UNIVERSIDAD AUTÓNOMA CHAPINGO
UNIDAD REGIONAL UNIVERSITARIA DE ZONAS ÁRIDAS

UNIVERSIDAD DE CÓRDOBA
INSTITUTO DE ESTUDIOS DE POSGRADO

GLUTAMATE SUPPLEMENTATION, ENDOCRINE PROFILE, AND OVARIAN ACTIVITY INTERACTION IN CROSSBREED GOATS DURING THE SEASONAL ANESTROUS

SUPLEMENTACIÓN DE GLUTAMATO, PERFIL ENDOCRINO E INTERACCIÓN DE LA ACTIVIDAD OVÁRICA EN CABRAS CRIOLLAS DURANTE EL ANESTRO ESTACIONAL

Thesis in joint supervision to obtain the double degree of
Doctor on science

DOCTOR EN CIENCIAS EN RECURSOS NATURALES EN RECURSOS NATURALES Y MEDIO AMBIENTE EN ZONAS ÁRIDAS (UACH-URUZA, MEXICO)

DOCTOR EN BIOCIENCIAS Y CIENCIAS AGROALIMENTARIAS (UCO-IdEP, SPAIN)

By:

LUIS ANTONIO LUNA GARCÍA

Under supervision of:

Ph.D. César A. Meza-Herrera
(UACH-URUZA, Mexico)

Dr. Carlos C. Pérez-Marin
(UCO-IdEP, Spain)

Bermejillo, Durango, Mexico, February 2025

GLUTAMATE SUPPLEMENTATION, ENDOCRINE PROFILE, AND OVARIAN ACTIVITY INTERACTION IN CROSSBREED GOATS DURING THE SEASONAL ANESTROUS

Thesis carried out by **LUIS ANTONIO LUNA GARCÍA** under the supervision of the indicated Advisory Committee, approved by the same and accepted as a partial requirement to obtain the degree of:

DOCTOR EN CIENCIAS EN RECURSOS NATURALES Y MEDIO AMBIENTE EN ZONAS ÁRIDAS

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Bermejillo, Durango, Mexico, February 2025.

UNIVERSIDAD DE CÓRDOBA

PROGRAMA DE DOCTORADO EN BIOCIENCIAS Y CIENCIAS AGROALIMENTARIAS

TESIS DOCTORAL

Suplementación de glutamato, perfil endocrino e interacción de la actividad ovárica en cabras criollas durante el anestro estacional

Presentado por **LUIS ANTONIO LUNA GARCÍA** en satisfacción de los requisitos necesarios para la obtención del grado de **DOCTOR EN BIOCIENCIAS Y CIENCIAS AGROALIMENTARIAS.**

Los Directores:

Ph. D. Carlos C. Pérez-Marín
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Cordoba, Spain, February 2025.

EXTERNAL REVIEWER

Educación
Secretaría de Educación Pública

TECNOLÓGICO NACIONAL DE MÉXICO

Instituto Tecnológico de Torreón
Subdirección Académica

Torreón Coahuila, Mexico, February 10, 2025

ASUNTO: ***Doctoral Thesis Report, presented by: LUIS ANTONIO LUNA GARCÍA***

DR. MAXIMINO HUERTA BRAVO
COORDINADOR GENERAL DE ESTUDIOS DE POSGRADO
UNIVERSIDAD AUTONOMA CHAPINGO

With my first comment, I would like to thank you for the distinction you have given me by inviting me to participate as an **EXTERNAL READER** of the Doctoral Thesis: **GLUTAMATE SUPPLEMENTATION, ENDOCRINE PROFILE AND INTERACTION OF OVARIAN ACTIVITY IN CREOLE GOATS DURING THE SEASONAL ANESTRUS.** After having read and analyzed the aforementioned thesis, I tell you that it is very complete research, with the inclusion of IX Chapters. The Thesis begins with a very adequate Introduction that focuses on the idea of the use of exogenous glutamate and its possible effect on the endocrine profile and the interaction of ovarian activity in anovulatory Criollo goats. The Research Topic is innovative, pertinent while shows a great feasibility of applying its results from a practical point of view, since the experimental design is aimed at generating strategies that counteract the effects of reproductive seasonality. As we know, this reproductive seasonality causes goat products to be concentrated at one time of the year, a situation that affects the economy of goat farmers. It is in this phase that a productive-reproductive-biological problem emerges as a challenge to face the impact that this research also has from a socio-economic perspective. The objectives and the working hypothesis are duly aligned with the title of the study, which clearly demonstrates that it adheres perfectly to the scientific method. Besides, the chapter that addresses the State of the Art was developed in a broad, sharp and critical format by the Doctoral Student, since it addresses the necessary areas to give scientific support to the question or scientific gap of this study, which denotes the scientific rigor that every Doctoral Thesis must have.

Chapter V contains TWO articles published in international journals, with a high impact factor. The first of them published in the journal ***BIOLOGY,*** with an impact factor of 3.1, and is entitled: **"Goats as valuable animal model to test, the targeted glutamate supplementation upon antral follicle number, ovulation rate and LH-pulsatility".** From this study it is concluded that glutamate supplementation in goats increased both total ovarian activity as well as LH pulses, which could lead us to replace the use of exogenous hormones. The second article was published also in the journal **BIOLOGY**, and is titled: **"Targeted glutamate supply boosts insulin concentration, ovarian activity, and ovulation rate in yearling goats during the anestrous seasonal".** From this article it is concluded that an increase in serum insulin concentration, an escalation in ovarian activity and an enhancement in the ovulation rate in goats were promoted during the seasonal anestrus season. In both articles, **Luis Antonio García-Luna** is the main author.

2025 Año de La Mujer Indígena

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If we consider that the journal in which the PhD student's articles were published have a high impact factor, the significant quality of the research carried out in this Doctoral Dissertation is undoubtedly supported. Moreover, these scientific studies achieved the recognition of the International Scientific Community, as these articles are submitted to a Peer Review. Therefore, this Dissertation fully complies with all the international requirements that a Doctoral Thesis must have.

Chapter VI is devoted to a General Conclusion, which is also aligned with the objective of this study, as indicated by the scientific method, considering the results obtained in both studies. Regarding the use of exogenous glutamate in goats in seasonal anestrus, it is concluded that an effective activation of the reproductive axis was promoted, resulting in a significant increase in the levels of the reproductive hormone LH, suggesting a reduction in the effect of negative feedback of estradiol on the hypothalamic-pituitary axis, increasing the ovulatory rate, all of the above escorted by a serum increase in the metabolic hormone insulin.

In Chapter VII, a graphic summary is shown, which in an innovative and illustrative format, involves the main activities across time, developed in both scientific studies.

In Chapter VIII, the bibliographic references that support the development of the Thesis are cited.

Finally, in chapter IX, an Appendix shows a series of three complementary investigations that used mice as an experimental animal model. These studies continue with the evaluation of the use of exogenous glutamate in a murine model, which will undoubtedly generate important information to try to understand in a more complete way, how glutamate affects the hypothalamic-pituitary-gonadal axis in mammals. In the same sense, this series of new experiments highlights the scientific commitment of the PhD student, Luis Antonio Luna-Garcia, in developing these additional works, which will undoubtedly complement the information previously generated regarding the use of exogenous glutamate in goat experimental models, and its effect on reproduction.

After having read and analyzed this doctoral thesis, I consider that it meets the national and international requirements, to be satisfactorily approved.

Therefore, my decision is:
DEFINITIVELY APPROVED

EDUCACIÓN SECRETARÍA DE EDUCACIÓN PÚBLICA | TECNOLÓGICO NACIONAL DE MÉXICO
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CONTENT

LIST OF ABBREVIATIONS

AANAT	Arylakylamine N-acetyltranferase
ARC	Arcuate Nucleus
cAMP	Cyclic Adenosine Monophosphate
CL	Corpus Luteum
CNS	Central Nervous System
DDC	Dopa Dercarboxylase
Dio2	Deiodinase 2
Dio3	Deiodinase 3
E2	Estradiol
eCG	equine-Chorionic Gonadotropin
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
Eya3	Eyes Absent Homolog 3
FSH	Follicle Stimulant Hormone
GH	Growth Hormone
GnRH	Gonadotropin Releasing Hormone
hCG	human-Chorionic Gonadotropin
HIOMT	Hydroxyindole-O-methyltransferase
HPG	Hypothalamus-Pituitary-Gonadal
HPT	Hypothalamus-Pituitary- Thyroid
KNDy	KisspeptinNeurokininB-Dynorphin
LH	Luteinizing Hormone
ME	Median Eminence
MSG	Monosodium Glutamate
PGF2α	Prostaglandin F2 α
PMR	Premmamilary Nucleus
PRL	Prolactin

PT	*Pars Tuberalis*
PVN	Paraventricular Nucleus
Rch	Retrochiasmatic Area
SCN	Suprachiasmatic Nucleus
SON	Supraoptic Nucleus
T3	Triiodothyronine
T4	Thyroxin
TSH	Thyroid-Stimulating Hormone
TSHR	Thyroid-Stimulating Hormone Receptor

DEDICATED TO

To my Son, ***Luis Antonio Luna Del Valle***
To my Wife, ***Maria Fernanda Del Valle Diaz***

Thank you for your continued support, your courage, and for inspiring me to improve every day. Your belief in me has made it all possible.

I Love You Deeply

ACKNOWLEDGEMENTS

I want to sincerely thank the **National Council for Humanities, Science, and Technology (CONAHCYT)** for granting me the scholarship that made it possible for me to pursue my postgraduate studies.

To the **Doctorate Program in Natural Resources and Environment in Arid Zones, the Regional University Unit of Arid Zones**, and the **Autonomous Chapingo University,** thank you for giving me the chance to join your program and grow academically.

To the **Doctorate Program in Biosciences and Agri-Food Sciences** and **the University of Córdoba,** thank you for welcoming me into your program and allowing me to continue learning and developing my skills.

To my thesis advisor, **Ph.D. César A. Meza-Herrera**, I'm deeply grateful for your support, encouragement, and guidance throughout this journey. Your advice and motivation made all the difference during this important chapter of my life.

To my co-advisor, **Dr. Carlos C. Pérez Marin**, thank you for always being there to guide me, share your knowledge, and support me during the entire process, especially during my time in Cordoba, Spain.

Sincere thanks to **Dr. Manuel Tena-Sempere** for welcoming me into his lab and giving me the opportunity to carry out my research stay. I also want to thank everyone in the **GC-10 Laboratory for Hormonal Regulation of Energy Balance, Puberty, and Reproduction**, **UCO**, especially **Dr. Rafael Pineda**, for their help, advice, and attention during my time there.

To the **Maimonides Institute for Biomedical Research of Córdoba (IMIBIC, Spain)**, thank you for providing everything I needed to complete my pre-doctoral internship.

I'm so grateful to **Dr. Rebeca Corona Garcia-Cabral** for being part of my advisory committee and for welcoming me into the **D-05 Neuroendocrinology and Functional Neuroanatomy**

Laboratory. Thank you for your guidance, friendship, and support during my research stay and for always being available to help.

I also want to thank **Dr. Teresa Morales Guzman** for allowing me to work in her lab and for her support during my stay. And to **Dr. Veronica Viñuela Berni** for your friendship, advice, and the fun moments we shared during our research work will always mean so much to me. Also, thanks to **MVZ Javier Rodriguez Jiménez** for you technical assistance, good humor, and the great moments during my stay.

To the **Institute of Neurobiology (INB), National Autonomous University of Mexico (UNAM)**, thank you for making my research stay possible. Special thanks to the **Vivarium staff**, and **to Dr. Maria Antonieta Carbajo** and **Dr. Alejandra Castilla** for their technical support and help during my research work.

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To **Dr. Evaristo Carrillo**, for his support and guidance along with my academic formation, and for accepting to be my External Reviewer.

To **Dr. Carlos Iglesias-Pastrana**, thank you for your friendship, support, and advice during my time in Córdoba.

To my classmates in the Graduate Program in Natural Resources and the Environment in Arid Zones, especially **M.C. Lizbeth Palma-Gaytan**, thank you for being part of this experience and for sharing this journey with me.

Finally, and most importantly, I want to thank my parents **Rosa Delia Garcia Montelongo** and **Juan Ramon Luna-Orozco** for their endless love and support throughout every step of my education. Also, thanks to my brother **Juan Ramon Luna-Garcia**, for always encouraging me, and to my whole family, thank you for always being there, encouraging me, and believing in me during this amazing chapter of my life.

BIOGRAPHICAL DATA

PERSONAL INFORMATION

Name: Luis Antonio Luna-García

Date of birth: October 08, 1994

Place of birth: Gomez Palacio, Durango, Mexico

RFC: LUGL941008PX2

CURP: LUGL941008HDGNRS05

Profession: Veterinarian

ACADEMIC FORMATION

High School: Center of Agricultural Technology Baccalaureate, No. 1.
Class: 2010-2013.

Bachelor´s degree: Veterinarian by the Antonio Narro Agrarian Autonomous University.
Class: 2013-2018.

Master of Science: Agricultural Production by the Antonio Narro Agrarian Autonomous University.
Class: 2018-2020.

SCIENTIFIC DEVELOPMENT

Author and co-author of scientific articles on topics related to animal production and reproduction. Participant in national and international conferences, including the presentation of a scientific poster at the Congress of the Academy of Research in Reproductive Biology (AIBIR) and an oral presentation at the Scientific Congress for Researchers in training (PIF) at the University of Córdoba.

RESUMEN GENERAL

Suplementación de glutamato, perfil endocrino e interacción de la actividad ovárica en cabras criollas durante el anestro estacional

La reproducción estacional en cabras requiere la integración de señales internas y externas para regular el eje reproductivo. Estos cambios estacionales en el eje afectan la producción, generando un patrón discontinuo que impacta negativamente en las ganancias y rendimientos de los productores caprinos. En mamíferos, el glutamato desempeña un papel clave en la modulación de procesos neuroendocrinos y reproductivos. Para evaluar su posible efecto en el perfil metabólico y reproductivo durante el anestro estacional, se realizaron dos experimentos (Exp. 1 & Exp. 2) utilizando cabras jóvenes de un año (n = 20), divididas en dos grupos experimentales: (1) GLUT; n = 10; peso vivo (PV) = 29,1 ± 1,02 kg; condición corporal (CC) = 3,4 ± 0,2 y (2) CONT; n = 10; PV = 29,2 ± 1,07 kg; CC = 3,5 ± 0,2. En el Exp. 1, se evaluó el patrón de secreción de hormona luteinizante (LH) y la actividad ovárica total (AOT), definida como la suma de folículos antrales (FA) y la tasa ovulatoria (TO). En el Exp. 2, se analizaron los niveles séricos de insulina y la AOT. Los resultados del Exp. 1 mostraron que el grupo GLUT presentó un mayor número de pulsos de LH (5,0 vs. 2,2 pulsos/6 h) y una AOT significativamente superior en comparación con el grupo CONT ($p < 0,05$). Asimismo, en el Exp. 2, el grupo GLUT exhibió un aumento en los niveles séricos de insulina y en la AOT durante el anestro estacional respecto al grupo CONT ($p < 0,05$). Estos hallazgos sugieren que el glutamato podría ser una estrategia prometedora para mitigar los efectos de la estacionalidad reproductiva en pequeños rumiantes, al mejorar parámetros reproductivos y metabólicos durante la estación no reproductiva.

Palabras Clave: cabras, glutamato, anestro estacional, actividad ovárica

Tesis de Doctorado en Ciencias en Recursos Naturales y Medio Ambiente en Zonas Áridas
UNIVERSIDAD AUTÓNOMA CHAPINGO Unidad Regional Universitaria de Zonas Áridas
Autor: M.C. Luis Antonio Luna García
Director de tesis: PhD. Cesar Alberto Meza Herrera

GENERAL *ABSTRACT*
Glutamate supplementation, endocrine profile, and ovarian activity interaction in crossbreed goats during the seasonal anestrous

Seasonal reproduction in goats requires the integration of internal and external signals to regulate the reproductive axis. These seasonal changes affect production in the axis, resulting in a discontinuous pattern that negatively impacts the profits and yields of goat producers. In mammals, glutamate plays a key role in modulating key neuroendocrine and reproductive processes. To evaluate its possible effect on the metabolic and reproductive performance during the seasonal anestrus, two experiments (Exp. 1 & Exp. 2) were conducted using yearling goats (n = 20), divided into two experimental groups: (1) GLUT; n = 10; live weight (LW) = 29.1 ± 1.02 kg; body condition score (BCS) = 3.4 ± 0.2, and (2) CONT; n = 10; LW = 29.2 ± 1.07 kg; BCS = 3.5 ± 0.2. In Exp. 1, the secretion pattern of luteinizing hormone (LH) and the total ovarian activity (TOA) defined as the sum of antral follicles (AF) and ovulation rate (OR), were evaluated. In Exp. 2, serum insulin levels and TOA were analyzed. In the Exp. 1, the GLUT group showed a higher ($p<0.05$) number of LH pulses (5.0 vs. 2.2 pulses/6 h) and an augmented ($p<0.05$) TOA as compared to the CONT group. Besides, in Exp. 2, the GLUT group exhibited increases ($p<0.01$) in both serum insulin levels and TOA during regarding to the CONT group. These findings support glutamate supplementation as a promising strategy to mitigate the effects of reproductive seasonality in small ruminants, improving both reproductive and metabolic parameters during the non-reproductive season.

