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REVIEW ARTICLE

THE ESTROUS CYCLE IN BITCHES: MECHANISMS, INFLUENCING FACTORS, AND CLINICAL RELEVANCE

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ABSTRACT

The estrous cycle of female dogs is a complex, hormonally regulated process comprising proestrus, estrus, diestrus, and anestrus. Its variability is influenced by breed-specific genetics, environmental factors, and microbial dynamics within the reproductive tract. Methods of estrus determination, including vaginal cytology, hormonal assays, ultrasonography, and thermography, remain integral to reproductive management, while emerging artificial intelligence-based approaches offer novel advancements in cycle prediction and monitoring. Despite extensive research on hormonal mechanisms, significant gaps remain in understanding the interplay between endocrine regulation, microbial colonization, and genetic predisposition in cycle variability. The use of hormonal contraceptives and gonadectomy introduces long-term physiological alterations, including chronic luteinizing hormone elevation, which has been linked to metabolic disorders, orthopedic complications, and neoplasia. Additionally, microbial influences on reproductive health, particularly the role of vaginal and uterine microbiota in fertility and disease susceptibility, require further investigation. The prolonged anestrus phase in canines poses a significant challenge for assisted reproductive technologies, limiting the efficacy of artificial insemination and embryo transfer. This review critically examines current methodologies for estrus cycle determination, explores factors contributing to interindividual variability, and evaluates the long-term implications of reproductive interventions on fertility and overall canine health.

Keywords: Female dogs, reproductive phases, reproductive health

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INTRODUCTION

The estrous cycle of female dogs, often interchangeably used with the term reproductive cycle, consists of four well-defined phases: proestrus, estrus, diestrus, and anestrus. Unlike the menstrual cycle in humans, the canine estrous cycle is characterized by distinct physiological and hormonal changes that regulate fertility and reproductive potential. Most bitches are monoestrous, physiologically entering estrus once or twice per year, with cycle length and frequency varying across breeds (Concannon, 2011). Small and toy breeds (e.g., Chihuahuas, Toy Poodles) cycle every 4–6 months, whereas large breeds (e.g., Mastiffs, Great Danes) cycle annually. Certain breeds, such as Basenjis and Tibetan Mastiffs, have ability to display seasonal estrus, typically in spring or autumn (Root Kustritz, 2012). Regulated by the hypothalamic-pituitary-gonadal axis, estrous transitions involve estrogen, progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), influencing ovulation timing and fertility (as discussed in Kutzler, 2007). In addition to hormonal dynamics, vaginal and uterine microbiomes are important for reproductive health. It is well documented that the vagina harbors many bacterial species in contrast to the uterus. Dominant vaginal commensals include Staphylococcus spp., Streptococcus spp., and Escherichia coli (Maksimović et al., 2012). Vaginal mycoplasmas (Mycoplasma spp., Ureaplasma spp.) are also frequently detected and may contribute to subclinical infections, infertility, or pregnancy loss (Maksimović et al., 2018). The uterus of healthy bitches remains a sterile environment despite the consistent presence of bacteria in the vagina (Maksimović et al., 2012). However, hormonal fluctuations can predispose bitches to infections such as pyometra and vaginitis. Another major consideration regarding reproductive system health status is the decision to spay a female dog. Spaying has direct implications on the estrous cycle. Removing the ovaries eliminates the source of estrogen and progesterone, thereby halting the cycle entirely. Veterinarians often recommend early spaying of pet dogs that are not intended for breeding to prevent the inconvenience of heat/estrus periods and avoid reproductive health risks. Nevertheless, a recent study (Kutzler, 2020) highlights concerns regarding gonadectomy (spay/neuter) and its impact on luteinizing hormone (LH) and prolactin regulation. After removal of the gonads, the pituitary continues to secrete LH at high levels due to the loss of negative feedback. This chronic LH elevation may contribute to several long-term health effects in the bitch. Despite the primary hormonal mechanisms governing estrus are well understood, significant variability exists in the timing and duration of each phase across breeds. Further research is needed to elucidate the influence of environmental, genetic, and nutritional factors on these fluctuations (Yaeger and Fales-Williams, 2025). Reproductive technologies such as artificial insemination, embryo transfer, and superovulation remain less effective in dogs than in livestock due to challenges in estrus synchronization, largely attributed to the prolonged anestrus phase (McRae et al., 2025). It remains unclear how fluctuations in vaginal microbiota throughout the estrous cycle may impact fertility and reproductive health, though the extent of their influence on conception rates and pregnancy outcomes (Maksimović et al., 2012; Maksimović et al., 2018; Egyptien et al., 2024). The long-term effects of hormonal contraceptive-induced estrus suppression and gonadectomy warrant further investigation, as some evidence suggests potential impacts on ovarian function and fertility (Bawaskar, Lakde and Raghuwanshi, 2024). In this review, we address these gaps, their influence on the canine estrus cycle, breeding outcomes and long-term reproductive health.

Canine Estrous Cycle

Based on the annual frequency of estrous cycles, mammals are categorized as polyestrous, seasonally polyestrous, or monoestrous. Polyestrous species exhibit a continuous and evenly distributed estrous cycle throughout the year, while seasonally polyestrous species undergo estrous cycles only during specific times of the year (mostly spring, summer or autumn). Domestic dogs (*Canis lupus* *familiaris*) are classified as monoestrous, as they typically experience a single estrus per breeding season, which may occur at any point during the year. The canine estrous cycle is a hormonally regulated process comprising four distinct phases: proestrus, estrus, diestrus, and anestrus, each characterized by specific endocrine, physiological and behavioral changes (Feldman and Nelson, 2004; Concannon, 2011). Determining the phase of the estrus cycle is critical for breeding management, diagnosing reproductive disorders, and optimizing artificial insemination timing. Various techniques, including vaginal cytology, hormone assays, ultrasonography, and thermography, are employed to identify the specific cycle stage. Each method for determining the estrus cycle in dogs has its own advantages and limitations. Vaginal cytology is commonly used due to its affordability and accessibility, while hormonal assays provide more precise ovulation detection. Ultrasonography is useful for confirming ovulation and assessing ovarian health, and infrared thermography offers a non-invasive alternative. Artificial intelligence-based detection uses deep learning to analyze cytology images and accurately predict estrus cycles. It minimizes observer bias and can be integrated into mobile apps for field use, though it requires extensive data training and is still experimental (Rajan et al., 2024).

