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# Breeding Strategies for Healthy and Sustainable Development of Animal Husbandry

*Edited by Xiaojun Liu and Hong Li*

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IntechOpen Book Series

# Veterinary Medicine and Science

Volume 18

## Aims and Scope of the Series

Paralleling similar advances in the medical field, astounding advances occurred in Veterinary Medicine and Science in recent decades. These advances have helped foster better support for animal health, more humane animal production, and a better understanding of the physiology of endangered species to improve the assisted reproductive technologies or the pathogenesis of certain diseases, where animals can be used as models for human diseases (like cancer, degenerative diseases or fertility), and even as a guarantee of public health. Bridging Human, Animal, and Environmental health, the holistic and integrative "One Health" concept intimately associates the developments within those fields, projecting its advancements into practice. This book series aims to tackle various animal-related medicine and sciences fields, providing thematic volumes consisting of high-quality significant research directed to researchers and postgraduates. It aims to give us a glimpse into the new accomplishments in the Veterinary Medicine and Science field. By addressing hot topics in veterinary sciences, we aim to gather authoritative texts within each issue of this series, providing in-depth overviews and analysis for graduates, academics, and practitioners and foreseeing a deeper understanding of the subject. Forthcoming texts, written and edited by experienced researchers from both industry and academia, will also discuss scientific challenges faced today in Veterinary Medicine and Science. In brief, we hope that books in this series will provide accessible references for those interested or working in this field and encourage learning in a range of different topics.

# Meet the Series Editor

Rita Payan Carreira earned her Veterinary Degree from the Faculty of Veterinary Medicine in Lisbon, Portugal, in 1985. She obtained her Ph.D. in Veterinary Sciences from the University of Trás-os-Montes e Alto Douro, Portugal. After almost 32 years of teaching at the University of Trás-os-Montes and Alto Douro, she recently moved to the University of Évora, Department of Veterinary Medicine, where she teaches in the field of Animal Reproduction and Clinics. Her primary research areas include the molecular markers of the endometrial cycle and the embryo–maternal interaction, including oxidative stress and the reproductive physiology and disorders of sexual development, besides the molecular determinants of male and female fertility. She often supervises students preparing their master's or doctoral theses. She is also a frequent referee for various journals.

# Meet the Volume Editors

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Dr. Hong Li is an Associate Professor of Animal Genetics at Henan Agricultural University, China. Previously, she was a visiting scholar at Oklahoma State University, USA. She received her Ph.D. in Animal Husbandry from Henan Agricultural University, China in 2016. Since then, she has worked at the College of Animal Science and Technology at the same university. Her research interests include poultry genetics and molecular biology. She has chaired or participated in several scientific research projects, such as the National Natural Science Foundation of China, and published 80 research papers and edited several books.

# Contents

# Preface

Techniques involved in animal genetics and breeding are the most important factors affecting genetic improvement and production efficiency of livestock. It is due to the continuous development and extensive application of techniques, such as performance testing, estimation of breeding value, analysis of the genetic mechanism of complex traits, sex control, and so on, that we can meet the needs for more high-quality livestock products to meet the needs of the world's increasing population. With the advent of the post-genome era, multiomics and bioengineering technologies are being combined with computer-based statistical analysis methods, resulting in new approaches to improving techniques used in animal genetics and breeding, and thus, promoting the development of animal husbandry. This book provides a comprehensive overview of the traditional and current state-of-the-art techniques in animal genetics and breeding, from both theoretical and practical viewpoints. The introductory chapter describes applications of omics techniques in livestock genetics and breeding. Subsequent chapters address such topics as techniques for genetically selecting highly productive animals while producing less greenhouse gas (GHG) emissions, breeding soundness (BSE) of rams and bucks using community-based breeding programs (CBBPs), how bovine species respond to in vitro thermal stress stimulation using peripheral blood monoclonal cells as the cellular system, and semen characteristics of wool-breed ram lambs raised in high altitudes. These techniques are important for the healthy and sustainable development of animal husbandry, especially for animals living under specific climatic and geographical environmental conditions.

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Section 1

# Introduction

Chapter 1

# Introductory Chapter: Applications of Omics Techniques on Livestock Genetics and Breeding

*Hong Li and Xiaojun Liu*

## 1. Introduction

Livestock has been domesticated for thousands of years, and generated into various breeds under a long artificial selection. They provide economic and high quality animal-derived proteins to meet the human nutrition requirement. The process of artificial selection has significantly enhanced crucial traits in agricultural animals [1, 2]. However, the genetic potential of farm animals has not yet been fully exploited. The quantitative trait is determined by multiple genes and regulated by the interplay of genetics, environment and their interaction [3]. The underlying biological mechanisms governing these phenotypic characteristics remain poorly understood. Therefore, the investigation into the formation mechanism of such intricate traits has consistently garnered significant attention within the realm of animal genetics and breeding.

Due to the limited number of molecular markers available for gene mapping, few breakthroughs have been made in the fine mapping of quantitative traits. Although quantitative genetics has been applied in animal breeding, leading to a technological revolution in the past century, selecting certain complex traits based solely on pedigree-derived breeding remains challenging due to the intricate nature of animal genetics and developmental mechanisms. The related concept and technology completion of the Human Genome Project has greatly promoted farm animal genomic research. With the completion of major livestock and poultry breed genome sequencing projects, coupled with the continuous emergence of high-throughput sequencing technologies (omics), agricultural animal genetic breeding research methods and means have gradually evolved from traditional conventional breeding to the integration of various omics technologies. The integration of diverse omics data for analyzing important economic traits aids in accurately and comprehensively revealing the formation mechanism.

## 2. Application of omics enhances the progress of animal selection and breeding

The omics mainly includes genomics, transcriptomics, proteomics, epigenomics and metabolomics. The application of them in livestock can improve the detection efficiency in the subtle changes of phenotypic [4, 5]. In animal genetics and breeding, integrative analysis of omics data can promise to deliver comprehensive insights

into the biological systems under study, and contribute to the identification of causal mutations, thereby enhancing the accuracy of genetic selection [6]. Additionally, it has contributed to the estimation of more accurate breeding values (BVs) and facilitated the selection of genetically superior animals at an early stage, thereby enhancing genetic gain [7, 8]. This, in turn, leads to improved animal productivity and profitability.

*Genomics* Deciphers the origin of agricultural animal domestication has been paid much attention by researchers. Understanding the origin of modern domestic animals helps us understand the history of breed and population formation, animal adaptability to the environment, the basic characteristics of genetic background shaping, and the molecular mechanism of the formation of main traits, which can provide basic information for the rational development of molecular breeding. Whole genome resequencing (WGS) has been widely used in detecting the molecular signatures, origin of domestication, and genetic variation of economic traits of agricultural animals [9, 10]. The genome-wide association studies (GWAS) was a kind of method, that firstly genome-wide genotyping through high-throughput sequencing technology, and then the phenotype and genotype of each marker were sequentially regressed to determine whether each marker was significant. With the development of sequencing technology and the reduction of cost, GWAS has become a new strategy and mainstream method to identify complex (quantitative) traits in the world [11].

*Transcriptomics* Transcriptome sequencing (RNA-seq) is widely recognized as the predominant method for investigating RNA functions. It can help the researchers to deepen the elucidation of the gene function, and analyze the possible intrinsic connections between gene expression alteration and animals' phenotype [12]. The integrated analysis between the RNA-seq and GWAS can reveal the key genes and their complex interactions mechanism involved in the concerned phenomenon [13]. In addition, the early selection of individuals based on multi-omics data obtained during early sexual maturity may contribute to an increased genetic gain by effectively reducing the generation intervals [14].

*Proteomics* Proteomics essentially refers to the study of protein characteristics at a large-scale level, including protein expression levels, post-translational modifications, protein-protein interactions, etc. It could decrease the sample analysis time while increasing the depth of proteome coverage when proteomics combined with advanced bioinformatic tools [15]. Proteomic studies primarily focus on characterizing the proteome of a specific organ, tissue, cell type, or organism under particular conditions or by comparing differential protein expression across two or more selected scenarios [16]. It has been commonly used to identify the candidate protein markers of fertility and reproductive problems [17], early growth and development [18], and meat quality [19] for molecular breeding in animal science. To identify the genetic variants with desirable traits for selection and breeding, proteomics has been used in different animal products such as meat, milk and cheese [20].

*Metabolomics* The field of metabolomics offers valuable insights into the intricate biochemical pathways underlying diverse physiological processes. It can identify metabolic pathways that play important roles in life processes, such as growth and development. For example, metabolomics approach was employed to investigate the impact of bone quality on productivity in chickens [21].

*Epigenomics* Epigenetic mechanisms encompass post-translational modifications of histones, DNA methylation, chromatin conformation and non-coding RNAs, and mainly participate in the processes of DNA repair, regulating gene expression and

homologous recombination [22]. Epigenetic changes are important for understanding complex trait variation and inheritance. Revealing the epigenomic constituents across diverse cell types facilitates the identification of numerous potential regulatory elements [23]. The methylation level of *SLCO1B3* gene identified through the whole-genomic bisulfate sequencing was associated with the changes in eggshell color in Lushi blue-eggshell chickens [24]. Candidate epigenetic regions or biomarkers for pig fertility were also identified by using the genome-wide DNA methylation method [25]. A new insight into the molecular mechanism of adaptation to physiological changes in liver of hens at the pre-laying and peak-laying stage was revealed by liver proteome and acetyl-proteome [26].

## 3. Conclusion

With the fast development of modern technology, modern animal breeding programs are constantly evolving with advances in breeding theory, biotechnology, and genetics. The application of the omics approach has the potential to revolutionize animal breeding practice, shifting it from a simplistic "black box" methodology to one that incorporates an understanding of regulatory networks and pathways that underlie the expression of crucial phenotypes. It establishes the groundwork for further investigations into the molecular mechanisms governing quantitative trait regulation and the development of molecular markers applicable to breeding practices. Therefore, the integration of Omics data to enhance livestock production is promising.

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Section 2

# Breeding Strategies for Specific Goals

Chapter 2

# Technology for Carbon Neutral Animal Breeding

*Getahun Belay Mekonnen*

## Abstract

Animal breeding techniques are to genetically select highly productive animals with less GHG emission intensity, thereby reducing the number of animals required to produce the same amount of food. Shotgun metagenomics provides a platform to identify rumen microbial communities and genetic markers associated with CH4 emissions, allowing the selection of cattle with less CH4 emissions. Moreover, breeding is a viable option to make real progress towards carbon neutrality with a very high rate of return on investment and a very modest cost per tonne of CO2 equivalents saved regardless of the accounting method. Other high technologies include the use of cloned livestock animals and the manipulation of traits by controlling target genes with improved productivity.

**Keywords:** breeding, GHG, animals, cloned, technology, carbon neutral

## 1. Introduction

A serious, systemic problem that affects us now, not in the future, is climate change [1]. C-neutral farming collaborates with farmers and agri-food companies to develop a technological solution that lessens the impact on the environment. All industries, including agriculture, must significantly reduce their emissions if we are to reach net-zero emissions. Achieving net-zero emissions will have an impact on productivity, the environment, and land use, though the precise effects on the livestock industry are unknown. To achieve carbon neutrality, it is necessary to change dietary habits, increase the value of food and agricultural waste, switch from fossil fuels to renewable energy, develop low-carbon technologies and low-carbon agriculture, build resilient cities and buildings, implement decentralized energy systems, and electrify the transportation industry [2]. To enable SHF to realize its climate-resilient dairy development strategies, interventions at various points along the dairy value chain are required [3]. The importance of raising the carbon peak, pursuing a strategy that is carbon-neutral, and supporting the long-term development of animal husbandry [4].

The idea of modifying an animal to make it more environmentally friendly raises questions about its wider sustainability and ethical implications, even though there are still significant gaps in the evidence proving the effectiveness of the solutions being advanced.

Perhaps the most significant result of relying on climate engineering to provide low-cost and straightforward ways to control our climate is the failure to critically

examine, much less address, the constantly increasing demand, production, and food waste. The already shaky political will for other important and radical climate change responses may also be weakened as a result. I illustrate my point by making a comparison between the extensive measures taken to change a cow's regular behaviour and the major efforts made to meaningfully challenge the regular actions, consumption patterns, and dietary choices of the public [5].

The continued increase in global population, the unequal distribution of wealth, and the rising demand for socially and nutritionally sustainable livestock products will shape the future of livestock. Other uses of land and water are predicted to compete fiercely, making more socially acceptable, efficient, and sustainable livestock production necessary. Climate change, environmental mitigation, and animal adaptation are recent issues in the field of animal breeding that have new demands on breeding procedures and research [6]. However, putting a negative economic value on methane would encourage action and help to reach the reduction goal in fewer generations. Therefore, it seems that including methane in the breeding objective will aid dairy cattle in more quickly reducing their methane emissions [7].

## 2. Efficient and robust animals

Sustainability in animal breeding is defined as the on-going availability of breeding animals and their germinal products for commercial production, which now and in the future meet the needs of a wide range of stakeholders, including breeders, farmers, livestock keepers, producers, consumers, and others while promoting more animal welfare-conscious agriculture. The implementation of international agreements encourages the development of sustainable breeding and production policies for animals. Long-term policy perspectives are necessary for animal breeding and livestock development strategies because poor choices can have negative long-term consequences [8].

The management and breeding of dairy cattle for a reduced impact on the environment are the two most significant applications of CH4 proxies. Single or multiple proxies can be used as indirect criteria for the breeding objective when selecting traits with lower environmental impact, but care must be taken to prevent unfavourable correlated responses.

Finally, even though combinations of proxies seem to offer the most accurate estimates of CH4, their current greatest drawback is the fragility of their general applicability. Therefore, future work should focus on creating proxy combinations that are reliable and usable in a variety of production systems and environments [9].

Additionally, genetically modified animals have significant positive effects on human health and the environment because they are more effective at turning feed into animal protein and produce less waste. In vitro methods for studying genes and their regulation, various methods for gene therapy, and the development of novel strains of existing microorganisms for use in medicine or industry all fall under the umbrella of genetic engineering. There are an increasing number of useful applications for genetic engineering in animal production, including the creation of transgenic animals that are disease-resistant, raising animal productivity, treating genetic disorders, and creating vaccines [10]. This entails creating novel heritable genetic material combinations using recombinant nucleic acid (DNA or RNA) techniques and then incorporating that material either directly through micro-injection, macro-injection, or micro-encapsulation techniques or indirectly through a vector system [11].

Animals that are consistently able to increase their output per unit of input because they are less susceptible to diseases and changes in their environment and management are the focus of breeding and reproduction organizations. Farmers can now request that breeding organizations label their products based on their use of resources, susceptibility to disease or stress, and climate adaptability. In Europe, there are voluntary codes of good practice for breeding organizations. The advantages are long-lasting and accumulate over time: genetic advancement currently accounts for 0.5–1% of an increase in animal productivity annually. Targeted breeding programs can help to increase this even more, but the context and farming system will determine the suitability of particular breeds, their ability to mitigate risks, and any trade-offs with other breeding goals. Emerging issues in the field of animal breeding include climate change, environmental mitigation, and animal adaptation, which place new demands on breeding practices and research [6]. Regardless of the accounting method, breeding, despite its slowness, is a realistic option for moving closer to carbon neutrality because it offers a very high rate of return on investment and a very low cost per tonne of CO2 equivalents saved [12]. Agricultural areas produce the most carbon emissions from animal husbandry, followed by agro-pastoral areas (which are on the decline) and pastoral (with a rising trend) [13].

Breeding objectives are set to support sustainability's many facets, including quality, diversity, acceptability, the environment, and economics (Elzbieta [8]) states that the implementation of international agreements aids in the development of policies for sustainable animal breeding and production. Better health, reproduction, feed efficiency, heat stress, and other adaptation traits are likely to be prioritized over higher production in countries where cattle production has already been intensified. This could necessitate the use of cutting-edge phenotyping technologies as well as additional new big data techniques to extract data for breeding [14].

