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Stress Response at different Ages of Weaning in Cattle

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***Abstract*— *Weaning is an inherent husbandry practice in cow-calf beef production systems that impose physical, psychological, and nutritional stressors on calves. In order to characterize the main stressors and corresponding stress responses of calves, two weaning schemes were studied. One of conventional weaning (6 month calves) and another of early weaning (1 month calves). Blood samples were analyzed in an hematology analyzer, the plasma was separated and an analysis of total protein and of cortisol levels was carried. Finally, the response to a vaccine was assessed by analyzing antibody response titers. A significant decrease in the number of lymphocytes and increase in the number of granulocytes was observed in the conventionally weaned group leading to an elevated GR:LY ratio. On day one post-weaning, plasmatic proteins showed a significant increase of similar range in both groups. With regard to cortisol levels, there was a strong increase at day one corresponding to a clear stress response in the conventional weaning group, wheras in the group of early weaning animals only a marginal increase which difficult returned to baseline levels could be observed. As to the antibody response to vaccination, early weaning animals did not show a satisfactory response as was observed in calves subjected to conventional weaning. In conclusion, leukocyte subsets, plasmatic proteins, cortisol levels, and immune status were altered after weaning challenge in both studied age groups of calves. Results indicate weaning as a stressor, regardless of age.*

Keywords*— *early weaning, cattle, cortisol, immune response, stress response.

I. INTRODUCTION

Weaning is an inherent husbandry practice in cow-calf beef production systems that imposes physical, psychological, and nutritional stressors on calves. Weaning involves separation of the calf from its dam, resulting in the split of the maternal-offspring bond and removal of milk from its diet. Thus, the weaning procedure can be an acute stressful event in young calves (O'Loughlin, McGee, Waters, Doyle, & Earley, 2011). In beef herds, calves remain with their cows until they are weaned, which traditionally occurs at six to seven month of age. Time of weaning has an impact on cow and calf performance as well as health and productivity of the native range or pasture. In an intense production system, the negative pressure exerted on animal comfort is potentially higher. To apply the concept of animal welfare, these interventions involve situations that are associated with different stress levels that possibly include different negative effects. With the emergence of early weaning (EW) and hyper-early weaning (HEW) systems, it is important to evaluate how much stress is experienced by calves and their mothers by these production systems. Both practices are used in a large commercial scale in Argentina and other Mercosur countries since the early 90's (Vittone, MORANO, SILVEIRA, & Romera, 2012). In Argentina, two systems have been implemented for weaning, EW involves the

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removal of the calf from the cow at 60 to 70 days of age while in HEW the calf is weaned at 30 to 45 days of age (Gonzalez et al., 2013). Early weaning is a management practice that reduces grazing pressure on pastures by decreasing the nutrient requirements of cows. In addition, it can result in improved conception rates, provided the calves are weaned during the breeding season. Calves that are weaned early are relatively efficient at converting feed to gain, and can weigh as much, or more, than calves allowed to remain with their dams until a conventional weaning age is reached (Rasby, 2007). The practice of EW tends to bring animal production to one of its most clear objectives, which is to produce a calf per cow per year.

The goal of low-stress weaning techniques is to minimize negative stress caused by nutritional, environmental, physical, and social changes experienced by calves through weaning (WSU, 2013)., Calves respond to abrupt weaning with increased vocalizations, agitation, walking activity, and reduced feed intake and rumination (Haley, Bailey, & Stookey, 2005; Veissier, Le Neindre, & Trillat, 1989). Furthermore, they show alterations in hormonal mediators of stress (Blanco, Casasús, & Palacio, 2009) and immune function (Arthington, Eichert, Kunkle, & Martin, 2003; Hickey, Drennan, & Earley, 2003) resulting in a suppressed immune system and an enhanced disease susceptibility (Lambertz, Farke-Röver, Moors, & Gauly, 2015). Accordingly, EW will be more successful and less stressful when adequate attention to nutrition, health, management, and facilities are considered.