Vaginal cytology is a widely used method that evaluates the morphology of epithelial cells to determine the estrus cycle stage. Proestrus is characterized by intermediate and superficial cells, while estrus shows a predominance of cornified cells. Diestrus features a sharp decline in superficial cells with increased parabasal cells, and anestrus consists mostly of basal and parabasal cells (Kutzler, 2018). This method is cost-effective but requires expert interpretation. Progesterone and LH testing are gold standards for ovulation timing. A progesterone rise above 2 ng/mL signals ovulation, while the LH peak occurs 24-48 hours before ovulation (Meyers-Wallen, 2007). Though highly accurate, hormonal assays are costly and require laboratory analysis. Ultrasonography allows real-time assessment of ovarian structures. Proestrus presents with the small follicles, estrus shows fluid-filled follicles, and diestrus is marked by corpora lutea formation (Miller et al., 1992). It is a reliable but equipment-dependent method requiring trained personnel. Infrared thermography is a non-invasive method that detects temperature changes in the perineal region, correlating with estrus due to hormonal changes (Olğaç et al., 2017). However, environmental factors can impact accuracy, making it less reliable than hormonal assays.

Phases of Canine Estrus Cycle

Proestrus

Duration: 7–10 days (range: 3–17 days)

Key Characteristics

- Physical/clinical signs: Vulvar swelling, serosanguinous vaginal discharge, and increased attractiveness to males, although females refuse mating (Concannon, 2011).
- Hormonal changes: Rising estrogen levels from developing ovarian follicles stimulate epithelial proliferation in the uterus and vagina (as outlined by Feldman and Nelson, 2004). Estrogen peaks at the end of proestrus, triggering the preovulatory luteinizing hormone (LH) surge (as previously described by Concannon, 2009).
- Ovarian activity: Follicles growth, mature and reach a diameter of 6–10 mm before ovulation (Johnston et al., 2001).
- Vaginal cytology: Predominantly non-cornified epithelial cells and red blood cells (Kutzler, 2018; Root Kustritz, 2006).

Estrus

Duration: 5–9 days (range: 3–21 days)

Key Characteristics

- Behavioral receptivity: The female allows mating, indicated by a posture with an elevated pelvis and deviated tail (as outlined by Feldman and Nelson, 2004).
- Time of ovulation: Occurs 2–3 days after

the LH surge. Oocytes are immature at ovulation and require 48–72 hours for meiosis completion (postovulatory maturation) before fertilization (as previously described by Concannon, 2009).

- Hormonal changes: Estrogen declines sharply after the LH surge, while progesterone rapidly increases, reaching a peak of 15–90 ng/mL during diestrus (Feldman & Nelson, 2004).
- Vaginal cytology: The predominance of superficial cornified epithelial cells (>90%) anuclear or with picnotic nuclei indicates optimal timing for mating (Root Kustritz, 2006; Kutzler, 2007; Kutzler, 2018).

Diestrus

Duration: ~60 days (regardless of pregnancy status)

Key Characteristics

- End of sexual receptivity: The female no longer accepts the male. Vulvar swelling subsides, and serosanguinous discharge ceases. Diestrus is defined as the first day after estrus when the female refuses the male (Feldman and Nelson, 2004; Maksimović, 2010).
- Luteal phase: The corpus luteum secretes progesterone, maintaining levels >2 ng/mL for 30–60 days to support pregnancy (Feldman and Nelson, 2004).
- Progesterone decline: Levels drop to <1 ng/mL by day 60, triggering parturition in pregnant dogs or terminating pseudopregnancy (Concannon, 2009, 2011).
- Pseudopregnancy: Non-pregnant females exhibit a hormonal profile very similar to that of pregnant individuals and may display maternal behaviors (e.g., nest-building, mammary gland development, lactation) (Johnston et al., 2001).
- Health risks: Elevated progesterone increases the risk of pyometra, particularly in older females (Root Kustritz, 2006).
- Vaginal cytology: This phase is characterized by a marked decrease in cornified superficial cells and an increase in non-cornified parabasal and intermediate cells. Additionally, there is a notable influx of neutrophils during beginning of this stage. The abrupt decline in superficial cells, accompanied by the reappearance of parabasal cells and neutrophils, is a hallmark of the diestrual shift (Root Kustritz, 2006; Kutzler, 2018).

Anestrus

Duration: 3–4 months (longer in large and giant breeds)

Key Characteristics

- Reproductive inactivity: Anestrus is a phase of reproductive dormancy with no clinically observable changes or behavioral alterations. Although considered a resting phase, some studies suggest that the ovaries and pituitary gland remain active during this period (Maksimović, 2010).
- Hormonal quiescence: Ovarian inactivity, low estrogen levels (<15 pg/mL), and low progesterone levels (<1 ng/mL) (Johnston et al., 2001).
- Uterine recovery: The endometrium undergoes repair and involution (requires 85-90 days due to the type of placenta) during this phase (Feldman and Nelson, 2004).
- Breed variability: Smaller breeds (e.g., Chihuahuas) may cycle every 4–6 months, while larger breeds (e.g., Great Danes) often have intervals of 8–12 months (Root Kustritz, 2006).
- Vaginal cytology: Anestrus consists mostly of basal and parabasal cells (Root Kustritz, 2006; Kutzler, 2007; Kutzler, 2018).

Machine Learning and Artificial Intelligence-Based Estrous Cycle Prediction in Bitches

The application of machine learning (ML) and artificial intelligence in veterinary reproductive science is rapidly evolving. These advanced computational techniques aim to improve the accuracy and efficiency of estrous cycle detection in bitches, reducing human error and enabling real-time monitoring. Machine learning models can analyze vaginoscopic images, hormonal levels, and physiological indicators to predict different stages of the estrous cycle. The fundamental principle of ML-based estrous prediction is the ability of artificial intelligence systems to detect subtle reproductive changes that may not be easily identified by human observation of clinical signs (Rajan et al., 2024). Artificial intelligence and machine learning hold tremendous potential for revolutionizing estrous cycle prediction in bitches. By incorporating vaginoscopic imaging, hormonal analysis, and cytological evaluation, AI-driven models provide rapid, precise, and objective monitoring of reproductive cycles (Rajan et al., 2024). Although challenges such as data availability and computational costs persist, advancements in deep learning and mobile integration are poised to make artificial intelligence-assisted evaluation or detection, not limited only to estrus cycles, but also to become a standard tool in veterinary practice in the near future.