Key words: goats, glutamate, seasonal anestrous, ovarian activity

Doctorate Science Thesis on Natural Resources and Environment in Arid Lands.
UNIVERSIDAD AUTÓNOMA CHAPINGO Unidad Regional Universitaria de Zonas Áridas
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CHAPTER I:

INTRODUCTION

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INTRODUCTION

Goat farming is an activity of significant economic and social importance in many regions of the world, particularly in arid and semi-arid climates where goats play a fundamental role in the production of meat, milk, and other products (Baraza *et al*., 2008). In developing countries, this activity not only contributes to food security but also serves as a vital source of income for small-scale rural producers (Escareño *et al*., 2013; Kumar and Roy, 2013). Mexico is no exception, being one of the main goat milk producers in Latin America, with production systems ranging from extensive to intensive, depending on the climatic and socioeconomic conditions of each region (Escareño *et al.,* 2012).

In Mexico, the Comarca Lagunera has established itself as a key point for goat farming as the main milk-producing region in the national territory (Isidro-Requejo *et al.,* 2018). Its importance lies in its ability to combine traditional and modern production techniques, despite the challenges posed by an arid climate and limited water resources (Gomez-Pasten *et al.,* 2010). However, goat production in this region, as in others, is strongly influenced by seasonal factors, especially with regard to reproduction.

Seasonal reproduction is a phenomenon regulated by neuroendocrine mechanisms that respond to environmental stimuli, such as photoperiod, affecting gonadotropin secretion and, consequently, reproductive activity (Chemineau et al., 2008; Duarte *et al.,* 2008). This seasonal reproduction results in seasonal production, which translates into discontinuous income for producers (Chemineau *et al*., 2008; Muñoz *et al*., 2019; Veliz-Deras *et al*., 2023). Previously, seasonal reproduction was addressed with various protocols based on exogenous hormones, substances that are now considered a risk to human and animal health. Therefore, it is urgent to develop new alternatives to control seasonal reproduction that in addition to being environmentally friendly reproductive methods, are cost-effective and improve production efficiency and

adhere to the principles of sustainability and ethical acceptability, thus eliminating dependence on exogenous hormone treatment (Dumont *et al.,* 2014; Dardente *et al*., 2016).

In this context, the study of exogenous glutamate administration in the regulation of reproductive seasonality can help to achieve advances in the control of goat reproduction. The administration of exogenous glutamate in small ruminants has demonstrated its effect on productive and reproductive parameters, both in reproductive and anestrus periods (Lincoln and Wu, 1991; Gazal *et al*., 2002; Sanchez *et al*., 2010; Meza-Herrera *et al*., 2020). Therefore, the management of seasonal reproduction through biotechnological techniques is fundamental to increase the efficiency of production systems and adjust them to market demands (Delgadillo *et al.,* 2011).

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CHAPTER II:

OBJECTIVES

CHAPTER II:

OBJECTIVES

2.1 General objective

- To evaluate the possible effect of exogenous glutamate in yearling goats during the anestrus period (April-May), to reduce the negative feedback effect of estradiol on the hypothalamic-pituitary axis.

2.2 Specific objectives

- To test the effect on the pulsatility and the pattern of LH secretion

- To evaluate the effect on metabolic hormones such as insulin and its role in the ovulation rate

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CHAPTER III:

HYPOTHESIS

CHAPTER III:

HYPOTHESIS

H1. Exogenous glutamate will have a positive effect on the pattern of gonadotropic hormone LH secretion, inhibiting the negative feedback of estradiol and increasing ovarian activity and ovulatory rate in yearling goats during seasonal anestrus.

H2. Administration of exogenous glutamate will have a positive effect on the concentration of insulin levels, increasing ovarian activity in yearling goats during seasonal anestrus.

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CHAPTER IV:

STATE OF THE ART

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STATE OF THE ART

4.1. Goat production

There are approximately 1.3 billion goats worldwide, half of which are distributed in the arid and semi-arid regions of Asia and Africa (Vazquez-Rocha *et al*., 2024). Moreover, Asia and Africa account for just over 90% of the world's total goat population, at 52% and 39%, respectively, while Europe accounts for 5%, the Americas for 4%, and Oceania accounts for only 1% of the total goat milk population (Lu *et al*., 2023).

Goat milk production has grown at a faster rate globally (1.8%) compared to human population growth (1.4%) and cow milk production (0.2%). Although it accounts for only 2% of total world production, goat milk is very important in Africa, Asia and the Mediterranean (Isidro-Requejo *et al*., 2018).

In Latin America, some countries such as Argentina, and Chile practiced the goat production, but, the countries that stand out for this activity are Brazil in the first place, and Mexico as the second producer (Escareño *et al*., 2013). In some countries, mainly in developing countries such as Mexico, the growth in the goat population is largely due to the multiple attributes of goats, such as: reliable producers in bad times, fast breeders, lower nutritional needs, curious eating habits and, good market price, therefore, they are assets that can be easily liquidated for cash in times of need (Escareño *et al*., 2015; Hossain *et al.,* 2004).

Goats are raised in all Latin American countries, whit the creole breed being the most prevalent, playing a significant role in the rural communities in developing countries, primarily by generating employment opportunities and enhancing household incomes of low and medium-input farmer through the sale of live animals and animal by-products (Boyazoglu *et al.,* 2005; Tesema *et al*., 2018; Muñoz *et al*., 2019). In Mexico, there are approximately 494,000 goat farms,

with nearly 1.5 million individuals involved in goat farming as a complementary economic activity (Tajonar *et al.,* 2022). Goats are primary raised in arid and semi-arid regions characterized by scarce water resources, prolonged droughts, and low socioeconomic conditions (Baraza *et al*., 2008), acting as the primary source of livelihood for approximately 80% of producers (Guerrero-Cruz, 2007).

Due to their adaptability to harsh climates, goats are an ideal species for enhancing animal production in challenging environments and can play a vital role in food security, particularly in poorer regions where adverse climatic conditions and growing food demand (Kumar and Roy, 2013). Goats demonstrate their highest production potential in regions with adverse climatic conditions, this adaptability is largely attributed to their small body size, low metabolic demands, capacity to lower metabolic and nutritional requirements, and superior digestive efficiency, mainly their ability to utilize high-fiber forages with tannin content, providing advantages over other livestock species (Silanikove, 2000).

All these characteristics make goats particularly adaptable to diverse animal production systems, which are classified according to the type of resources utilized, such as pasture or crop residues, the intensity of resource use (intensive or extensive), and the primary product generated, weather milk, meat or dual-purpose outputs (Escareño *et al.,* 2012). This adaptability is reflected in three main production systems employed in national goat farming: intensive, semi-extensive, and extensive systems (Miller and Lu, 2019). The intensive system is located in northern Mexico, where farmers invest significantly in animal housing and farms infrastructure, this system is primarily focused on milk production and manufacturing of dairy products where goats receive total mixed diets, access to veterinary consultations, advanced technologies for genetic improvement, and support from government programs (Oliveros-Oliveros *et al*., 2009;Tajonar *et al*., 2022).

Among these, the extensive system is the most widely adopted because of its low production costs (Escareño *et al.,* 2012). This cost efficiency is attributed to its reliance on natural pasture as the primary source of animal feed (Salina-Gonzalez *et al.,* 2016). This system is particularly well suited for farmers facing agronomic and economic constraints (Vazquez-Rocha *et al*., 2024). Within the extensive production system, three distinct subsystems can be identified based on the utilization of grazing land. The first is the free-grazing subsystem, where animals feed on fields with crop residues, often integrated with other livestock species, such as cattle and sheep. The second subsystem involves grazing combined with nighttime sheltering, the production and sale of dairy products and live animals constitute the primary sources of income for farmers. The third subsystem, known as transhumance, is characterized by seasonal goat migration during the spring and autumn, the primary objective of this subsystem is meat production, employing large herds of goat that traverse mountains terrains for extended periods, this system predominantly relies on a local goat breed referred to as Mexican Pastoreña Goat (Blanca pastoreña) (Dominguez *et al*., 2018). Cantú et al. (1989) reported that 64% of goats in Mexico are located in arid zones, with their production occurring through extensive systems, while the remaining 36% are maintained under intensive production systems in temperate zones.

Milk production is predominantly derived from small-scale producers that serve as a supplementary activity to primary agricultural and livestock practices (Morales-Pablo *et al*., 2012). In recent years, as in the rest of the world, the demand for goat dairy products has increased (Escareño *et al*., 2011). This increase could be attributed to the unique characteristics of goat milk compared to those of bovine milk. Goat milk contains lower lactose levels, more easily digestible fat molecules, and a higher concentration of total solids, rendering it a more nutritious and economic product (Sanchez *et al*. 2003), and this activity in Mexico, the demand for goat dairy products is increasing, driving the integration of production and commercialization in certain regions, such as the Comarca Lagunera (Escareño *et al.*, 2011).

4.2. Goat Production in the Comarca Lagunera

Comarca Lagunera is a semi-arid region situated in northern Mexico, comprising 20 municipalities distributed between the states of Coahuila and Durango. Goat farming is one of the primary livestock industries in this region, with a population of 404,369 goats (SIAP 2022). This area represents the most significant goat milk production zone in the country, involving approximately 9000 small-scale production units (Isidro-Requejo *et al.,* 2018). Escareño et al. (2011) report that individual goat produce an average of 1.5 L of milk per day, with mean daily milk yield per herd estimated at 56.9 L. Approximately 60% of the goat milk produced is utilized in cheese production, while the remaining 40% is processed into milk-based confections, such as *cajeta* (Tovar-Luna, 2009). Additionally, goat milk serves a key ingredient in the production of various value-added products, including cosmetics, soaps, shampoos, body creams, flans, yogurts, and gelatin (Santos-Lavalle *et al.,* 2018).

Goat herds are predominantly composed of animals referred to by producers as “criollo”; this term can be substituted and accepted by the term “local” (Montaldo and Meza-Herrera, 1999; Mellado, 2008; Montaldo *et al*., 2010). The local goat of the Comarca Lagunera is one of the few species capable of adapting to extreme environmental conditions, including high summer temperatures above 40 º C and low annual rainfall about 250 mm (Isidro-Requejo *et al*., 2024). Another adaptability characteristic of these goats is their ability to resist prolonged periods of malnutrition (Gomez-Pasten *et al.,* 2010). Despite these challenges, the adaptability of goats is exemplified by the significant contribution of the Comarca Lagunera region to national goat milk production, accounting for approximately 35% of the country's total production (SIAP, 2020).

The production systems in this region are diverse, with few technologies and a lack of organization being susceptible to environmental and market factors (Salinas-Gonzalez *et al*., 2016). The most commonly used production system in this region is the semi-extensive system, that is, producers supplement their

goats diet with concentrates following grazing on communal lands (Echavarria et al., 2006). This is mainly because the goats feed on the local vegetation of the region such as mesquite (Prosopis spp.), huizache (Acacia spp.), and gobernadora (Larrea tridentate), this diet change during the rainy season to herbaceous species, including trompillo (Solanum elaeagnifolium), malva (Sphaeralcea angustifolia), and rodadora (Salsola jail), also, goats may supplement their intake with agricultural residues (Maldonado-Jaquez *et al.,* 2017).

Prominent commercial companies have successfully implemented intensive technologically advanced production system using specialized European dairy goat breeds, in contrast, small-scale producers face a lack of systematic improvement programs specifically designed to address their unique production conditions (Escareño *et al*., 2011). Moreover, previous government initiatives that distributed purebred European goats promoting inadvertently encouraged indiscriminate crossbreeding with local Creole goats, which constitute the traditional foundation of goat production in Mexico (Mellado, 2008). Comparative analysis of the two primary production systems reveals that the semi-extensive system contributes approximately 75.6% to total production, whereas intensive production units account for nearly 18% of total production (Tovar-Luna, 2009).

In the Comarca Lagunera, goat milk is the primary source of income in goat production system, followed by goat meat, knowing that this region represent the most significant goat milk production region in Mexico, producers derive 79% of their total income from milk sales and 21% from the sale of meat, this include both kid and adult animals, which are rarely sold and traded with intermediaries based on live weight when necessary (Escareño *et al*., 2011; Salinas-Gonzalez *et al*., 2015).

Small-scale producers in this region perceive that milk commercialization does not pose significant challenges, however, the major companies responsible for

purchasing milk often pay low and unfair prices, particularly during period of overproduction (Escareño *et al.,* 2011). Despite efforts by smallholder to intensify their production processes and capitalize on market opportunities, productivity remains low due to deficiencies across various aspect of animal production.

In the Comarca Lagunera, goat reproduction exhibits a clear seasonal modulation, with an anestrous period occurring from March to May, a reproductive phase extending from August to February, and a transitional reproductive period observed during June and July (Duarte *et al.,* 2008). Therefore, the number of offspring born, weaned, and successfully raised to maturity mainly determines the economic viability of a production unit. Hence, the improvement of the efficiency of animal reproduction is a key strategy for increasing meat and milk production (Alexandre and Mandonnet, 2005; Casey and Webb, 2010).

However, the most extensive and semi-extensive production systems are often limited by several factors. Among these, delayed age at puberty, low kid production and growth rates, prolonged kidding intervals, limited prolificacy, reduced milk yield due to short lactation periods and pronounced reproductive seasonality. So, it is clear that reproductive seasonality impact in a negative fashion to goat production, because this type of reproduction is misaligned with market demand throughout the year; this scenario is generally observed in goats under subtropical and temperate latitudes (Mellado, 2008; Escareño *et al*. 2011; Muñoz *et al*., 2019; Veliz-Deras *et al*., 2023). To address this challenge in goat reproduction, advancements in reproductive research have produced interesting strategies through the application of biotechnological tools (Tesema *et al.,* 2018).

4.3. Seasonal reproduction of the female goat

Animals exhibit a diverse variety of physiological and behavioral adaptations to seasonal variations; these are evident in processes such as growth rate, metabolic adjustments, immune function, migration, hibernation, and reproductive activity (Guh *et al*., 2019). Seasonal reproduction is an adaptive process through which species adapt their physiology to change environmental conditions (Beltran-Frutos *et al*., 2022). This type of adaptation enables animals to synchronize their kidding with periods of abundant food availability, optimizing milk production (Nishiwaki-Ohkawa and Yoshimura, 2016). A determinant of the timing of the breeding season is the length of the gestation period, as it ensures synchronization with optimal environmental conditions for offspring survival. However, circadian rhythms and seasonal variations in day length are fundamental factors influencing reproductive activity in both male and female animals (Guh *et al.,* 2019). Consequently, photoperiod acts as the primary bioregulator of the central nervous system (CNS), influencing the activity of the hypothalamus, the pituitary gland, and the pineal gland (Vasantha, 2016).

Based on their physiological response to seasonal variations in daylight, animals can be classified as long-day or short-day breeders. Long-day breeders are fertile during period of increasing day length, while short-day breeders are fertile during decreasing day length (Nishiwaki-Ohkawa and Yoshimura, 2016). This type of reproduction has been studied in several animal species, but there has been an increased interest in farm species, such as sheep and goats (Dardente *et al*., 2016).

Goats are polyestrous animals; with seasonality related to shorts days that alternate two mainly reproductive phases annually; the breeding season and the anestrous season (Savoric *et al.,* 2024). The breeding season is characterized by regular estrous cycles and ovulation approximately every 21-22 days (Gomez-Brunet *et al*., 2012). This phase typically begins in early autumn and extends until late winter (Delgadillo *et al*., 2004).

Studies conducted in Alpine goats has shown variability in the length of estrous cycles, approximately 77% of goats exhibit normal estrous cycles ranging from 17 to 25 days, meanwhile, 14% experience shorts cycles, averaging 8 days, and only 9% present extended cycles, averaging 39 days in duration (Fatet *et al*., 2011). During this phase the ovaries undergo a series of morphological, biochemical and physiological changes leading to the ovulation (Sharma *et al.*, 2019). The sum of all these changes is called the ovarian cycle. The ovarian cycle in goats comprises two main stages: the follicular and the luteal phases (Fatet *et al*., 2011; Sharma *et al.,* 2019). During the follicular phase, follicular growth occurs, characterized by what is known as a follicular wave, which involves a progression of events regulated by the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), including recruitment, selection and dominance (Evans, 2003). Studies in goats have demonstrated that, during the estrous cycle, three to four follicular waves can occur per cycle, culminating in the maturation of the ovulatory follicle (Sharma *et al.,* 2019).

The development of ovarian follicles is driven by FSH, which is secreted by the anterior pituitary gland. As these follicles grow and mature, the larger follicles begin to secrete increasing concentrations of estradiol-17β, which triggers the estrous behavior and exerts a positive feedback effect on the hypothalamic-pituitary-gonadal axis, inducing a surge in GnRH secretion, leading to a pre-ovulatory surge of LH (Herbison, 2020). The LH surge initiates ovulation and promote the luteinization of follicular cells to form the CL (Webb *et al*., 2004). The follicular phase begins with proestrus, leading to the maturation of dominant follicles, followed by the estrous phase, characterized by the onset of behavioral estrus and culminating in ovulation (Fatet *et al*., 2011).