Precision animal breeding will be made possible by incorporating thorough mechanistic models of animal performance in a given environment into genetic evaluation techniques that allow the prediction of genetic merit for underlying biological traits [15]. The idea of telos, which was previously primarily discussed in discussions about traditional genetic engineering, has been applied to genome editing and genomic selection to enhance animal welfare. It contests prevalent understandings of telos and offers a substitute theory that can be applied to recently developed breeding technology applications. This account rejects both removing the desire to pursue characteristic activities and altering animal bodies in ways that compromise their ability to perform such activities, while conditionally allowing increasing robustness against environmental stress [16]. The identification of genes and genetic markers suggests that it is possible to design strategies for breeding cows with the desired microbiota composition associated with phenotypes [17]. Wheat inclusion in the dairy cow diet could be an effective strategy for significantly lowering methane emissions; it also reduced milk fat percentage and milk fat and energy-corrected milk production [18].

## 3. Improved performance on low-quality feed

A significant portion of the global GHG emissions related to livestock production is caused by the production and feeding of animal feed. Though current research identifies traits for selecting animals that show excellent performance on lower-quality feed, most animals perform better on high-quality feed. Once they have been located, breeding organizations can choose these animals for their breeding and

reproduction programs and sell them. Monogastric animals that thrive on subpar feed should be commercially available in five years. This should take 8–10 years for cattle.

This development benefits both extensive systems that depend on lower-quality feed and the intensive livestock industry by allowing adjustments to current feeding regimens. Enhancing efficiency is one of the best ways to lower emissions from the production of beef. "Improved efficiency" can refer to better feed utilization, less need to clear more land, and fewer emissions of greenhouse gases per kilogram of beef produced. Researchers are working on techniques to breed animals for lower emissions after discovering that enteric methane intensity is a genetic trait. These technologies are still being developed, though [19].

When nitrates and vegetable oils were added to the diet, they both reduced enteric CH4 yield by 6-20%. Under smallholder conditions, cattle can be fed condensed tannins, saponin, and starch found in the leaves, pods, and seeds of tropical trees and shrubs, along with nitrates and vegetable oils. Strategies for enteric CH4 mitigation in cattle grazing poor-quality tropical forages can successfully boost productivity while lowering enteric CH4 emissions overall and per unit of product (such as meat or milk), thereby lowering the contribution of ruminants to GHG emissions and consequently to climate change [20]. In high-yielding dairy cows fed a partial mixed ration based on maize silage without access to pasture, the longer rumination time is associated with lower methane emissions as well as lower methane production per milk unit [21].

## 4. Selecting for low-methane producing ruminants

In milk production systems, enteric methane is a significant source of greenhouse gas emissions [22]. An additional cost-effective, long-lasting, and cumulative mitigation strategy involves breeding animals that take advantage of the natural variation in CH4 emissions. Selective breeding can reduce CH4 intensity by 24% in 2050 if the Dutch breeding goal is expanded to include CH4 production. This demonstrates that breeding is a valuable addition to the full range of mitigation tactics that could be used to meet the objectives for 2050 set by the EU. If it is determined that using animal breeding techniques will reduce enteric CH4 production while also having the desired effect on breeding [23]. Another effective, long-lasting, and cumulative mitigation strategy is animal breeding, which takes advantage of natural variations in CH4 emissions [23].

When the cost of feed in the breeding objective is high, multiple-trait selection can reduce overall GHG emissions while improving the economic performance of beef cattle at a low carbon price. Both the overall and per-unit GHG emissions of the product were decreased. Any plan to lower beef cattle's GHG emissions must include selection. When the cost of feed is low, selecting beef cattle without considering the cost of emissions will significantly increase GHG emissions [24]. Breeding makes a significant contribution to the overall arsenal of mitigation tactics that could be used to meet the EU's goals for 2050. If animal breeding techniques are chosen to reduce enteric CH4 production and have the desired effect on breeding [23]. A potential strategy to lessen the contribution of the dairy industry is the genetic selection of low-CH4-emitting cows [25].

Genetics can also influence the parameters that determine herd structure, such as cow replacement rates or calf death rates. The herd structure or the relative proportions of each animal type within the herd, influences the overall amount of emissions

and meat or milk produced [26]. Recent research suggests that genetically improving cattle can significantly reduce emissions at a negative cost, i.e., while providing net financial benefits. The use of concentrates may have to be increased as a result of improved genetics, which would reduce the use of fiber. As a result, it is clear that traits related to the feed efficiency of the bird are the key determinants of changes in EI and how they can be influenced by animal breeding. Broiler birds' daily feed intake has increased as a result of breeding, in order to support their faster growth. The ability to increase growth rate and daily feed intake influences the future potential of breeding to reduce GHG emissions associated with broiler production. By switching to slower-growing birds, feed efficiency will inevitably decrease, increasing GHG emissions and nutrient excretion. Over the years, breeding has significantly increased potential productivity (the number of eggs per hen per year), improved feed efficiency, and lowered the intensity of GHG emissions. Further emissions reductions through breeding, however, are probably going to be less than 10% below the current level as productivity is getting close to its biological limits [26].

To increase our understanding of the taxonomic and functional profiles of microbes connected to this rare and endangered pig breed, we studied the faecal microbiome of a local pig breed [27]. The industry's importance is evidenced by the rise in investment in genomic technologies in Canada, which aim to increase feed efficiency and cut greenhouse gas emissions [28]. The most optimistic predictions for advancements in genomic technologies have been exceeded, allowing for the industrial application of genomic selection. There are already a wide variety of analytical tools available, and many more will be created thanks to advancements in sensor technology and artificial intelligence. Possibly the biggest revolution will be the explicit inclusion of high-dimensional phenomics in animal breeding methods. Phenomics data will undoubtedly improve our understanding of the biological principles underlying phenotypes in the interim [29].

Although breeding is an effective strategy for reducing methane yield, traits like wool, live weight, and fat deposition may be impacted over time and should be watched closely [30]. Genetic selection for residual feed intake is an indirect method for reducing enteric methane (CH4) emissions in beef and dairy cattle (RFI). If enteric CH4 production is measured directly, it should be expressed as residual CH4 production or as CH4 production (g/animal per day) after accounting for body size, growth, body composition, and dry matter intake (DMI). Additionally, RFIfat cattle may benefit from a 1% to 2% increase in dry matter and CP digestibility compared to +RFIfat cattle due to lower DMI, shorter feeding intervals, improved rumen fermentation, and a different rumen bacterial profile. The rate of genetic change using this method is expected to boost feed efficiency and reduce enteric CH4 emissions from cattle by 0.75–1.0% per year with equal levels of body size, growth, and excess weight when compared to cattle not selected for RFIfat [31]. To lessen the impact of dairy cattle products on the environment, phenotypes must be chosen for emitting animals. This includes a direct selection for breath measurements, in addition to indirect selection using traits such as feed intake, milk spectral data, and rumen microbial communities. Even with a few registrations, it is still possible to include methane emission as a breeding goal trait with genomic selection. Many of these characteristics are either expensive or difficult to record. If methane emission reduction became a reality, there would be little disagreement about which phenotype to choose: methane in grams or liters per day, methane in liters per kilogram of energy-corrected milk or dry matter intake, or a residual methane phenotype, where methane production is adjusted for milk production and cow weight [32]. Rumen microbial biomarkers have been linked

to methane production in dairy cows; if heritable, these biomarkers could be used for targeted methane-reduction selection programs in the dairy cattle industry [33]. It is also discussed how the systems biology approach can be used to integrate and assess various levels of biological data, which can help with understanding the genetic underpinnings and biology of traits that cause ruminants to produce CH4 and reduce agriculture's overall environmental impact [34]. In particular, the order Veillonellales and the phylum Proteobacteria were found to be enriched in low emitters, while the order Desulfovibrionales and the order Proteobacteria were found to be enriched in high emitters [35]. Consequently, it is possible to target the rumen microbiome and cow genome separately by breeding low-methane-emitting cows and concurrently by looking into potential methods that target changes in the rumen microbiome to reduce CH4 emissions in the cattle industry [36].

As predicted for Australian macro pods, lower emissions were accompanied by increased Succinovibrionaceae abundance, changes in acetate and hydrogen production, and decreased methanogens. Numerous predicted protein sequences were different between cattle that emit more and less methane [35]. Propionate pathway enhancement in high-quality forage diets serves as a hydrogen sink for methanogens. In the propionate pathway, which is enhanced by high-quality forage-based diets, betaproteobacteria genes were found to be present, suggesting a syntrophic relationship may be at play to lower methane emissions in beef cattle [37]. The distinct group of rumen methanogens whose transcriptional profiles along the ethnogenesis pathway correlate with methane yields and offer fresh options for reducing CH4 at the levels of microbiota composition and transcriptional control [38]. Metagenomics has recently been the main technology used to describe the GI microbiome and its connection to host nutrition and health [39]. As predicted for Australian macropods, lower emissions were accompanied by increased Succinovibrionaceae abundance, changes in acetate and hydrogen production, and decreased methanogenesis. Between high and low methane-emitting cattle, there were differences in a significant number of predicted protein sequences. Ninety-nine percent were unidentified, indicating a promising future resource [35].

A thorough and high-quality protein sequence database that enables accurate protein identification and quantification, representative samples, precise protein extraction, and fractionation are all essential for conducting meaningful and accurate metaproteomic analyses [40]. These findings demonstrate that using conventional PETs improved animal performance while reducing the environmental impact of the feedlot cattle industry. As a result, eliminating them would result in an increase in the environmental impact of beef produced for both domestic and foreign markets [41].

A sophisticated technique called transgenesis allows for targeted gene modification and has the potential to boost genetic diversity by producing animals with improved productivity, reduced environmental impact, and disease resistance. The ability to alter a single gene is becoming more feasible as more data from genomic sequencing projects becomes available. A tool to address new issues and global challenges facing production agriculture could be the use of transgenic technologies in the production of farm animals. However, proponents of biotechnology tools like cloning and transgenesis will probably encounter resistance from the public at large, which does not understand or accept these reproductive methods for producing animals [42]. Although cloning is a potent tool for creating genetically identical copies of desired donor animals, its effectiveness is still debatable. For a variety of reasons, many scientists and regular people are against cloning. Due to the high failure rate of cloned animal growth from fetus to adulthood, it has been deemed an ineffective

technique up until this point [43]. In this instance, selective breeding was successful in reducing methane production by 20% over the course of ten years, but at the cost of increasing the ad hoc weight of methane in the selection index to 33% and slowing the genetic gain for production traits from 6 to 18%. This demonstrates the feasibility of incorporating environmental characteristics into the selection indices while maintaining populations that are profitable for producers [44].

In contrast to selection based on measured CH4 using respiration chambers (13%), which was used in our population, selection based on the abundances of the 30 most informative microbial genes offered a mitigation potential of 17% of mean CH4 emissions per generation. This shows the great potential of microbiome-driven breeding to reduce CH4 emissions over time and slow down climate change. Marker-assisted and genomic selection could be used to improve phenotypes like PME that are challenging and expensive to measure. Additionally, the ability of VFA indicators to predict methane emissions may help to increase the size of the reference population needed for genomic selection and genome-wide association studies [45].

If they are heritable, the rumen microbial biomarkers linked to dairy cows' methane production could be used for targeted methane-reduction selection programs in the dairy cattle industry [33]. Wide phenotypic variation and a lack of accurate methane measurements at the individual level are the main obstacles to the implementation of reduced methane emission traits in breeding programs. CH4 production trait heritability is generally moderate, and breeding programs can use it to target changes in microbial composition to decrease CH4 emission in the dairy industry for long-term environmental benefits at the expense of a minimal genetic gain reduction in production traits [46]. The current meta-analysis demonstrated that dairy cow's exhibit additive genetic variation for methane emission traits that could be used in genetic selection strategies [47]. The intensity of CH4 would be drastically reduced to about 0.2 kg CH4/kg LW gain, as observed in some intensive feeding systems, by optimizing the LW gain of grazing sheep and cattle to thresholds of 0.14 and 0.7 kg/day, respectively. This might indicate a 55% mitigation potential for livestock products in pasture-based systems. Our findings add fresh information to the discussion about reducing the negative environmental effects of pastoral ecosystems [48].

Nitrates, essential oils, and tannins are rumen environment modifiers that influence methanogens and reduce the availability of fermentation products required for CH4 formation. Breeding interventions may also be used to directly or indirectly select low-CH4-emitting animals, and genome-wide association studies are predicted to help with this process. Overall, dietary changes and the addition of feed additives have short-term, reversible effects, whereas selective breeding results in long-term, cumulative reductions in CH4 emissions [49]. The rumen microbiome of cows likely has no genetic influence on the variation in CH4 emission. As a result, breeding low-methane emitting cows while simultaneously researching potential strategies that target changes in the rumen microbiome to reduce CH4 emissions in the cattle industry allows for separate targeting of the rumen microbiome and cow genome [36].

Wide phenotypic variation and a lack of accurate methane measurements at the individual level are the main obstacles to the implementation of reduced methane emission traits in breeding programs. CH4 production trait heritability is generally moderate, and breeding programs can use it to target changes in microbial composition to decrease CH4 emission in the dairy industry for long-term environmental benefits at the expense of a minimal genetic gain reduction in production traits [46]. Since residual methane and feed intake have a moderate correlation and a positive correlation response, including residual feed intake in the breeding goal could further

reduce methane. A significant reduction in methane emissions could be achieved while maintaining an increase in milk production by adding a negative economic value for methane [50].

Future breeding goals should take into account how both traits differ along with (and across) lactation(s) and how they correlate with various production, maintenance, and intake traits [51]. Dairy cows that were given concentrates while grazing produced more milk overall and produced less CH4 per unit of milk [52]. In this instance, selective breeding was successful in reducing methane production by 20% over the course of ten years, but at the cost of increasing the ad hoc weight of methane in the selection index to 33% and slowing the genetic gain for production traits from 6% to 18%. This study demonstrates the feasibility of incorporating environmental characteristics into selection indices while maintaining populations that are profitable for producers [44]. The current meta-analysis demonstrated that dairy cattle exhibit additive genetic variation for methane production traits that could be used in genetic selection strategies [47].

Feed is an important factor in breeding goals because it makes up a significant portion of the variable costs linked to dairy systems. As a result, traits that indicate feed efficiency are increasingly in demand for genetic analysis. Many countries already have an idea of how much energy is required for milk production, maintenance, and so on, their breeding goals are to take feed efficiency into account. Currently, it is not possible to take actual feed intake variation into account when determining traits like residual feed intake (RFI), which is the difference between actual and predicted feed (or energy) intake. Given the high cost of accurately measuring feed intake in numerous cows, phenotypes derived from it are obvious candidates for genomic selection, provided that the trait is heritable and the accuracy of genomic predictions is acceptable to those using the breeding values. If breeding values are estimated for heifers rather than cows, the traits of the heifer and cow must be correlated. According to research on beef and dairy cattle, genomic predictions of dry matter intake (DMI) and RFI have an accuracy of about 0.4. There are ways to improve prediction accuracy; for instance, it has been demonstrated that combining data from three research herds (in Australia and Europe) can raise DMI genomic prediction accuracy from 0.33 within the country to 0.35 using a three-country reference population. Genetic correlations with other traits must first be estimated before RFI is included as a selection objective. Because of the mathematical relationship between RFI and energy balance calculation, failure to properly account for the mobilization of body reserves may result in the selection of a trait that is similar to the selection for a reduced energy balance.

Therefore, if RFI is to become a selection objective, it should be incorporated into a multi-trait selection index with net profit as the breeding objective, as this would allow genetic correlations with other traits to be properly taken into account. RFI is an obvious breeding goal if genetic parameters are accurately predicted. In the event that these are uncertain, DMI may be preferred [53].

Reduced CH4 emissions from ruminants may be achieved through the adoption of genetic selection and, in the future, genomic selection. Short-term (a few minutes to several hours) and long-term (days) feed intake is closely related to CH4 emissions. Even though there is less genetic variation than there is for CH4 emissions, CH4 yield (MY, g CH4 per kg dry matter intake) is a heritable and repeatable trait when measured over the medium term. Individual animal CH4 emissions are only moderately repeatable across diets and feeding levels when measured in respiration chambers. Short-term measurements have lower repeatability, possibly as a result of changes in the amount of feed consumed before the measurement and variations in time.

