the homeostasis of the animal, the coordination of different systems is necessary to return the animal physiology to equilibrium. The responses to the same stressors – considered as any stimulus that triggers a stress response- might differ among different individuals, both in magnitude and duration, depending on their previous experience with that stressor and their temperament (Earley et al. 2010; McEwen and Wingfield 2003). However, other factors such as gender, age, or physiological reproductive status modulate the response to stressors (Freitas-de-Melo and Ungerfeld 2016a; Mormedè et al. 2007; Dallman et al. 2002).

Depending on the intensity of the stressor and the lapse during which it acts, the response can be classified as acute or chronic. When the stressor induces a short response enough to get back to the homeostatic status, it is classified as acute. However, if the stressor continues acting for longer or triggers an intense response with which the animal cannot cope, this is considered a chronic stress response.

### ***1.4.1 Acute Stress Response***

There are many different types of acute stressors, but in general, it is assumed that an acute stressor acts from minutes to hours, and the animal should cope with it, returning quickly to the status that it had before the action of the stressor. Acute stressors simultaneously trigger both responding pathways, including the sympathetic autonomic nervous system and the neuroendocrine system (Carrasco and Van de Kar 2003; O'Connor et al. 2021). The sympathetic system is activated immediately after a stressful situation, displaying the main responses a few seconds or even minutes after the perception of the stressor (Charmandari et al. 2005). However, its' action is of short duration, ending quickly. On the other hand, although the neuroendocrine response takes longer to display the main effects, it is maintained during extended periods, from some minutes to hours. Both systems act simultaneously and synergistically, as glucocorticoids are synthesized and released into the blood; this pathway requires more time to be evidenced (Carrasco and Van de Kar 2003; Matteri et al. 2000). The activation of both responses leads to physiological and behavioral changes necessary to cope with the stressor (Mormedè et al. 2007).

The activation of the sympathetic autonomic nervous system provokes the release of catecholamines into the sympathetic synapses, including epinephrine and norepinephrine. The chromaffin cells located in the medulla of the adrenal glands produce both catecholamines and release them into the bloodstream. In general, catecholamines increase the heart and respiratory rates, as well as the body temperature. Catecholamines also stimulate glycogenolysis and lipolysis, increasing glycemia and energy availability. The activation of the sympathetic autonomic nervous

system also causes mydriasis, vasodilation in skeletal muscle and peripheral vasoconstriction (Matteri et al. 2000). Therefore, the activation of the sympathetic autonomic nervous system produces a blood redistribution to prioritize the perfusion of the brain, heart, and skeletal muscles. Catecholamines also modify the activity of the animal, increasing its' alertness, vigilance, and animal excitement (Sabban 2010).

When the animal perceives the stressor, the hypothalamus releases corticotrophin-releasing hormone and vasopressin into the portal system. Corticotrophin-releasing hormone acts at the adenohypophysis, stimulating the release of the adrenocorticotrophic hormone into the bloodstream (Matteri et al. 2000). The adrenocorticotrophic hormone stimulates the secretion of glucocorticoids, mainly cortisol in domestic ruminants and corticosterone in rodents, in the cortex of the adrenal gland (Matteri et al. 2000). Glucocorticoids stimulate gluconeogenesis, lipolysis, and the catabolism of proteins, increasing glycemia and energy availability (Kudielka and Kirschbaum 2007). Glucocorticoids also promote an increase in cerebral perfusion, the use of glucose, an increase of blood pressure in most blood vessels, and stimulate heart frequency and cardiac output (Sapolsky et al. 2000). Glucocorticoids also act on the immune system, causing lymphopenia, eosinopenia, and neutrophilia (Griffin 1989). The glucocorticoids exert a negative feedback at the hypothalamus and the pituitary gland, inhibiting the secretion of corticotrophin-releasing hormone, vasopressin, and adrenocorticotrophic hormone (Sabban 2010; McEwen 2007). Therefore, even if the stressor continues acting longer, the high concentrations of glucocorticoids are not maintained, so the decrease in its concentration does not necessarily indicate that the stressful situation ended.