Algorithms and Methodologies

AI algorithms used for estrus detection often rely on a combination of sensor data, computer vision, and machine learning models. The primary approaches include Computer Vision and Deep Learning, Wearable and Sensor-Based Systems and Time-Series and Predictive Models.

Convolutional Neural Networks (CNNs) and machine vision systems are widely applied for image and video analysis to detect behavioral changes associated with estrus (Neethirajan, 2022). For instance, CNNs can process video feeds to identify increased mounting behavior in cattle or restlessness in dogs, which are common estrus indicators.

Wearable and Sensor-Based Systems - Wearable sensors, such as accelerometers and temperature monitors, are commonly integrated with AI algorithms to track physical activity, body temperature, and posture changes (Sharifuzzaman et al., 2024). These systems can provide continuous, real-time data, improving estrus detection accuracy.

Time-Series and Predictive Models - Recurrent Neural Networks (RNNs) and Long Short-Term Memory (LSTM) networks are also employed to analyze time-series data from sensors, capturing subtle patterns in movement and physiological changes over extended periods (Gulzar and Hussain, 2023).

Natural Language Processing (NLP) for Vocalization Analysis - AI models can analyze vocalizations in animals, such as dogs, which tend to increase during estrus. This less common but emerging approach leverages speech recognition algorithms adapted for animal vocal patterns (Burrai et al., 2024).

Multimodal Data Fusion - Recent studies focus on integrating multiple data sources, including video, sound, and biometric data, to enhance predictive accuracy. This approach reduces false positives and negatives by cross-referencing different estrus indicators (Mohebbi et al., 2025).

Limitations and Challenges

Despite their promise, AI-based estrus detection systems face several challenges. The setup for AI systems, including sensors, cameras, and computational infrastructure, can be prohibitively expensive for small-scale farms (Amer et al., 2024). AI models rely on vast amounts of high-quality data for training. Inconsistent or insufficient data can lead to poor model performance and unreliable predictions (Nair, 2024). Maintaining AI systems requires technical expertise, regular updates, and robust cybersecurity to protect sensitive animal data (Zhang et al., 2024). There are ongoing debates about animal privacy, the ethical implications of continuous monitoring, and the potential stress on animals due to wearable devices (Gupta, 2024).

AI systems can be sensitive to environmental conditions, which may affect their reliability. For instance, weather, barn design, and herd size can all impact sensor performance (Cho and Kim, 2023).

Hormonal Factors Influencing the Estrous Cycle

The regulation of the estrous cycle in dogs is controlled by a complex interplay of endocrine signals involving the hypothalamic-pituitary-gonadal (HPG) axis. The major hormones responsible for coordinating estrous transitions include gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), estrogen, progesterone, and prolactin (as previously described by Concannon, 2009). Hormones regulating estrus cycle in dogs are presented in Table 1.

Hypothalamic Control and Gonadotropin Regulation

The cycle begins with GnRH release from the hypothalamus, which stimulates the anterior pituitary to secrete LH and FSH. These gonadotropins promote follicular development and maturation as well as estrogen synthesis within the ovaries. LH surge is the key trigger for ovulation, occurring approximately 48 hours before follicular rupture and oocytes release. Studies have shown that LH concentrations exhibit pulsatile release during anestrus, but increase in frequency as proestrus begins, reaching peak values just before ovulation. FSH, while also involved in follicular recruitment, appears to play a more limited role in ovulation in dogs compared to other species. The FSH profile during the cycle remains relatively stable except for a minor peak at the beginning of proestrus (Concannon, 2009).

Estrogen and Progesterone Dynamics

Estrogen, primarily estradiol-17β, is synthesized by the granulosa cells of ovarian follicles in response to FSH and plays a crucial role in stimulating the behavioral and physiological signs of estrus. Estrogen levels rise throughout proestrus, peaking just before the LH surge, then decline sharply as ovulation occurs. High estrogen levels induce the characteristic cornification of vaginal epithelial cells, which is used clinically to determine estrous status through cytology (Concannon, 2009; Kutzler, 2007). Progesterone, produced by the corpora lutea after ovulation, maintains pregnancy and regulates cycle length. In the dog, unlike most mammals, progesterone secretion begins before ovulation, concurrent with the LH surge (Concannon, 2009). Serum progesterone concentrations increase significantly after ovulation, reaching peak levels during diestrus, and decline gradually unless pregnancy occurs (Hinderer et al., 2021). Progesterone's early rise makes it a valuable marker for ovulation timing in breeding management (Concannon, 2009).

Prolactin and Luteal Function

Prolactin, a pituitary hormone, plays an essential role in luteal maintenance in the dog. Unlike many species where luteal support depends on LH, prolactin becomes the primary luteotropic hormone after 20 days of estrus (Concannon, 2009). Experimental suppression of prolactin via dopamine agonists (e.g., cabergoline) leads to premature luteolysis and shortened diestrus, which is why prolactin inhibitors are commonly used for estrus induction in bitches with prolonged anestrus (as discussed in Kutzler, 2007).

Influence of Thyroid Hormones on Estrous Regulation

There is increasing evidence that thyroid dysfunction affects estrous cycling in bitches. Hypothyroidism, a common endocrine disorder in dogs, has been associated with prolonged anestrus, irregular cycles, and reduced fertility. The mechanism is likely linked to thyroid hormones' regulatory role in metabolic and reproductive function, including ovarian steroidogenesis (Hinderer et al., 2021).

Role of Cortisol and Stress in Cycle Disruptions

Elevated cortisol, a marker of stress, has been shown to interfere with pulse frequency of GnRH and LH secretion, leading to suppressed estrous cycles. Chronic stress and high glucocorticoid levels may prolong anestrus or prevent normal follicular development, highlighting the importance of minimizing stressors in breeding animals (Calabrò et al., 2021).

Estrous Cycle Variability - Hormonal, Microbial, Genetic, and Environmental Factors

The domestic female canine is a non-seasonal, monoestrous species with an interestrus interval averaging about 7 months (31 weeks), but with high individual variability (range ~4–13 months) (Concannon, 2009). This variation arises largely from differences in the length of anestrus, which have a genetic basis (Kutzler, 2007). Some bitches have consistent cycle intervals (e.g. every 6–7 months), whereas others exhibit irregular timing over their lifetime. At least one breed (the Basenji) has retained a seasonal, photoperiod-regulated cycle, typically coming into heat only once annually in the autumn (Concannon, 2009). In most breeds, however, photoperiod is not overtly entrained to the cycle, though an intrinsic "free-running" circannual rhythm is suspected to influence cycling. Environmental cues can still play a role – for example, housing intact females together can sometimes lead to synchronized or altered cycle timing (towards dominate alpha female), suggesting pheromonal stimulation among them (Concannon, 2009). Extreme stress or poor body condition may also delay or disrupt estrous cycling, while optimal nutrition supports regular cycles (Calabrò et al., 2021). Notably, the uterine lining requires ~135 days post-estrus for complete histologic repair, so attempting to induce a new cycle too soon can reduce fertility (Kutzler, 2007). Thus, both innate hormonal rhythms and external factors (season for certain breeds, pheromones, stress, nutrition) contribute to estrous cycle variability in dogs.