Even though repeated measurements are beneficial, it is best if they are taken at least three to fourteen days apart. But in order for short-term measurements to be helpful for genetic evaluation, we believe that a number (between 3 and 20) of measurements taken over a long period of time will be necessary (weeks to months). There are opportunities to use short-term measurements to measure CH4 in standardized feeding situations, such as breath "sniffer" devices attached to milking parlors or total mixed ration feeding bins [54].

The potential to reduce national livestock emissions by implementing these dietary interventions could be estimated using the confidence intervals derived for the mitigation efficacy [55]. The potential to reduce national livestock emissions by implementing these dietary interventions could be estimated using the confidence intervals derived for the mitigation efficacious [56].

When nitrates and vegetable oils were added to the diet, they both reduced enteric CH4 yield by 6–20%. Condensed tannins, saponins, and starch found in the leaves, pods, and seeds of tropical trees and shrubs can be fed to cattle under smallholder conditions, along with nitrates and vegetable oils. Strategies for enteric CH4 mitigation in cattle grazing low-quality tropical forages can successfully increase productivity while reducing enteric CH4 emissions overall and per unit of product (such as meat or milk), thereby lowering the contribution of ruminants to GHG emissions and subsequently to climate change [20].

Consuming milk products from cows fed nitrate may be safe in terms of residual nitrate and nitrite levels and the linseed plus nitrate combination may have a long-term CH4-mitigating effect on dairy cows. To prevent decreased cow performance, more work needs to be done to optimize the linseed and nitrate doses [57]. Diets had little effect on protozoa concentration or rumen fermentation parameters. Tea saponin is ineffective in this experiment's conditions at lowering dairy cows' methane emissions [58].

Ruminant feeding of whole-plant oat forage may reduce CH4 emissions, but lower biodegradability may also hurt animal performance. In contrast, feeding barley forage may reduce emissions without hurting animal performance [59]. Rumen fermentation profiles and enteric CH4 emissions per unit of ECM, GEI, and ADG demonstrate excellent potential for enteric CH4 emissions estimation [60]. By decreasing methane emissions by 40% + and 90%, respectively, the supplements 3-nitrooxypropanol and the seaweed Asparagopsis increased animal productivity with negligible effects on animal health or product quality. Methane emissions were reduced by 10% or less using biochar, nitrate, grape marc, vaccination, genetic selection, or vaccination. Cattle browsing legumes, such as Desmanthus or Leucaena species, and best management practices increase animal productivity and mitigate methane to a small extent. Large daily doses of ground wheat fed to dairy cows reduced methane emissions by about 35%, but the reduction was not long-lasting [61].

The gas emitted by ruminants that has the biggest negative impact on the environment is methane from enteric fermentation. It may be possible to reduce rumen methane emissions by adding lovastatin (Lv) to feedstocks, which would reduce the number of methanogenic archaea (MA). However, in vivo tests showed that there was a decline in VFA production. During in vitro and in vivo tests, Lv had no detrimental effects on the digestibility of dry matter; in fact, there is evidence that it may even increase digestibility [62].

Although their long-term impact has not been well established, some feed supplements have had the potential to lower ruminant CH4 emissions, even though some of them are toxic or may not be practical from an economic standpoint [63]. A potential feed ingredient for reducing goats' enteric methane emissions is red yeast

rice. However, it needs to be used carefully because it might stop some nutrients from being digested [64].

A potential feed ingredient for reducing goats' enteric methane emissions is red yeast rice. However, it must be used with caution as it may prevent some nutrients from being digested [65]. With low feed inclusion, Asparagopsis retains its significant methane-mitigating potential in a commercial feedlot setting [66]. Tea saponin alone, when added to pelleted concentrates, had no effect on reducing enteric methane emissions in non-lactating dairy cows under experimental conditions [67].

## 5. Finding new traits for GHG emissions

The potential for breeding and selection programs to choose for lower-emitting animals increases with any variation in emissions among individual animals; these are already being studied. The makeup of the microbial ecosystems in the animal's stomach and the structure of the stomach serves as the foundation for additional factors affecting the animal's emissions. For instance, early-life feeding practices may have a lasting impact on the rumen microbial composition and, consequently, methane emissions throughout an animal's productive life. Currently, research is being done on the possibility of altering the rumen microbial composition in lambs and calves after weaning to reduce methane production in adulthood. Genome editing will help us achieve these goals only if global regulatory and policy frameworks allow their use in agricultural breeding programs and deployment to farms. The regulatory environment for genome editing products is rapidly changing on a global scale, with an increasing number of nations putting more emphasis on product qualities and whether they could be achieved through conventional breeding than on the technologies involved in their creation [68].

One of the tasks assigned to the committee was to produce a report evaluating methods for identifying potential unintended compositional changes in the range of messenger ribonucleic acid (mRNA), proteins, metabolites, and nutrients that may occur in food derived from cloned animals that have not had their genes altered through the use of genetic engineering techniques. The committee was also tasked with researching ways to spot the unintended negative health effects of foods made from cloned animals [69].

The direct selection of a residual methane production trait would favorably influence all other methane traits. The large standard errors emphasize the need to increase data sets by assessing the methane emissions and DMI of more animals or by investigating proxy traits and combining data through international cooperation [70].

According to this meta-analysis, sheep have low to moderate genetic control over their gas emission traits. When accurate phenotypic records or genetic parameter estimates for traits related to gas emissions are unavailable, the average genetic parameter estimates that were obtained could be taken into account in genetic selection programs for sheep [47].

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Chapter 3

# Breeding Soundness Evaluation in Ram and Bucks under Community-Based Breeding Program (CBBP) Sites of the Amhara Region, Ethiopia

*Assemu Tesfa, Mesfin Lakew, Chekole Demis, Mulatu Gobeze and Alayu Kidane*

## Abstract

The objective of this study was to evaluate the breeding soundness (BSE) of rams and bucks used in community-based breeding programs (CBBPs). The evaluation was done in April 2022. The data were analyzed using the general linear model (GLM) procedures of the SPSS (version 22). Based on the criteria set for physical soundness, 88.89% and 87.32% of rams and bucks were satisfactory. The overall semen volume per ejaculation in small ruminants under study was $0.67 \pm 0.04$ ml with a minimum of 0.1 ml in buck and 1.2 ml both in rams and bucks. The average gross semen motility score was $3.55 \pm 0.09$ (>70% of sperm cells are active). A significant ($P < 0.05$) difference was observed between ram and buck semen concentrations, which was $4.06 \pm 0.42$ ($10^9$) and $3.89 \pm 0.23$ ($10^9$), respectively. Based on the selected examination parameters, 84.23% of the mating males of small ruminants were satisfactory for breeding, from which rams and bucks contribute to 86.48% and 82.18%, respectively. Rams and bucks above 22 cm of scrotal circumference at two and lower age, alert and active with no feet, eye, and conformation abnormalities can be selected for mating. In CBBP sites, it is better to furnish semen evaluation equipment and technical capacity to implement artificial insemination.

**Keywords:** breeding soundness, bucks, rams, satisfactory, semen characteristics

## 1. Introduction

Reproductive capacity of the herd/flock is influenced by numerous factors such as reproductive health, fertility, prolificacy, the ability to mount, and the nutritional level of individuals [1]. A successful breeding period relies on mating an appropriate number of sound males to reproductively active females and monitoring to identify any problems [2]. In fact, 50% of the reproductive potential and genetic change of a flock is

provided by the mating male animal [3, 4], care and strategic management of them is required. To help identify males that are capable or not capable of settling females, producers can perform breeding soundness examinations (BSE). Breeding soundness examination is an overall assessment of a male's potential ability to service and impregnate a given number of females during a given period of time [3]. The evaluation consists of a physical examination, body condition score, scrotal circumference, inspection of the reproductive organs, semen evaluation [5, 6], libido assessment [3], and screening for sexually transmitted disease [4]. Measurement of scrotal circumference reflects the weight of the gonad and therefore the ability of sperm production [7], and it has a great value as an indicator of the onset of puberty, total semen production, semen quality, pathological conditions of testes, and the potential subfertility or infertility [8].

Breeding soundness examination should be performed at least two months before breeding season [9] to allow animals to recover from pathologies or poor physical conditions [3], and it also should be a routine activity in breeding programs [2, 4]. Periodical BSE identifies the main causes of ram/buck failures, making it an important tool to increase the reproductive efficiency of the herd [10]. Rams/bucks are subsequently classified as sound/satisfactory, temporarily unsound/questionable, or unsound [2]. The satisfactory rams will achieve good reproductive performance if joined to ewes at a ratio of 1:50 for 60 days [5, 11].

The selection and distribution of rams/bucks for mating in the existing CBBP sites of Ethiopia were based on physical evaluation, pedigree information, and breeding values for selected target traits. And they are handled at farmers' hands with varying management levels. Both of these methods do not guarantee the fertility of these animals. Currently, the number of CBBP sites has increased, and scaling-out plans of the CBBP were also implemented. In the document [12], rams to be distributed for the scaling out sites were sourced from existing CBBPs, and as these animals are genetic materials, their failure to mate after distribution costs the program. Therefore prior to distribution, BSE should be done as a routing activity. This paper, therefore, was initiated to address the following objectives:

- To determine the effect of breed, body condition, and scrotal circumference on different semen parameters of mating animals in CBBP sites of small ruminants;
- To evaluate the breeding soundness of rams and bucks used in the existing CBBPs;
- To set standards for the use and distribution of satisfactory rams and bucks.

## 2. Material and methods

### 2.1 Working sites and breeds

The activity was conducted at established community-based breeding program sites of the Washera breed at the Sekela district, Simien sheep at the Dabat district, and Central highland goat at the Gondar Zuria district (**Figure 1**).

**Sekela district:** It is located 160 km away to the South East from Bahir Dar, the capital of the Amhara National Regional State, and 74 km away North East from Finote Selam, the capital town of West Gojjam Zone. The estimated total area coverage of the district is 6534.5 hectares, from which 70%, 18%, and 12% were highland (Dega), midland (Woynadega), and lowland (Qola) agroecologies. It is located at an

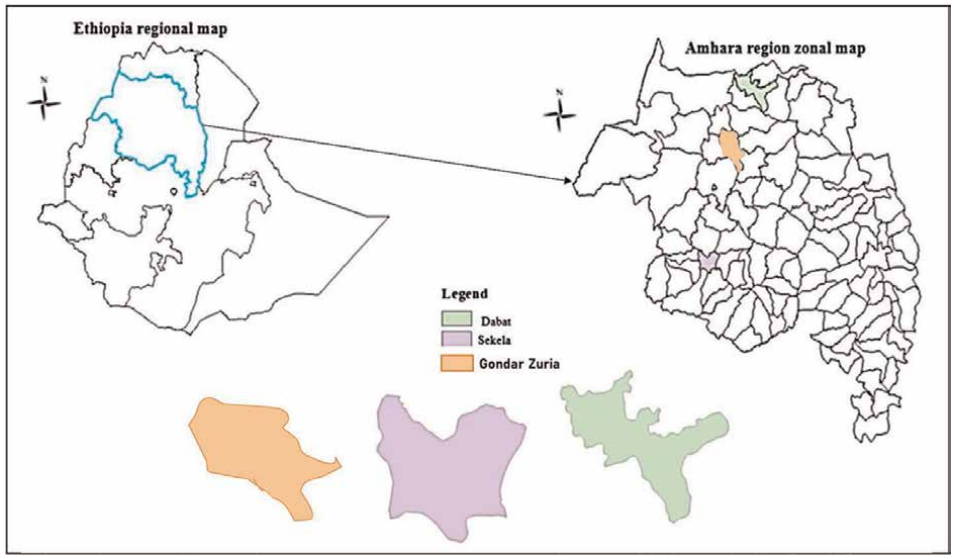


**Figure 1.**
*Map of the study districts.*

elevation of 3062 meters above sea level and 10°55′0″ N latitude and 37°31′60″ E longitude. The average annual rainfall of the area ranges from 1600 mm to 1800 mm with an average temperature of 18°C [13].

**Dabat district:** It is located at 12°59′3″ N and 37°45′54″ E in Amhara National Regional State, North Gondar Zone. It receives an average annual rainfall of about 1100 mm with the main rainy season extending from June to October. The average annual maximum and minimum temperatures are 19.9°C and 8.58°C, respectively [14].

**Gondar Zuria district:** It is located in the Central Gondar Zone of the Amhara Regional State, northwest Ethiopia. The District is among the 11 districts of the Central Gondar zone and has 41 rural and three urban Kebeles. The total area of the district is around 48,204 $km^2$. The district receives monthly average maximum and minimum temperatures of 29.96°C and 15.72°C, respectively. The altitude ranges from 1500 to 3200 m above sea level. Agroecologically, the district falls into two zones: Weyna Dega (72%) and Dega (28%). Mixed farming is predominant in the district (i.e., crop production and livestock rearing (90%)) [15].

## 2.2 Source and type of data

Semen evaluation was done in April 2022. A total of 63 and 92 body measurements from ram and bucks, respectively, were collected. The rams were Washera and Simien sheep breeds, while the bucks are Central Highland goats. Semen parameter evaluation was done from 16 rams and 15 bucks, which are under mating at CBBP sites.

## 2.3 Data collection procedures

### *2.3.1 Physical soundness examination*

The physical soundness examination includes symmetricity of testicles (1 = symmetric and 2 = nonsymmetric), shape of testicles (1 = normal and 2 = abnormal) as


**Figure 2.**
*Ways of measuring SC (A) and different scrotal abnormalities (B) [3].*

indicated (**Figure 2B**), firmness of the scrotum (1 = firm rubber ball and 2 = extremely hard and very soft), body condition score (thin (1–2 score), moderate (2–3), and fattened (above 3)), rear leg conformation (1 = desirable and 2 = camped behind, bowleggedness (base narrow) and toed-out stance (base wide)), general health condition of eye, feet, head and neck, nasal cavity and alertness (1 = healthy and alert and 2 = nonhealthy and inactive). These parameters were collected with a degree of acceptance. Each level of the evaluation was done based on the reference standards used for BSE [3, 16], and the interpretation was done [11].

### *2.3.2 Scrotal and other linear body measurements*

The scrotal circumference was measured at the widest part of the scrotum and recorded in centimeters (**Figure 2A**). Body measurements of heart girth (cm), weight (kg), height at prepuce (cm), rump height (cm), body length (cm), height at weather (cm), and face length (cm) were collected from rams at the CBBP sites. Estimated body weight was calculated with the following formula [17]:

$$\mathrm{BW} = \frac{HG\ (inch)^2 * BL\ (inch)}{300} \tag{1}$$

where BW is estimated body weight in pound, HG is heart girth by inch, and BL is body length by inch.

## 2.4 Semen analysis

**Semen collection:** Semen was collected by an artificial vagina (AV) with temperature of 42–43°C. Prior to collection, the prepuce of the ram was cleaned to prevent contamination of the semen. The collection was performed in the morning and shade areas to avoid tiredness of rams and sperm death due to direct sunlight. The libido of the ram was recorded during semen collection and scored from 5 (excellent) to 1 (very poor) [11, 18].

**Semen evaluation:** The color of semen was scored subjectively and classified as: milky, watery, thin creamy, creamy, and thick creamy [18]. Semen volume was recorded using a graduated collecting glass (0.1 mL accuracy). While being processed, ejaculates were placed in a thermos flask containing water at 35–37°C. Sperm mass motility was estimated subjectively by using a phase contrast microscope. For that semen was taken with a pipette, dropped on the slide and covered with a cover slip and observed with 10× magnification on the objective lens. The mass motility was graded from 0 to 5 scores based on the passion of the wave motion [18].

Measurement of the sperm concentration was done by using a portable spectrophotometer pre-calibrated for ram semen (Ovine-caprine Accuread photometer; IMV®, France). Sperm cell concentration was estimated using a micropipette to take normal saline (0.9%) and put 4 ml of normal saline and 10 microliters of fresh semen on the UV Macro cell (UV Macro Cell 2.5 ml– 4.5 ml, Great Britain) and mix gently and measure the concentration using Accu Read IMV Technologies SA, 232 Spectrophotometer.