### ***1.4.2 Chronic Stress Response***

A chronic stress response is a consequence of a continuous or intermittent but repeated exposure of an animal to stressors during several days (Pacák and Palkovits 2001). Chronic stress response frequently occurs in farm animals, as the general allocating conditions, pathologies, or other types of stressors usually remain active for days or weeks. As it might be expected, a sustained action of glucocorticoids over several days has harmful consequences on the productive and reproductive results, affecting animal health and welfare. If the animal cannot cope with the chronic stressor, it might have several negative consequences, including the animal's death. One consequence of repeated exposure to stressors is the recurrent increase in glucocorticoid concentrations, which negatively affect the normal function of the immune system. Therefore, a chronic stress response might cause immunosuppression, making the animal more susceptible to diseases (Griffin 1989). In this situation, animals lose the appetite, decrease the food conversion, and at the same time, prioritize the catabolic pathways, negatively affecting its' general body condition. Frequently, chronically stressed animals are also more anxious, nervous, and restless, display stereotypic movements, and reduce their sexual behavior (Maniam and Morris 2012; Sapolsky et al. 2000).

Although it is assumed that the stress response is necessary for the animal to cope with stressors, facilitating its adaptation, if the stressor is maintained over time, or the intensity of its effect generates an intense or sustained response, the final result can be harmful to the animal. Therefore, if the reactions displayed by the animal are not enough to recover the homeostasis or the action of the stressor does not stop, the general status of the animal may continuously deteriorate until death. In many husbandry conditions, the stress response cannot modify the stressful situation (Moberg 2001). Therefore, the animal can reestablish its homeostasis only when the stressor disappears.

Some common chronic stressful situations in farm animals are early artificial weaning, transport, and shearing or mixing animals from different groups. Artificial weaning involves several stressors, as the mother-offspring bond is suddenly broken, the physical and social environment is new for the animal, and the animal has to adapt its feeding behavior and digestive processes to the replacement of milk by solid food (Freitas-de-Melo et al. 2022; Freitas-de-Melo and Ungerfeld 2016b). During transportation, the animals are allocated in trucks with high density, provoking injuries and subjecting them to prolonged fasting. Shearing is another stressful situation, as animals are moved from their paddocks, usually by dogs or unknown humans, and located in smaller pens close to the shearing shed. In this condition, they are exposed to high noise levels produced by the shearing machine. During the process, those animals entering to shearing are withdrawn from the group by humans, which implies taking out other group members, which also is a stressful situation for those animals. After shearing, mixing recently shorn animals with those still waiting to be shorn implies the introduction of stressed animals, who are also not easily recognized by non-shorn animals (Ungerfeld et al. 2018). After winter shearing, the stressful situation remains longer with the increase in thermoregulatory demands (Carcangiu et al. 2008; Hargreaves and Huston 1990a, b). Other examples are changes in the social environment, as happens after social grouping when the animals are forced to cohabit with previously unknown individuals (Giriboni et al. 2015), or after social isolation in gregarious species as farm ruminants (Freitas-de-Melo et al. 2016).

In summary, during a chronic stress response, the physiological and behavioral responses are costly with harmful consequences for the animals as the responses cannot modify the cause of the stress. Therefore, it is essential to reduce how the animal perceives the stressor and its response to improve animal welfare.