The luteal phase is characterized by continued follicular growth, although ovulation is inhibited due to elevated progesterone levels (Sharma *et al.,* 2019). Approximately 16-18 days after estrus, prostaglandin F2α secreted by the non-pregnant uterus induces luteolysis, decreasing progesterone synthesis and triggering the onset of a new follicular phase (Baril *et al*., 1993). The luteal

phase can be divided in metaestrus when progesterone concentrations begin to rise, and diestrus, when peripheral concentrations of progesterone are high up to the start of luteolysis (Arroyo, 2011; Fatet *et al*., 2011).

The second phase is the anestrous season marked by the absence of sexual and ovulatory activity (Gomez-Brunet *et al*., 2012). Despite the lack of corpora lutea, follicular wave patterns persist in the ovaries during this phase (Schwartz *et al*., 2002; Sharma and Sood*,* 2019). The anestrous season typically occurs during spring/summer months and is highly variable across and within goat breeds (Duarte *et al*., 2008). This variability is influenced by factors such as the geographic origin as well as the timing and length of the seasonal reproductive cycles (Gomez-Brunet *et al*., 2012). Goats originated from latitudes greater than 35º North or from subtropical regions, located between latitude 35 and 25º, exhibit distinct breeding season, while those goats from tropical regions, the reproductive seasonally of local small ruminants tends to fade (Leboeuf *et al*., 2008; Fatet *et al*., 2011; Gomez-Brunet *et al*., 2012).

Seasonal reproductive patterns in goats are closely regulated by variations in photoperiod (Fatet *et al*., 2011). The transition from longer daylight hours to shorter days promotes reproductive activity, primarily due to an increase in melatonin secretion (Vasantha, 2016). Elevated melatonin levels stimulate the reproductive axis by promoting the pulsatile release of GnRH, which in turn enhances the secretion of LH and FSH, ultimately triggering ovulation (Malpaux *et al*., 2001; Kopycińska *et al*., 2016). This activation marks the onset of the breeding season in seasonal breeders and is analogous to the process of puberty, as animal moves from a transition-sexually quiescent state to an active reproductive state (Smith, 2012). Conversely, goats experience a period of reproductive inactivity during extended photoperiods, typically from late winter to mid-summer. This anestrous season is characterized by a reduction in the pulsatile secretion of GnRH/LH, primarily attributed to the heightened negative feedback effects of estradiol (Chemineau *et al*., 2010; Smith and Clarke, 2010).

4.4. Neuroendocrinology of seasonal reproduction

In goats, photoperiod regulates reproductive physiology by influencing the perception of light intensity and converting this environmental signal into a neurohormonal message through a complex polysynaptic pathway (Vasantha, 2016). This pathway originates in the eye; the retina detects light through specialized photoreceptor cells containing rhodopsin, the proteins responsible for light absorption (Correa and Fernández, 2017). The critical role of the eye as a photoreceptive organ is evidenced by the complete loss of photoperiodic responses following ocular removal (Vasantha, 2016). The light signal is then transmitted via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) in the hypothalamus (Hannibal *et al*., 2014; Dardente and Simmoneaux, 2022). As the primary circadian pacemaker, the SCN synchronizes biological rhythms and converts the light signal into a neurohormonal output (Reiter, 1991; Bellastela *et al*., 2014). From the SCN, the signal is relayed to the pineal gland through a sophisticated neurohumoral pathway involving multiple brain nuclei (Weems *et al.,* 2015; Dardente and Simmoneaux, 2022). The SCN plays a pivotal role in seasonal regulation by controlling nocturnal melatonin secretion from the pineal gland. This melatonin release conveys daily and seasonal photoperiodic information to melatonin receptor-expressing tissues, thereby modulating physiological responses associated with seasonality (Pevet and Challet, 2011).

Melatonin is an indoleamine neurohormone synthesized from the amino acid tryptophan (Tamura *et al*., 2019; Gorman, 2020). It is found in almost all living organisms (Amaral and Cipolla-Neto, 2018). In mammals, melatonin is mainly secreted by the pineal gland, an epithalamic gland composed of pinealocytes, astrocytes and other neuronal cell types. The pineal gland plays an important role in the neuroendocrine translation of photoperiod, increasing nocturnal melatonin secretion by activating enzymes such as tryptophan hydroxylase, which converts tryptophan to 5-hydroxytryptophan, this is then converted to serotonin by dopa decarboxylase (DDC), then N-acetyltransferase (AANAT),

located in the pineal gland, acetylates and converts serotonin to N-acetyl-serotonin. In addition, melatonin synthesis is regulated by adrenergic receptors (α1 and β1), which phosphorylate the enzyme AANAT via cAMP-dependent protein kinase (Lépinay *et al*., 2021). Finally, N-acetyl-serotonin is converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT). All these enzymes are key to melatonin synthesis and are under the control of the neuroendocrine system, which regulates the timing and amount of secretion (Vasantha, 2016; ViviD and Bentley, 2018).

Melatonin synthesis follows a circadian pattern characterized by nocturnal elevation, regulated by the SCN and synchronized with the light/dark cycle, resulting in increased melatonin production during the night, which returns to basal levels during daylight hours (Dardente *et al*., 2016). That is, a light stimulus mainly in the blue wave rage activates melanopsin in the retinal ganglion cells and reaches the hypothalamus via the hypothalamic retinal tract, inhibiting melatonin synthesis (Reiter, 1991). Variations in the pattern of daily melatonin secretion result in an endocrine representation of the year, i.e., melatonin levels are elevated in winter as opposed to summer when melatonin levels are lower in response to fewer hours of darkness (Gorman, 2020). In short-day breeders such as goats, the decrease in daylight hours increases melatonin secretion, resulting in a stimulatory signal; conversely, the short duration of the signal in melatonin production translates into long days, which are inhibitory (Malpaux *et al.,* 1998).

Therefore, the secretion and synthesis of melatonin coincides with the duration of the night and is translated as a neuroendocrine message responsible for the effects observed in the physiology of the animal, affecting reproductive function, regulating the frequency and amount of hormones of the reproductive axis (Helfer *et al*., 2019; Szpregiel and Wroska, 2020). This is supported by studies demonstrating that the removal of the pineal gland inhibits both reproductive and metabolic responses regulated by photoperiod in various species, including

sheep and goats (Reiter, 1980; Nakane and Yoshimura, 2018). In pinealectomized animals, these effects can be reversed through the administration of melatonin (Goldman, 2001). In the case of short-day breeders, melatonin has a pro-gonadotropic function as it helps regulate reproductive hormone levels, in contrast to long-day breeders, where melatonin has an anti-gonadotropic effect due to its role in decreasing reproductive hormones (Hoffman, 1981).

Data in sheep indicates that melatonin does not cause seasonal changes in reproductive physiology, but plays a key role in synchronizing endogenous circadian rhythms (Weems *et al.,* 2015). Synchronization is achieved by melatonin interacting with cognate receptors at key sites and intervening in various seasonal responses (Nakao *et al.,* 2008). In mammals, two types of G protein-coupled melatonin receptors are known, called MT1 and MT2 (Scherbarth and Steiniechner, 2010). These receptors are widely expressed in various peripheral tissues and brain areas of reproductive interest, such as the hypothalamic regions that control the release of GnRH and gonadotropins in the anterior pituitary, with the MT1 receptor being the most studied (Dardente *et al.,* 2016; Klosen *et al*., 2019; Tamura *et al*., 2019). According to Gorman (2020), MT1 receptors are expressed in the SCN, suggesting that melatonin may alter circadian function. It has also been shown that MT1 receptors, but not MT2 receptors, are directly involved in the control of seasonal responses, including reproductive responses (Yasuo *et al*., 2009; Klosen *et al*., 2019). Similar to SCN, removal or injury of the pineal gland blocks seasonal responses such as reproductive and metabolic responses and can be reversed by exogenous melatonin administration (Goldman, 2001; Tamura *et al.,* 2019).

Melatonin is not stored in the pineal gland, once produced it is released into the bloodstream and distributed to different body fluids such as bile, cerebrospinal fluid, saliva, semen, amniotic fluid and ovarian follicular fluid, acting on different target organs through specific receptors (Tamura *et al.,* 2019). There is much

controversy regarding the site of action of melatonin in seasonal reproductive responses, with some studies suggesting activation of melatonin receptors in the Pars Tuberalis (PT), and conflicting studies demonstrating involvement of the pre-mammillary region (PMR) of the hypothalamus in melatonin receptor-mediated seasonal effects (Malpaux *et al.,* 1998; Weems *et al.,* 2015). The PT is in the rostral part of the adenohypophysis, and its location places it in close proximity to key regions in reproductive physiology, such as the median eminence (ME), glial cells of the third ventricle, and portal vasculature (Korf, 2018). Although there are controversial results regarding the involvement of the PT in seasonal responses, some authors claim that melatonin controls PT activity through MT1 receptors, which are highly expressed in the PT (Chemineau *et al*., 2010; Weems *et al*., 2015). Within PT, Thyroid-Stimulating Hormone (TSH) is locally produced in a manner independent of the hypothalamic-pituitary-thyroid (HPT) axis. Its synthesis is modulated by nocturnal melatonin secretion, highlighting the role of melatonin in regulating TSH production within this region (Dardente *et al*., 2016; Nakane and Yoshimura, 2019).

In addition, PT thyrotropin does not express receptors for TRH (Tthyrotropin-Rreleasing Hhormone) (TRH) and therefore does not activate the hypothalamic-pituitary-thyroid axis (Weems *et al.,* 2015). Interesting data in mice show that this type of TSH, through a post-translational glycosylation process, prevents thyroid stimulation and generates a PT-dependent TSH (Ikegami and Yoshimura, 2016). This glycosylation process affects the half-life and bioactivity of glycoprotein hormones; therefore, PT-produced TSH has a longer half-life and greater stability compared to pituitary-produced TSH (Nakane and Yoshimura, 2018). TSH produced by PT serves important functions in a paracrine manner by acting on receptors that control the activation and inhibition of Dio2 and Dio3 deiodinases in the tanycytes of the basal medial hypothalamus (MBH) (Dardente *et al.,* 2016). The tanycytes are a type of glial cell that line the third ventricle and send terminals to different nuclei in the hypothalamus depending on their

location (Ikegami and Yoshimura, 2016; Nakane and Yoshimura, 2018). Therefore, the PT and tanycytes are considered to be regions involved in the integration of photoperiodic information and the regulation of seasonal physiology (Wood and Loudon, 2018).

In tanycytes, TSH binds to its cognate receptor (TSHR) and increases the transcription of enzymes called deiodinases (Weems *et al.,* 2015, Dardente *et al.,* 2016). These deiodinase enzymes (Dio2 and Dio3) appear to be critical for the regulation and control of thyroid hormones; Dio2 converts the relatively inactive form of T4 (thyroxine) to its active form T3 (triiodothyronine), while Dio3 degrades both T4 and T3 to rT3 and T2, respectively (Nakao and Yoshimura, 2008). These enzymes are expressed in some brain regions, but the region with the highest expression is the tanycytes, which send their projections to areas such as in close contact with the ME, the arcuate nucleus (ARC), and the ventromedial and ventricular nuclei of the hypothalamus (Rodriguez *et al.,* 2010; Nakane and Yoshimura, 2018). The expression of the Dio2 enzyme is increased during long days, leading to an increase in T3 production through the activation of TSH receptors expressed in tanycytes, whereas Dio3 is increased during short days (Hanon *et al.,* 2008; Dardente and Simonneaux, 2022). Studies in sheep have shown that TSH gene expression is strongly regulated by photoperiod, with higher expression during long days compared to short days (Ross *et al.,* 2011).

Long photoperiods increase the expression of the transcription factor eya3 in the PT (Weems *et al.,* 2015). This gene is a clock gene whose peak expression occurs about 12 hours after the peak of melatonin, i.e. when melatonin concentrations are low, the expression of this gene increases; in addition, this gene has been observed to stimulate TSH expression (Dardente *et al*., 2010). In addition, since melatonin controls TSH production, TSH is increased during long summer days, whereas its production is decreased during short winter days in the northern hemisphere (Dardente *et al*., 2016). Therefore, in species such as the goat, thyroid hormones are essential for the transition to anestrus, as

thyroidectomized animals remain in a persistent state of reproductive activity (Dahl *et al.,* 1994).

4.4.1. Thyroids hormones in seasonal reproduction

Thyroid hormones have been shown to play a key role in some seasonal adaptations, i.e., basal metabolic rate, changes in body weight, thermogenic capacity, as well as changes in reproductive physiology (Dardente *et al*., 2014). The key role of thyroid hormones is supported by work in different seasonal species, such as Syrian and Siberian hamsters, rats, mice (i.e., long-day breeders), as well as sheep and goats (i.e., short-day breeders) (Nakane and Yoshimura 2018). It has also been observed that thyroid ablation suppresses the normal physiological response of the HPG axis, which can be reversed by exogenous T4 administration (Chemineau *et al.,* 2010). In addition, the absence of thyroid hormone inhibits the refractory response, suggesting that T3 signaling is important in the control of seasonal reproductive responses (Dardente and Simmoneaux, 2022). Similarly, work in sheep has demonstrated the fundamental role of thyroid hormones, as changes in the concentration of these hormones, especially T3, lead to changes in neuronal plasticity in key areas involved in reproduction (Bernal, 2002; Dardente *et al*, 2014; Dardente *et al*., 2018). That is, ewes exposed to long photoperiods stimulate Dio2 expression, and T4 administration terminates the reproductive season by decreasing LH concentration; therefore, during long days, Dio2 increases the conversion of T4 to T3 to terminate the reproductive season in ewes (Yasuo *et al*., 2006; Weems *et al*., 2015; Guh *et al*., 2019).

The pre-mammillary region (PMR), situated within the hypothalamus at the base of the brain and bordered by the fornix and mammillary bodies, has been identified as a critical site for reproductive regulation (Malpaux *et al*., 1998). Studies in sheep using melatonin microimplants in the PMR have shown a significant advancement in the onset of the reproductive season, attributed to the stimulation of LH secretion (Goodman *et al.,* 2010). These findings suggest

that melatonin exert its regulatory role in seasonal reproduction by acting on this specific hypothalamic region (Malpaux *et al*., 1998; Malpaux *et al.,* 2002; Weems *et al*., 2015). Based on this evidence, it is proposed that PMR plays a critical role in the reactivation of reproductive activity in small ruminants, whereas the PT may be primarily involved in the transition from the reproductive season to anestrus along with thyroid hormones, this effect is comparably analogous to the effects of negative feedback of E2 (Lehman *et al.,* 2010; Weems *et al*., 2015).

4.4.2. Negative feedback of estradiol: The main mechanism responsible of seasonal reproduction

The negative feedback of Estradiol (E2) on the HPG axis serves as a key neural mechanism responsible for the effects on seasonal reproduction (Weems *et al*., 2015). This process is characteristic of the anestrus period in the goat and is associated with an increase in the sensitivity of E2 to suppress GnRH and LH secretion (Goodman *et al.,* 2010). During this phase, GnRH secretion is modulated by two distinct negative feedback mechanisms: progesterone (P4) primarily inhibits GnRH pulse frequency, whereas E2 suppresses GnRH pulse amplitude (Chemineau *et al.,* 2010). Moreover, the persistently low levels of E2 during anestrus exert a potent inhibitory effect on GnRH secretion, thereby preventing the LH surge required for the preovulatory peak (Weems *et al.,* 2015).

During the anestrus period, the negative feedback effects of E2 suppress ovulation; interestingly the positive feedback mechanism that increases GnRH levels remains functional and is not inhibited. Consequently, the administration of exogenous E2 during this period leads to an increase in GnRH and LH secretion (Karsch *et al.,* 1980). These observations suggest that the negative feedback of E2 responsible for the inhibitory effects during the anestrus period presents a seasonal control (Weems *et al.,* 2015). E2 exerts its effects through nuclear estrogen receptors (ERα and ERβ), which are widely distributed throughout the CNS. However, GnRH neurons lack ERα, receptor critical for

mediating E2 negative feedback, indicating that the seasonal regulation of GnRH secretion operates independently of direct steroidal influence (Weems *et al*., 2017). Study by Meyer and Goodman (1985) demonstrated that dopamine D2 receptor antagonist blocks the inhibitory effects of E2 on GnRH/LH secretion during anestrus in ewes. The retrochiasmatic area (RCh) contains a population of dopaminergic neurons located in the A15 region, which play a crucial role in the regulation of seasonal GnRH secretion (Goodman *et al*., 2012). Studies have confirmed that A15 dopaminergic neurons project to key hypothalamic regions involved in reproductive control, such as the ARC nucleus (Goodman *et al*., 2010; Weems *et al.,* 2017; Nestor *et al*., 2023).