Microbial Factors

In healthy bitches, the reproductive tract harbors a resident microbiome throughout the cycle (Maksimović et al, 2012; Lyman et al., 2019). Disruptions or infections in this microbial ecosystem can impair fertility. Certain pathogens are recognised as significant contributors to infertility in dogs. Among these, Brucella canis, a zoonotic bacterium, is particularly notable for causing abortions and persistent reproductive failure (Graham and Taylor, 2012). In contrast to opportunistic bacteria, *B. canis* stands out as a notable primary pathogen of canine infertility (Graham and Taylor, 2012). Routine screening for *B. canis* and prompt treatment of uterine/vaginal infections are, therefore, essential in breeding animals (Pretzer, 2008). While *Neospora caninum* is a well-known pathogen in cattle, its impact on canine reproductive health should not be overlooked. Vertical transmission, pregnancy complications, and the risk of congenital infections highlight the importance of monitoring and preventive measures in breeding dogs (Dubey 2003; Dubey et al., 2007). Maksimović et al. (2012) investigated the vaginal and uterine bacterial flora in clinically healthy bitches during different stages of their reproductive cycle. The most identified bacteria were *Streptococcus spp*., coagulase-negative *Staphylococcus spp*., and *Escherichia coli*. The study found that bacteria were isolated from 77.5% of vaginal swabs, with isolates more frequently detected in pure culture than in mixed cultures. It was previously proposed that vaginal microbiome is usually mixed in clinically healthy bitches, and the isolation of bacteria in pure culture suggests pathogens have overgrown normal flora (Olson et al., 1986; van Duijkeren, 1992). Maksimović et al. (2012) observed that bacterial isolates were more frequently obtained in pure culture during proestrus and anestrus, whereas mixed bacterial cultures were predominantly detected during estrus and diestrus. These findings suggest that the isolation of a pure bacterial culture does not inherently indicate an infectious process but may instead reflect normal variations in the vaginal microbiota across different stages of the estrous cycle. Notably, all uterine samples tested in the study were negative for bacterial growth, reinforcing the notion that the uterus of healthy bitches remains a sterile despite the consistent presence of bacteria in the vagina. In contrast, Paudel et al. (2023) focused on bitches diagnosed with pyometra, a common reproductive disorder characterized by uterine infection. The study revealed that *E. coli* was the most prevalent pathogen, isolated in 35.55% of cases, followed by *Pseudomonas spp*. (26.66%). More recently, Xavier et al. (2024) conducted a study analyzing clinical data, histopathological alterations, and microbiological findings in dogs with pyometra. The study revealed that *E. coli*, especially strains belonging to phylogroup B2, were the most common isolates. These strains harbored virulence genes associated with adhesion (fimH, focG, and papC) and serum resistance (traT). Notably, the presence of the papC gene was linked to higher necrosis scores in the endometrium. These findings suggest that papC-positive *E. coli* strains play a significant role in the severity of pyometra in dogs. The identification of specific virulence factors, such as papC, in *E. coli* isolates from pyometra cases underscores the importance of targeted therapeutic strategies. Recognizing the association between these virulence genes and disease severity can aid veterinarians in prognostic assessments and in tailoring more effective treatment plans for affected dogs.

Hormonal Factors

Proper endocrine function underlies normal cycling, coordinated rise and fall of progesterone, estrogen, LH, FSH, and prolactin drive cycle transitions (Concannon, 2009). Even subtle hormonal imbalances (e.g. hypothyroidism or luteal insufficiency) can lengthen interestrus period or cause pregnancy failure. For example, inadequate progesterone support in pregnancy (hypoluteoidism) leads to embrionic death and resorption or abortion if unrecognized (Hinderer et al., 2021).

Genetic Factors

Influence the onset and frequency of reproductive cycles in dogs. Breed and lineage differences play a significant role. Smaller breeds typically reach puberty earlier and cycle more frequently, exhibiting two or even three heats annually. In contrast, giant breeds or specific lineages may cycle only once per year. Research indicates that within the same litter, some bitches consistently experience longer anestrus phases than others, suggesting heritable variations in cycle length. Bitches with unusually long interestrus intervals naturally have fewer breeding opportunities and may display lower lifetime fertility. Conversely, very short interestrus intervals (less than approximately 4-5 months) can lead to uterine disease due to insufficient post-estrus involution, reducing fertility if breeding occurs too frequently (Kutzler, 2007).

Environmental Factors

Just like photoperiod, pheromones, management, and nutrition are crucial. A study found that improving the diet of breeding bitches with more protein sources, essential fatty acids, and various vitamins for two months before estrus led to better estrus presentation and early pregnancy outcomes (Calabrò et al., 2021). This highlights the importance of balanced nutrition and health in cycle regularity and fertility.

Table 1 Hormones involved in the estrus cycle of dogs

Hormone	Origin	Function	Level During Estrus Stages
Estrogen	Ovaries (Graafian follicles)	Stimulates the development of follicles in the ovaries, promotes sexual receptivity, and triggers physical signs of estrus (e.g., vulvar swelling, bloody discharge).	Proestrus: High, rising to peak; Estrus: Peaks then drops sharply; Diestrus: Low; Anestrus: Low

Hormone	Origin	Function	Level During Estrus Stages
Progesterone	Ovaries (Corpus luteum)	Maintains pregnancy, supports the development of the endometrium, and inhibits further estrous cycles.	Proestrus: Low; Estrus: Starts to rise; Diestrus: High, peaking mid-diestrus; Anestrus: Low
Luteinizing Hormone (LH)	Pituitary gland (anterior lobe)	Triggers ovulation and formation of the Corpus luteum, critical for the release of the mature egg.	Proestrus: Low; Estrus: Peaks sharply around the time of ovulation; Diestrus: Low; Anestrus: Low
Follicle-Stimulating Hormone (FSH)	Pituitary gland (anterior lobe)	Stimulates the growth and maturation of ovarian follicles and increases estrogen production.	Proestrus: Moderate, supporting follicle growth; Estrus: Drops just before ovulation; Diestrus: Low; Anestrus: Low
Prolactin	Pituitary gland (anterior lobe)	Supports milk production and maternal behaviors, also plays a role in maintaining the Corpus luteum in some species.	Proestrus: Low; Estrus: Low; Diestrus: Moderate to high (especially in pregnant dogs); Anestrus: Low
Oxytocin	Pituitary gland (posterior lobe)	Stimulates uterine contractions during labor and milk letdown during nursing.	Proestrus: Low; Estrus: Low; Diestrus: Low; Anestrus: Low; Parturition: High