## **1.5 Influence Sex Steroids on Stress Response and Animal Welfare**

Several studies demonstrate that the concentration of sexual steroids modifies how an animal perceives a stressor and the endocrine stress response itself. In general, it is assumed that progestogens reduce the stress response, inducing calm and relaxed states, while acute progesterone withdrawal and estrogens increase the responses to

stressors (Freitas-de-Melo and Ungerfeld 2016a). There are two main metabolites derived from progesterone, allopregnanolone and pregnanolone, which are called neuroactive metabolites, and are responsible for reducing the stress response (Liang and Rasmusson 2018; Barbaccia et al. 2001). These neuroactive metabolites lessen the animal's perception of the stressor, as they present an anxiolytic effect (Wang 2011), and they also act decreasing the response of the hypothalamic-pituitary-adrenal axis (Brunton et al. 2009; Patchev et al. 1996). All these mechanisms reduce the animal's behavioral and physiological stress response (Bitran et al. 1993, 1995). The acute decrease of progesterone concentrations, as occurs immediately after luteolysis or after the withdrawal of progesterone intravaginal devices, increases anxiety and the sensitivity to stressors (Hantsoo and Epperson 2020; Smith et al. 2007). Furthermore, estrogens also increase anxiety and endocrine stress response in rodents (Figueiredo et al. 2007; Jasnow et al. 2006; Morgan and Pfaff 2002). Surprisingly, the effects of the physiological reproductive status and hormonal treatments on stress response are commonly not considered when female farm animals are subjected to stressful situations.

### ***1.5.1 Mechanisms of Action of Progesterone, Progestagens, and Estrogens***

Progestagens and estrogens act through genomic mechanisms, modifying the expression of specific genes, stimulating or inhibiting gene transcription, and synthesizing specific proteins (Schumacher et al. 1999). The effect of this pathway requires from minutes to days to be noted (McEwen 1991). Progesterone can also provoke short-term effects, acting through its' neuroactive metabolites. Briefly, progesterone is metabolized mainly in the liver (Pluchino et al. 2009) to various compounds, including dihydro-progesterone, allopregnanolone, and pregnanolone (Corpechot et al. 1993; Seamark et al. 1969). These compounds can cross the blood-brain barrier (Pluchino et al. 2009). Furthermore, both progesterone and dihydro-progesterone can also be metabolized in the brain in allopregnanolone and pregnanolone (Compagnone and Mellon 2000). Therefore, these neuroactive metabolites can arrive from the blood to the central nervous system, or be produced *in situ* (Sze and Brunton 2019), achieving high concentrations in the brain (Paul and Purdy 1992).

Allopregnanolone and pregnanolone can bind to the aminobutyric acid type A (GABA<sub>A</sub>) and glycine (Gunn et al. 2011; Sarkar et al. 2011; Jiang et al. 2006), which are both brain's inhibitory receptors. Allopregnanolone and pregnanolone can also inhibit excitatory pathways through the nicotine, serotonin, and glutamate receptors (Sedláček et al. 2008; Kaura et al. 2007; Bullock et al. 1997). These neuroactive metabolites are GABA<sub>A</sub> positive allosteric modulators, reducing neuronal excitability (Paul et al. 2020; Lambert et al. 2009). The union of these neuroactive metabolites on GABA<sub>A</sub> receptor induces quickly anxiolytic, sedative, and analgesic

effects (Akk et al. 2007; Wang 2011), exerting a stress-protective influence (Brunton et al. 2009; Ma et al. 2005). Allopregnanolone also decreases the synthesis of mRNA for corticotropin-releasing hormone and the secretion of both adrenocorticotrophic hormone and glucocorticoids in male rats (Brunton et al. 2009). The concentration of allopregnanolone increases along gestation in rats, significantly decreasing just before delivery (Concas et al. 1998). The increase in the concentration of allopregnanolone might be related to the availability of progesterone as a substrate and the increase in the concentration of enzymes that synthesize allopregnanolone (Brunton et al. 2005).