For spermatozoa live/dead ratio (semen morphology), semen was stained with eosin-nigrosin stain followed by microscopic examination (40×). Spermatozoa with red head were counted as dead cells and the colorless ones as live spermatozoa [11]. The proportion of morphologically abnormal spermatozoa was determined by examining 200 spermatozoa in an eosin-nigrosin smear under the same magnification. The spermatozoa were evaluated for vitality (percentage of live spermatozoa) and abnormal percentage (head, midpiece, and tail abnormal). The semen quality analysis was done in collaboration with Debre Berhan Agricultural Research Center (DBARC).

### 2.5 Statistical analysis

Breed, body condition score, scrotal circumference, libido, and age were used as a factor to evaluate the semen characteristics. The data were analyzed using the general linear model (GLM) procedures of the SPSS (version 22). Post-hoc least significant difference (LSD) tests were used to assess differences between means. The results are presented as mean (±SE), and the level for statistical significance was set to $P < 0.05$.

$$Y_{ijklmno} = \mu + M_i + L_j + C_k + S_l + A_m + B_n + e_{ijklmno}. \quad (2)$$

where

$Y_{ijklm}$ = semen characteristics (volume, motility, color, concentration, vitality, and abnormality),

$\mu$ = overall mean,

$M_i$ = effect of ith mating male animals (ram and buck),

$L_j$ = effect of jth libido score (3, 4, and 5),

$S_k$ = effect of $k^{th}$ body condition score (medium (2–3 BCS) and good (>3BCS)),

$C_l$ = effect of lth scrotal circumference (acceptable (≤20 cm), satisfactory (21–23 cm), and excellent (>23 cm)),

$A_m$ = effect of mth age (0PPI, 1PPI, 2PPI, and 3PPI),

$B_n$ = effect of nth birth type (single and multiple), and

$e_{ijklm}$ = residual effect.

## 3. Result and discussions

### 3.1 Physical soundness of rams and bucks at CBBP sites

Based on the criteria set for physical soundness, 88.10% (88.89% of rams and 87.32% of bucks) were satisfactory (**Figure 3**). The observed result in the current study is a good indicator of satisfactory ram, which is capable to mount and mate female animals, and an indication of the care during ram and buck selection as a replacement at the same CBBP and for distribution to other sites. The observed lower percentage in firmness of the scrotum is an indicator of the absence of reproductive organ palpation during selection and this should get attention during selection. Good

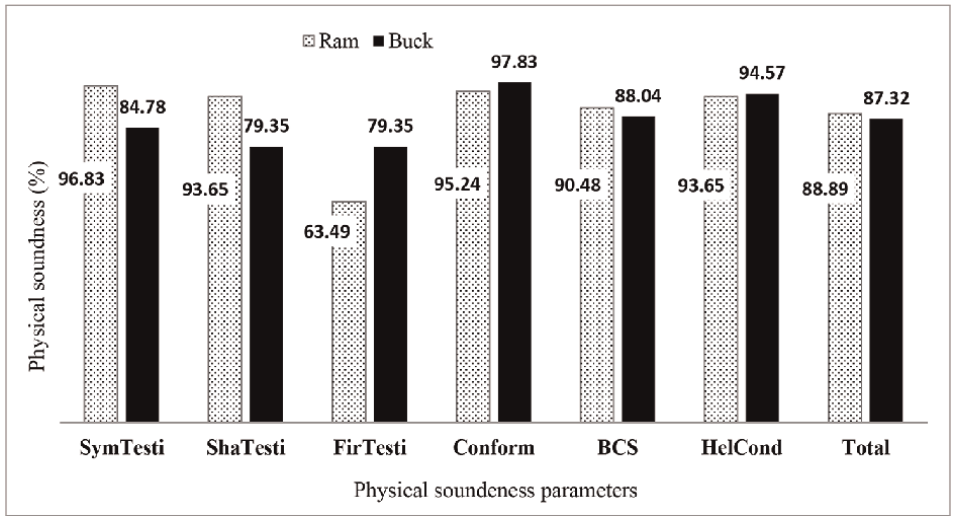


**Figure 3.**
*Physical soundness examination result for ram and buck at CBBP sites. SymTesti = symmetricity of testicles; ShaTesti = shape of testicles; FirTesti = firmness of the testicles; Conform = rear leg conformation; BCS = body condition score (above 2); HelCond = health condition.*

physical soundness is an indicator of the ram to deliver semen to ewes and management level of the producers [19]. Physical problems such as lameness, blindness, and penile or perpetual problems may not interfere with semen production or quality, but rams will not be able to find estrous ewes and/or mate them, resulting in poor reproductive performance [11]. During the physical examination, the body condition of the ram during the breeding season is an indicator of its breeding efficiency [10].

### 3.2 Effect of fixed factors on semen quality parameters

#### *3.2.1 Semen volume*

The mean (±SE) semen volume per ejaculation in small ruminants under study was 0.67 ± 0.04 ml with a minimum of 0.1 ml in buck and 1.2 ml in both rams and bucks. The volume of semen could be higher if there would be training of the artificial vagina before a day or two. The body condition score and libido had shown a significant ($P < 0.01$) effect on semen volume. The best animals in libido had higher volume per ejaculation (**Table 1**). A significant difference between semen volume and age was also reported [1, 20] for different sheep breeds of Spain. The average volume of semen per ejaculate (ml) was comparable with Menz sheep ram (0.7 ml) [18], Abergelle buck (0.64 ± 0.03 ml) [21], 0.5 ± 0.3 ml reported by Siddiqua et al. [22]; higher than 0.27 ± 0.12 ml [23] ranging from 0.43 ± 0.03 to 0.45 ± 0.22 in black Bengal bucks [24]; and lower than 1.0 ± 0.2 ml in Norduz goats [25]. The difference in semen volume within the same species was due to breed differences.

#### *3.2.2 Gross semen motility score*

The average gross semen motility score was 3.55 ± 0.09, which is above 70% of sperm cells are active (**Table 1**). All the factors considered in the current study had no significant ($P > 0.05$) difference in semen motility. Nonsignificant difference of breed

| Parameters | N | Volume/ejaculation (ml) | Motility (1–5) | Color | Concentration ($10^9$) | Vitality (%) | Abnormal (%) |
|---|---|---|---|---|---|---|---|
| Overall | 31 | 0.67 ± 0.04 | 3.55 ± 0.09 | 2.68 ± 0.27 | 3.98 ± 0.24 | 90.71 ± 0.36 | 9.00 ± 0.24 |
| Animal | | | | | * | | |
| Ram | 16 | 0.71 ± 0.05 | 3.50 ± 0.13 | 2.94 ± 0.37 | 4.06 ± 0.42 | 90.53 ± 0.54 | 8.81 ± 0.33 |
| Buck | 15 | 0.63 ± 0.07 | 3.60 ± 0.13 | 2.40 ± 0.40 | 3.89 ± 0.23 | 90.90 ± 0.49 | 9.20 ± 0.34 |
| BCS | | * | | * | | | |
| Medium | 17 | 0.62 ± 0.05 | 3.53 ± 0.12 | 2.35 ± 0.38 | 3.80 ± 0.40 | 91.03 ± 0.44 | 9.03 ± 0.33 |
| Good | 14 | 0.74 ± 0.06 | 3.57 ± 0.14 | 3.07 ± 0.37 | 4.19 ± 0.23 | 90.32 ± 0.59 | 8.96 ± 0.35 |
| SC | | | | | | | |
| Acceptable | 8 | 0.74 ± 0.07 | 3.50 ± 0.19 | 2.25 ± 0.62 | 4.12 ± 0.51 | 90.19 ± 0.72 | 9.50 ± 0.48 |
| Satisfactory | 8 | 0.71 ± 0.08 | 3.63 ± 0.18 | 2.63 ± 0.53 | 3.87 ± 0.60 | 90.88 ± 0.69 | 9.00 ± 0.35 |
| Excellent | 15 | 0.61 ± 0.06 | 3.53 ± 0.13 | 2.93 ± 0.37 | 3.96 ± 0.30 | 90.90 ± 0.55 | 8.73 ± 0.37 |
| Libido score | | * | | ** | *** | * | |
| 3 | | 0.59 ± 0.11b | 3.43 ± 0.20 | 1.43 ± 0.43b | 3.58 ± 0.27b | 89.79 ± 0.71 | 8.93 ± 0.49 |
| 4 | | 0.62 ± 0.04b | 3.64 ± 0.15 | 2.73 ± 0.45ab | 3.26 ± 0.44c | 91.64 ± 0.69 | 9.09 ± 0.26 |
| 5 | | 0.76 ± 0.07a | 3.54 ± 0.14 | 3.31 ± 0.38a | 4.80 ± 0.30a | 90.42 ± 0.45 | 8.96 ± 0.47 |
| Age | | | | | | * | ** |
| 0PPI | 6 | 0.68 ± 0.07 | 3.67 ± 0.21 | 2.50 ± 0.67 | 3.67 ± 0.37 | 91.25 ± 0.77a | 8.75 ± 0.62b |
| 1PPI | 13 | 0.58 ± 0.06 | 3.62 ± 0.14 | 2.31 ± 0.44 | 3.79 ± 0.47 | 90.62 ± 0.58ab | 9.46 ± 0.27a |
| 2PPI | 8 | 0.74 ± 0.08 | 3.38 ± 0.18 | 3.13 ± 0.48 | 4.18 ± 0.40 | 91.44 ± 0.61a | 8.13 ± 0.54c |
| 3PPI | 4 | 0.80 ± 0.15 | 3.50 ± 0.29 | 3.25 ± 0.75 | 4.63 ± 0.55 | 88.75 ± 0.85b | 9.63 ± 0.31a |
| Birth type | | | | | | | * |
| Single | | 0.66 ± 0.05 | 3.58 ± 0.12 | 2.74 ± 0.36 | 4.11 ± 0.27 | 90.34 ± 0.46 | 8.74 ± 0.28 |
| Multiple | | 0.69 ± 0.07 | 3.50 ± 0.15 | 2.58 ± 0.43 | 3.76 ± 0.45 | 91.29 ± 0.57 | 9.42 ± 0.39 |

*BCS = body condition score; SC = scrotal circumference; 0PPI = shoat with milk teeth (>about 9 months); 1PPI = shoat with 1 pair of permanent incisor (PPI) and the like.*
*Means with the same letter are not significantly different.*
*$^{*}P < 0.05$.$^{**}P < 0.01$.$^{***}P < 0.001$..*

**Table 1.**
*Mean (±SE) of values of semen quality parameters across different fixed factors.*

and BCS on semen motility was reported [1]. The mass motility score was comparable with the Menz sheep ram (3.17) [18].

### *3.2.3 Semen concentration*

The average semen concentration ($10^9$) reported in the current study was 3.98 ± 0.24. A significant difference was observed between ram and buck semen concentrations (**Table 1**), which was 4.06 ± 0.42 ($10^9$) and 3.89 ± 0.23 ($10^9$), respectively. Besides, the respective minimum and maximum concentrations were observed in rams 7.7 ($10^6$) of Simien sheep and 6.98 ($10^9$) of Washera sheep. Libido score had also a significant ($P < 0.001$) effect on sperm concentration (**Table 1**); the higher the libido score the higher the concentration. The observed concentration has given a good insight to conduct artificial insemination in the genetic improvement programs. As indicated by Larsen [26], 300 million spermatozoa were used for a single insemination in small ruminants; based on this, with the average 3.98 ± 0.24 ($10^9$) number of spermatozoa recorded in the current study, 13 ewes can be inseminated. The average semen concentration ($10^9$) recorded in the current study was higher than Menz (2.44), Awassi cross Menz (3.34) [18], and Abergelle bucks (3.14 ± 0.11) [21]. Significantly lower sperm cell count ($0.98 \times 10^9$) in the testicles of West African dwarf bucks was reported [27]. The concentration of the current study was considered as normal based on the study of Faigl et al. [28], which reported a concentration range from 3.5 to 6.0 billion as normal. A similar report on a nonsignificant difference of breed and BCS on sperm concentration was reported [1].

### *3.2.4 Semen morphology*

The morphology analysis was done for vitality and abnormality percentage (**Table 1**). The average vitality and abnormality percentage of the current study was 90.71 ± 0.36 and 9.00 ± 0.24, respectively. The head, midpiece, and tail abnormalities for ram were 0.12, 0.51, and 8.41%, and for bucks were 1.0, 1.0, and 8.0%, respectively; the proportion is presented in **Figure 4**. Age had shown a significant effect on semen vitality ($P < 0.05$) and abnormality ($P < 0.01$), and libido score and birth type had a significant ($P < 0.05$) effect on semen vitality and abnormality, respectively. The current result was higher than the reports of Faigl et al. [28] and Goshme et al. [18] who reported an average vitality range of 70–80 and 84.04% for different breeds, respectively. Varying level of head, midpiece, and tail abnormality in sperm cell was reported (**Figure 5**) [29]. Based on the study by Petrovic et al. [30], sperm

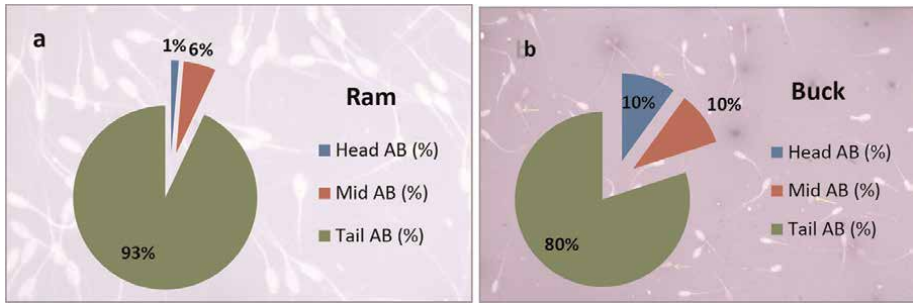


**Figure 4.**
*Proportions of sperm morphological abnormality (%) of ram (a) and buck (b).*

abnormality is varied depending on the seasons of the year on which higher abnormality was recorded during hot seasons. **Figures 6** and **7** present sperm abnormalities at different parts.

### *3.2.5 Semen color*

The average value for semen color was 2.68 ± 0.27, which is characterized as a thin creamy from the five color ranges (**Figure 5**). The color observed in the current study was in line with the finding of Pankaj et al. [31] who reported a color range of 1.9 ± 1.0

**Figure 5.**
*Reading: semen volume (left) and concentration (right).*

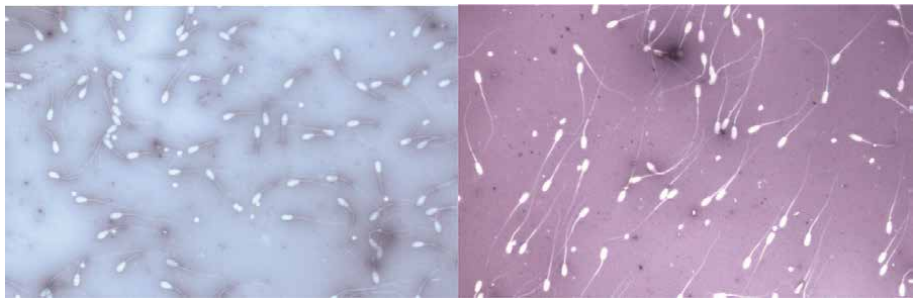

**Figure 6.**
*Morphologically normal sperm cells (eosin-nigrosin stain).*

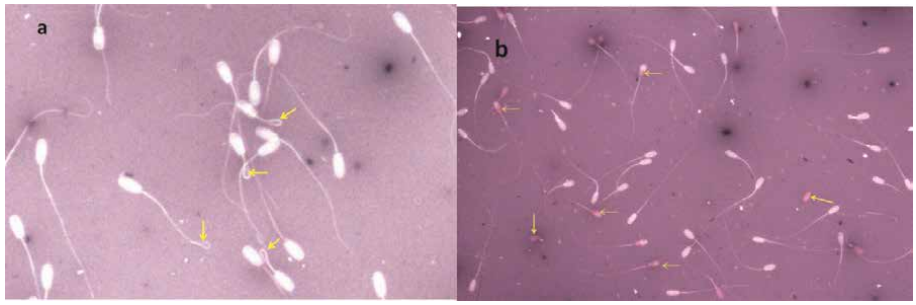


**Figure 7.**
*Morphologically abnormality of sperm cells: (a) bended and terminally coiled tails and (b) abnormal head (eosin-nigrosin stain).*

to 4.0 ± 0.0. Color is an indicator of injury or infection in the reproductive tract [31] and sperm concentration. Body condition and libido score had shown a significant ($P < 0.05$) effect on semen color (**Table 1**).