Long-Term Effects of Hormonal Contraceptives and Gonadectomy on Reproductive Health

Recent research of Kutzler (2020) highlights concerns regarding gonadectomy (spay/neuter) and its impact on luteinizing hormone (LH) regulation. After removal of the gonads, the pituitary continues to secrete LH at high levels due to the loss of negative feedback. This chronic LH elevation may contribute to several long-term health effects, including:

- Urinary incontinence: Higher LH levels post-gonadectomy are associated with estrogen-sensitive urinary sphincter incompetence, particularly in female dogs.
- Orthopedic disorders: Persistently elevated LH may affect joint development, increasing the risk of cranial cruciate ligament rupture and hip dysplasia.
- Obesity and metabolic changes: LH elevation may contribute to altered metabolism and weight gain in neutered animals.
- Neoplasia: Hormone-sensitive cancers such as lymphoma, hemangiosarcoma, and mast cell tumors have been linked to early-age gonadectomy, possibly due to prolonged LH stimulation of non-gonadal tissues.

Given these potential risks, Kutzler (2020) suggests that veterinarians and breeders consider alternative sterilization methods, such as ovary-sparing spay (hysterectomy without ovary removal) or vasectomy in males. These techniques prevent reproduction while preserving natural hormone regulation, mitigating some of the negative effects linked to LH hypersecretion. Integrating these findings into reproductive management strategies can help optimize canine health while maintaining effective population control.

CONCLUSION

The estrous cycle in female dogs consists of four hormonally regulated phases: proestrus, estrus, diestrus, and anestrus. Cycle variability is influenced by genetic, environmental, microbial, and endocrine factors. While traditional methods such as vaginal cytology and hormonal assays remain essential for estrus detection, artificial intelligence-based approaches offer promising advancements in cycle prediction.

Despite extensive research on hormonal regulation, gaps remain in understanding the interplay between endocrine function, microbial colonization, and genetic predisposition. The vaginal and uterine microbiome significantly impact reproductive health, with pathogens like *Escherichia coli* and *Brucella canis* linked to infertility and disorders, such as pyometra.

The prolonged anestrus phase complicates assisted reproductive technologies, limiting the success of artificial insemination and embryo transfer. Additionally, hormonal contraceptives and gonadectomy result in chronic luteinizing hormone elevation, increasing the risk of metabolic disorders, orthopedic issues, and hormone-sensitive neoplasia. Alternative sterilization methods, such as ovary-sparing spay, may help mitigate these risks.

Further research is needed to clarify the complex interactions between endocrine regulation, microbial dynamics, and genetic factors influencing cycle variability. Advancements in artificial intelligence-driven estrus detection and reproductive management strategies will be key to optimizing fertility and overall canine health.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTIONS

Concept and Design – AM, BP, BČ; Supervision – AM, BČ; Literature review, analysis and interpretation of data - AM, SF, BP,BČ; Writing and Critical review - AM, SF, BP, BČ

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ESTRUSNI CIKLUS KOD KUJA: MEHANIZMI, FAKTORI UTJECAJA I KLINIČKI ZNAČAJ

SAŽETAK

Estrusni ciklus ženki pasa je složen, hormonalno reguliran proces koji obuhvata proestrus, estrus, diestrus i anestrus. Njegova varijabilnost je pod utjecajem genetskih faktora pasmine, sredine i mikrobne dinamike reproduktivnog trakta. Metode otkrivanja estrusa, uključujući vaginalnu citologiju, hormonske eseje, ultrazvuk i termografiju i dalje predstavljaju osnove upravljanja reproduktivnim zdravljem, dok noviji pristupi zasnovani na vještačkoj inteligenciji omogućavaju napredak u polju predviđanja ciklusa i praćenja. Uprkos opsežnom istraživanju hormonskih mehanizama, postoji značajan raskorak u razumijevanju odnosa između endokrine regulacije, mikrobne kolonizacije i genetske predispozicije u varijabilnosti ciklusa. Upotreba hormonalnih kontraceptiva i gonadektomija izazivaju dugotrajne fiziološke promjene, uključujući porast luteinizirajućeg hormona povezanog s metaboličkim poremećajima, ortopedskim komplikacijama i neoplazijama. Pored toga, utjecaji mikroba na reproduktivno zdravlje, posebno ulogu vaginalnog i uterinog mikrobioma na fertilnost i prijemčivost za bolest, zahtjevaju daljnja istraživanja. Prolongirana faza anestrusa kod pasa predstavlja značajan izazov za tehnologije potpomognute oplodnje ograničavajući efikasnost vještačke oplodnje i embriotransfera. Ovaj pregled daje kritički osvrt na postojeće tehnologije otkrivanja estrusnog ciklusa, razmatra faktore koji utječu na interindividualnu varijabilnost i evaluira dugotrajne implikacije reproduktivnih intervencija na fertilnost i cjelokupno zdravlje pasa.

Ključne riječi: Reproduktivne faze, reproduktivno zdravlje, ženke pasa

RESEARCH ARTICLE

EVALUATING THE IMPACT OF SEASON AND BREEDING AREA ON BLOOD PARAMETERS IN PRAMENKA SHEEP EXPOSED TO THERMAL STRESS CONDITIONS

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ABSTRACT

This study aimed to investigate seasonal variations in blood parameters of two Pramenka sheep strains, Dubska and Hercegovacka, across and within four areas in Bosnia and Herzegovina under thermal stress conditions. It included 48 sheep from Vlasic and Kupres (breeding areas of the Dubska strain), and Nevesinje and Podvelezje (breeding areas of the Hercegovacka strain). The investigation was conducted across both summer and winter seasons, encompassing a total of eight herds (two per area). Hematological parameters included erythrocyte, leukocyte, and platelet counts, mean platelet volume, the total count of granulocytic and agranulocytic leukocytes, hemoglobin concentration, red blood cell distribution width and erythrocyte parameters. Biochemical analyses covered total bilirubin, total cholesterol, glucose, urea, creatinine, creatine kinase, non-esterified fatty acids, beta-hydroxybutyrate, proteins, liver enzymes and electrolytes. Results revealed significantly higher values of red blood cell parameters during the summer period in the area of Nevesinje ($P<0.05$) compared to the Podvelezje area. Additionally, it's important to emphasize significantly increased levels of basophils during the summer season across all areas, except for Vlasic, when compared to the winter season. Furthermore, the amounts of total proteins, albumin and globulin were significantly increased in all areas during the summer compared to winter. The study findings underscore the substantial influence of the breeding area, encompassing its geological composition, spatial distribution, and land cover, on specific blood parameters investigated in sheep during periods characterized by adverse temperature conditions.