Stress alters the internal animal homeostasis inducing changes in the hypothalamic-pituitary adrenocortical (HPA) axis and the sympathoadreno-medullary system. The endocrine activation promotes the release of cortisol as the principal stress marker hormone and several other hormones. Although different authors have used several blood constituents to determine stress, cortisol, despite its variability and short life, is still one the most used indicators of stress (Romero Peñuela, Uribe-Velásquez, & Sánchez Valencia, 2011). The interaction between the immune system and stress is complex; it is an important subject of investigation since it is vitally important for both understanding the biology of animals as well as for the study of many diseases and imbalances. Young animals without a developed adaptive immune system rely on the HPA axis, which serves as the primary regulator and modulator of immunity.

Previous studies have evaluated biochemical, immunological, and behavioral stress markers in the weaning period (Enríquez, Hötzel, & Ungerfeld, 2011; Hulbert & Moisá, 2016; Lambertz et al., 2015; O'Loughlin et al., 2011). There are also studies that evaluate the stress response in early weaning designs (Coppo, 2007; Rasby, 2007). Our objective was to analyze and compare biochemical and immunological markers in a conventional and an early weaning system that are both used in animal husbandry in order to characterize the main stressors and stress responses. The identification of biomarkers of stress may allow to improve management in order to maximize production, meat quality, and animal welfare.

II. MATERIALS AND METHODS

Experiments were conducted in Concepción del Uruguay INTA's Experimental Station, Province of Entre Rios, Argentina. Hereford and Polled Hereford cows and their respective calves raised at the Experimental Station were used. Cows were vaccinated 30 days antepartum and calves at weaning and 21 days post-weaning using a commercial vaccine (Biopoligen HS, Biogénesis-Bagó) containing inactivated IBR (BoHV1 and BoHV5), Bovine Viral Diarrhea Virus (BVDV1 and BVDV2), Para-Influenza Virus (PI3), *Haemophilus somnus*, *Pasteurella multocida*, and *Mannheimia haemolytica* (*Pasteurella haemolytica*).

Experimental Design

At weaning, calves were housed in four corrals, each covering a 50 m^2 area equipped with waterers and feed bunks. Calves were allotted to two experimental groups according to age and weight. One groups representing Early Weaning calves (EW: 56.2±6.84 days old; weight: 64.2±9.93 kg) and another group was composed of Conventional Weaning calves (CW: 218.2±11.4 days old; weight: 135.1±25.44). Each group was placed in two pens (5 animals/corral). One corral from each group was immunized with 2 dosis (at weaning and 21 days post-weaning) of a commercial vaccine. The animals from the other corral of each group remained unvaccinated.

Calves were weaned following the management requirements for EW and CW according to the Manual of early weaning in beef herds (Galli, Monje, Vittone, Sampedro, & Busto, 2005). All calves were offered ad libitum a calf ruminal starter (Ruter®, Asociación de Cooperativas Argentinas) and water.

Sample Collection

Peripheral blood samples were taken at different time points with respect to weaning at -7, 0 (weaning), 1, 7, 14, 21, 35, and 42 days via jugular venipuncture using sodium citrate 3.8 % as anticoagulant. Samples collected were analyzed in the hematology analyzer Nihon Kohden Celltac alpha MEK-6450 equipped with software for bovine blood. Then,

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after centrifugation at 1000 g for 30 minutes, the plasma was collected and stored at -20°C until further use.

Cortisol levels

Plasmatic cortisol concentration was determined by high performance liquid chromatography (HPLC). Cortisol was extracted from plasma by adding diethyl ether:dicloromethane (60:40) and the organic phase was evaporated. As an internal standard, Dexamethasone was added to each tube. Samples were resuspended with methanol, vortexed, and injected into the HPLC system. The guard column Venusil XBP C18 (particle size 3 µm, 150 x 4.6 mm) was equilibrated using HPLC-grade acetonitrile-water (40:60 v/v). The mobile phase was a mixture of distilled water:methanol (45:55) and the flow rate was set to 1 ml/min.