Although it is not wholly well established in the literature, the reduction of stress response could be mediated by the action of progesterone on intracellular receptors. Some studies using mice knock-out for the intracellular receptors of progesterone reported a decline in the stress or anxiety responses, suggesting that progesterone also has a stress-protective effect throughout its receptors (Reddy et al. 2005). However, when rats were treated with progesterone and a progestin receptor antagonist before applying a stressor, the reduction in the stress response was still observed (Bitran et al. 1995). In female ovariectomized rats, the treatment with medroxyprogesterone, a progestin that does not produce the neuroactive metabolites, also reduces the stress response during restraint (Hassell et al. 2011). It has been suggested that progestagens, progesterone, or dihydro-progesterone could directly join the intracellular receptors located in the amygdala and the bed nucleus of the stria terminalis (Brinton et al. 2008). These brain regions are related to the stress response, fear, or anxiety (Walker et al. 2003), modulating the stress response. Progesterone and medroxyprogesterone differ in their effects on the expression of a subunit of GABA<sub>A</sub> receptors in the hippocampus, which have important implications for the modulation of anxiety (Pazol et al. 2009), and in the effects of both hormones in cognition in rats (Frye et al. 2013). Overall, progestagens and the neuroactive metabolites of progesterone may reduce the perception of stressors, and thus, the endocrine stress response.

In contrast, the acute decreases in progesterone concentration and its neuroactive metabolites after chronic exposure can regulate the expression of a specific subunit of GABA<sub>A</sub> receptor (Smith et al. 1998, 2007). Progesterone withdrawal leads to an increase in the display of behaviors related to anxiety (Gulinello et al. 2002; Gallo and Smith 1993) and individual risk-taking in rats (Löfgren et al. 2006). In the same direction, the treatment with estradiol benzoate enhances anxiety behavior in three anxiogenic behavior tests in female mice (Morgan and Pfaff 2002). Furthermore, estrogens increase the expression of corticotrophin-releasing hormone mRNA in the central nucleus of the amygdala (Jasnow et al. 2006), and increase the glucocorticoid response to an acute stressor in rodents (Figueiredo et al. 2007; Burgess and Handa 1992).

### ***1.5.2 Action of Sexual Steroids in the Stress Response in Ruminants***

There are few studies in ruminants regarding the effects of sex steroids on the stress response, and even a few studies related to the possible mechanisms of action. The concentration of allopregnanolone in the cerebrospinal fluid increases during gestation, reaching a maximum concentration in the last month and decreasing during the first month of lactation (Misztal et al. 2020a). Misztal et al. (2020b) reported that administration of allopregnanolone reduces corticotrophin-releasing hormone and vasopressin mRNA expressions in the paraventricular nucleus of isolated and restrained ewes. Consequently, administration of allopregnanolone to those ewes also reduces the corticotrophin-releasing hormone, adrenocorticotrophic hormone, and cortisol concentrations (Misztal et al. 2020b). Furthermore, there is another possible pathway for the allopregnanolone anxiolytic and sedative effects in this species, as at least in sheep central nervous system-isolated tissue, *in vitro* allopregnanolone binds to the GABA<sub>A</sub> receptor (Crossley et al. 2000).

In rodents, the sensitivity to stressors decreases by the end of pregnancy according to the progesterone or allopregnanolone availability (Brunton 2010; Douglas et al. 2005). Although there are scarce studies, pregnant ewes also decrease their sensitivity to stressors. In effect, pregnant ewes have slighter behavioral responses than non-pregnant ewes to social isolation or a surprise test (Viérin and Bouissou 2001) and shearing (Ungerfeld and Freitas-de-Melo 2019). Pregnant ewes also respond with lower cortisol concentrations than non-pregnant ewes to the shearing handling (Ungerfeld and Freitas-de-Melo 2019). Progesterone concentration was negatively related to the intensity of behavioral stress responses (Viérin and Bouissou 2001), suggesting that the inhibitory effect is associated with the progesterone availability. The sensitivity of cows also varies according to the reproductive status, with a lower reaction of pregnant cows to human handling (Freitas-de-Melo et al. 2019).