*3.2.6 Correlation between semen quality parameters*

A positive significant ($P < 0.05$) correlation was observed in body weight with semen volume ($P < 0.01$) and color ($P < 0.05$); libido score with semen volume, color, and concentration; and body condition with semen volume and color (**Table 2**). Besides, there is also a correlation between semen volume with color and concentration, color with motility and concentration, and motility with concentration. A similar significant difference between age and semen volume; scrotal circumference with age and BCS was reported [1].

*3.2.7 Scrotal circumference and mating test (libido score)*

The average scrotal circumference, body condition score, and libido score were 22.14 ± 0.23, 2.06 ± 0.04, and 4.19 ± 0.14, respectively. There was a significant ($P < 0.05$) difference in SC and BCS between mating animals. Higher scrotal circumference (22.56 ± 0.49) was observed in rams compared with bucks (21.86 ± 0.21). Similarly higher body condition score (2.18 ± 0.07) was observed in rams than in bucks (1.98 ± 0.05). In considering the scrotal circumference for classifying mating ram and buck, it is paramount important to consider age, breed, season, nutrition and other diseases, and previous reproductive history [2]. The libido score for rams and bucks was 4.31 ± 0.19 and 4.07 ± 0.21, respectively, and there is no significant difference ($P < 0.05$) between mating animals. The observed higher libido score in the study breeds was an important indicator in the efficiency of the rams and bucks to deliver semen for females [11], and poor libido was reported as a cause of infertility or reduced fertility [30].

The average scrotal circumference observed in rams was lower than 27.5 ± 1.29 cm [32] and 24.2 ± 1.8 cm [33]. For Ethiopian sheep breeds, average scrotal circumference range from 25 cm at one year to 30 cm at four years of age was reported [4]. Besides, considerably higher (22.52 ± 3.61 cm) and lower (17.25 ± 0.76 cm) scrotal circumference was reported for Algeria Indigenous Bucks [34] and West African dwarf bucks [27], respectively. Compared with these findings and the guideline [6, 11], the average scrotal circumference recorded in the study of rams and bucks can be categorized as satisfactory for breeding purposes.

*3.2.8 Breeding soundness examination (BSE) and interpretation*

Based on the BSE, rams and bucks are classified into three, *viz*, satisfactory, questionable, and unsatisfactory [2]. The category was based on physical examination score, scrotal circumference, and semen characteristics [11]. In the current study, to evaluate the satisfactory rams, finding above average were considered as cutoff values. Based on the selected examination parameters, 84.23% of the mating males of small ruminants were satisfactory for breeding (**Table 3**), from which rams and bucks contribute to 86.48% and 82.18%, respectively. The main reasons contributing to the failure of physical examination were body condition, scrotal circumference, and semen color. Relatively higher ram BSE failure (22.15%) was reported [10]. Similarly, confirmation as a reason for ram BSE failure [1] and body condition and semen

| | Age | SC | BW | LS | BCS | SV | Color | MS | Concn | Viability | AP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Age | | 0.188 | 0.538*** | 0.028 | 0.342* | 0.394* | 0.146 | 0.019 | 0.215 | −0.243 | 0.075 |
| SC | | | 0.435** | 0.198 | 0.271 | −0.181 | 0.244 | 0.118 | 0.053 | 0.209 | −0.183 |
| BW | | | | 0.289 | 0.721*** | 0.401** | 0.316* | −0.099 | 0.183 | −0.200 | 0.026 |
| LS | | | | | 0.550*** | 0.313* | 0.455** | 0.076 | 0.418** | 0.073 | 0.024 |
| BCS | | | | | | 0.435** | 0.422** | −0.069 | 0.253 | 0.026 | −0.081 |
| SV | | | | | | | 0.333* | 0.253 | 0.478*** | −0.184 | 0.106 |
| Color | | | | | | | | 0.529*** | 0.603*** | 0.007 | 0.002 |
| MS | | | | | | | | | 0.450** | −0.012 | −0.066 |
| Concn | | | | | | | | | | −0.243 | 0.032 |
| Viability | | | | | | | | | | | −0.194 |
| AP | | | | | | | | | | | |

*SC = scrotal circumference; BW = body weight; LS = libido score; BCS = body condition score; SV = semen volume; MS = mass motility; Concn = concentration; AP = abnormality percentage.*

**Table 2.**
*Partial correlation between semen quality parameters.*

| Average values for satisfactory ram | Category | | Overall |
|---|---|---|---|
| | Ram | Buck | |
| Physical examination (above 80% sound) | 88.89 | 87.32 | 88.10 |
| Body condition score (above 2) | 90.48 | 88.04 | 89.26 |
| SC (above 22 cm) | 63.49 | 53.26 | 57.42 |
| Semen color (thin to thick creamy) | 62.5 | 46.67 | 54.84 |
| Sperm morphology (≥70%) | 100 | 100 | 100 |
| Sperm motility (≥30% progressive motility | 100 | 100 | 100 |
| Abnormal sperm cells (‘15–20%) | 100 | 100 | 100 |
| Overall average (%) | 86.48 | 82.18 | 84.23 |

**Table 3.**
*Breeding soundness examination parameters for satisfactory rams.*

character [10], and physical abnormalities [11] was reported. The satisfactory rams can successfully serve above 30 ewes in an unsynchronized free-grazing flock.

The aforementioned failure causes for BSE in mating animals are highly correlated with the season of the year and nutrition [18, 35–37], which indicated that these mating animals can be satisfactory if they are well managed and fed. Poor management may result in rams or bucks that are either not sound for breeding or are culled or die well before the end of their productive lives [2]. Rams and bucks evaluated under these circumferences, therefore, are categorized as questionable those need further evaluation. Besides these, the satisfactory ram should be good in general health, good conformation, normal genital tract, and no previous history of infertility [11]. **Table 3** indicates the average values set for satisfactory mating animals based on different evaluation criteria [1, 4, 6, 11].

## 4. Conclusion and recommendations

Implementing BSE as a routine activity under CBBP sites can improve the productivity of participant farmers under the program through the introduction of fertile ram and buck. About 15.77% of the mating animals at CBBP sites of sheep and goats failed in the general breeding soundness examination. The main reasons were physical examination, body condition, scrotal circumference, and semen color, which all can be improved through successful management. The semen characteristics and libido observed in both breeds were better, and it allows conducting artificial insemination to fasten the genetic and economical gain from the program. In areas where there is no laboratory support for semen evaluation, rams and lambs above 22 cm of scrotal circumference at two and lower age, alert and active with no feet, eye, and conformation abnormalities can be selected for mating. If there is a lab facility to evaluate the semen, the above-indicated cutoff values can be considered as a minimum standard for satisfactory ram and buck under BSE. Besides these BSE parameters, rams and bucks with better breeding values based on target traits should also be considered for selection.

- Better management throughout the year could better be implemented for mating and candidate rams and bucks.

- In CBBP sites, it is better to furnish semen evaluation equipment to better evaluate mating animals, and technical capacity on artificial insemination had better be developed to speed up the achievement and gain from the CBBP sites.

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Section 3

# Characteristics of Animals under Specific Environmental Conditions

**Chapter 4**

# Techniques of Using Peripheral Blood Mononuclear Cells as the Cellular System to Investigate How of the Bovine Species (Indian Zebu-Jersey Crossbreds) Responds to *in vitro* Thermal Stress Stimulation (Thermal Assault/Heat Shock)

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**Abstract**

Animal production is negatively impacted by global warming and is subject to serious consequences for livestock production systems. In order to understand how PBMCs of Indian Zebu-Jersey crossbreds respond to various levels and durations of thermal assault and heat shock, in this chapter we will discuss techniques involving *in vitro* thermal stress stimulation (TSS) to stimulate bovine peripheral blood mononuclear cells (PBMCs) under various thermal assault conditions (TACs), including normal to extreme temperatures and varying durations of thermal exposure (DTEs). The consequences of thermal stress on bovine species can be lessened and managed with an understanding of how PBMCs as a cellular system respond to in *vitro* heat shock and thermal assault. To learn more about how Indian Zebu-Jersey crossbreds respond to *in vitro* thermal conditions, it may also be possible to explore the relationship between the decrease in PBMCs count during *in vitro* TSS and the expression of the heat shock protein genes (HSPs) such as *HSPs* 70 and 90 genes. This will be exploited to discover how Indian Zebu-Jersey crossbreds respond *in vivo* to diverse environmental thermal conditions and will further enable *in vivo* understanding of the

potential for thermotolerance in bovine species for better adaptability, survival, and production performance.

**Keywords:** heat shock, PBMCs, thermal assault, cellular system, Zebu Cattle, heat stress

## 1. Introduction

Livestock animals are required to raise their respiratory rate and peripheral blood flow in the tropics due to the harsh weather conditions, which has a detrimental effect on physiological and production performance, including poor milk quality [1]. Environmental thermal conditions have made it difficult for both humans and animals to survive in the face of the existential danger posed by climate change, which has had a variety of negative effects on performance, production, and food security [2]. Cattle as well as other animals can suffer severe effects from thermal stress (TS), including decreased feed intake, low milk production, stunted growth, poor health, decreased activity, and poor performance [3], they also succumb to hyperthermia if thermal assaults are not mitigated [3].

Furthermore, livestock animals are compelled to adapt and survive under assault of thermal conditions or extreme environmental conditions in the tropics, which has a negative impact on their physiology and production performance. The ability of cattle and other animals to maintain homeostasis is negatively impacted by changes in external temperatures and relative humidity, which forces them to actively maintain the internal body temperature (IBT) required for their survival and productivity [4]. Homeothermy is the ability of an animal to regulate its internal body temperature (IBT) in the face of thermal challenges from the environment [1]. TS is attained when an animal's BT is raised over its usual physiological range. The condition results in increased management expenses, lowers food security, and has a negative influence on income production, all of which create economic loss [5].

According to earlier research findings [6–9], variations in thermal assault conditions (TACs) and heat shock have an impact on cellular integrity, proliferation, and viability as well as RNA concentration, making animals more susceptible to opportunistic infections caused by weakened immune systems and a decline in productivity and reproduction [1]. Because RNA is a heat-labile nucleic acid, it is extremely unstable when exposed to harsh environmental conditions, especially heat or thermal assault and as such the degradation of RNA nucleotides and subsequent mutational damage to the structure of nucleic acids that affect nucleic acid synthesis and functions have also been linked to harsh environmental TACs [1]. Previous studies revealed that temperature variations had a significant impact on the production and proliferation of RNA nucleotides [1]. For instance, a moderate 37°C *in vitro* temperature mimics and resembles the BT of mammalian species and increases RNA synthesis and proliferation as well as cell survival [1, 8, 10]. In order for cattle to perform better in terms of production and reproduction abilities, it is necessary to mitigate the effects of harsh environmental conditions [3].

In order to obtain biological information about how cellular systems react to heat shock following exposure to TACs, Onasanya and his team [1] performed *in vitro* TSS of PBMCs on PBMCs of Indian Zebu-Jersey crossbreds maintained under stressful thermal conditions. In this chapter, the methods and techniques employed by the authors will be adequately discussed.

## 2. Blood sample collection and experimental animals

Seventy (70) Indian Zebu-Jersey crossbred animals were between the age of four and six years. Blood samples (10 mL per animal) were taken aseptically in EDTA bottle (**Figure 1**). After blood collection, the samples were transported in cooled iced-packed box and PBMCs were isolated within two hours of collection [1]. The reason for this is that, immediately the blood is collected the cells will continue to survive on the blood glucose, once the blood glucose is exhausted the cells will begin to die and they won't be able to respond to *In vitro* TSS.

## 3. Procedures for isolation of peripheral blood mononuclear cells

Ten (10) mL of animal blood samples were homogenized and properly mixed. Then, homogenized blood was added in an equal V/V ratio to 10 mL of previously prepared phosphate-buffered saline (PBS) (HiMedia Laboratories, Mumbai, India). A gentle homogenization and thorough mixing with pipetting up and down followed. A new 50 mL conical tube containing 3 mL of Histopaque®-1077 (Sigma-Aldrich Co. LLC, Darmstadt, Germany) was then carefully filled with the blood-PBS mixture, and the tube was centrifuged at 400 × g for 20 min in a REMI R-4C laboratory centrifuge (Goregaon East, Mumbai - 400 062, India) [1].

Using Histopaque®-1077 (Sigma-Aldrich Co. LLC), fresh whole blood was fractionated, and four separate layers were visible: the top layer was yellowish plasma, the bottom layer was milky PBMCs, and the top layer was histopaque. The top layer

**Figure 1.**
*Showing India Zebu–Jersey crossbred cattle.*

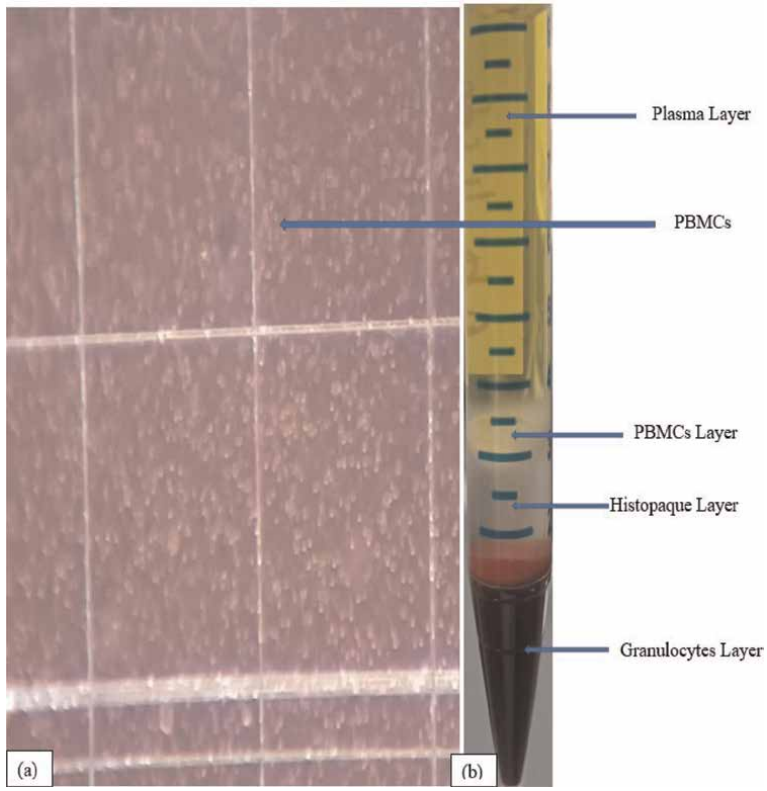


**Figure 2.**
*(a) showing the hemocytometer's ability to detect peripheral blood mononuclear cells under a microscope; (b) shows the fractional separation of a blood sample, which reveals peripheral blood mononuclear cells as well as other fractional layers.*

contained erythrocytes and granulocytes (**Figure 2b**). **Figure 2a** show the detection of the PBMCs under microscope.

After centrifugation, PBMCs were collected with the least amount of plasma (and Histopaque®-1077) and transferred into a clean15 mL conical tube. The PBMC suspension was then extensively homogenized by pipetting up and down after adding 10 mL of PBS (PBS was used to wash the PBMCs), followed by 10 min of centrifugation at 100 × g. After that, the supernatant was discarded in order to recover the PBMC pellet. The conical tube was then flicked until the PBMC pellets were fully resuspended in the remaining PBS solution. 10 mL of PBS solution was added to the pellet and thoroughly mixed by up-and-down pipetting. After centrifuging the mixture at 100 × g for 10 min, the supernatant was discarded.