A series of standards (rat normal plasma with cortisol, SIGMA 31719) covering the range of 5-500 ng/ml were used. The concentration was determined from a calibration curve, made on the basis of the ratio of cortisol vs. dexamethasone peak-height.

Plasmatic total proteins

The total protein concentration was measured using the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951). A standard curve was estimated based on a series of dilutions of bovine serum albumin (BSA) (0, 0.10, 0.25, 0.50, 1.00, 2.00 mg/ml) that were in duplicate. Samples were diluted so that they would fall within the BSA standard range. Biuret reagent was added to each tube and mixed thoroughly. The mixture was then allowed to incubate at room temperature for 15 minutes prior to the addition of Folin & Ciocalteu's reagent. The absorbance was measured at 650 nm and blanked on water as a control.

Detection of Antibodies (Ab) against BoHV1

Total Ab in bovine serum were determined using an indirect ELISA as described previously (S. Romera et al., 2014; S. A. Romera et al., 2000). Briefly, polystyrene microtiter ELISA plates (Immulon 1B, Dynatech Laboratories) were coated with 50 µl of positive (concentrated and semi-purified BoHV1 reference strain Los Angeles containing 109 DICT50mL-1) or negative antigen (non-infected MDBK cells) in carbonate buffer of pH 9.6 and incubated for 12 h at 4°C. Samples were tested at a dilution of 1:40 in three serial four-fold dilutions. A peroxidase-labeled affinity purified goat anti-bovine IgG (Kirkegaard and Perry Laboratories, KPL) diluted 1:2000 was used as conjugate. After each incubation, plates were washed four times with PBS-T. The reaction was developed using hydrogen peroxide/2,2B-azino-bis 3-ethylbenzthiazoline- 6 Sulfonic Acid (ABTS) in citrate buffer,(pH 5) as the substrate/chromogen system. The Ab titer of each sample was expressed as the $\log_{10}$ of the reciprocal of the serum dilution. A serum was considered positive when after correction for optical densitiy its value was above the cut off value of the assay.

Statistical Analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Differences among groups were evaluated by two-way ANOVA under a model of repeated measures throughout time. All data were checked for adherence to normal distributions. The effects of treatment, sampling time and the possible interactions were listed in the model statement. When the interaction was significant, simple effects were performed. When there was no interaction, the post hoc Tukey test was performed.

The Infostat statistical program was used (InfoStat Group, National University of Córdoba; http://www.infostat.com.ar/). Values of $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = *** were considered significant.

III. RESULTS

Cortisol

Weaning as a stressor can be identified by the observation of a strong stress response as indicated by the large increase of plasmatic cortisol on day one. It can be seen in Fig. 1 that the level of cortisol (ng/ml) is largely increased on day one both in conventional ($p<0.001$) and early weaning ($p<0.001$), but with differences in the type of response. In early-weaned calves, a peak characteristic for an acute stress response is not seen, but a downward curve most characteristic for chronic stress is observed. The acute stress response in the conventional weaning group is clearly visible, whereas in early-weaning animals it is hardly seen.