4.4.3. Dopamine and kisspeptin in seasonal reproduction

Studies in sheep have demonstrated that the administration of dopamine (DA) D2 receptor antagonists during anestrus blocks the inhibitory effects of E2 on GnRH and LH pulses (Meyer and Goodman, 1985). Similarly, lesion in this neuronal population during anestrus increases LH pulse frequency (Havern *et al.,* 1994), providing evidence for the involvement of dopamine in the regulation of seasonal reproduction (Weems *et al*., 2015). Dopaminergic neurons implicated in this process originate from a specific group in the RCh, referred to as the A15 neuronal group (Goodman *et al*., 2012).

These A15 neurons exhibit estrogen sensitivity, as their inhibitory effect on GnRH and LH secretion is E2-dependent (Meyer and Goodman, 1985). However, A15 neurons themselves do not express estrogen receptors (ERα or ERβ) (Lehman *et al.,* 1993). Instead, E2 influences A15 neurons indirectly, likely via glutamatergic neurons located in the preoptic area (POA), which are both E2-sensitive and closely associated with A15 neurons (Singh *et al*., 2009). During anestrus, the synaptic connectivity between these two neuronal populations increases, suggesting synaptic plasticity as a mechanism for heightened A15 neuronal activity and subsequent suppression of GnRH pulse frequency (Pompolo *et al.,* 2003).

Another hypothesis posits that A15 dopamine neurons establish axo-axonal contacts with GnRH terminals in the ME, directly modulating GnRH release (Kuljis and Advis, 1989). While evidence supports interactions between A15 neurons and GnRH neurons, recent studies have highlighted KNDy (Kisspeptin, Neurokinin B, and Dynorphin) neurons as the key mediators of dopamine's effects on GnRH secretion (Weems *et al.,* 2015, 2017). This is consistent with the established role of KNDy neurons in both seasonal and non-seasonal reproductive regulation (Lehman *et al*., 2010; Weems *et al*., 2015).

KNDy neurons are highly sensitive to E2. A decline in E2 levels, characteristic of anestrus, reduces the expression of kisspeptin and diminishes the connectivity between KNDy and GnRH neurons (Smith *et al*., 2008). Notably, KNDy neurons express dopamine D2 receptors (D2R), with their expression being upregulated twofold during anestrus compared to the reproductive period (Goodman *et al.,* 2012). Dopamine binding to D2 receptors inhibits kisspeptin release, which in turn suppresses GnRH pulse frequency (Weems *et al*., 2015). This indicates that KNDy neurons in the ARC are critical for transmitting the negative feedback effects of E2; increased dopamine secretion from A15 neurons inhibits GnRH secretion during anestrus by reducing KNDy neuronal activity (Weems *et al.,* 2017; Nestor *et al*., 2023).

4.5. Control of seasonal reproduction

Reproductive activity fluctuations lead to significant variations in product availability in the market, causing price increases and irregular income for farmers. To address these challenges, farmers have implemented various strategies to active the reproductive axis during the anestrous period (Dardente *et al*., 2016). The management of sexual activity in goats during seasonal anestrus can be achieved by inducing estrus and ovulation through three approaches: socio-sexual interactions, hormonal treatments, and light treatments (Martin *et al.,* 2004; Fatet *et al*., 2011; Dardente *et al.,* 2016).

4.5.1. Socio-Sexual Interactions

Socio-sexual interactions between males and females can be used to interrupt the natural inhibition of reproductive activity (Delgadillo *et al*., 2020). The introduction of a sexually active male to a group of females in anestrus stimulates LH secretion, induces estrous behavior, and results in ovulation within the first 5 days after this interaction (Bedos *et al*., 2014). This phenomenon is known as the "male effect" (Delgadillo *et al*., 2009). This technique represents one of the oldest ancient practices used by farmers to induce breeding during the anestrous season in goats (Underwood *et al.,* 1994 cited in: Dardente *et al.,* 2016). Initially, females must be isolated for a minimum period of 40 days prior to their exposure to male (Chemineau and Malpaux, 1998). Comparing the exposure of females to sexually active and sexually inactive males evidenced the effect. The presence of a sexually active male significantly stimulated LH hormone secretion within the first 15 minutes of introduction, and this response persist for 12 hours, while, females exposed to a sexually inactive male did not exhibit any increase in LH secretion (Muñoz *et al.,* 2017). Therefore, the introduction of a male can induce a pre-ovulatory LH surge within 1 to 3 days, followed by ovulation approximately 22 hours after the LH peak (Fernandez *et al.,* 2011). This response highlights the complex nature of the male effect, which relies on a combination of sensory stimuli, including visual, tactile, and olfactory cues (Gelez and Fabre-Nys, 2004).

The response to the introduction of a sexually active male varies among goat breeds and depends primarily on the quality of the anestrus period (Ungerfeld *et al*., 2004). In Mexican local or Creole goats, the male effect fails to produce the desired response when females are in the mid-anestrous season, however, at the end of the anestrous period, this strategy can advance the onset of the breeding season by a few weeks (Delgadillo *et al*., 2015). This indicate that the introduction of a sexually active male reduces the negative feedback exerted by E2 on the hypothalamic-pituitary-gonadal axis, acting on different types of neuronal cells (Muñoz *et al.,* 2017). The male effect was further enhanced through the application of photoperiodic treatments, where male or females were exposed to artificial photoperiod regimes, effectively reactivating the

reproductive axis (Abecia *et al.,* 2015; Delgadillo *et al*., 2015; Zaragoza *et al.,* 2019).

4.5.2. Photoperiod Treatments

Artificial photoperiodic cycles initially developed for use in the poultry industry and later adapted for rams, allows the regulation of sexual activity in seasonally breeding animals (Luo *et al*., 2019). Since goat reproduction is regulated by the duration of daylight hours, treatments based on alternating long and short days can be used in both females and males (Gómez-Brunet *et al.,* 2012). Implementing alternating periods of 1 to 2 months of long days (16 hours of light and 8 hours of darkness) followed by 1 to 2 months of short days (8 hours of light and 16 hours of darkness) effectively reduces the seasonal fluctuations in the reproductive activity of bucks, increasing plasma testosterone concentrations (Delgadillo *et al*., 1991; Delgadillo *et al.,* 2002; Leboeuf *et al.,* 1998). It has been demonstrated that photoperiodic treatments are an effective method for stimulating endocrine activity and enhancing sexual behavior in males from various latitudes during reproductive rest period (Simões *et al*., 2021). Photoperiod-treated bucks have similar capacity as melatonin-treated bucks to induce reproductive responses of female goats during spring (Zaragoza *et al.,* 2019). Sexually active bucks exposed to light treatment can effectively induce ovulation in most females during the anestrous season (Bedos *et al.,* 2014; Muñoz *et al.,* 2017). The incorporation of sexually active bucks as a reproductive management strategy holds significant potential for controlling goat reproduction in both intensive and semi-extensive systems, representing a sustainable, environmentally friendly and easily implementable option, offers an effective solution for improve goat reproduction during anestrous season (Simões *et al*., 2021).

4.5.3. Hormonal Treatments

Hormonal protocol utilized in goat reproduction typically rely on progestagens, equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG) and

prostaglandins (PGF2 alpha analogue), to achieve estrus and ovulation synchronization, these methods are effective during both the breeding and non-breeding seasons (Gómez-Brunet *et al*., 2007; Fatet *et al.,* 2011). Conventional progestagen-based protocol for goat reproduction usually involves the use of vaginal sponges impregnated with 20-45 mg of fluorogestone acetate, which are maintained for a variable duration of 6-12 days (Fatet *et al.,* 2011). Within these protocols, eCG is administered either two days before at the time of sponge removal in doses from 250-600 IU (Martemucci and D´Allessandro, 2011). One notable advantage of these protocols is their applicability throughout the year. Alternative protocols include the use of implants containing 3-6 mg of norgestomet or controlled internal drug-releasing devices (CIDR) with 330 mg of progesterone (Fatet et *al*., 2011). Additionally, single injections of progesterone combined with eCG or hCG have demonstrated favorable results (Gomez-Brunet *et al*., 2007; Alvarado-Espino *et al.,* 2019). These treatments efficiently synchronize ovulation, achieving fertility rates of approximately 60% kidding (Gómez-Brunet *et al.,* 2012).

Innovative protocols have also emerged, using novel substitutes such as kisspeptin analogues (C6), this compound effectively induce ovulation during anestrus, making them promising candidate for estrus synchronization in small ruminants (Dardente and Simmoneaux, 2022). A single intramuscular injection of C6 containing 15 nmol/animal given 24 hours after the end of progestagen treatment induce a peak of LH and FSH, moreover, the application of this kisspeptin analog improve fertility when is compared with eCG (Decourt *et al.,* 2019).

Given the growing awareness of climate change and its profound impact on wild and domestic species as well as biodiversity, the use of exogenous hormones such as estrogens, progestagens, and eCG, is increasingly regarded as hazardous to both consumers and the environment due to their role as endocrine disruptors (Roy *et al*., 1999; Martin *et al*., 2012; Dardente *et al*., 2016). Consequently, there is an urgent need to develop eco-friendly

reproductive methods that are cost-effective, enhance production efficiency, and adhere to principles of sustainability and ethical acceptability, thereby eliminating reliance on exogenous hormonal treatment (Dumont *et al*., 2014; Dardente *et al.,* 2016).

4.6. Glutamate in the control of the reproductive function of small ruminants

Glutamate is a non-essential amino acid that serves as a substrate or precursor molecule in various metabolic pathways within the body (Yelamanchi *et al*., 2016). Within CNS, glutamate is the most abundant amino acid and serves as the primary fast excitatory neurotransmitter, mediating the majority of excitatory synaptic transmissions within the brain (Bearss *et al.,* 2024). Besides, it plays a crucial role in numerous biological processes, including cellular proliferation, apoptosis, neuronal survival, and neurogenesis, highlighting its significance in the proper maintaining both physiological and developmental functions in the CNS (Brann and Mahesh 1997; Iremonger *et al.,* 2010). These functions are mediated through the activation of glutamate receptors in key brain regions. Glutamate receptors are classified into two main categories of excitatory amino acid receptors: ionotropic receptors, which form ligand-activated ion channels, and metabotropic receptors, which are G-protein-coupled receptors (Mahesh and Brann, 2005; Bearss *et al*., 2024). Ionotropic actions trigger the rapid activation of electrical phenomena, whereas metabotropic actions are prolonged (Medina-Marin and Escobar, 2002).

Evidence suggests that these receptors may play a significant role in modulating reproductive processes such as hormone secretion, puberty, and ovulation, since they are expressed in key hypothalamic nuclei that regulate these functions (Dhandapani and Brann, 2000; Meza-Herrera *et al*., 2011, Meza-Herrera, 2012). Research conducted on rats and monkeys has identified a high density of glutamate receptors in several hypothalamic nuclei, including the paraventricular nucleus (PVN), suprachiasmtic nucleus (SCN), supraoptic

nucleus (SON), ARC, ME (Mahesh and Brann, 2005). These findings emphasize the widespread distribution of glutamate receptors in various brain regions, besides, highlight glutamate´s dual role as both a principal neurotransmitter in the mammalian central nervous system and a critical modulator of the HPG axis (Meza-Herrera, 2012; Bearss *et al*., 2024). It has been observed that, the decrease in hypothalamic glutamate release in the mediobasal and anterior preoptic area was related to a reduction in GnRH, LH, FSH and steroid concentrations, compromising the normal function of the HPG axis (Cardoso *et al.,* 2010). Likewise, others authors have reported that glutamate and its ionotropic receptors not only regulate LH secretion but also play a role in modulating the release of GH and PRL (Estienne *et al*., 1990; Barb *et al*., 1996; Jiang *et al*., 1997; Gazal *et al*., 2002). These effects on the endocrine system have been evaluated following both systemic and intracerebroventriuclar administration (Brann and Mahesh, 1997).

As mentioned above, glutamate and its agonist modulate reproductive processes (Dhandapani and Brann, 2000; Meza-Herrera *et al*., 2007). Studies in diverse animal models have confirmed the role of glutamate in puberty, demonstrating that endogenous glutamate levels increase in the POA during this stage (Parent *et al*., 2005). During the transitional stage, hypothalamic regulation of the reproductive cycle involves a dynamic balance between excitatory glutamatergic signaling and inhibitory GABAergic signaling (Ford and Ebling, 2000; Han *et al.,* 2002). Based on these findings, pharmacological studies have demonstrated that acute treatment of agonist of glutamate such as N-methyl-D-aspartate (NMDA), accelerates pubertal onset in rodents, primates and sheep (Plant and Barker-Gibb, 2004), while their peripheral administration modulates LH release without directly affecting pituitary function (Ondo *et al*., 1976). Besides, Intracerebroventricular administration of glutamate receptor antagonist has been shown to reduce both the pulsatile secretion and pre-ovulatory LH surge (Ping *et al.,* 1995).

GnRH neurons situated in the hypothalamus receive limited ionotropic glutamate inputs (Iremonger *et al.,* 2010). This indicates that the activation of the reproductive axis and its role in increasing LH levels, both in terms of pulsatile secretion and pre-ovulatory surge, occurs through indirect mechanism. A likely candidate for mediating this process in the hypothalamus particularly is the anteroventral periventricular nucleus (AVPV) and ARC (Nestor *et al*., 2023). Evidence from rodent studies suggests a potential interaction between kisspeptin and neurotransmitter such as glutamate (García-Galiano *et al*., 2012). Glutamatergic transmission to kisspeptin neurons in the hypothalamus is a critical component for mediating both positive and negative feedback responses. This is because estrogen receptors (ER) in kisspeptin neurons modulate the upstream glutamatergic network (Wang *et al*., 2018).

KNDy neurons are modulated by glutamate, which they use as a co-transmitter. These neurons establish intricate circuits, forming synapses with other neuronal populations, including the kisspeptin neurons of the AVPV (Cravo *et al*., 2011; Qui *et al*., 2016),

Activation of the kisspepetin neurons and increased glutamatergic neurotransmission are two excitatory events that stimulate both the onset of puberty and seasonal reproductive activity (Meza-Herrera, 2012; Meza-Herrera and Tena-Sempere, 2012). Several authors suggest that the mechanism leading to puberty is similar to that of the transition from anestrus to the reproductive epoch, as the two shares an increased negative feedback effect (Foster, 1988, Clarke, 1988, Smith and Clarke, 2010).

Knowing this and the previous effects of glutamate on pubertal advancement, it is appropriate to think that glutamate could advance the reproductive time in seasonal animals. Glutamate has been tested in several animal models, mainly in laboratory animals, indicating a potential role for glutamate and its agonists in the control of seasonal reproduction (Brann and Mahesh, 1997). These studies focus on the stimulatory effect on LH secretion during anestrus in small ruminants, both in males and females. Studies with male goats and rams have

demonstrated the effect of glutamate on LH and testosterone secretion during the reproductive season and seasonal arrest (Lincoln and Wu, 1991; Gazal *et al*., 2002; Sanchez *et al*., 2010). Also, previous studies in goat females during the anestrus period have demonstrated the role of glutamate as a modulator of both ovarian function and metabolic hormone synthesis, and that glutamate is involved in decreasing the negative feedback exerted by estradiol on the hypothalamic-pituitary axis, a key factor for the preovulatory LH peak (Meza-Herrera *et al*., 2014; Meza-Herrera *et al*., 2020).

It is important to mention that several studies in animal models have reported adverse effects of glutamate on the HPG axis, with particular emphasis on the use of monosodium glutamate (MSG). Most of these studies have been carried out in murine models, both in males and females, and have shown that chronic MSG administration led to neurotoxicity through excitotoxicity (Nnadozie *et al*., 2019). This process is characterized by overstimulation of glutamatergic receptors, particularly those of the NMDA type, resulting in neuronal damage in key regions for the regulation of the HPG axis (Olney, 1969; Perez-Vega *et al*., 2020). In addition, chronic MSG exposure has been reported to alter gonadotropin secretion, which negatively affects spermatogenesis in males and ovarian function in females (Sharma and Deshmukh, 2021). Moreover, glutamate-induced oxidative stress has been identified as an underlying mechanism in the disruption of cellular homeostasis in reproductive tissues, which aggravates damage at the testicular and ovarian levels (Eweka *et al*., 2010). Notwithstanding the evidence supporting the adverse effects of MSG on reproductive function, it is crucial to consider that the doses used in rodent's studies are often substantially higher relative to their body weight. Consequently, extrapolation of these findings to both animal of zootechnical interest and the human population from a translational perspective present significant limitations and should be approached with caution (Walker and Lupien, 2000; Zanfirescu *et al.,* 2019).