Keywords: Blood parameters, sheep, Pramenka, thermal stress, season

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INTRODUCTION

Animal husbandry serves as the primary source of animal protein, including eggs, meat, and milk, crucial for the sustenance and nutrition of impoverished households in low- and middle-income countries. Additionally, it provides a primary or supplementary source of income for farmers (Leroy et al., 2022). Domestic sheep, particularly autochthonous breeds, are widely distributed species around the world. Due to their distinct biological traits, such as short gestation periods, high fertility, quick development rates, high feed conversion efficiency, and high disease resistance, certain animal types are commercially of particular importance in this context (Adams and Ohene-Yankyera, 2014). In Bosnia and Herzegovina (BiH), the importance of sheep breeding is constantly rising. The autochthonous Pramenka breed is predominantly represented in BiH and Serbia, though it is also reared across the Balkans. Historically, the Pramenka breed was widespread throughout Europe, however, it has been largely replaced by breeds with more pronounced productive performance. Originating from the mouflon (*Ovis musimon*), as indicated by Cinkulov et al. (2008), this breed offers significant economic advantage and exhibits remarkable resilience, thriving even in demanding conditions related to feeding and housing, as highlighted by Katica et al. (2004). According to data sourced from the Breeding Program for Sheep in the Federation of BiH, there are four distinct autochthonous strains of Pramenka sheep in BiH, namely: Dubski, Privorski, Kupreski, and Hercegovacki (Breeding Program for Sheep in the Federation of Bosnia and Herzegovina, 2018). However, Adilovic and Andrijanic proposed a different classification, identifying two additional strains, i.e., the Podveleski and Varcarski strains (Adilović and Andrijanić, 2005). Animal husbandry, especially the breeding of resilient autochthonous Pramenka breed, plays a crucial role in supporting the livelihoods of farmers in BiH, since these sheep are renowned for their genetic and phenotypic traits. However, while these traits are well-documented and have been pivotal in their widespread use, the effects of climate change on these valuable characteristics remain largely unknown.

The increasingly pronounced effects of climate change present a significant threat globally, affecting both human well-being and livestock productivity. The Intergovernmental Panel on Climate Change (IPCC) reports a steady increase in Earth's temperature by 0.2℃ per decade, with projections indicating a potential rise in the global average surface temperature to between 1.4℃ and 5.8℃ by 2100 (IPCC, 2023). Climate change impacts animal husbandry through direct effects like heat stress, extreme weather events, and reduced productivity, especially in "water-stressed regions", as well as indirect effects such as increased water requirements, the emergence of new diseases, and economic disruptions (Godde et al., 2021). BiH follows this global trend with projected temperature increases. Climatological analyses suggest a trajectory of temperature elevation until 2030 compared to the baseline period of 1961-1990, with an anticipated annual increase of up to 1.0°C across the entire state territory (Radusin et al., 2022). These changes pose challenges to animal husbandry, including sheep farming, altering climate patterns and affecting ruminants through physiological changes. Thermal stress, a prominent consequence of climate change, affects livestock when ambient temperatures exceed upper or lower critical thresholds for the internal temperature of domestic animals, leading to heat or cold stress (Collier et al., 2019). Understanding how climate change influences the favorable Pramenka traits is essential, as rising temperatures, altered precipitation patterns, and increased frequency of extreme weather events could significantly impact their health and productivity, as previously demonstrated (Cwynar et al., 2014; Rana et al., 2014). Inspired by similar study designs, we focused on evaluating how seasonal variations affect the hematological and biochemical parameters of two Pramenka strains, Dubska and Hercegovacka, across different breeding areas of BiH under thermal stress conditions.

MATERIAL AND METHODS

Ethics Committee Approval

This study is under the Law of Protection and Welfare of Animals, ("Sl. glasnik BiH", no. 25/2009 and 9/2018), and approved by the Ethical Committee of the University of Sarajevo - Veterinary Faculty (no. 01-02-18-27/20).

Study area and sample collection

This study was conducted in two distinct regions of BiH, focusing on Dubska and Hercegovacka strains of Pramenka sheep. Dubska strain was examined in the central region, including Vlasic mountain (Mudrike village - 44.306747°N,17.536004°E) and Kupres plateau (Zvirnjaca village - 43.824834°N, 17.365428°E), involving four distinct flocks. Figure 1 illustrates the spatial representation and distribution of the agricultural zones, forested regions and semi-natural habitats of Mudrike and Zvrinjaca villages. Conversely, the Hercegovacka strain was examined in the southern region, covering Podvelezje (Kruzanj village - 43.281656°N, 7.950202°E) and Nevesinje areas (Ziljevo village - 43.256564°N, 18.124937°E), with two flocks in each area. Figure 2 provides a spatial distribution of urban, agricultural and forested land cover categories in the areas of Kruzanj and Ziljevo villages. The geographical selection criteria included altitude (Kupres and Vlasic), above-average summer and winter temperatures, and the presence and abundance of the Pramenka sheep strains in these areas.