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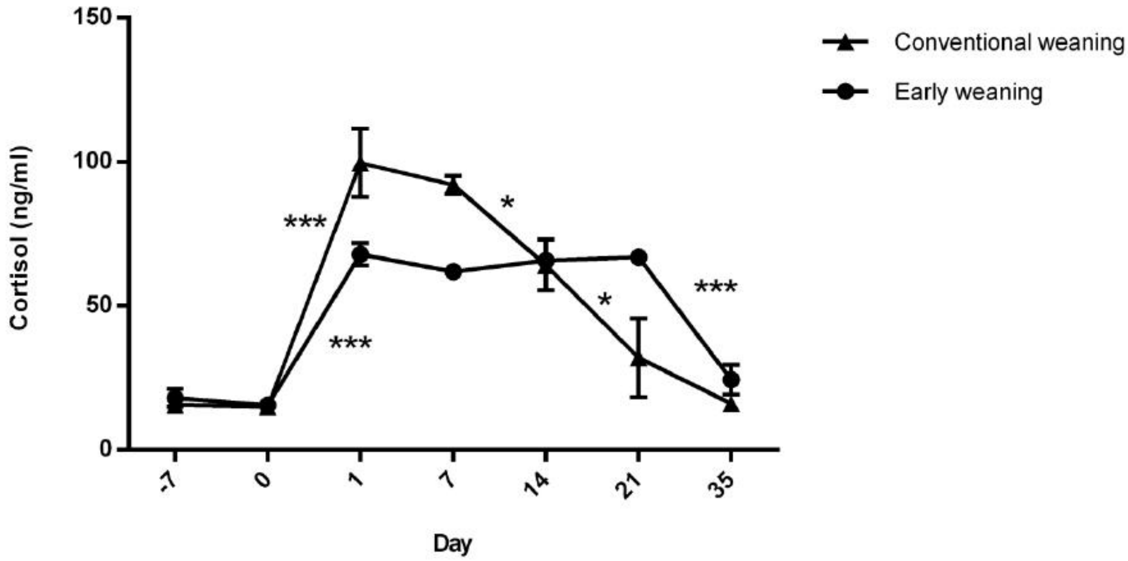

*Fig.1: Determination of plasmatic levels of cortisol by HPLC (ng/ml) (n=6). All data are expressed as mean ± standard error of the mean (S.E.M.); two-way ANOVA under a model of repeated measures; * p<0,05; ** p<0,01; *** p<0,001*

Total proteins

We noted in conventional and early weaning generated a response with a significant increase of plasmatic protein levels at day 1 ($p<0.01$) followed by a decrease at day 7 ($p<0.01$) and a return to the baseline at day 21 ($p<0.01$) (Fig. 2). The baseline ranges of protein levels observed comply with reference values (Rivas, Rossini, & Salvador, 2006).

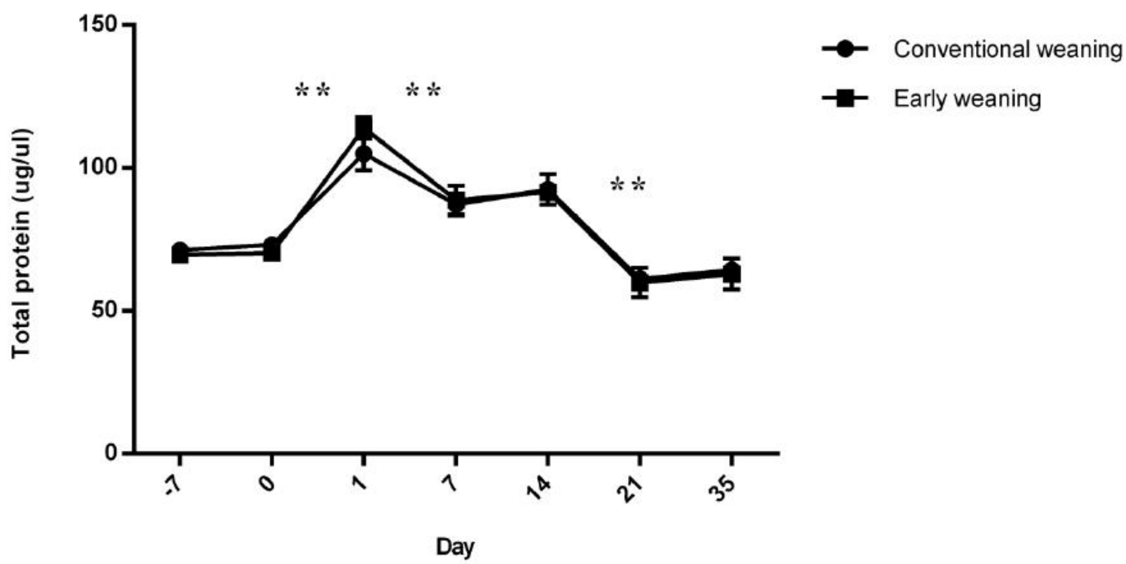

*Fig.2: Determination of total plasmatic proteins (µg/µl) by Lowry method (n=10). All data are expressed as mean ± standard error of the mean (S.E.M.); two-way ANOVA under a model of repeated measures; * p<0,05; ** p<0,01; *** p<0,001.*