UNIVERSIDAD AUTÓNOMA CHAPINGO
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UNIVERSIDAD DE CÓRDOBA
INSTITUTO DE ESTUDIOS DE POSGRADO

Thesis in joint supervision to obtain the double degree of Doctor of Sciences:

GLUTAMATE SUPPLEMENTATION, ENDOCRINE PROFILE, AND OVARIAN ACTIVITY INTERACTION IN CROSSBREED GOATS DURING THE SEASONAL ANESTROUS

SUPLEMENTACIÓN DE GLUTAMATO, PERFIL ENDOCRINO E INTERACCIÓN DE LA ACTIVIDAD OVÁRICA EN CABRAS CRIOLLAS DURANTE EL ANESTRO ESTACIONAL

CHAPTER V:

SCIENTIFIC ARTICLES

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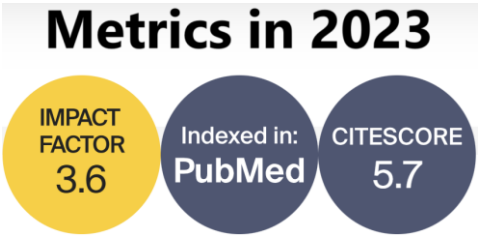

ARTICLE 1:

Article

Goats as Valuable Animal Model to Test the Targeted Glutamate Supplementation upon Antral Follicle Number, Ovulation Rate, and LH-Pulsatility

Luis A. Luna-García [1,2], César A. Meza-Herrera [1,*], Carlos C. Pérez-Marín [2], Rebeca Corona [3], Juan R. Luna-Orozco [4], Francisco G. Véliz-Deras [5], Ramón Delgado-Gonzalez [5], Rafael Rodriguez-Venegas [5], Cesar A. Rosales-Nieto [6], Jorge A. Bustamante-Andrade [7] and Ulises N. Gutierrez-Guzman [7]

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Simple Summary: Exogenous glutamate administration to yearling-anestrous goats enhanced not only ovarian function (i.e., ovulation rate and antral follicle number) but also denoted undoubted effects at the hypothalamic-pituitary level, augmenting LH pulsatility. Our results show not only the use of glutamate as a clean alternative but also as an interesting reproductive strategy to amplify ovarian function, considering goats as an animal model. While this should enlarge the possibility of escalating our knowledge regarding the hypothalamic-pituitary-gonadal modulation by glutamate, such research outcomes may also embrace potential translational implications.

Abstract: The potential effect of intravenous administration of glutamate on the ovarian activity and the LH secretion pattern, considering the anestrous yearling goat as an animal model, were assessed. In late April, yearling goats (n = 20) were randomly assigned to either (1) Glutamate supplemented (GLUT; n = 10, Live Weight (LW) = 29.6 ± 1.02 kg, Body Condition (BCS) = 3.4 ± 0.2 units; i.v. supplemented with 7 mg GLUT kg^{-1} LW) or (2) Non-supplemented (CONT; n = 10; LW = 29.2 ± 1.07 kg, BCS = 3.5 ± 0.2 units; i.v. saline). The oats were estrus-synchronized; blood sampling (6 h × 15 min) was carried out for LH quantification. Response variables included pulsatility (PULSE), time to first pulse (TTFP), amplitude (AMPL), nadir (NAD), and area under the curve (AUC) of LH. Ovaries were ultra-sonographically scanned to assess ovulation rate (OR), number of antral follicles (AF), and total ovarian activity (TOA = OR + AF). LH-PULSE was quantified with the Munro algorithm; significant treatment x time interactions were evaluated across time. The variables LW and BCS did not differ (p > 0.05) between the experimental groups. Nevertheless, OR (1.77 vs. 0.87 ± 0.20 units), TOA (4.11 vs. 1.87 ± 0.47 units) and LH-PULSE (5.0 vs. 2.2 pulses 6 h-1) favored (p < 0.05) to the GLUT group. Our results reveal that targeted glutamate supplementation, the main central nervous system neurotransmitter, arose as an interesting strategy to enhance the hypothalamic–hypophyseal–ovarian response considering the anestrous-yearling goat as an animal model, with thought-provoking while promising translational applications.

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1. Introduction

In goats, the seasonal variations in ovarian activity are the direct result of changes in the release of the gonadotropin-releasing hormone (GnRH) secretion, mainly exerted through the changing actions of the photoperiod [1,2]. Indeed, the photoperiod is the main environmental cue that modulates reproductive seasonality [3,4], altering the negative feedback exerted by estradiol (E2) upon the luteinizing hormone (LH) secretion [5,6]. The increased negative feedback of E2 on LH secretion has been accepted as the mechanism responsible for seasonal reproduction in most small ruminants [5,6]. In addition, glutamate (GLUT) has been recognized as the main, fast-acting excitatory neurotransmitter of the central nervous system (CNS), regulating most of the excitatory synaptic transmissions in the brain, while also involved in several biological processes [7]. GLUT-receptors have been localized in the diverse hypothalamic nuclei, some of which are key to the reproductive and neuroendocrine functions [8]. Moreover, the functional glutamatergic systems have been demonstrated in non-neural peripheral tissues, such as the heart, kidney, and gonads [7,8]. At a central level, the glutamatergic systems (i.e., ligand-receptors), have been involved in the control of pulsatile GnRH secretion, and the pre-ovulatory surge of gonadotropins (LH and FSH) [9]. Interestingly, the glutamatergic neurons that have been associated in the control of the GnRH neurons are also responsive to kisspeptin, an essential peptide in the regulation of the hypothalamic–pituitary–gonad (HPG) axis, promoting an increase in GnRH pulses [8–10]. While domestic sheep and goats have shown great potential as large animal models, sheep have been mainly used to perform preclinical and translational studies in reproductive research, while goats have been used in a more limited fashion [10]. We hypothesized a positive effect of the intravenous GLUT-supplementation upon ovarian function through an improved release profile of LH, considering the anestrous-yearling goat as the animal model. Thus, this study was designed to solve such a working hypothesis.

2. Materials and Methods

2.1. Location, Ethical-Welfare Issues, and Animal Management

The present study was carried out in northern Mexico (26° N, 103° W; 1120 m), in an intensive, commercial goat production system. Yearling anestrous Alpine-Saanen-Nubian × Criollo goats (n = 20), with an average live weight (LW) of 29.17 ± 1.02 kg and body condition score (BCS) of 3.45 ± 1.02 units, were involved. The experiment was conducted along the natural anestrous season, during April and May, i.e., under long-day photoperiodic conditions. The LW and BCS (from 1 = emaciated to 5 = obese) were weekly recorded before feeding by an experienced technician. All of the experimental procedures were completed following the recommendations for ethical use, care, and welfare of animals in research at global [11] and national [12] levels, and they were institutionally authorized (UACH-DGIP-REBIZA-IBIODEZA/15-510-400-2).

2.2. Experimental Design

At the end of April, the animals were individually housed in pens and they were randomly assigned to two experimental groups: (1) Glutamate (GLUT; n = 10) and (2) Control (CONT; n = 10). The LW and BCS were similar in both GLUT (29.1 ± 1.02 kg, 3.4 ± 0.2 units) and CONT (29.2 ± 1.07 kg, 3.5 ± 0.2 units) groups. All of the animals received a basal diet twice per day (07:00 and 16:00), consisting of alfalfa hay (14% crude protein, 4.7 MJ/kg), corn grain (11.2% crude protein, 9.9 MJ/kg), and corn silage (8.1% crude protein, 6.7 MJ/kg), balanced to cover their net energy requirements for maintenance [13]. Additionally, the GLUT-goats were supplemented every third day during the experimental period, with an intravenous injection of glutamate (L-glutamate, Merck-C5H9NO4-art-101791; from day 34 pre-estrus to day 17 post-estrus). Animals had ad libitum water access and shaded areas.

The composition values of the components of the basal diet (Dry Matter (DM)% basis) were obtained from representative samples taken throughout the experimental period and analyzed based on the formerly defined techniques [14].

2.3. Estrus Synchronization, Blood Sampling, and LH Determinations

The estrus synchronization was initiated 23 days after the beginning of the experiment. Intravaginal progestogen-impregnated sponges containing 45 mg of fluorogestone acetate (Chronogest®; Intervet International B.V., Boxmeer, Holland) were inserted for 10 days; one day before the sponge withdrawal, the goats received an i.m. dose of 75 µg of D-cloprostenol (Prosolvin-C®, Intervet International B.V., Boxmeer, Holland). Five goats per group were randomly selected 24 h after the sponges' withdrawal (−1 day); blood samples were collected at 3 h after the morning feeding. A volume of 10 mL of blood was collected every 15 min for 6 h by jugular venipuncture using sterile vacuum tubes (Corvac; Kendall Health Care, St. Louis, MO, USA) and allowed to clot at room temperature for 30 min. The blood was centrifuged (1500× *g*, 15 min) to obtain serum, and then it was decanted and stored into polypropylene microtubes (Axygen Scientific, Union City, CA, USA) at −20 °C until assayed. The peripheral serum LH concentrations were measured in duplicate by radioimmunoassay, as previously described [15]. The assay sensitivity was 0.2 ng/mL and intra-assay variation coefficient for LH quantification was 10%; the Munro algorithm was used to identify the LH pulses [16]. While the LH basal levels were quantified as the average of the lowest obtained values [17,18], the area under the curve (AUC) of LH was also determined.

2.4. Ultrasonographic Evaluation of Ovarian Activity

On day 17 post-estrus (coincident with the end of the luteal phase), an ultrasonographic assessment was carried out by a qualified operator to monitor the ovarian activity, using an ultrasound scanner (Toshiba Medical Systems Ltd., Crawley, UK), equipped with a 7.5 MHz linear-array transducer. The ovaries were scanned to record the number of corpus luteum (which indicate the ovulation rate, OR) and the antral follicles (AF; those ≥ 5 mm) [19]. Then, the total ovarian activity (TOA) was considered as AF + OR, recorded in both of the ovaries in each animal within the experimental group. The main activities performed during the experimental period are depicted in Figure 1.

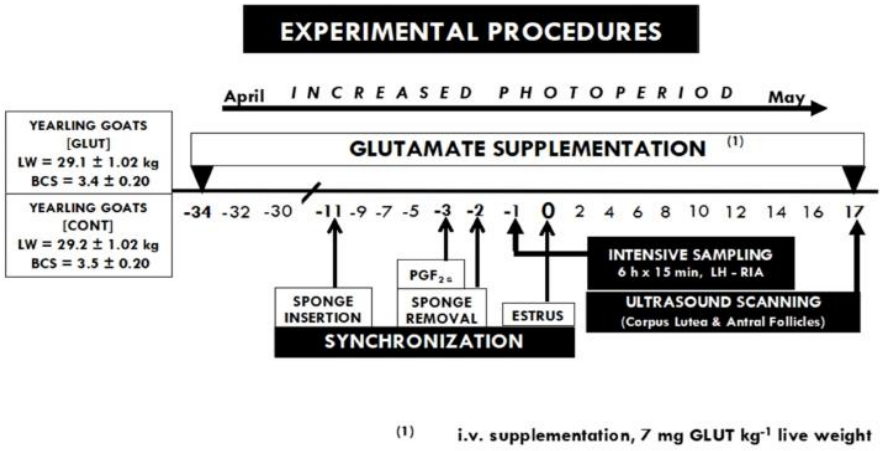

Figure 1. Time-line of activities performed during the experiment, including the estrous synchronization protocol and the targeted glutamate i.v.—supplementation every third day along with the experimental period, (L-glutamate, from day 34 pre-estrus to day 17 post-estrus). Blood sampling (every 15 min × 6 h) for LH quantification was performed 24 h prior to the estrus day (day 0). Later, ovarian ultrasonographic assessment was carried out on day 17 post-estrus to study the relationship between the TOA and LH secretion pattern. All the experimental units had ad libitum water access and shaded areas in each pen.

2.5. Statistical Analyses

The variables LW, BCS, OR, TOA, and serum LH concentrations were evaluated using the PROC-MIXED of SAS (SAS Institute Inc., Cary, NC, USA), for repeated measures across time in the same animal. Each goat within the experimental group was defined as the experimental unit. The experimental treatment (i.e., GLUT or CONT) and the sampling day (i.e., Time) were analyzed using mixed linear model procedures and the estimation technique of restricted maximum likelihood (PROC MIXED). While time was considered as the repeated measure, the treated goats were defined as the repeated subject and regarded as the random error term [20]. In the case of mean significant differences of the analyzed response variables across time, these were solved through the LSMEANS-LSD option of PROC GLM. Since the LH pulse frequency showed a non-parametric distribution, a Kruskal–Wallis test was used to analyze the variable LH-PULSE. The response variables were evaluated for normality using the Shapiro–Wilk test, and $\log^{10}$, transformed for basal and mean LH concentrations as well as for LH pulse amplitude. When a significant effect of the treatment x time interaction occurred, the data were compared across time. Pearson's correlations were used to check the associations among the variables LW, BCS, and OR. Non-transformed data are shown and expressed as least-square means ± standard error (SE). All of the statistical analyses were carried out using the procedures and options of SAS (SAS Inst. Inc., V9.1, Cary, NC, USA); significant differences between means were set at $p < 0.05$.

3. Results

The comparison of LW and BCS at the beginning (29.4 ± 1.02 kg and 3.4 ± 0.17) and the end of the experimental period (35.13 ± 1.07 kg and 3.4 ± 0.2) did not differ between the experimental groups ($p > 0.05$). Interestingly, however, the differences ($p < 0.05$) were observed for OR and TOA (1.77 vs. 0.87 ± 0.20) and (4.11 vs. 1.87 ± 0.47), respectively, favoring the GLUT-treated group. Moreover, an increased LH-PULSE (5.0 vs. 2.2 pulses 6 h^{-1}, $p < 0.05$) also favored the GLUT-treated group (Table 1). The LH-pattern release across time, along with OR and TOA, are shown in Figure 2. Further, positive correlations occurred between LW1 and BCS1 ($r^2 = 0.71$; $p < 0.01$), BCS and TOA ($r^2 = 0.7$, $p < 0.05$), AF and OR ($r^2 = 0.61$, $p = 0.01$), and OR and TOA ($r^2 = 0.87$, $p = 0.001$).

Table 1. Least-square means regarding LW and BCS at the onset of treatments (-initial) and at the ultrasound scanning (-ultrasound), ovulation rate (OR), total ovarian activity (TOA), and LH profile across time (pulsatility, time to first pulse, amplitude, nadir and AUC) in goats supplemented with glutamate (GLUT) and non-supplemented (CONT) groups.

	GLUT	CONT	S.E. [1]
LW-initial (kg)	29.60 [a]	29.24 [a]	1.02
BCS-initial (units)	3.4 [a]	3.5 [a]	0.17
LW-ultrasound (kg)	35.06 [a]	35.21 [a]	1.07
BCS-ultrasound (units)	3.5 [a]	3.2 [a]	0.20
Ovulation rate (units)	1.77 [a]	0.87 [b]	0.20
Total ovarian activity (units)	4.11 [a]	1.87 [b]	0.47
LH pulsatility, pulses/6 h (units)	5.0 [a]	2.2 [b]	0.60
LH time to first pulse (min)	35.0 [a]	81.0 [a]	31.99
LH amplitude (ng)	2.35 [a]	1.16 [a]	0.70
LH nadir (ng)	0.43 [a]	0.20 [a]	0.11
LH AUC (arbitrary units)	72.6 [a]	40.0.0 [a]	21.5

[a,b] Least-square-means without a common superscript, differ ($p < 0.05$) [1] Most conservative standard error is presented.

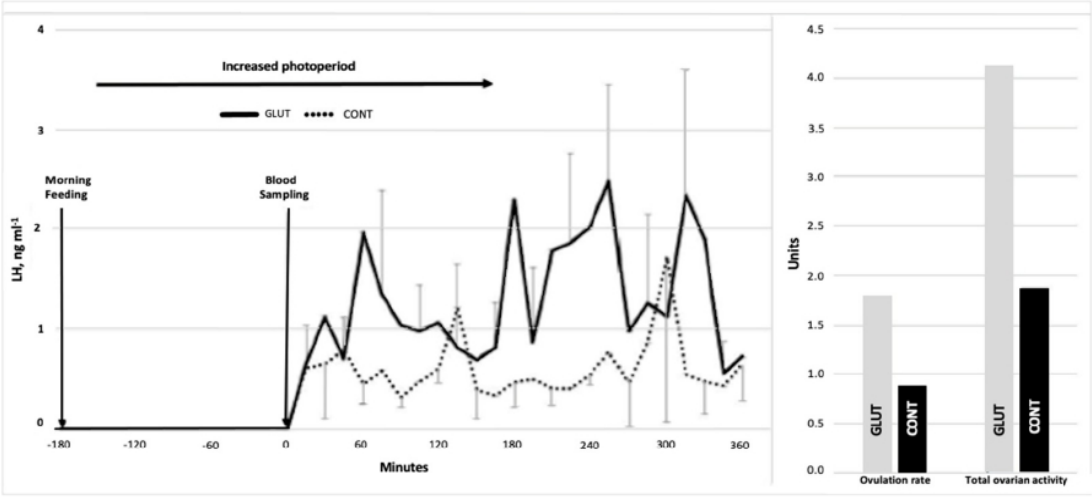

Figure 2. Serum LH concentrations (ng/mL) across time (**left**), and OR (units) and TOA (units) (**right**) in glutamate-supplemented (GLUT) and non-supplemented (CONT) goats.