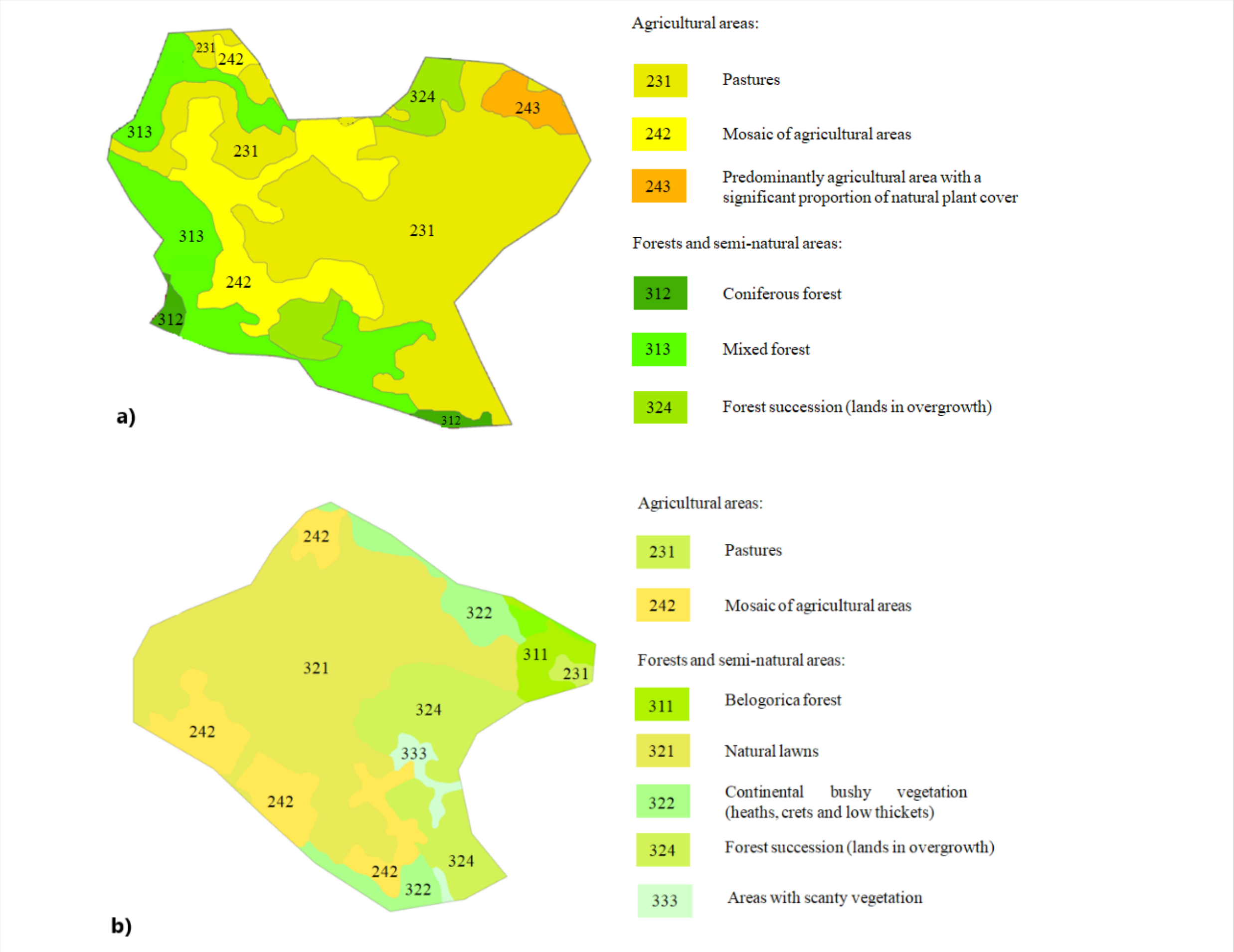

Figure 1 Spatial distribution of land cover and land use patterns in Vlasic (a) and Kupres (b) areas based on Corine Land Cover (CLC) data

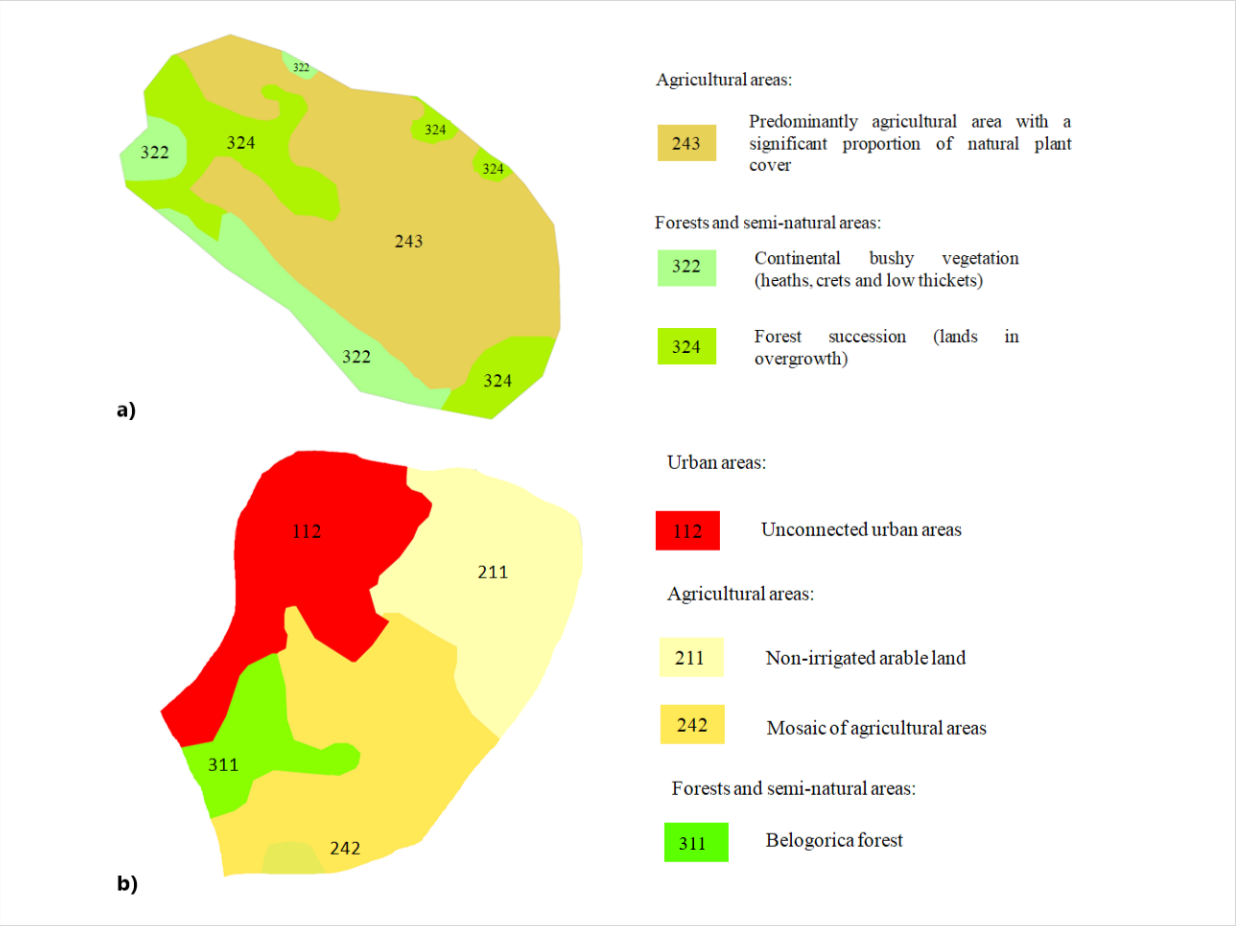

Figure 2 Spatial distribution of land cover and land use patterns in Podvelezje (a) and Nevesinje (b) areas based on Corine Land Cover (CLC) data