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Hematologic parameters

Table.1: Effect of weaning on total lymphocyte and granulocyte number

	Granulocyte number (cel/ul)		Lymphocyte number (cel/ul)	
	CW	**EW**	**CW**	**EW**
Day				
-7	2,2 [a]	2,3 [a]	4,3 [a]	3,4 [a]
0	2,6 [a]	2,6 [a]	4,2 [a]	4,2 [a]
1	6,4*,[b]	3 [a]	1,7 *,[b]	3,7 [a]
7	6*,[b]	2,4 [a]	3,6 [a]	4,1 [a]
14	5,8*,[b]	3 [a]	3,25 [a]	3 [a]
21	6,1*,[b]	1,9 [a]	4,5 [a]	3,8 [a]

Two-way ANOVA under a model of repeated measures throughout time
*p≤0.05 group factor. a, b: time factor

Following weaning, in conventional weaned calves the lymphocytes number decreased from the baseline level at day one (p < 0.05, Table 1), whereas the granulocytes increased (p < 0.05, Table 1). Importantly, granulocytes remained elevated throughout the study while lymphocytes returned to the baseline by day 7. Thus, the post-weaning GR:LY ratio increased (p < 0.01, Fig. 3) and did not return to the pre-weaning ratio level during the evaluation period. Early weaning had no effect (p > 0.05) on any measured parameter and accordingly, the number of cells corresponding to reference baseline values were observed (Table 1).

No effect (p > 0.05) of weaning on the number of red blood cells, hemoglobin concentration, hematocrit percentage, mean corpuscular hemoglobin concentration, and platelet number was seen as all parameters stayed within reference values (data not shown).

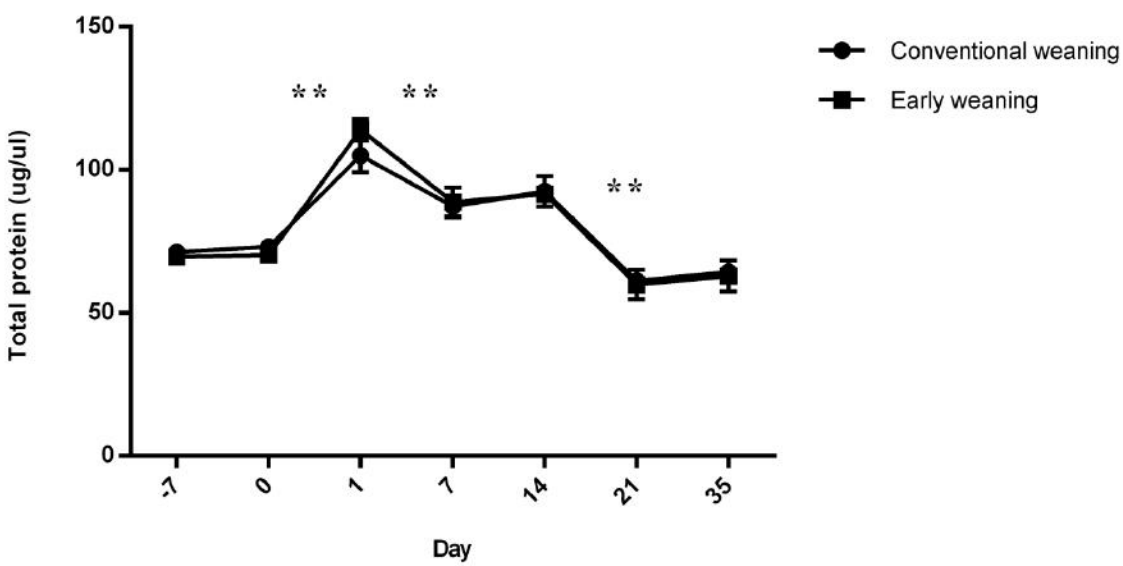

*Fig.3: Effect of weaning on granulocyte: lymphocyte (GR:LY) ratio (n=10); two-way ANOVA under a model of repeated measures; * p<0,05; ** p<0,01*