The method (washing with PBS, mixing, and centrifuging at 100 × g for 10 min was performed three times) to recover the PBMCs pellet. The PBMCs were then resuspended in 1 mL of a mixture consisting of 100 mL of fetal bovine serum (FBS) and 900 mL of basic medium (RPMI-1640): 900 mL (HiMedia, Laboratories), and gently mixed by up and down pipetting in the FBS-RPMI mixture. Trypan blue dye exclusion method was used to count and confirm the viability of the isolated PBMCs, and TSS was immediately performed.

## 4. Procedures for generating various thermal assault conditions

From the previously published study, 70 animals were placed into seven groups with 10 individuals each. In total, 70 Indian Zebu-Jersey crossbred cattle breed's blood samples yielded 70 aliquots of PBMCs, both stressed and unstressed cells. With the exception of the 0°C TAC, the PBMCs were exposed to each of the four TACs for 3 h and 6 h (0, 37, 40, and 45°C) (**Figure 2**). Before the TSS procedure, the number of viable cells were estimated to be between $7.04 \times 10^6$-$2.56 \times 10^7$ cells/mL. About 1 $\times 10^6$ PBMCs/mL were present in each aliquot of 500 μL [1].

All PBMC aliquots were first stabilised for 30 min. at 37°C in a 5% $CO_2$ incubator with nutritive medium (RPMI 1640; Cole-Parmer Binder C170UL-120V-R CO2 Incubator, Mumbai, India). After 30 min of initial stabilisation of both stressed and unstressed samples in nutrient media at 37°C in 5% $CO_2$ incubator, the control sample labelled unstressed was immediately harvested.

## 5. Procedures for thermal stress stimulation of PBMCs

Isolated PBMCs were divided into two groups, one of which underwent TS and the other of which was not. Initially, aliquots of (500 μL) PBMCs were cultured in a nutrient medium (RPMI 1640) at 37°C for 30 min. in a 5% $CO_2$ incubator for stabilization. Different TACs and DTEs were used to conduct an in vitro TSS of PBMCs. The PBMC aliquots (500 L) were labelled and put through four different TACs in a circulating REMI RSB-12 water bath, as illustrated in **Figure 3**, No TS: Control, 37°C: Normal temperature, 40°C: Moderate heat, and 45°C: Extreme heat) included of four treatment groups, whereas two DTEs were also included (3 and 6 h).

After TSS was completed, the stressed PBMCs were given time to recover at 37°C for 30 min in an incubator with 5% $CO_2$ before being trypsinized and harvested. Unstressed control samples (500 μL) on the other hand, were stabilized for 30 min before being harvested. They were also not subjected to TAC or DET (0°C or 0 h). Both stressed and unstressed PBMCs will be used for total RNA isolation for heat shock protein gene expression analysis, including *HSP* 70 and 90 genes or other downstream analyses.

## 6. Evaluation of the PBMC count and viability

Using the Trypan blue dye exclusion method, PBMC number and viability were calculated after PBMC isolation. The trypan exclusion dye method involved the staining of the PBMCs with trypan blue dye such that the viable PBMCs were not stained but dead cells were stained and excluded when observed under a microscope on a haemocytometer (Microyn Improved Neubauer Haemocytometer, Hunt Valley,

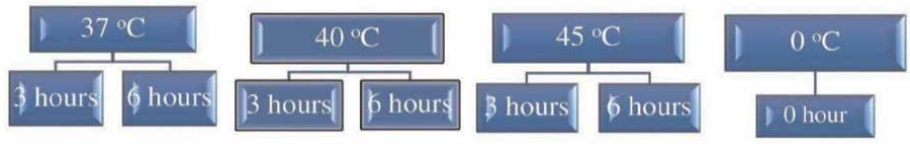


**Figure 3.**
*Illustration of an experimental design with varied thermal assault conditions and durations thermal exposure for stressed and unstressed peripheral blood mononuclear cells of Indian Zebu-Jersey crossbred cattle [1].*

Maryland, USA) (CELESTRON Labs CB2000C Compound Microscope, Celestron, LLC., California, USA) (**Figure 2a**). Before TSS, it was estimated that there were 7.04 $\times 10^6$ to 2.56 $\times 10^7$ viable cells per millilitre. Total RNA was extracted from the isolated PBMCs. Using a Thermo-Scientific-Nano Drop 2000 spectrophotometer, total RNA was quantitated (Shimadzu co-operation, Kyoto, Japan).

## 7. Estimation of viable peripheral blood mononuclear cells and quantitation of viable peripheral blood mononuclear cells

As earlier published by Onasanya and his co-workers [1], the PBMCs were estimated, and viability was checked using various parameters shown in Eqs. (1)–(4).

$$\%\text{Viability of PBMCs} = \text{Total viable PBMCs/Total number of PBMCs(Viable + Dead)} \times 100 \quad (1)$$

$$\text{Average number of viable PBMCs per square} = \text{Total number of viable PBMCs in 4 Squares}/4 \quad (2)$$

$$\text{Dilution factor} = \text{Total volume}\left(\text{Volume of PBMCs} + \text{Volume of trypan blue dye/Volume of PBMCs}\right) \quad (3)$$

$$\text{Concentration of Viable PBMCs/Square} = \text{Average number of viable PBMCs} \times \text{Dilution factor} \times 10^4 \quad (4)$$

For this study, two dilution factors were used.

In their previously published data (**Table 1**) on the TSS of PBMCs, Onasanya and his coworkers [1] found that heat shock/thermal assault at 45°C for 6 h-DTE had the greatest impact on PBMCs of Indian Zebu-Jersey crossbreds than at any other thermal conditions examined.

## 8. Processing and preservation of Isolated PBMCs for the extraction of total RNAs and mRNA expression analyses

PBMCs to meant for total RNA isolation, especially for gene expression analyses, should be aliquoted into convenient volumes, such as 250 μL of equal PBMCs per

| TAC/DTE | Concentration of PBMCs (Cells/mL) | Concentration of total RNA (ng/μL) |
|---|---|---|
| 37°C/3 h | $1.64 \times 10^6 \pm 0.12^{b}$ | $9.89 \times 10^3 \pm 0.15^{a}$ |
| 37°C/6 h | $9.19 \times 10^5 \pm 0.11^{c}$ | $6.91 \times 0^3 \pm 0.13^{c}$ |
| 40°C/3 h | $7.80 \times 10^5 \pm 0.22^{d}$ | $1.88 \times 10^3 \pm 0.22^{d}$ |
| 40°C/6 h | $4.00 \times 10^5 \pm 0.33^{f}$ | $7.23 \times 10^2 \pm 0.44^{e}$ |
| 45°C/3 h | $6.04 \times 10^5 \pm 0.45^{e}$ | $7.57 \times 10^2 \pm 0.32^{e}$ |
| 45°C/6 h | $3.59 \times 10^5 \pm 0.34^{g}$ | $6.99 \times 10^2 \pm 0.51^{f}$ |
| 0°C/0 h | $2.56 \times 10^7 \pm 0.22^{a}$ | $8.95 \times 10^3 \pm 0.15^{b}$ |

*[a–g]Means within the same column having different superscripts are significantly different [***]P < 0.001; TACs: Thermal Assault Conditions; DTEs: Durations of Thermal Exposures.*

**Table 1.**
*Mean values for PBMCs and RNAs after TSS at different TACs and DTEs in Indian-Jersey Crossbreds [1].*

treatment group. The purpose of this is meant to eliminate error of orthogonality that could arise from variation in the numbers of PBMCs among treatment groups, so that variation in RNA concentration will not be due to differences in PBMC numbers among the treatment groups. Thereafter, the PBMC was centrifuged at 8000 rpm for 5 min, gently pipette out the supernatant without disturbing the pellet, add 250 μL of RNAlater® and store at 4°C for 24 h to stabilize the RNA and internal environment of the cells. After the cellular environment of the PBMC has stabilised for 24 hours, centrifuge the PBMCs at 10,000 rpm for 10 min to remove the RNAlater® gently without disturbing the PBMCs pellet and store the recovered PBMCs at −80°C for downstream analyses.

## 9. Computation of equal number for PBMCs across the treatment groups

How to guarantee that each treatment group has equal number of PBMCs is shown in **Table 2**. For instance, the maximum PBMC count in the four treatment groups is $3.08 \times 10^7$. Note that, the treatment group with the highest PBMC count will be used as the benchmark for the computation to guarantee that PBMC counts are equal across treatment groups. Eqs. (5–7) demonstrate the various equations to make the estimations.

$$\text{Numbers of PBMCs in 250 μL} = \frac{\text{PBMCs in 250 μL}}{250} \tag{5}$$

$$\text{Numbers PBMCs per } 3.08 \times 10^7 = \frac{3.08 \times 10^7}{\text{PBMCs/μL}} \tag{6}$$

Volume (μL) to remove from PBMCs of each treatment to generate equal number PBMCs across the treatment groups

$$= 250 - \text{PBMCs per } 3.08 \times 10^7 \tag{7}$$

## 10. Procedures for isolation of total RNA from PBMCs pellet

1. Spin the PBMC pellet at 10 000 rpm for 10 to recover the PBMCs pellet (for freshly isolated PBMCs)

2. For the already processed PBMCs pellet, add the PBMCs pellet + 1 mL of Trizol in 1.5 μL eppendorf tube, mix gently by up and down pipetting for 5 times,

| TAC (°C/Min) | PBMCs/250 μL | PBMCs/μL | PBMCs per $3.08 \times 10^7$ | Vol of PBMCs to remove |
|---|---|---|---|---|
| 37/15 | $3.68 \times 10^7$ | 147, 200 | 209 | 250–209 = 41 |
| 45/15 | $3.25 \times 10^7$ | 130, 000 | 237 | 250–237 = 13 |
| 37/30 | $3.55 \times 10^7$ | 142, 000 | 217 | 250–217 = 33 |
| 45/30 | $3.08 \times 10^7$ | 123, 200 | 250 | 250–250 = 0 |

**Table 2.**
*Computation of equal PBMC count across the treatment groups.*

subsequently cover the tube and mix thoroughly for 5 times in the right – left direction.

3. Incubate at room temperature (15–25 °C) for 10 min
4. Add 200 µL of chloroform into the above mixture, mix gently for 5 times in the right– left direction for 5 times
5. Centrifuge the above at 10 000 rpm for 20 min at 4°C
6. After spinning, transfer the supernatant into a new 1.5 µL eppendorf tube and add ethanol (100%) in equal V/V ratio. The supernatant is the clear transparent/ upper layer while the Trizol is the bottom layer found beneath the supernatant layer
7. Mix the above mixture thoroughly for 5 times in the right – left direction and fetch 500 µL of supernatant-ethanol mixture
8. Transfer the above mixture into spin column placed in the collection tube
9. Incubate at room temperature (15–25°C) for 5 min
10. Centrifuge the above at 10 000 rpm for 2 min at 4°C
11. Discard the flow-through and re-use the collection tube
12. Then, transfer the spin column back into the same collection tube
13. Add the remaining supernatant-ethanol mixture obtained in (7) above into the spin column and collection tube at (12) above
14. Incubate at room temperature (15–25°C) for 5 min
15. Centrifuge the above at 10 000 rpm for 2 min at 4°C
16. Discard the flow-through and re-use the collection tube
17. Place the spin column back into the same collection tube
18. Add 700 µL RWI wash buffer into the above spin column and collection tube
19. Centrifuge the above at 10 000 rpm for 2 min at 4°C
20. Discard the flow-through and re-use the collection tube
21. Place the spin column back into the same collection tube
22. Add 500 µL RPE wash buffer into the above spin column and collection tube
23. Centrifuge the above at 10 000 rpm for 2 min at 4°C

24. Discard the flow-through and re-use the collection tube

25. Place the spin column back into the same collection tube

26. Centrifuge the empty column obtained above at step 25 and centrifuge at 10 000 rpm for 5 min at 4°C

27. Discard the flow-through and the collection tube

28. Transfer the empty spin column into a new 1.5 μL eppendorf tube

29. Add 15 μL nuclease free water (65°C sterilized in dry bath or water bath for 10 min) into the empty spin column

30. Centrifuge at 10 000 rpm for 5 min at 4°C

31. Repeat step 29 by adding 10 μL sterilized nuclease free water into the empty spin column placed same 1.5 μL eppendorf tube obtained at step 28

32. Incubate the above at room temperature (15–25°C) for 5 min

33. Centrifuge at 10 000 rpm for 5 min at 4°C

34. Discard the collection tube and keep the eppendorf tube

35. Total RNA is isolated into 1.5 μL eppendorf tube

36. Quantitate the concentration of RNA and estimate the purity (1.7–2.0 optical density is consider good for gene expression heat shock protein genes)

37. Store at −80°C for gene express analyses of heat shock protein genes

## 11. How to preform TapeStation quantity control check for total RNA integrity for preparation of mRNA library and mRNA expression

A 4150 TapeStation System (Catalog: G2992AA, Agilent) that is intended for analysing Eukaryote and Prokaryote RNA can be used to perform the RNA quality assessment [10]. Total RNA molecules with lengths between 50 and 6000 nt are used to compute the RNA integrity (RINe) values, which are used to assess the quality of the total RNA. 3μL of RNA ScreenTape were combined with 1 μL of total RNA sample. Sample buffer was heated at 72°C for 3 min to denature it, after which the sample was immediately put on ice for 2 min before being loaded onto the Agilent 4150 TapeStation equipment. The software assigns total RNA integrity number (RINe) that indicates the integrity of the total RNA [10]. RINe values were graded from 1 to 10, with values between 1 and 5 indicating fully degraded total RNA, 5–7 indicating moderately degraded total RNA, and values above 8 indicating high-quality total RNA. Total RNA whose RINe number falls within the range of 6 and above are of good quality hence they are recommended for preparation mRNA library and gene expression.

## 12. Procedures for total RNA quantitation

RNA concentration was determined on Qubit® 3.0 Fluorometer using the Qubit™ RNA BR Assay Kit (Catalog: Q10211, ThermoFisher Scientific), which contains RNA reagents consisting of buffers, dye that binds specifically to RNA with linear fluorescence detection in the range of 20 ng/ul to 1000 ng/ul and two RNA standards [11]. The dye and the buffer were diluted at 1:200 ratio and 1 μl of the RNA sample was mixed with the dye mix and incubated at RT for 2 min and the readings were taken in the Qubit.3 Fluorometer. Prior to the sample's measurement, the instrument was calibrated using the two standards provided in the kit [11] (**Table 3**).

## 13. Conclusion

The cellular systems of livestock animals are exposed to heat shock under prolonged and extreme TACs such high tropical temperatures, which prevents proper cell performance and may even cause cell death or prompt apoptosis. Consequently, severe TAC-DTE combinations have an adverse effect on cell count and survival by causing prompt apoptosis. Therefore, PBMCs can be employed as a cellular model or biological indicator to learn more about how animals' response to thermal assault conditions both *in vitro* and/or *in vivo*. In order to better understand how livestock animals, react to *in vitro,* it will be established in the future whether there is a relationship between the decreased PBMC count following *in vitro* TSS and the expression of the heat shock protein genes. This will enable better understanding of the thermotolerance ability of bovine species and other livestock animals under real-life scenarios/conditions for improved adaptability, survivability, and production performance, the biological data obtained from such study will be used to understand the *in vivo* response of livestock animals to different environmental TACs.