Recently we observed that estrous ewes stayed more time standing up immobile and alert during social isolation than ewes in their luteal phase (Freitas-de-Melo et al. 2022). Pinto-Santini and Ungerfeld (2019) observed that ewes were probably more sensitive to stressors during the follicular than during the luteal phase, as while during the first there was a clear circadian pattern in cortisol secretion, with a rise during the early morning, but this pattern disappeared during the mid-luteal phase. Furthermore, the surface temperature, total serum protein, globulin, and plasma glucose concentrations were greater in ewes in the follicular phase than in ewes in the luteal phase (Freitas-de-Melo et al. 2022; Pinto-Santini and Ungerfeld 2019). Nevertheless, Kilgour and Szantar-Coddington (1997) did not find any differences between the behavioral response of estrous and non-estrous ewes during social isolation. Furthermore, Orihuela et al. (2002) did not observe differences in the cortisol concentration in diestrous or proestrus ewes after transportation.

The effect of progesterone administration to evaluate ruminants' response to different stressors was studied in some experiments. In particular, long-term treatment



with progesterone reduces ewes' responses to weaning (Freitas-de-Melo et al. 2013). Ewes treated with intravaginal devices impregnated with progesterone displayed lesser behavioral changes after weaning. After artificial weaning, treated ewes also present lower globulins concentration than untreated ewes, suggesting a possible protective effect of the treatment. Therefore, to simplify the potential handling of animals, we administered an injection of oil-based progesterone to ewes immediately before weaning, and although it decreased the behavioral response (Freitas-de-Melo and Ungerfeld, unpublished data), the decrease was not as strong as that previously reported with longer treatments (Freitas-de-Melo et al. 2013). On the other hand, the acute withdrawn of progesterone concentrations increase the sensitivity to stressors. In effect, anestrus ewes secrete more cortisol after being isolated 24 h after ending the treatment with progesterone than remaining untreated (Freitas-de-Melo et al. 2016). Similarly, heifer response to human handling is greater after progesterone withdrawal than in untreated animals (Freitas-de-Melo et al. 2019). Although the effects of estrogens on stress response in ruminants were studied to a lesser degree, ewes treated with estradiol benzoate respond to an ACTH challenge with greater cortisol concentrations than untreated ewes (Van Lier et al. 2014).

In this context, although more research is required, there is room to consider treatments with progesterone as practical alternatives to reduce the stress responses to routine handlings in ruminants, thereby improving their well-being. The need for long-term treatments appears as a significant limitation, as it implies the use of intravaginal devices to ensure sustained progesterone concentrations during several days. However, new long-action progesterone formulas might be tested to avoid using devices, which are not allowed in some countries due to the residues in the environment. Table 1.2 summarizes the main effects of progesterone or the reproductive physiological state on the stress response in female ruminants.

## 1.6 Conclusions

According to our knowledge, there are no studies on how the sensitivity of the animals to stressors is affected by administering sexual steroids during standard reproductive practices. Moreover, although the effectiveness of administering different progestogens (Ungerfeld et al. 1999) or estradiol esters (Melo et al. 2016) on the reproductive responses have been compared, there are no studies relating the possible effects of these treatments with the stress responses. For example, in some studies, it has been reported that the pregnancy rate of ewes is greater when estrus is synchronized with progesterone than with medroxyprogesterone (Santos-Neto et al. 2015). The greater pregnancy rate was related to the characteristics of the intravaginal device or its' effects on follicular dynamics. However, the insertion and withdrawal of the device imply repeatedly moving the animals, taking them to the farm facilities, contacting unknown technicians, and sometimes grouping with other unknown individuals, which are all stressful events. In this sense, as progestagens