Total immune response to anti-BoHV1 vaccine

All animals of the conventional weaning group responded successfully to vaccination (5 of 5). In contrast, of the early weaning group only two animals showed a response. In conventional weaned animals antibody titers ranged between 1.6 to 2.8. The maximum titer was seen on day 35

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and lasted until the end of the evaluation period. In the early weaning group, only two vaccinated animals showed a humoral response with lower antibody titers (2 of 5) though the observed range of titers between the two animals was almost the same as in the group subjected to conventional weaning (Fig. 4).

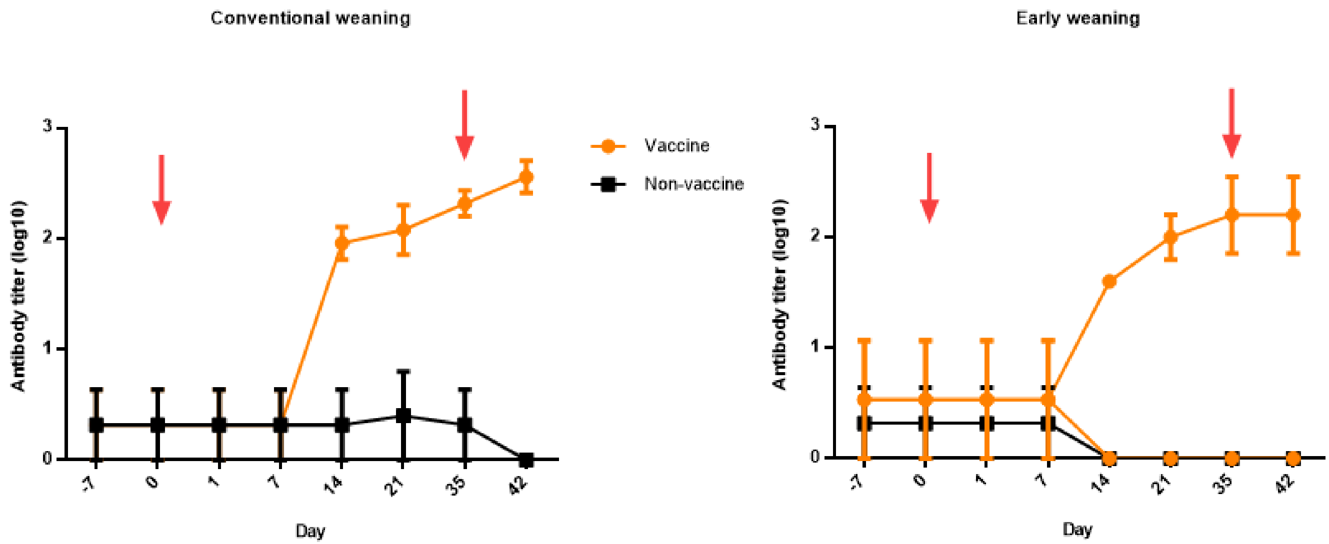

Fig. 4: Total antibody titers (log10) against Bovine Herpes Virus (BoHV) in weaned calves, both early (A) and conventional weaning (B). Vaccines administrations are indicated with arrows (from the total of 10 calves, 5 were vaccinated; orange line).

IV. DISCUSSION

The impact of early calf weaning on the reproductive performance of cows has been demonstrated under research and trade conditions in Argentina and other countries in the last thirty years (Galli et al., 2008). EW and HEW implementation has shown to increase the reproductive efficiency by accelerating postpartum rebreeding of the dam leading to increased pregnancy rates. As shown by our results, weaning generates a very marked stress response, with a cortisol peak and an increase of total plasmatic proteins at day one in both groups. As weaning practices are probably widely applied, a critical issue for the success of the practice is a correct nutritional and sanitary management of weaned calves (Diego Daniel Gonzalez et al., 2013).