Finally, researches, academics, and livestock farmers will all profit greatly from the *in vitro* thermal stimulation and associated methodologies /procedures as presented in this chapter regarding the function PBMCs can play as a biological indicator in the monitoring and control of heat stress challenges in farm animals.

| Sample details | | TapeStation QC | | QUBIT quantification | | Test results | |
|---|---|---|---|---|---|---|---|
| S/N | Sample ID | RIN | 28S/18S Ratio | Conc. (ng/ul) | Volume (ul) | Total RNA mass (ng) | QC status |
| 1 | S1 | 7 | 1.3 | 9.74 | 40 | 389.6 | Pass |
| 2 | S2 | 6.3 | 1.2 | 13.8 | 40 | 552 | Border-Line |
| 3 | S3 | 7.5 | 1.9 | 10.7 | 40 | 428 | Pass |
| 4 | S4 | 7.9 | 1.6 | 15.8 | 40 | 632 | Pass |
| 5 | S5 | 7.2 | 1.1 | 17.3 | 40 | 692 | Pass |
| 6 | S6 | 7.5 | 0.7 | 6.08 | 40 | 243.2 | Pass |

**Table 3.**
*TapeStation quality control check for total RNA quantitation and total RNA integrity values.*

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## Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

# Factors Characterizing Puberty in Ram Lambs of Four Breeds Raised under High Altitude Conditions

*Harvey Lozano, Jimmy Vargas, Liliana Chacón and Nathalie Kirschvink*

**Abstract**

The aim of this study was to compare ram lambs of four Colombian wool breeds raised under high altitude conditions to describe evolution of semen characteristics, body development, and libido and plasma testosterone. Corriedale, Hampshire, Romney Marsh, and Creole rams were enrolled since the age of 4 months for libido and testosterone (maximum, mean and amplitude) assessment, whereas semen collection was performed between 6 and 11 months of age by use of electro-ejaculation. Beside analysis of variables in function of breed and over time, a semen maturity score, considering semen volume, mass motility, individual progressive motility, concentration and % of living spermatozoa was established in function of adult rams' reference data. Colombian Creole displayed significantly higher results regarding all variables and showed the most important body development at each time point of the study.

**Keywords:** male lamb, non-seasonal, puberty, maturity, semen, libido, testosterone, breed

## 1. Introduction

Puberty corresponds to the transition into adulthood and can be defined in rams as the time when fertile spermatozoa are present in the ejaculate [1]. For some authors it is defined as the time when rams show 'interest' in females in estrus by successive mounting with ejaculation [2]. It is also described as the stage of sexual maturation when a ram can display complete sexual behaviour, to produce and to release gametes [3]. Another definition of puberty in ram lambs is based on a sustained rise in plasma testosterone concentrations over three consecutive blood samplings performed in one week and confirmed by the presence of spermatozoa in the ejaculate with at least 30% mass motility [1, 4]. In general, sexual development of ram lambs appears to be more closely associated with body growth than with chronological age. Body weight can be a good criterion for the achievement of puberty than chronological age alone [2]. Rate of testis growth is reported to be more rapid in lambs of highly prolific breeds such as the Finnish Landrace than in less prolific breed [5]. Louda et al, after observing testis growth and small but consistent differences in the development of sexual activity and

of sperm production, suggested that young rams of prolific breeds (Romanov and Finnish Landrace) may differ in their future reproductive performance. The early pubertal development associated with increased body weight is desirable in terms of improved reproductive performance [5, 6]. Broad information about the onset of puberty is of considerable importance for successful reproductive management [7]. On one hand, it is important to avoid inbreeding by separating prepubertal rams from ewes, especially in counties with extensive breeding practices and poor separation between animal groups of different ages or different reproduction status. On the other hand, insufficient sexual maturity of young rams may lead to reduced flock fertility. A few information is available in terms of testosterone levels, semen quality, libido and sexual performance at high altitude conditions in a non-seasonal country [8].

Seasonality is a main effect in the onset of rams' puberty. Animals live under the influence of seasonal fluctuations of environmental conditions with variable amplitudes frequently more marked in the higher latitudes and altitudes [9, 10]. Photoperiod is the key environmental signal timing in the reproductive cycle because the effect of season and/or day length has been studied as a main factor in onset of puberty in different breeds of young rams. Rams' sensitivity to photoperiod is different from ewes. Reproductive season can be influenced by birth date and puberty time. During spring and summer, a persistent hyper prolactinaemia was associated with low circulating FSH concentrations in Texel rams [11]. Young rams may reach puberty by 4 months of age during the first fall season, while the onset of puberty may be delayed until 9–12 months for rams born late in the lambing season and if additionally, the situation is accompanied of poor feeding and poor climatic conditions [12, 13]. In Karakul rams in southern Iran is observed how lambs born at the beginning of spring is sexually more precocious than lambs born later [14].

It is well known that interactions between body and testis growth, sperm production and testosterone secretion is complex since early pre-pubertal ages and, in many aspects, such events can be influenced by both the genetic background of animals and the environment where they are raised [2]. Plasma FSH concentrations were lower in Texel rams than in Suffolk and Ile-de-France rams during both pre-pubertal and pubertal periods [11]. Emsen [5] showed that crossing Awassi sheep with a native breed Redkaraman with relatively higher growth rate can considerably improve the early pubertal development of rams. Puberty of Awassi ram lambs in improved flocks, started around seven months of age and at on average weight of 34.6 kg [15]. In general, sexual development of ram lamb appears to be more closely associated with body growth than with chronological age and the live weight of the ram lambs at puberty is probably related to the genotype [2]. In that research ram lambs of breeds Friesland and Chios attained puberty when they reached approximately 50% of their adult body weight. By comparing different breeds and hormone levels during pre and pubertal time it was shown that Canadian ram lambs had significantly lower FSH levels than other breeds like Finnish Landrace. However, as adults the same group of Canadian rams had larger testicles and better semen quality [12]. In pubertal development, scrotal circumference was highly and positively correlated with live weight, but negatively correlated with inhibin and FSH concentrations in rams of Suffolk and DLS (Dorset × Leicester × Suffolk) [13]. Rate of testis growth was more rapid in lambs of Finnish Landrace with high prolificacy than a less prolific breed such as Merino [16].

Onset of puberty time in ram lambs is considerably influenced by nutrition. Prepubertal nutritional restrictions delay testicular growth and the rate of sexual development in ram lambs is highly dependent on energy intake and live weight gain [17].

Nutritional restrictions can influence the activity of the hypothalamus-pituitary axis and thus reduce gonadotropin levels in ram lambs. It can even be influenced during prenatal period based on mother's nutrition during pregnancy reducing lamb's pituitary capacity to liberate gonadotropins like LH [17]. The scrotal circumference of rams under an intensive feeding management increased by 4.4%, while decreased for rams under extensive conditions by 2% due to poor nutritional conditions [18].

The social environment in which male lambs are reared appears to influence some aspects of reproductive behavior: the exposure to cyclical ewes during the first 6 months of life is fundamental to induce an increase in testosterone concentrations and in testicular size and also the social rank of male lambs during pre-pubertal development affected reproductive performance of adult rams [19].

Sexual performance of rams having previously direct contact with females at 7–9 months of age was enhanced in comparison with rams without that previous experience [20]. In a study developed to evaluate the possible influence of litter size on the onset of puberty and hormone levels it could be realized how lambs born as singletons had lower testosterone levels at 8 months of age than those born as twin or triplets [12]. Ungerfeld and González-Pensado [21] reported a study in which intensive male-male sexual behavior is described in pubertal lambs with approximately the same age (less than 6 months) and it was shown how more dominant lambs displayed more intensive sexual behavior toward subordinate males and cyclical ewes, whereas more subordinate males received more mounts from other males and were less active to mount. Ram lambs of the breed Polish Milk Sheep, which is more prolific and attains puberty earlier, were more active in sexual play than ram lambs of the Polish White-headed Mutton [22].

## 2. Materials and methods

### 2.1 Animals and location

The study was conducted between July 2012 and April 2013 at the experimental farm of the National University of Colombia at 2650 m of altitude at 4°42′ latitude north and 74°12′ longitude west, near Bogotá (Colombia).

Twenty-four ram lambs, aged 3–4 months at the beginning of the trial and from four different breeds (6 per breed) were enrolled in the study. Animals belonged to the native Colombian Creole and three foreign adapted breeds that are frequently used by Colombian breeders: Hampshire, Corriedale and Romney Marsh. The animals were born in the center and were selected of base of a selection index. They were grazing all the time of the experiment and each animal received daily 200 g of a pelleted concentrate mixture and 300 g of trefoil hay. Mineral salts and water were provided ad libitum.

The protocol was approved by the Bioethics committee of the Faculty of Veterinary Medicine and Animal Science of the National University of Colombia (Acta: CD-071-2014).

### 2.2 Assessment of semen characteristics in ram lambs

Once per month, ram lambs were collected by electro ejaculation, penis was exposed from the prepuce cavity and the urethral process was gently introduced into a conical tube previously to start ejaculation process. Semen was placed in a water bath

at 37°C and subjected to the following tests: (1) Volume was measured in a conical glass tube graduated with 0.1 mL optically visible intervals; (2) motility (Mass: semen was assessed for semen wave motion graded on a subjective scale ranging from 1 to 5, where 1 was scored when there was no mass movement and 5 represented vigorous waves of sperm motion and Individual progressive motility %).

Semen concentration was determined using a standard spectrophotometer (540 nm). (4) The proportion of live and dead spermatozoa was determined using the nigrosine-eosin staining technique by counting at least 200 spermatozoa under oil immersion objective (1000×) random fields. The proportion of morphologically abnormal spermatozoa was also determined by examining 200 spermatozoa in an eosin-nigrosine smear under the same magnification. Abnormal spermatozoa were then classified into proportion of spermatozoa with head abnormalities, midpiece abnormalities, tail abnormalities, proximal droplet, distal droplet, detached heads or tailless spermatozoa [23]. All examinations were performed by the same operator.

### 2.3 Plasma testosterone concentration

Blood samples were collected every month during 5 hours at 30 minutes intervals using heparinized venoject® tubes and centrifuged (1500 × g for 15 min). The plasma was recovered and stored at −70°C. The concentration of testosterone in the plasma samples was measured in duplicate by an adapted enzyme immunoassay using a diagnostic commercial kit (DS-EIASteroid®-Testosterone RH-353). A calibration curve was used in order assess concentrations ranging from 1.25 to 40 nmol/l of testosterone in plasma. Known samples of a mature adult ram and of an ovariectomized ewe were used as positive and negative control respectively on each plate. Lower detection limit was 0.2 nmol/l and inter-and intra-assay were 6.7 and 5.5%, respectively. In each ram, testosterone curves were plotted over each 5 h period to determine the maximum value. The difference between recorded maximum and minimum concentration allowed to calculate testosterone amplitude.

### 2.4 Body measurements

Body weight was assessed once monthly using an electronic balance. Scrotal circumferences using a scrotal tape was evaluated also monthly.

## 3. Statistical analysis

Semen characteristics, testosterone concentration (peaks, amplitude, mean) were analyzed using a generalized linear model (GLM) for repeated measures analysis. The model contained effects due to breed and period. Mean differences in body weight (kg), scrotal circumference (cm) and scrotal circumference/body weight was compared by Duncan's method. The level of significance was set at $P < 0.05$ for all tests. Data was analyzed using SAS System (SAS version 9.12, SAS Institute Inc.). A semen maturity score, aiming at comparing the global semen quality of young rams in function of semen quality of adult rams of the same breed, was established as follows: Semen characteristics of five adult rams of each breed collected over a period of one year was averaged and considered as reference value. These breed-specific reference values were established for semen volume, concentration, mass motility (MM), individual progressive motility (IPM) and percentage of living spermatozoa. At each

month of collection, semen variables of the ram lambs were expressed as % in function of the adult rams' reference value. This percentage was classified in function of three semen maturity levels: when 50, 75 or 100% of the adults' value were reached, a note of 1 was accorded to this semen variable. If the % was lower than 50, 75 or 100%, a note of 0 was attributed. At each month of sampling, the maturity score establishing the sum of the notes attributed for semen volume, concentration, MM, IPM and % of living spermatozoa for achievement for 50, 75 or 100% of adult rams' semen quality was calculated.

## 4. Results

**Table 1** shows semen characteristics and body measurements in function of breed and indicates significant time-, breed- or interaction effects. No breed effect was found for semen volume, whereas Corriedale rams showed lowest values for concentration, MM, IPM ad % of normal spermatozoa ($p < 0.05$). **Figure 1** displays detailed evolution of these variables (volume, MM, IPM, concentration and % of normal spermatozoa) over time and in function of breed. All variables achieved a stable level when the rams were 10 and 11 months old. Creole rams showed consistently higher values for all variables except semen volume, whereas Corriedale rams generally showed the lowest values.

**Table 2** shows the evolution of the semen maturity score per breed and over time. Although no significant differences between breeds were found, a clear trend for more rapid development were found in Creole and Romney Marsh ram lambs.

**Table 3** shows as the evolution of body development (expressed in function of adults' ram weight) over time. Creole and Romney Marsh display highest values at begin of the investigation whereas Creole rams maintained this rapid development until the age of 11 months.

**Figure 2a** and **b** display maximum testosterone concentration and testosterone amplitude in function of breed and over time. Creole ram lambs had significantly higher values. The timerelated increase was mostly observable in all breeds between 3 and 6 months of age. There were not correlations between testosterone levels and semen quality, sexual activity, or body measurements. In general, the first expressed signs of interest in the oestrous females occurred prior to the sustained rise in testosterone.

## 5. Discussion

This study aimed at describing semen characteristics, body development, and libido and plasma testosterone concentration in young rams of four economically important wool breeds in high altitude conditions in Colombia. Prior considering and discussing results in detail some drawbacks must be mentioned. Semen collection was performed by electro-ejaculation since the age of 6 months and as expected and described by others [1, 18], semen quality and testosterone levels recorded at this age suggested that puberty was already achieved by most of the rams. The relatively late start of semen collection was due to the fact that despite regular training of the rams, semen collection by use of an ewe in estrus was not successful in all rams, which meant that semen quality data were only available in a few rams. Therefore, the investigators decided to switch to semen collection by use of electro-ejaculation. Even

| | n | Creole | Romney M | Hampshire | Corriedale | Effect breed | Effect collect | Effect breed * collect |
|---|---|---|---|---|---|---|---|---|
| | | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | | | |
| | | 7 | 7 | 7 | 7 | | | |
| Semen character | | | | | | | | |
| Volume (ml) | 6 | 1.10 ± 0.3 | 1.10 ± 0.38 | 1.30 ± 0.4 | 1.20 ± 0.39 | NS | $P < 0.05$ | NS |
| Conc. (millions/ml) | 6 | 2431.4 ± 972.3[a] | 2512.4 ± 734.9[a] | 2494.7 ± 1021.6[a] | 2016.6 ± 1013.9[a] | $P < 0.05$ | $P < 0.05$ | NS |
| Mass motility (0–5) | 6 | 4.10 ± 0.6[a] | 3.90 ± 0.99[a] | 3.8 ± 0.85[a] | 3.20 ± 1.37[b] | $P < 0.05$ | $P < 0.05$ | NS |
| IPM (%) | 6 | 85.1 ± 7.4[a] | 80.9 ± 14.2[ab] | 79.4 ± 12.5[b] | 72.8 ± 16.54[c] | $P < 0.05$ | $P < 0.05$ | NS |
| N (%) | 6 | 85.4 ± 2.9[a] | 85.3 ± 3.12[a] | 85.1 ± 2.72[a] | 80.8 ± 6.24[b] | $P < 0.05$ | $P < 0.05$ | NS |
| Body measure | | | | | | | | |
| Scrotal circumference (cm) | 6 | 28.4 ± 2.2[a] | 32.3 ± 1.54[b] | 31.3 ± 2.13[c] | 29.1 ± 2.37[a] | $P < 0.05$ | $P < 0.05$ | NS |
| Body weight (Kg) | 6 | 34.9 ± 5.7[a] | 44.5 ± 5.58[b] | 39.7 ± 6.69[c] | 39.1 ± 6.83[c] | $P < 0.05$ | $P < 0.05$ | NS |
| SC/BW | 6 | 0.82 ± 0.09[a] | 0.74 ± 0.07[b] | 0.80 ± 0.11[a] | 0.76 ± 0.08[b] | $P < 0.05$ | $P < 0.05$ | NS |

*Values with the same letter are not significantly different, $p < 0.05$.*
*NS, not significantly different.*

**Table 1.**
*Mean (±SD) seminal characteristics and body measure evaluated in ram lambs in four breed (Creole, Romney, Corriedale and Hampshire).*