**Table 1.2** Effects of progesterone or the reproductive physiological state on the stress response in female ruminants

Model	Treatment	Physiological state	Stressor	Physiological response	Behavioral response	References
Ewe	-	Pregnancy	Shearing	↓Cortisol	↓Walking ↑Standing ↑Grazing	Ungerfeld and Freitas-de-Melo (2019)
Ewe	-	Pregnancy	Isolation, surprise and presence of a human	-	↓Walking ↓Immobilization ↓Squares crossed ↓Reared against the wall and pawed ↑Time in the central area of the test pen ↓Time near the entrance doors ↑Feating	Viérin and Bouissou (2001)
Cow	-	Pregnancy	Human handling and presence	-	↓Flight distance ↓Exit velocity	Freitas-de-Melo et al. (2019)
Ewe	Intravaginal progesterone releasing device for 32 days	Anestrus	Artificial weaning	↑Total protein ↑Globulin	↓Vocalization ↓Pacing	Freitas-de-Melo et al. (2013)
Ewe	Oil-based progesterone immediately before weaning	Anestrus	Artificial weaning	↓Cortisol	↓Pacing ↓Walking ↑Rumination	Ungerfeld and Freitas-de-Melo (unpublished data)
Heifers	Intravaginal progesterone releasing device for 5 days	Anestrus	Human handling and presence	-	↓Flight distance	Freitas-de-Melo et al. (2019)
Ewes	Intravaginal progesterone releasing device for 14 days + withdrawal	Anestrus	Social isolation	↑Cortisol	No difference was found	Freitas-de-Melo et al. (2016)

(continued)

Table 1.2 (continued)

Model	Treatment	Physiological state	Stressor	Physiological response	Behavioral response	References
Heifers	Intravaginal progesterone releasing device for 5 days + withdrawal	Anestrus	Human handling and presence	–	↑Flight distance	Freitas-de-Melo et al. (2019)
Ewe	–	Follicular vs luteal phase	Social isolation	No difference was found	Tended to ↑vocalization ↑immobilization during the follicular phase	Freitas-de-Melo et al. (2022)
Ewe	–	Follicular vs luteal phase	No stressor	↑cortisol during the follicular phase	–	Pinto-Santini and Ungerfeld (2019)
Ewe	–	Diestrous vs proestrus	Transportation	No difference was found	–	Orihuela et al. (2002)
Ewe	–	Estrous vs non-estrous	Social isolation	–	No difference was found	Kilgour and Szantarcoddington (1997)
Ewe	Estradiol benzoate	Anestrus	ACTH challenge	↑cortisol	–	Van Lier et al. (2014)

as medroxyprogesterone are not metabolized to the neuroactive steroids (Pluchino et al. 2009; Bernardi et al. 2006), it is possible that the effects of these hormones on the reduction of stress response also differ, and this might partially explain differences in fertility.

Therefore, considering that the devices are withdrawn close to the moment of artificial insemination, a period in which stress should be avoided to increase the pregnancy rates, the fertility might differ due to differences in the effects of different hormones in the sensitivity to practical handlings. Similarly, different estradiol esters have been used to modify the follicular growth pattern, and thus, the moment in which timed artificial insemination is performed in cattle. Estradiol benzoate is widely used instead of estradiol cypionate, although some studies were done expecting that the last would increase fertility due to its effects at the hypothalamus-pituitary-ovary axis, related to the longer half-life (Melo et al. 2016; Sales et al. 2012). However, the use of estradiol cypionate did not increase the pregnancy rates. Therefore, it should be studied if the sustained concentrations of estradiol during a longer time before the moment in which the cows are grouped, moved to the facilities, handled by the technicians, and inseminated, increase the sensitivity to these stressors, and this might partially explain the lower fertility rate.

It is known that individual temperament affects the results of these treatments (Mello et al. 2020), and it is also known that cows are more responsive to tests used to evaluate temperament after progesterone withdrawal (Freitas-de-Melo et al. 2019), raising the hypothesis that the estradiol ester used might affect the cow perception of stressors, and therefore, their fertility. These examples demonstrate a big room to work in the link between these two types of research areas, which have scarce contribution between them until now. Understanding the animal as an indivisible individual, in which a hormone administered to produce the desired reproductive effect and also influences other systems, might be the main direction to improve the animals' welfare and results of the reproductive biotechnologies.

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