The temporal course of total plasma protein levels did not vary between the two groups and in both a clear peak response was observed at day one post-weaning. This parameter seems to be a reliable weaning-stress marker and a clear response is seen.

With respect to plasmatic cortisol leves, early-weaned animals did not generate a typical acute stress response. Although, these animals showed a cortisol peak at day one values did not return to baseline and were still elevated at day 35 post-weaning. This result is important from the endocrinological point of view since cortisol impacts on many physiological functions of these animals. In particular, for the agricultural sector, its role in the immune system is very important since it directly impacts the health status of the animals. It is well established that incubation of cattle immune cells with physiologically relevant high concentrations of cortisol decreases lymphocyte proliferative responses. The immune response is one of the mechanisms through which animals defend themselves against environmental challenges. A common theory is that stress responses suppress immune activity, thus increasing susceptibility to disease. The underlying mechanisms of immune responses to stress remains unclear because of the complexity of the interrelation of the immune system and the stress responses, involving other physiological factors such as age, nutrition, genetics, and gender (Salak-Johnson, McGlone, & Norman, 1996). Extreme environmental conditions and stressful management practices influence the health and well-being of domestic farm animals. Converging evidence suggests that these stressors directly alter host immune function. These alterations have been linked to disturbances in the HPA axis and the hypothalamic autonomic nervous system (Moberg, 2012).

Regarding to the general parameters of the immune system we have measured the % of LY, GR, and the GR:LY ratio. We found that weaning influenced leukocyte populations in conventionally weaned calves but did not find any alterations in animals subjected to EW. Correspondingly, conventional weaned calves showed significantly elevated GR:LY ratios due to a significant reduction in the %LY accompanied by an elevation of

%GR. Previously it has been demonstrated that dexamethasone induces an elevation of GR:LY ratios in cattle (Anderson, Watson, & Colditz, 1999). Furthermore, it has been shown that the stress of weaning influences serum levels of acute-phase proteins, iron-binding proteins, inflammatory cytokines, cortisol, and leukocyte subsets in calves (Kim et al., 2011) suggesting that glucocorticoids are a contributing factor to the alteration of the GR:LY ratio. Our findings corroborate this previous studies that suggest that weaning affects leukocyte levels and that the GR:LY ratio may be an effective biomarker of the stress response in calves of six months of age. However, our findings also showes that in animals subjected to early weaning at an age of 60 days this effect is not seen. This difference may be attributed to developmental age differences of the cortisol response or the HPA and/or the immune system. Kampen et al., (2006) demonstrated that the percentages of CD4+, CD8+ and TCR+ lymphocytes stabilized during the first 10 to 12 weeks of life. The non-response of the GR:LY ratio of younger animals of 60 days of age may be due to the presence of still immature the leukocyte subsets or, alternatively, to an immature HPA system. At the moment, we can not identify a single factor responsible for this effect and/or draw any conclusions, but our results indicate that when using the GR:LY ratio as a stress indicator the animal age needs to be taken into account.

The HPA axis regulates the immune system mainly by the release of glucocorticoids. These exert a variety of effects on the immune system through signaling mechanisms mediated by steroid hormones. In rodents it has been demonstrated that increased glucocorticoids produce an increase in the release of mature neutrophils from bone marrow the magnitude of which decreases over time (Alvarez, 2002; Tadich, Kruze, Locher, & Green, 2003). In addition, it has been shown that in chronic stress neutrophilia-mediated leukocytosis occurs, whereas the number of lymphocytes and eosinophils decreases as long as glucocorticoid concentrations remain high (Alvarez, 2002; Yagi et al., 2004). Our results reproduce these observation in the group subjected to conventional weaning, but we emphasize the difference in the response according to age. It would be interesting to elucidate in the two animal age groups, on one hand the difference in the mechanism of interaction between the HPA axis and the immune system and, on the other hand the capacity of the HPA axis to generate immune responses and adaptations .