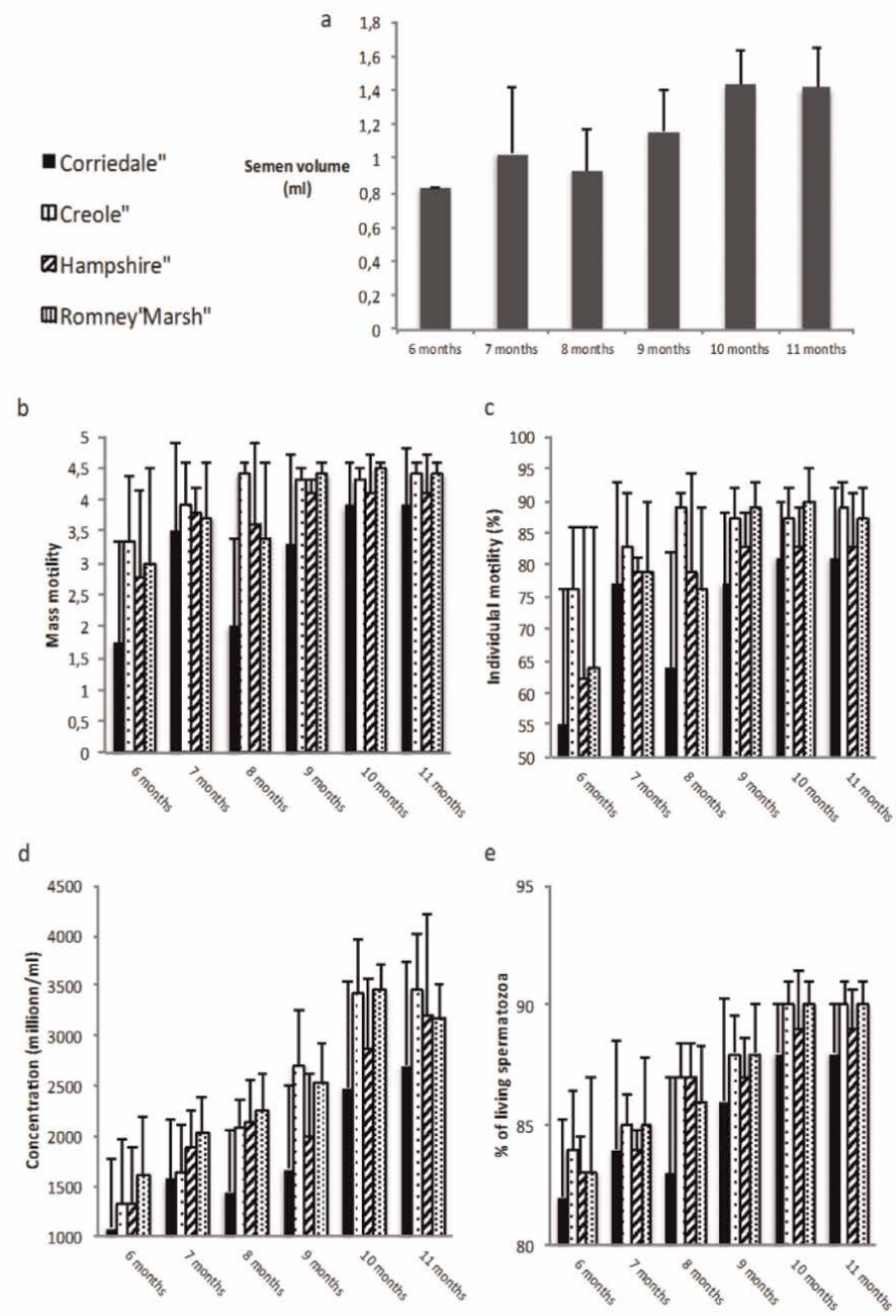


**Figure 1.**
*Evolution of semen quality in rams of four breeds (7 animals/breed) over time in rams aged between 6 and 11 months. A significant time effect was found for semen volume (a), whereas significant time and breed effects were found for mass motility (b), individual progressive motility (c), concentration (d) and % of living spermatozoa (e). Data are shown as mean and standard deviation.*

| Age | Number of rams with a maturity score > 50% | | | | Number of rams with a maturity score > 75% | | | | Number of rams with a maturity score > 100% | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Months** | **CO (n = 7)** | **CR (n = 7)** | **HA (n = 7)** | **RM (n = 7)** | **CO (n = 7)** | **CR (n = 7)** | **HA (n = 7)** | **RM (n = 7)** | **CO (n = 7)** | **CR (n = 7)** | **HA (n = 7)** | **RM (n = 7)** |
| 6 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 2 | 2 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 3 | 4 | 3 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 4 | 7 | 5 | 7 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 4 | 7 | 6 | 7 | 2 | 5 | 4 | 7 | 0 | 2 | 0 | 0 |
| 11 | 5 | 7 | 6 | 7 | 4 | 6 | 6 | 7 | 0 | 2 | 0 | 0 |
| Total (n) and % of observations fulfilling maturity | 19 | 28 | 23 | 32 | 6 | 15 | 10 | 14 | 0 | 4 | 0 | 0 |
| | 45% | 67% | 55% | 76% | 14% | 36% | 24% | 33% | 0% | 10% | 0% | 0% |

*Maturity scores consider semen quality corresponding in terms of volume, concentration, mass motility, individual progressive motility and % of normal spermatozoa when 50, 75 or 100% of adults' semen quality is achieved. Data per line are shown as number of rams whose semen corresponds to the fixed maturity score.*
*CO, Corriedale; CR, Creole; HA, Hampshire; RM, Romney Marsh.*

**Table 2.**
*Semen maturity scores of Corriedale, Creole, Hampshire and Romney Marsh ram lambs tested between 6 and 11 months of age.*

| Age expressed in months | CO (n = 7) | CR (n = 7) | HP (n = 7) | RM (n = 7) | Breed effect |
|---|---|---|---|---|---|
| 6 | 47.5 ± 8.4 | 56.5 ± 5.0 | 46.8 ± 4.3 | 51.0 ± 4.3 | $P < 0.05$ |
| 7 | 48.3 ± 7.9 | 58.2 ± 4.6 | 47.7 ± 3.7 | 51.6 ± 4.0 | $P < 0.05$ |
| 8 | 50.3 ± 7.5 | 67.7 ± 5.8 | 52.2 ± 5.8 | 53.6 ± 4.5 | $P < 0.05$ |
| 9 | 54.7 ± 7.3 | 75.1 ± 7.9 | 57.8 ± 7.5 | 57.2 ± 5.3 | $P < 0.05$ |
| 10 | 55.9 ± 8.1 | 79.4 ± 8.5 | 60.6 ± 11.8 | 62.0 ± 5.9 | $P < 0.05$ |
| 11 | 56.4 ± 11.8 | 82.1 ± 8.6 | 65.4 ± 9.7 | 64.6 ± 4.9 | $P < 0.05$ |

*Data are expressed as % in functions of adult rams' body weight of the same breed. Means and SD are shown for each breed.*
*CO, Corriedale; CR, Creole; HA, Hampshire; RM, Romney Marsh.*

**Table 3.**
*Evolution of body development in function of breed and over time.*

if the semen characteristics do not importantly change when natural versus electric ejaculation are compared this methodological difference must be kept in mind when ram lamb semen data are expressed in function of adults' semen data, leading to an underestimation of young rams' semen quality. Although the semen quality score allowed to consider simultaneously all important semen characteristics (volume, MM, IPM, concentration and % of living spermatozoa) and thereby allowed to assess the ability of young rams at the end of the puberty (rather than to consider each variable separately), it remains a matter of fact that onset of puberty occurred before or just around first semen collection and that a precise age of onset of puberty could not be established for each ram and/or each breed. Regarding the end of the study, it appears that albeit a stabilisation since at least two months of all parameters (except semen concentration), it is impossible to define time point sexual maturity. As evidenced by the semen quality score, only two Creole rams showed semen characteristics that perfectly corresponded to those of adults of the same breed. The study should have been prolonged to assess when semen maturity was reached. Nevertheless, the present study describes that Colombian Creole appears as the most precocious breed: indeed, semen variables, semen maturity score, behavioural aspects as well as plasma testosterone levels were significantly increased in comparison to the other breeds, especially to Corriedale rams whose performances were almost always lower than in the other breeds. At the same time, it became apparent that development was more important in Creole rams whose relative body weight equalled 55–56% at the age of 6 months (versus 47–51% in the other breeds) and 80–85% at the age of 11 months (versus 55–65% in the other breeds). Moreover, when considering semen quality data (**Figure 1**), it can be said that since the age of 8 months almost all rams, except some Corriedale rams whose semen concentration remained below 2000 million spermatozoa/ml, were able to ensure reproduction. Indeed, the only limiting factor in terms of reproductive capacity seemed to be concentration.

It is interesting to state that this local breed appears to be optimally adapted to high altitude Andes conditions, whereas the foreign breeds, although imported since more than 50 years, show significant differences. They mostly account for a larger body size, meaning that the minimal % of body development is achieved later point, but seem also to depend on other factors. Indeed, testosterone levels were significantly higher in Creole rams and might impact semen quality. Another aspect was evaluated,

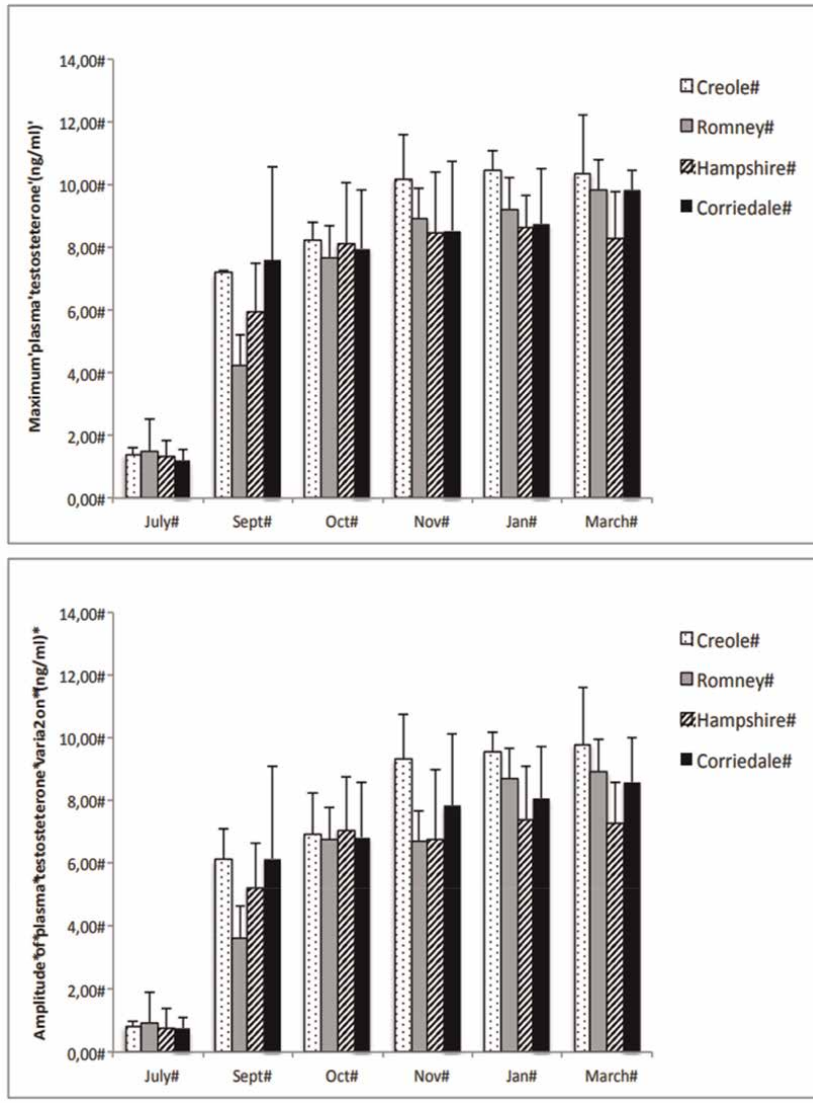


**Figure 2.**
*(a) Maximum plasma testosterone concentrations assessed between the age of 3 (July data) and 10 (March data) months in Creole, Romney Marsh, Hampshire, and Corriedale ram lambs. Data are shown as means with SD. A significant collection effect was recorded over time, as well a significant breed effect with Creole rams showing significantly higher testosterone levels, $p < 0.05$. (b) Plasma testosterone amplitude assessed between the age of 3 (July data) and 10 (March data) months in Creole, Romney Marsh, Hampshire, and Corriedale ram lambs. Data are shown as means with SD. A significant collection effect was recorded over time, as well a significant breed effect with Creole rams showing significantly higher testosterone levels, $p < 0.05$.*

but results are not presented here, it is about sexual behavior of the ram lambs since they were three until one year of age.

In Colombia some time after this research was developed, this group could perform a trial under low altitude conditions and ram lambs belonged to hair breeds instead of wool, finding some results in concordance to these results, especially about testosterone levels during ram lambs growing up. Into that hair groups experiment, time of evaluation was shorter than this research, but some parameters could be compared and the new information was useful for the good understanding of puberty

in ram lambs in a country as Colombia where there is different altitude in farms despite of being a non-seasonal country [24].

In conclusion, this investigation describes how semen quality, libido and plasma testosterone evolve over time an in four Colombian wool breeds and allows to point out the importance of body development to achieve satisfying reproductive abilities. It was shown that almost all variables were improved in Colombian Creoles, whereas Corriedale showed the lowest development.

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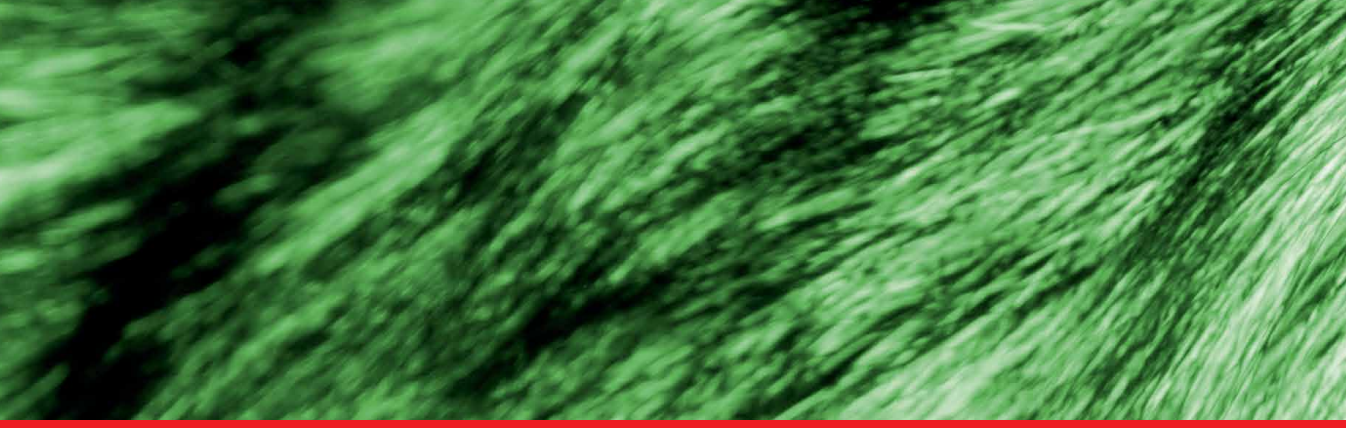

*Edited by Xiaojun Liu and Hong Li*

Healthy and sustainable animal husbandry is the goal of animal breeding. This book provides a detailed overview of applications of post-genome era techniques for livestock genetics and breeding. It also describes techniques for genetically selecting highly productive animals without producing large amounts of greenhouse gas emissions, community-based breeding programs for breeding rams and bucks, the response of bovine species to in vitro thermal stress stimulation, and the semen characteristics of wool-breed ram lamb-raised in conditions of high altitude. The book not only presents advanced animal genetic and breeding methods but also highlights useful, practical technology for animal breeding under specific climate, geographic, and environmental conditions.

*Rita Payan Carreira,*
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