4. Discussion

The results obtained backs both our working hypothesis as well as our animal model; the intravenous supply of glutamate to anestrous-yearling goats as an animal model endorsed the increases in both ovulation rate and total ovarian activity, while it simultaneously generated rises in LH pulsatility, considering the anestrous-yearling goat as an animal model. Whereas, no differences occurred between the experimental groups regarding LW and BCS, in addition, the response variables time of the first LH pulse, the LH amplitude, LH-nadir, and LH-AUC were also not different between the groups. This neuroendocrine and physiologic scenario suggests that the glutamate supplementation to yearling-anestrous goats exerted a positive effect upon the hypothalamic centers responsible for GnRH, and, thereafter, upon the anterior pituitary, augmenting the LH pulsatility. The specific site of action of glutamate along the hypothalamic–pituitary–ovarian continuum, as well as its role within the neuronal pathway responsible for the inhibitory effects of E2 during seasonal anestrus in goats, await to be elucidated.

The ability of E2 to inhibit the release of GnRH and LH is the mechanism responsible for the variation in ovarian activity during anestrous [21]. Such E2-negative action upon the LH-pulse generator is facilitated by a group of neurons located in the retrochiasmatic area of the hypothalamus, known as the A15 dopaminergic neurons [22]. These neurons release dopamine and through the dopamine type 2 receptor (D2R), which are present in the kisspeptinergenic neurons of the arcuate nucleus (ARC), inhibit the release of the peptide kisspeptin, resulting in a decrease in GnRH and LH pulses during the anestrous season [5]. Most of the brain cells use glutamate as the main fast-acting excitatory neurotransmitter [7]. This neuroexcitatory amino acid and its receptors are distributed throughout the CNS and diverse parts of the body, including the ovary [23]. Likewise, glutamatergic and kisspeptinergenic neurons have been involved in different physiological processes, including reproductive ones, such as the activation of the hypothalamic axis, particularly in the production of the preovulatory GnRH-LH peak [24].

The GnRH neurons are stimulated by glutamate through the activation of the ionotropic receptors, as N-methyl-D-aspartate (NMDA), and amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) [25]. In goats, the glutamate administration has shown positive effects upon diverse reproductive outcomes. The previous studies of our group have exposed that the glutamate supply diminishes the E2-negative feedback exerted on the hypothalamus–pituitary axis, modulating ovarian function and metabolic hormone synthesis [10]. The results of our study suggest that the responses generated by glutamate administration could have acted upon the ionotropic ovarian receptors [26,27], increasing the ovulatory response.

Besides, the glutamate supply may have promoted the increase in metabolic hormones and growth factors key to ovarian function, increasing follicular cell proliferation, and augmenting E2 synthesis [10]. Such a neurophysiological and endocrine scenario should have promoted a positive E2-effect upon the hypothalamus–pituitary axis, increasing the GnRH-LH pulsatility, and activating, in turn, ovarian activity.

Indeed, these results suggest that, in yearling-anestrous goats supplemented with intravenous glutamate, the inhibitory E2-feedback upon the hypothalamus–pituitary axis would be considerably diminished. Such an administration of glutamate probably acted somewhere along the neuronal continuum responsible for blocking GnRH secretion during the natural anestrous season, decreasing the action of dopamine on the kisspeptin-producing neurons, while enhancing the activation of the hypothalamic centers responsible for GnRH synthesis and secretion. The last, in turn, may have increased the LH-pulse, while augmenting the ovarian activity. Whereas diverse studies support the positive effects of glutamate administration upon the reproductive outcomes in diverse species, either male [28] or female [10,24,29,30], other studies have also demonstrated an interesting interplay between glutamatergic signaling and sexual male-to-female behavior [28,31].

Some components of the rams' sexual behavior such as ano-genital sniffing, vocalizations, mounting, intromission, and ejaculation are also observed in rodents [32–34]. Preceding studies of our research group demonstrated that parenteral glutamate supply enhanced male sexual behavior, both appetitive and consummatory, either in adult [32] or pubertal [33] rams. Recent reports have also shown that glutamatergic neurons in the ventral tegmental area of the lateral hypothalamus are activated by, and required for, innate defensive reactions [35]. Moreover, GnRH stimulates the LH release via glutamatergic receptors expressed in the hypothalamic kisspeptin neurons in both peripubertal [36] and adult [37] females. Such amplified glutamatergic neuron transmission effect is enhanced because of the co-expression of kisspeptin, neurokinin-B, and dynorphin, the so-called glutamatergic KNDy neurons, which drive the episodic release of GnRH [38,39]. Besides, increases in the ARC Kiss1, and Pdyn expression were positively correlated not only with gonadotropin secretion and follicular growth, but also with an augmented positive energy balance [40].

Based on the obtained research outcomes from our study, and merged with those generated by others previously discussed, a sensible question is, how to align these outcomes with a translational perspective? In humans, female infertility is one of the main public health problems worldwide [41]; whereas female infertility represents 37% of the causes in infertile couples [42], the most common factors causing women reproductive dysfunctions mainly involve ovulatory disorders (25%) and ovarian dysfunctions (50%) [43]. Such women's reproductive failures have also been linked, unfortunately, to mental, emotional, and physical issues [44]. The clinical management of infertility has included active ovarian stimulation treatments [45] to lessen a poor ovarian response or primary ovarian insufficiency; the key aim is to diagnose and then be able to manage women's infertility [46]. As noted, ovine and, to a lesser extent, caprine, have been used as large animal models for diverse research purposes. The use of the former ranges from the generation of therapeutic agents in the mammary glands, up to their use in human genetic disorders and regenerative medicine, as well as the edition of their genomes using CRISPR-based systems for diverse translational purposes [47]. Hence, building on the diverse research outcomes generated from the various goat research models presented along with this discussion, and considering the need to develop basic biomedical research for studying the neurophysiological functions and dysfunctions along with the hypothalamic–pituitary–ovarian axis to upgrade women's reproductive fitness, our research outcomes undoubtedly unveiled the interesting role that goats can play as a successful experimental animal model.

5. Conclusions

The targeted glutamate intravenous administration to anestrous-yearling goats positively influenced not only the ovarian function (i.e., ovulation rate and antral follicle

number) but also undeniably denoted effects at the hypothalamic–pituitary level (i.e., an augmented LH pulse). Such a neurophysiological complex scenario was certainly not observed in the control group. Our results show the use of glutamate to be a clean, green, and ethical alternative, substituting the use of exogenous hormones, while providing an interesting reproductive strategy to augment the activity of the hypothalamic–pituitary–ovarian continuum (HPOC). Our research outcomes should enlarge the possibility of escalating the knowledge about the action of a targeted neurotransmitter supply, such as glutamate, upon the endocrine and physiological mechanisms involved in the enhancement of the HPOC response, through the use of a goat animal model. Certainly, the research outcomes that stemmed from this study, using the goat as an experimental model, should augment the confidence in the design of biomedical reproductive studies. Besides, a potential glutamate-modulated cross-talk among the CNS and the HPOC requires clarification. Undoubtedly, the obtained results from this study await and deserve to be tested in clinical trials with potential translational applications.

Author Contributions: Conceptualization, L.A.L.-G. and C.A.M.-H.; Data curation, L.A.L.-G., C.A.M.-H. and C.C.P.-M.; Formal analysis, L.A.L.-G., C.A.M.-H., C.C.P.-M. and R.C.; Funding acquisition, C.A.M.-H., R.C. and F.G.V.-D.; Investigation, L.A.L.-G., J.R.L.-O., R.D.-G., R.R.-V., C.A.R.-N., J.A.B.-A. and U.N.G.-G.; Methodology, J.R.L.-O., F.G.V.-D., R.D.-G., C.A.R.-N. and J.A.B.-A.; Project administration, R.D.-G., R.R.-V., J.A.B.-A. and U.N.G.-G.; Resources, R.C., J.R.L.-O., F.G.V.-D., R.D.-G., R.R.-V., C.A.R.-N., J.A.B.-A. and U.N.G.-G.; Software, C.A.R.-N.; Supervision, C.A.M.-H.; Writing—original draft, C.A.M.-H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All of the experimental procedures were completed in accordance with the recommendations for ethical use, care and welfare of animals in research at global (USA; 11) and national (Mexico; 12) levels, and they were institutionally authorized by Chapingo Autonomous University; the animal study protocol was authorized by the Institutional Review Board, with approval reference number: UACH-DGIP-REBIZA-IBIODEZA/20-510-400-2.

Informed Consent Statement: Not applicable.

Data Availability Statement: None of the data were deposited in an official repository, yet, information can be made available upon request.

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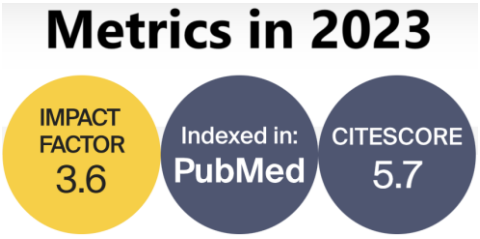

ARTICLE 2:

Article

Targeted Glutamate Supply Boosts Insulin Concentrations, Ovarian Activity, and Ovulation Rate in Yearling Goats during the Anestrous Season

Luis A. Luna-Garcia [1,2], Cesar A. Meza-Herrera [1,*], Carlos C. Perez-Marin [2], Angeles De Santiago-Miramontes [3], Jessica M. Flores-Salas [3], Rebeca Corona [4], Guadalupe Calderon-Leyva [3], Francisco G. Veliz-Deras [3], Cayetano Navarrete-Molina [5] and Ruben I. Marin-Tinoco [5]

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Simple Summary: Our research question considered whether exogenous glutamate administration would enhance serum insulin levels linked to augmented ovarian activity in yearling goats during the anestrous season. The obtained research outcomes support our initial working hypothesis. Certainly, we found that glutamate evokes serum insulin increases across time, enhancing in parallel the out-of-season ovarian activity, which is an augmented ovulation rate in yearling goats.

Abstract: The neuroendocrine regulation of the seasonal reproductive axis requires the integration of internal and external signals to ensure synchronized physiological and behavioral responses. Seasonal reproductive changes contribute to intermittent production, which poses challenges for optimizing goat product yields. Consequently, a significant objective in seasonal reproduction research is to attain continuous reproduction and enhance profitability in goat farming. Glutamate plays a crucial role as a modulator in several reproductive and metabolic processes. Hence, the aim of this study was to evaluate the potential impact of exogenous glutamate administration on serum insulin concentration and ovarian function during the out-of-season period in yearling goats. During the anestrous season, animals were randomly located in individual pens to form two experimental groups: (1) glutamate (n = 10, live weight (LW) = 29.1 ± 1.02 kg, body condition score (BCS) = 3.4 ± 0.2 units) and (2) control (n = 10; LW = 29.2 ± 1.07 kg, BCS = 3.5 ± 0.2), with no differences ($p < 0.05$) regarding LW and BCS. Then, goats were estrus-synchronized, and blood sampling was carried out for insulin quantification. Ovaries were ultrasonographically scanned to assess ovulation rate (OR), number of antral follicles (AFs), and total ovarian activity (TOA = OR + AF). The research outcomes support our working hypothesis. Certainly, our study confirms that those yearling goats treated with exogenous glutamate displayed the largest ($p < 0.05$) insulin concentrations across time as well as an augmented ($p < 0.05$) out-of-season ovarian activity.

Keywords: goats; glutamate; insulin; ovarian activity; anestrous

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1. Introduction

Changes in biological rhythms play a crucial role as adaptive and evolutionary mechanisms that promote reproductive success, individual survival, and species perpetuation [1].

Seasonal breeders show modifications in their physiology and behavioral responses to effectively adapt to seasonal variations in the environment. Among the various environmental cues, photoperiod stands out as the most reliable indicator for temporal prediction throughout the year [2]. This intricate process occurs through the retino-hypothalamic tract, a complex polysynaptic pathway that transforms external signals into neurohormonal signals. These neurohormonal signals subsequently act on specific brain nuclei responsible for the regulation of biological rhythms, resulting in increased melatonin release [3]. Melatonin levels affect reproductive function by regulating both the frequency and measure of reproductive hormones, which are regulated by the expression of melatonin receptors in hypothalamic nuclei that are involved in the control of GnRH release [1,2]. GnRH is the key master hormone in the hypothalamic–pituitary–gonadal (HPG) axis. GnRH neurons in the preoptic area release GnRH near the pituitary portal capillaries at the median eminence in a pulsatile manner modulating the HPG axis, which subsequently stimulates the pulsatile release of gonadotropins, i.e., luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary [4,5].

Gonadotropins drive gametogenesis in response to the synthesis of sex steroids and through feedback actions modulate the activity of GnRH in the HPG axis [6]. Ovarian steroids influence the reproductive system, but it is susceptible to other signals such as photoperiod, breed, age, stress, and metabolic status [7]. GnRH can be modulated in several ways by different molecules (i.e., neuropeptides and neurotransmitters); among these, kisspeptin is the essential molecule in the control of reproductive function, integrating hormonal, nutritional, and metabolic cues [5,8,9]. Kisspeptin can interact with other neuropeptides, hormones, and neurotransmitters to achieve its actions, including excitatory and inhibitory amino acids, such as glutamate and GABA, respectively [10].

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) and is involved in a myriad of brain functions, acting through kindred receptors expressed in central brain areas, including those related to reproductive function [11,12]. Numerous studies investigating the effect of glutamate have demonstrated its ability to modulate the HPG axis. Glutamate, along with its agonist, has been shown to increase the release of key reproductive hormones, including GnRH and LH [13,14]. Additionally, glutamate has been observed to act on non-neuronal cells, stimulating the release of gliotransmitters that in turn impact GnRH release [15]. Both glutamate and its receptors have been reported outside the CNS in various tissues such as the liver, kidney, skeletal muscle, and pancreatic islets, participating in diverse metabolic pathways [11,12]. The importance of exogenous glutamate has been demonstrated in previous works of our research team; it modulates reproductive neuroendocrine functions in small ruminants, such as puberty, sexual behavior, and reactivation of the reproductive axis and the out-of-season ovarian function, being an alternative for reproductive management [16–19].

Currently, achieving enhanced animal production and reproduction while simultaneously considering animal welfare and sustainable production has become a significant challenge. One key aspect of this challenge involves reducing the reliance on exogenous hormones in these processes [20–22]. Building on such findings, we hypothesized that the administration of exogenous glutamate promotes increased serum insulin levels and, in turn, promotes an augmented ovarian function in yearling goats during the anestrous season. Therefore, this study was designed to validate such a working hypothesis.

2. Materials and Methods

The research conducted in this study adhered strictly to established guidelines for the ethical treatment, care, and welfare of animals in research at both national [23] and international [24] levels. All procedures, methods, and management of the animals were carried out following these recognized recommendations. This study received institutional approval under the reference number UACH-DGIP-REBIZA-IBIODEZA/15-510-400-2.

2.1. Study Location, Environmental Conditions, Animal Selection, and Management

The research was conducted in the northern region of Mexico (26° N, 103° W; 1120 m). This study involved 20 yearling Alpine-Saanen-Nubian x Criollo goats in anestrus condition. The goats had an average live weight (LW) of 29.17 ± 1.02 kg and a body condition score (BCS) of 3.45 ± 1.02 units. This study was conducted during April, May, and June, which corresponded to the natural deep anestrous at 26° N. Before feeding, both the LW and BCS were recorded weekly. The body condition score was determined by an experienced technician using a five-point scale ranging from 1 (emaciated) to 5 (obese).

2.2. Experimental Design and Treatments

At the beginning of May, the animals were randomly assigned to individual pens forming two distinct experimental groups: (1) glutamate (GLUT; n = 10, LW = 29.1 ± 1.02 kg, BCS = 3.4 ± 0.2 units) and (2) control (CONT; n = 10; LW = 29.2 ± 1.07 kg, BCS = 3.5 ± 0.2 units). Both groups had similar average LW and BCS. The GLUT goats received an intravenous dose of 7 mg/kg of glutamate (L-glutamate, Merck-C5H9NO4-art-101791, Darmstadt, Germany) every third day throughout the entire experimental period, which lasted from 34 d pre- to 17 d post-estrus. The experimental groups were provided a basal diet twice daily (at 07:00 and 16:00 h). The diet consisted of a mixture of alfalfa hay (14% crude protein (CP), 4.7 net energy for maintenance (NEm) MJ kg^{-1}), corn silage (8.1% CP, 6.7 NEm MJ kg^{-1}), and corn grain (11.2% CP, 9.9 NEm MJ kg^{-1}), balanced to meet their net energy requirements for maintenance [25]. Both groups had *ad libitum* access to water and shaded areas. The composition values of the components in the basal diet (dry matter; % basis) were determined. Random samples of the diet were collected and subjected to further processing. The samples were initially reduced by scratching and finely chopped on clean paper. Subsequently, the processed samples were sieved using a 1 mm mesh sieve [26]. The specific activities conducted during the experimental period are illustrated in Figure 1.