The goal of low-stress weaning techniques is to minimize negative stress caused by nutritional, environmental, physical and social changes experienced by calves at weaning time. Stress increases cortisol levels, which reduces immune system performance and can predispose calves to illness from a variety of disease pathogens, particularly organisms that cause the bovine respiratory disease (BRD) complex (WSU, 2013). Much of the failure of vaccination may lie in the timing of administration, failure of stressed calves to respond properly to vaccination, and the increased susceptibility of stressed calves to all pathogens. Our results show that the health status of early-weaned animals is potentially worse than that of conventional weaned animals at the day of weaning, since lesser animals show a lower response to vaccination. Recently, has been observed that EW and HEW calves showed no seroconversion after vaccination against BoHV1 (Diego Daniel Gonzalez et al., 2013). This may be due to the quality of the vaccine, the age of the animals, the state of weaning stress or a combination of these factors.

Previous studies have shown that there are no differences between early and conventionally weaning calves in their behavioral response to the stress caused by weaning as assessed by food consumption, displacement, and vocalizations (Vittone et al., 2012). Furthermore they also showed that the re-breeding and fattening pens provided greater efficiency and speed to the meat production process and that early weaning improved breeding rates.

Weaning is an inherently stressful process because it is usually connected with major changes that include nutritional, physical, and psychological elements as stressors in modern cattle production systems (O'Loughlin et al., 2011). More specifically these multifaceted stressors involve numerous husbandry practices, including the abrupt separation of the calf from its dam, a nutritional adjustment to a non-milk diet, social reorganization, and often housing (Enríquez et al., 2011; Lynch, Earley, McGee, & Doyle, 2010). Reducing the stress of weaning or finding the most adequate weaning age will help to reduce the risk of calfhood BRD and keep calves eating, gaining, and growing. Careful monitoring of weaned calves is essential to allow intervention when stress-related conditions arise and to ensure the welfare of animals. This is not only an ethical issue and social responsibility but improves also the quality of products derived from these animals. Trying to favor the quality of life and adaptation of livestock to their environment in a system that increases productivity, such as early weaning, we can say that both groups are generating a stress response to weaning, regardless of age. However, there are differences in mechanisms, particularly in the immune system, which is why it is recommended to the sector to verify and reinforce vaccination schedules.

It is necessary to have solid indicators of the welfare state of the animals. As we show in this study, the ration of GR:LY, widely used as an indicator of stress, depends on calf age and is for this reason not a representative biomarker of stress across different calf ages. Therefore, we discourage its use to measure and compare stress associated events like weaning at different calf ages. Since Coppo, (2007) found a malnutrition status of early weaning calves resulting in lower weight gains, and low levels of nutritional indicators and we observed a deficient response to vaccination in EW animals. we believe it necessary to incorporate some additional immunological and nutritional indicators to those mentioned above in order to ensure a thorough surveillance of the sanitary or welfare state of animals and to act in consequence whenever necessary.

V. CONCLUSION

In this study, leukocyte subsets, plasmatic proteins, cortisol levels, and immune status were found to be altered after weaning challenge. Results indicated that weaning challenge represents an important stressor, regardless of age. Management strategies should be complemented with the screening of calf immunity to ensure the efficacy of implemented vaccination programs. We found that the conventional indicator (% GR: LY) is not very representative for the stress response at a younger calf age, and we recommend the use of cortisol plasma levels and total proteins as indicators of stress as more reliable biomarkers of stress across different calf ages.

It is necessary to have a robust group of biomarkers of stress that allows the assessment of calf welfare at different weaning ages associated with various production systems. This will facilitate to achieve two important goals, on one hand, the understanding of the relationship between stress and the immune system and, on the other hand, to analyze solidly the welfare state of weaning calves at different ages in diverse production systems.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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