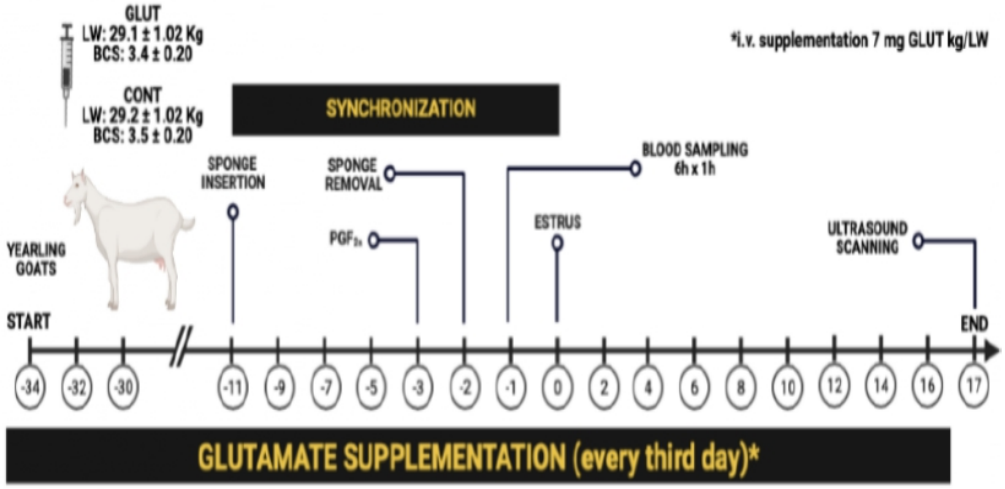

Figure 1. Timeline of activities performed during the experiment. Glutamate was supplemented (i.v. L-glutamate) every three days from d34 pre-estrus to d17 post-estrus throughout the experimental period. While the estrus synchronization protocol was conducted, an intensive blood sampling for insulin quantification was performed at 1 h intervals for 6 h, one day before the estrus. Ovarian ultrasonographic scanning was carried out on d17 post-estrus to assess the relationship between the TOA and serum insulin concentration. All the experimental groups had *ad libitum* water access and shaded areas in each pen.

2.3. Estrus Synchronization, Blood Sampling, and Insulin Measurement

On day −11 (according to Figure 1), i.e., 23 days after the beginning of this experiment, the estrus synchronization treatment was initiated using intravaginal sponges containing 45 mg of fluorogestone acetate (Chronogest®; Intervet International B.V., Boxmeer, Hol-

land). The sponges were left in place for 10 days. Nine days after sponge insertion (day −3; day 0 = estrus), goats received a single intramuscular dose of 1 mL prostaglandin F2α (0.075 mg D-cloprostenol/goat; Prosolvin-C®, Intervet International B.V., Boxmeer, Holland). Subsequently, on day −32, the sponges were removed. Twenty-four hours later (day −31), five goats from each group were randomly selected for intensive blood sampling. Blood samples were collected, by jugular vein puncture, using sterile tubes (Corvac; Kendall Health Care, St. Louis, MO, USA) every hour for 6 h, starting 3 h after the morning feeding. Serum was separated by centrifugation (1500× *g*, 15 min), decanted, transferred to polypropylene microtubes (Axygen Scientific, Union City, CA, USA), and stored at −20 °C until hormone analysis. Serum insulin concentrations were determined in duplicate by radioimmunoassay (RIA) in blood serum using components of a commercial solid-phase RIA kit (Diagnostic Products, Los Angeles, CA, USA), as previously outlined [27]. Basal insulin levels were quantified as the mean of the lowest points observed during the defined sampling period [28,29]. The intra-assay coefficient of variation value for insulin quantification was 10.0% with a detection limit of 0.2 ng mL^{-1}.

2.4. Ultrasonographic Scanning of Ovarian Activity

Transrectal ultrasonography was performed on all the goats included in the GLUT group (n = 10) and CONT group (n = 10) using a 7.5 MHz linear array transducer (Toshiba Medical Systems Ltd., Crawley, UK). A qualified operator performed the ultrasound scans on day 17 post-estrus, coincident with the end of luteal phase in mid-May, to assess ovarian activity. Each ovary was meticulously examined and the number of corpora lutea (CL) and antral follicles (AFs) were recorded following established procedures [30]. Subsequently, total ovarian activity (TOA) was calculated by summing the recorded numbers of AF and CL from both ovaries in each animal in the experimental group. Together, this composite measurement provided an overall assessment of ovarian activity throughout the study.

2.5. Statistical Analyses

A split-plot ANOVA for repeated measures was used to evaluate the effects of live weight, body condition score, ovulation rate, total ovarian activity, and serum insulin concentrations over time within the same animal. The main plot included treatments, with within-treatment animals as the error term. The subplot consisted of time and time × treatment, which were tested using the residual mean square [31]. Where significant F-values were observed, mean separation was performed using the LSMEANS-PDIFF option of PROC GLM. To assess normality, response variables were subjected to the Shapiro–Wilk test. Log10 transformation was applied to baseline and mean serum insulin concentrations to address the skewness of the data. When a significant interaction between treatment and time was detected, comparisons were made between different time points. These statistical analyses were performed using SAS GLM procedures (SAS Inst. Inc., V9.1, Cary, NC, USA). Pearson correlations were used to examine associations between live weight, body condition score, and number of luteal structures. Non-transformed data are presented for ease of interpretation and are expressed as least squares means ± standard error (SE). The most conservative SE is indicated, and the threshold for statistical significance was set at 0.05.

3. Results

No differences between experimental groups ($p > 0.05$) occurred when comparing LW and BCS, neither at the beginning (29.4 ± 1.02 kg and 3.4 ± 0.17) nor at the end of the experimental period (i.e., 35.13 ± 1.07 kg and 3.4 ± 0.2 units). Significantly greater OR (1.77 ± 0.20; $p < 0.05$) and TOA (4.11 ± 0.47; $p < 0.05$) were present in the GLUT-treated animals compared to the control animals (Table 1).

Table 1. Least squares means of live weight (LW), body condition score (BCS), ovulation rate (OR), and total ovarian activity (TOA) in glutamate-supplemented (GLUT) and non-supplemented (CONT) goat groups at the onset of treatments (INI) and ultrasound scanning (US).

	LW-INI (kg)	BCS-INI (Units)	LW-US (kg)	BCS-US (Units)	OR (Units)	TOA (Units)
GLUT	29.60 [a]	3.40 [a]	35.06 [a]	3.50 [a]	1.77 [a]	4.11 [a]
CONT	29.20 [a]	3.50 [a]	32.20 [a]	3.20 [a]	0.87 [b]	1.87 [b]
SE [1]	1.02	1.07	0.17	0.20	0.20	0.47

[a,b] Columns with distinct superscripts indicate significant differences ($p < 0.05$). [1] Most conservative standard error (SE) is presented.

Concerning the response variable serum insulin concentrations across time, the phenotypic values also favored ($p < 0.05$) the GLUT-treated group. Both the serum insulin concentrations across the experimental period along with the OR and TOA are shown in Figure 2. Positive correlations occurred between LW and BCS ($r^2 = 0.71$; $p < 0.01$), BCS and TOA ($r^2 = 0.7$, $p < 0.05$), and AF and OR ($r^2 = 0.61$, $p = 0.01$), as well as between OR and TOA ($r^2 = 0.87$, $p = 0.001$).

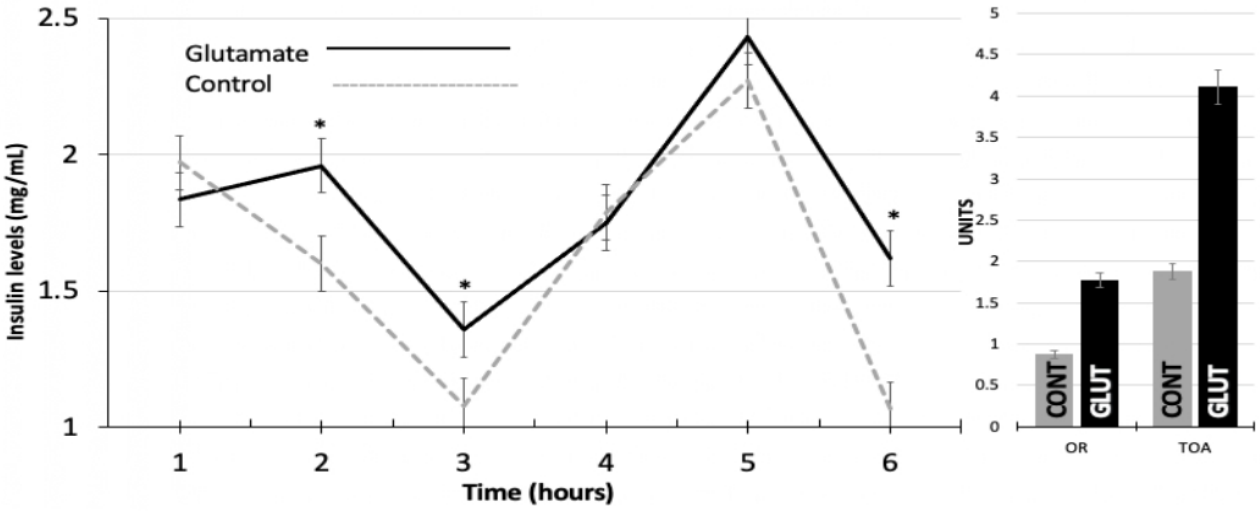

Figure 2. Serum insulin concentrations (mg mL^{-1}) across time (**left panel**) and ovulation rate (units) and total ovarian activity (units) (**right panel**) in glutamate-supplemented (GLUT) and non-supplemented (CONT) goats during the non-breeding season. * Indicates differences ($p < 0.05$) between treatments across time.

4. Discussion

Our research question considered whether exogenous glutamate administration would enhance serum insulin levels linked to augmented ovarian activity in yearling goats during the anestrous season. Certainly, the outcomes support the initial working hypothesis, and we observed that glutamate evokes serum insulin increases over time, enhancing in parallel the out-of-season ovarian activity in yearling goats. It is noteworthy that the differences observed in serum insulin levels among the groups were not influenced by LW and BCS, as there were no variations between the experimental groups for these variables. Based on our research findings, the increase in both serum insulin levels and ovarian activity in glutamate-treated goats indicates that the administration of exogenous glutamate to yearling goats during seasonal anestrous diminishes the inhibitory feedback of estradiol at the hypothalamic level. This, in turn, led to an increase in ovarian activity. This neuroendocrine response suggests an activation of the hypothalamic non-neuronal cells. On the other hand, exogenous glutamate administration suggests an augmented release pattern of the pancreatic release of insulin, accompanied by increases in the ovarian function of the glutamate-supplemented group. A third possible scenario involves a

direct effect of exogenous glutamate on ovarian function, acting as a co-gonadotropin and augmenting, in turn, ovarian function. Undoubtedly, the possible participation of the three previously suggested scenarios may also explain the amplified ovarian response shown by the glutamate-treated goats under the out-of-season reproductive response. These three scenarios generate new research questions while warranting new experimental challenges.

Glutamate is the main excitatory amino acid of the CNS, and its role as a neurotransmitter and neuromodulator is widely recognized in various brain processes [13]. Glutamate is synthesized and utilized as a substrate by various body tissues in key metabolic pathways for the proper functioning of the body [11,12]. Previous studies by our research team have demonstrated the role of exogenous glutamate and its positive effects on reproductive responses through the modulation of the synthesis of key metabolic and reproductive hormones [16–19]. Furthermore, the role of glutamate in the regulation of the function and survival of pancreatic endocrine cells has been unveiled in various animal models. Glutamate serves as a modulator of pancreatic function by interacting with ionotropic receptors located in the cell membrane, including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate receptor. These glutamate receptors are expressed in distinct pancreatic cell types (such as α, β, and γ cells), and upon activation, these receptors induce membrane depolarization by facilitating the opening of ion channels, leading to an influx of calcium ions into the intracellular environment [11,32]. Elevation in extracellular glutamate concentration activates the AMPA and kainate receptors; this activation evokes the stimulation of cGMP production, an increase in adenosine triphosphate (ATP) concentration, and the inhibition of ATP-sensitive K+ channels. Consequently, the plasma membrane is depolarized, and insulin secretion is activated in pancreatic β-cells [33].

In addition to participating in the regulation of glucose, insulin is also involved in the neuroendocrine regulation of the reproductive axis because insulin receptors are expressed along the reproductive axis, while the secretion of both GnRH and LH is stimulated in response to insulin administration [34,35]. Certainly, it has been proposed that insulin potentiates ovarian steroidogenesis in an LH-dependent fashion within ovarian theca cells [35,36]. The key role of insulin in reproductive function has been demonstrated in diabetic patients and in women with functional hypothalamic amenorrhea (FHA) who have hypogonadotropic hypogonadism and low fertility, respectively, with both pathologies coinciding with insufficient levels of insulin secretion [37–39].

The results obtained from our study indicate that the intravenous administration of exogenous glutamate resulted in an elevation of pancreatic glutamate concentration. This increase in glutamate concentration stimulated insulin secretion and may exert effects along with the HPG axis. Specifically, it is plausible that at the ovarian level, glutamate acts as a co-gonadotrophin, thereby enhancing the synthesis of ovarian sex steroids during the normal goat anestrous season at this latitude [36]. While our model supports the aforementioned response as the most compelling, it is important to acknowledge other intriguing pathways involving insulin and glutamate in the regulation of reproduction. Notably, insulin has been observed to exert its effect on non-neuronal populations by stimulating the release of gliotransmitters, specifically prostaglandin E2 (PGE2) [40–43]. This prostaglandin acts on GnRH neurons that express EP2 receptors, enhancing the secretion of GnRH [41,43]. Furthermore, it has been demonstrated that increased glutamate levels interact with astrocytes, leading to an elevation in PGE2 gliotransmission [15]. This mechanism potentially amplifies GnRH secretion, providing an additional link between glutamate and the modulation of reproductive processes.

This suggested mechanism of glutamate action on the pancreas to increase insulin release in conjunction with our results may be explained by the activation of a neuroendocrine pathway involving neuronal and glial cells, activating the reproductive axis. Additionally, exogenous glutamate has the potential to promote the reactivation of ovarian activity during seasonal anestrous, decreasing the inhibitory effect of estradiol negative feedback on the reproductive axis [16]. Under such a scenario, the augmented concentration of

serum insulin observed in our study may have generated a positive influence on ovarian function [36].

Certainly, it is necessary to conduct experiments to determine where exogenous glutamate acts within the reproductive axis. In addition, it is important to investigate whether the plausible effect mainly occurs at the brain level. This becomes particularly significant when considering that the majority of blood metabolites face significant challenges in crossing the blood–brain barrier (BBB) [44,45]. Further studies must, on the one hand, define whether and where glutamate reaches the brain and which neuronal populations might be activated, as well as what type of specific actions are triggered in response to exogenous glutamate. Another research approach could consider pharmacological studies using glutamate agonists and antagonists administered by different routes and therefore be able to better understand the dynamics of exogenous glutamate at different reproductive states. As for the suggested pathway involving the insulin's action on hypothalamic astrocytes, it might be an alternative pathway that may help to explain the action of glutamate and its key role in modulating out-of-season reproductive neuroendocrinology and should therefore be investigated. Considering these findings and merging them with our research outcomes, a hypothetical signaling pathway can be suggested in which exogenous glutamate promotes insulin release, generating, in turn, an augmented ovarian activity through the possible release of gliotransmitters that modulate GnRH and LH secretion, a neuroendocrine scenario previously reported by our research group [19] (Figure 3).

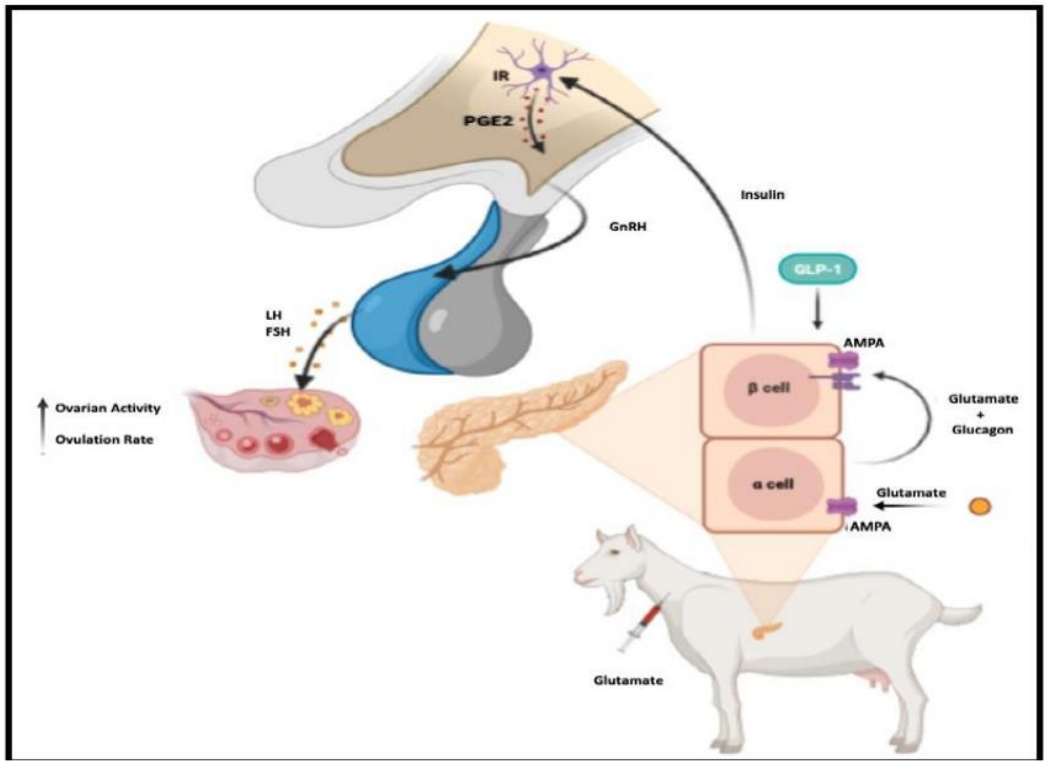

Figure 3. Proposed signaling pathway between insulin and hypothalamic astrocytes in response to exogenous glutamate signaling. The steps are explained in the text. Briefly, intravenous exogenous glutamate acts on glutamate receptors in pancreatic cells, increasing insulin secretion, which in turn enters the brain and binds to its receptor in hypothalamic astrocytes, generating an increase in GnRH and FSH and LH release, improving the ovarian activity in yearling goats during the anestrous season.

Moreover, and from a biomedical and translational perspective, these results generate new opportunities to investigate reproductive failures related to metabolic disorders that coincide with insulin imbalances, as in the case of diabetic patients and women with FHA; additionally, hyperinsulinemia also generates reproductive problems, as in the case of

women with polycystic ovary syndrome [32,35,36]. Therefore, understanding the mechanism of action of the glutamatergic system inside and outside the brain is central to the development of potential drugs to help the improvement of quality of life by decreasing the effects of metabolic imbalances and their deleterious actions upon reproductive function. All the proposed research approaches to solve the way and site of action of both exogenous glutamate administration linked to augmentations in serum insulin concentrations and an augmented ovarian function are, unquestionably, pending research assignments.

5. Conclusions

The obtained research outcomes support our working hypothesis; exogenous glutamate administration promoted an increased insulin concentration over the time, which was accompanied by augmentations in ovarian activity and ovulation rate in yearling dairy-type crossbred goats treated during the seasonal anestrous (i.e., April–June). Interestingly, the observed differences occurred between experimental groups irrespective of LW or BCS along with the experimental period. Understanding the mechanism of action of the glutamatergic insulin system either inside and/or outside the brain, considering a translational perspective, is central to the development of potential drugs that would help to improve quality of life through the lessening of neuro-metabolic imbalances and their interactions as well as their possible effects on ovarian function. Unquestionably, the use of inter-, multi-, and transdisciplinary approaches is certainly required to address such a crucial pending assignment.

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Institutional Review Board Statement: All of the experimental procedures were completed following the recommendations for the ethical use, care, and welfare of animals in research at global (USA; 11) and national (Mexico; 12) levels, and they were institutionally authorized by Chapingo Autonomous University; the animal study protocol was authorized by the Institutional Review Board, with approval reference number UACH-DGIP-REBIZA-IBIODEZA/20-510-400-2.

Informed Consent Statement: Not applicable.

Data Availability Statement: None of the data were deposited in an official repository, yet information can be made available upon request.

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Thesis in joint supervision to obtain the double degree of Doctor of Sciences:

GLUTAMATE SUPPLEMENTATION, ENDOCRINE PROFILE, AND OVARIAN ACTIVITY INTERACTION IN CROSSBREED GOATS DURING THE SEASONAL ANESTROUS

SUPLEMENTACIÓN DE GLUTAMATO, PERFIL ENDOCRINO E INTERACCIÓN DE LA ACTIVIDAD OVÁRICA EN CABRAS CRIOLLAS DURANTE EL ANESTRO ESTACIONAL

CHAPTER VI:

GENERAL CONCLUSIONS

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The use of exogenous glutamate as a modulator of reproductive processes has been studied for several decades. Previous research has demonstrated glutamate ability, as well as that of its agonists, to stimulate the activation of the reproductive axis in different species, mainly through the pre-ovulatory increase of LH. Studies from our research group have demonstrated the positive effect of exogenous glutamate on the activation of the reproductive axis in small ruminants, covering key stages such as puberty and sexual behavior, both in reproductive and seasonal anestrus periods, in both males and females. These results demonstrate the role of glutamate as an innovative and promising alternative to regulate reproductive processes and reduce the negative effects of seasonality.

Considering that seasonal reproduction is a main challenge for goat farming that limits the continuous availability of products, such as milk, meat and other goat by-products, resulting in intermittent production patterns that negatively affect producers' profits. Previously, seasonality was managed through strategies based on the use of exogenous hormones, which, although effective, pose significant risks to human and animal health. This highlights the urgent need to develop sustainable and safe alternatives to address this challenge efficiently.

Our results show that the administration of exogenous glutamate during seasonal anestrus in yearling goats is effective in activating the reproductive axis, resulting in a significant increase in LH levels, reducing the negative feedback effect of E2, increasing the ovulation rate and the number of pre-ovulatory follicles. Glutamate was also observed to promote an increase in serum levels of insulin, a key hormone in several metabolic and reproductive processes. These findings suggest that glutamate may provide an environmentally sustainable strategy to address the challenges associated with

reproductive seasonality, reducing reliance on exogenous hormone-based methods.

From a zootechnical and translational perspective, these results open up new research opportunities, not only in the field of animal reproduction, but also in human pathologies related to metabolic and hormonal imbalances. However, further research is essential, in particular to evaluate the effect of glutamate in males and its potential to enhance the 'male effect'. It is also essential to investigate its influence on production parameters such as milk quality. From a more basic approach, there is still a need to elucidate other issues such as a coherent explanation regarding the passage of glutamate across the blood-brain barrier as well as how it can exert its impact on different brain regions related to reproduction. Furthermore, it is crucial to evaluate and confirm the neurotoxic effect observed in various animal models, as excessive glutamate exposure may significantly affect the reproductive axis at various levels

Building on the above findings and pending research questions emanating from this thesis, a sequence of experiments was designed using a mouse model and considering male and female mice of the C57BL/6J strain. A total of three experiments were performed; the first experiment, considered a chronic treatment with glutamate for 10 days. Male and female mouse were divided into two groups: GLUT and CONT groups. The experimental mice received the treatment, and both olfactory and sexual behavior tests were performed at the end of the treatment. Animals from both groups were then subjected to a mating protocol to assess the effect of glutamate on reproductive performance. The second experiment included an acute glutamate treatment designed to assess neuronal activation in specific brain regions. In addition, LH and PRL concentrations were assessed in glutamate-treated males and females. The third experiment was a chronic treatment similar to the first, but without exposure to behavioral tests. The purpose was to obtain fresh tissue for subsequent evaluation of the expression of genes of interest related to

glutamate effects. Data from these experiments are currently being analyzed. Intergroup comparisons are underway to assess the effects of glutamate treatment on reproductive, neuroendocrine, and gene expression parameters (APPENDIX 1).

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CHAPTER VII:

GRAPHICAL ABSTRACT

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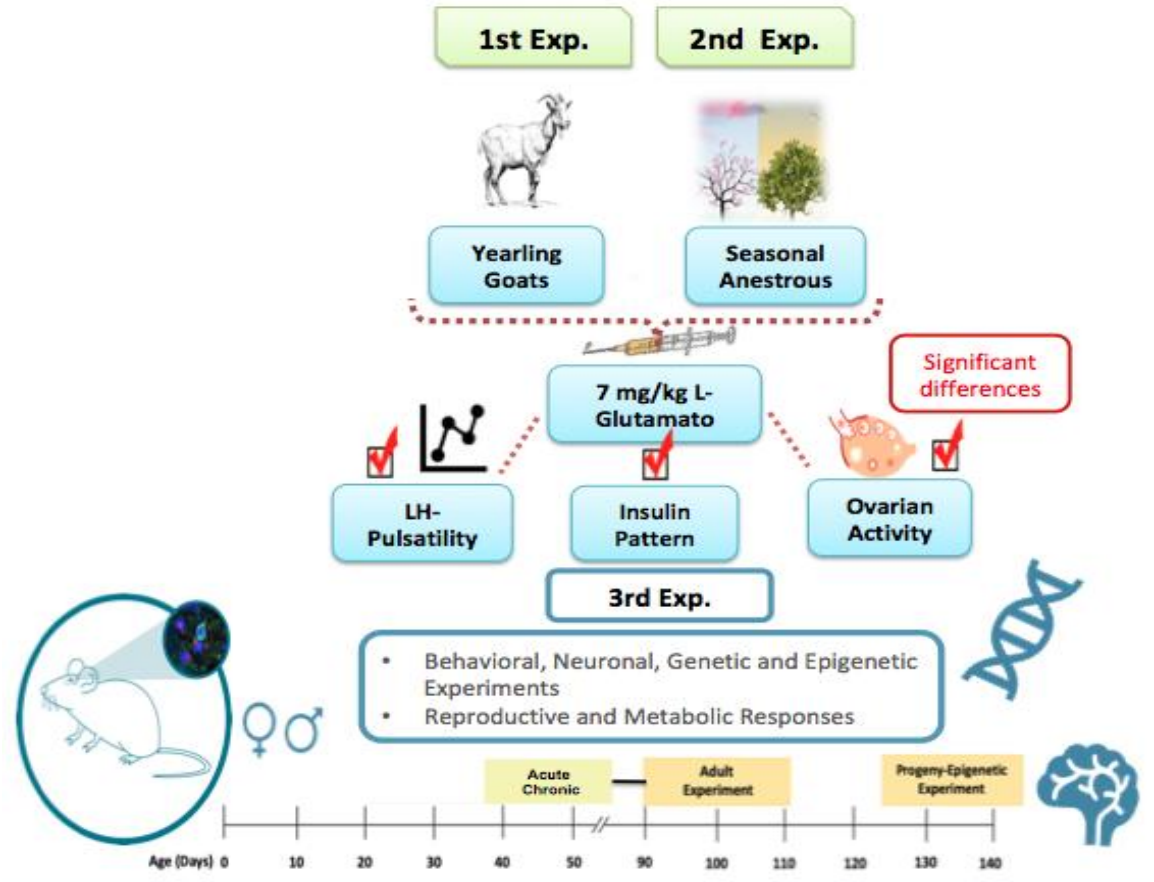

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CHAPTER VIII:

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Thesis in joint supervision to obtain the double degree of Doctor of Sciences:

GLUTAMATE SUPPLEMENTATION, ENDOCRINE PROFILE, AND OVARIAN ACTIVITY INTERACTION IN CROSSBREED GOATS DURING THE SEASONAL ANESTROUS

SUPLEMENTACIÓN DE GLUTAMATO, PERFIL ENDOCRINO E INTERACCIÓN DE LA ACTIVIDAD OVÁRICA EN CABRAS CRIOLLAS DURANTE EL ANESTRO ESTACIONAL

CHAPTER IX:

APPENDIX

APPENDIX

MOUSE MODEL EXPERIMENTS

FIRST EXPERIMENT:

Acute Treatment with Exogenous Glutamate on Neuronal Activation in Reproductive-Related Brain Areas

The objective of this experiment was to evaluate the effect of exogenous glutamate administration at different sacrifice intervals on neuronal activation in reproductive-related brain areas.

A total of 40 C57BL6J mice, 90 days old, were used. The sample consisted of 20 females and 20 males, equally distributed into four experimental groups with 5 subjects each, based on the time interval between glutamate administration and sacrifice:

Groups

Females

- Control group: n = 5 animals
- 30-minute group: n = 5 animals
- 60-minute group: n = 5 animals
- 90-minute group: n = 5 animals

Males

- Control group: n = 5 animals
- 30-minute group: n = 5 animals
- 60-minute group: n = 5 animals

- 90-minute group: n = 5 animals

For the females, daily monitoring of the estrous cycle was performed, and those animals in diestrus phase were selected to receive a dose of L-glutamate (10 mg/kg, intraperitoneally) according to their assigned group.

Males were chosen for L-glutamate administration under the same conditions and assigned to the appropriate group based on the post-administration sacrifice interval.

The experimental groups were defined by the interval between glutamate administration and the sacrifice of the animals (30, 60, or 90 minutes).

The following samples were collected:

- **Blood:** Immediately centrifuged at 3000 rpm for 10 minutes at 4 °C to obtain plasma.
- **Brain**
- **Gonads**

Brains and gonads were post-fixed for 24 hours, transferred to a 30% sucrose solution for cryoprotection, and stored at 4 °C. The obtained plasma was stored at -20 °C.

SECOND EXPERIMENT:

Glutamate Treatment and Reproductive Behavior in C57BL6J Mice.

The objective of this experiment was to evaluate the effect of chronic glutamate treatment on the display of preference behaviors, sexual motivation, and sexual behavior, as well as its impact on reproductive efficiency and potential effects on offspring. A total of 36 C57BL6J mice, 90 days old, were used and divided into two groups of females (n = 9) and two groups of males (n = 9) as follows:

Groups

Females:

- Glutamate group (Glut): n = 9 animals
- Control group (Ctrl): n = 9 animals

Males:

- Glutamate group (Glut): n = 9 animals
- Control group (Ctrl): n = 9 animals

During the treatment period, the body weight of all animals was recorded. The glutamate-treated group received L-glutamate at a dose of 10 mg/kg/day for 10 consecutive days. The control group was administered saline under the same conditions to maintain uniformity between groups.

Behavioral Testing

At the end of the 10-day treatment, males underwent a series of behavioral tests in the following order:

1. **Preference Test** (duration: 5 minutes)
2. **Sexual Motivation Test** (duration: 10 minutes)
3. **Sexual Behavior Test** (duration: 30 minutes)

The first two tests were conducted once, while the sexual behavior test was repeated three times. For these tests, stimulus females were used, which had been hormonally treated with estradiol and progesterone.

For the females, the estrous cycle was monitored throughout the treatment period. At the end of the 10 days, females in the estrus phase were selected to participate in behavioral tests with stimulus males. As with the males, the sexual behavior test was conducted three times, taking the estrous cycle into account.

After completing all behavioral tests, matings were carried out between males and females from both groups. Starting the day after pairing, daily monitoring of the females was conducted to check for the presence of vaginal plugs, which were recorded upon detection.

Eighteen breeding cages were used, with the following mating combinations:

MC: Male Control **MG**: Male Glutamate
HC: Female Control **HG**: Female Glutamate

- **Cage 1**: MC 1 – HC 1
- **Cage 2**: MC 2 – HC 2
- **Cage 3**: MC 3 – HC 3
- **Cage 4**: MC 4 – HC 4
- **Cage 5**: MG 5 – HC 5
- **Cage 6**: MG 6 – HC 6
- **Cage 7**: MG 7 – HC 7
- **Cage 8**: MG 8 – HC 8
- **Cage 9**: MG 9 – HC 9
- **Cage 10**: MG 1 – HG 5
- **Cage 11**: MG 2 – HG 6
- **Cage 12**: MG 3 – HG 7
- **Cage 13**: MG 4 – HG 8
- **Cage 14**: MC 5 – HG 1
- **Cage 15**: MC 6 – HG 2
- **Cage 16**: MC 7 – HG 3
- **Cage 17**: MC 8 – HG 4
- **Cage 18**: MC 9 – HG 9

Daily monitoring of the females was performed to check for the presence of vaginal plugs, with the date recorded upon detection. Males and females remained together from the day of mating until the weaning of the offspring. During this period, the following data were recorded:

- Nest formation
- Date of parturition
- Litter size
- Milk spot in pups
- Weaning date
- Puberty onset date in offspring

THIRD EXPERIMENT:

Chronic Glutamate Treatment on the Expression of Reproduction-Related Genes

The objective of this experiment was to evaluate the effect of chronic glutamate treatment on the expression of genes of interest. A total of 25 C57BL6J mice, 90 days old, were used. They were divided into two groups of females and two groups of males (n=6 per group), distributed as follows:

Females

- **Glutamate group (Glut):** n **=** 6 animals
- **Control group (Ctrl):** n = 6 animals

Throughout the treatment, the estrous cycle of the females was monitored, and treatment initiation required the subjects to be in the diestrus phase.

Males

- **Glutamate group (Glut):** n = 6 animals
- **Control group (Ctrl):** n = 6 animals

Animals in the treated group received L-glutamate at a dose of 10 mg/kg/day for 10 consecutive days. The control group was administered saline solution under the same conditions to ensure parity between groups. Additionally, the body weight of all animals was recorded during this period.

At the end of the 10-day treatment, monitoring of the estrous cycle in females continued. Sacrifice was performed when the females reached the proestrus-estrus phase. For the males, the first two subjects from each group were sacrificed on different days:

- **Control group males:** Sacrificed on day 12.
- **Glutamate group males:** Sacrificed on day 13.

The following samples were collected from the sacrificed subjects:

- **Blood** obtained directly from the trunk.
- **Gonads,** which were weighed and fixed in formalin for further analysis.
- **Fresh brain** samples, which were dissected to extract regions of interest: the **AVPV (anteroventral periventricular nucleus)** and the **ARC (arcuate nucleus).**

Blood samples were centrifuged for 10 minutes at 4°C. The resulting plasma was divided into two vials and stored in a freezer at -20°C. The dissected brain regions were homogenized and subsequently stored in a freezer at -20°C.