Sampling was performed using non-probabilistic approach with two-stage sampling design within each flock. Six healthy female sheep aged between one and six years were randomly selected from each herd. Sheep were raised on pasture during the summer and were provided with hay and silage during the winter, with *ad libitum* water access. Twelve blood samples were collected from each locality during each sampling period, resulting in 24 samples per strain across the two periods (summer and winter). In total, 48 samples were obtained for each of the two examined strains, amounting to 96 sheep blood samples overall. Blood samples were obtained from the jugular vein, utilizing 3 ml EDTA tubes (Ayset® Tube, EDTA 3K), 3.5 ml serum separation tubes (BD Vacutainer®SST™ II Advance), and 2 ml test tubes for glucose concentration (BD Vacutainer® containing NaF and Na_2EDTA). Blood samples intended for hematological analysis were stored at +4°C, while serum samples for biochemical analysis were stored at -20°C until laboratory processing.

Climatological data measurements

Air temperature and relative humidity data were sourced from the Federal Hydrometeorological Institute of Bosnia and Herzegovina for the Vlasic, Kupres and Podvelezje areas, and from the Republic Hydrometeorological Institute of Banja Luka for the Nevesinje area. Data were collected throughout the summer and winter seasons, as well as for the five days preceding blood sampling. To evaluate the influence of temperature on the hematological and biochemical blood parameters, the data on air temperature and relative humidity was used to calculate the Temperature-Humidity Index (THI), according to Finocchiaro et al. (2005):

$$THI = T - \left[\frac{0.55x(1 - RH)}{100}\right]x(T - 14.4)$$

where T stands for air temperature expressed in ℃; RH stands for relative air humidity (%).

The obtained values indicate the following: <22.2 = absence of heat stress; 22.2 to <23.3 = moderate heat stress; 23.3 to <25.6 = severe heat stress and 25.6 and more = extreme severe heat stress (Marai et al., 2007). According to Ramón et al. (2019) and Carabaño et al. (2021), the threshold value for cold stress was set at 9.8. More detailed information and a more comprehensive methodological approach regarding the climatological data are provided in our previously published study (Ohran et al., 2024).

Blood hematological and biochemical measurements

Hematological parameters assessed included the number of erythrocytes (RBC), hemoglobin concentration (Hb), packed cell volume (PCV), mean value of erythrocyte volume (MCV), mean value of hemoglobin concentration in the erythrocyte (MCHC), mean value of the amount of hemoglobin in the erythrocyte (MCH), red blood cell distribution width (RDW), total number of leukocytes (WBC), total number and percentage of neutrophils (Ne), eosinophils (Eo), basophils (Ba), lymphocytes (Ly) and monocytes (Mo). Additionally, platelet count (PLT) and mean platelet volume (MPV) were measured. Hematological analyses were performed using an ADVIA 120 automated veterinary hematology analyzer (Siemens, USA). Biochemical parameters analyzed included total protein (TP), albumin (ALB), globulin (GLO), total bilirubin (TB), total cholesterol (TC), glucose (GLU), urea (UREA), creatinine (CRE), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), creatine kinase (CK), non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), calcium (Ca), phosphorus (P), sodium (Na), chlorine (Cl) and magnesium (Mg). These analyses were conducted using an Olympus AU400 automated chemistry analyzer (Beckman Coulter, USA). The interpretation of hematological and biochemical blood parameters was based on reference values provided by Aiello et al. (2016).

Statistical analysis

To assess the differences in hematological and biochemical blood parameters between the examined areas, a one-way analysis of variance (ANOVA) was employed. For *post hoc* comparison between the groups, the Tukey's test was used (SPSS Statistics version 20, IBM Corp, USA). To compare hematological and biochemical parameters between seasons within the same area (summer vs. winter), a paired t-test was applied, as these are dependent samples (i.e., the same individuals observed in different seasons) (MedCalc Software Ltd. version 20.115, Belgium). The threshold for statistical significance was set a $P < 0.05$.

RESULTS

Climatological data

The climatological data for the examined areas are shown in Table 1. THIavg in investigated areas indicated no significant heat stress, except for Podvelezje, where mild heat stress was observed during summer. However, THIavg-5daysBC and THImax-5daysBC indicated strong to extremely strong heat stress observed during summer in the Podvelezje and Nevesinje areas, breeding areas of the Hercegovacka strain. Conversely, during winter, nearly all THI readings indicated cold stress in all areas, with extremely severe conditions in Vlasic and Kupres, breeding areas of the Dubska strain.

Table 1 Climatological data of the examined areas

	Hercegovacka strain				Dubska strain			
	PODVELEZJE		NEVESINJE		VLASIC		KUPRES	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
AvT (°C)	20.59	6.76	20.10	5.34	15.08	1.00	15.77	2.11
AvRH (%)	48.73	44.20	49.47	51.72	81.60	61.60	81.53	86.90
THIavg	22.22	4.95	21.62	2.82	15.38	-3.46	16.38	-3.70
MaT-5daysBC	29.76	10.61	31.55	10.55	30.45	7.70	29.39	5.99
MiT-5daysBC	15.56	-0.49	12.00	-3.80	2.85	-10.10	5.11	-8.89
AvRH-5daysBC	54.84	45.23	58.94	65.57	70.43	86.41	70.03	85.85
RHmax-5daysBC	100.00	79.03	94.50	77.57	99.02	99.82	98.93	99.45
THIavg-5daysBC	24.39	1.25	24.01	-1.51	16.58	-10.31	16.99	-11.31
THImax-5daysBC	38.12	8.98	40.37	8.93	39.10	4.06	37.46	1.44

Summer: June–September; Winter: November–February

AvT – average temperatures throughout the summer and winter season; AvRH – average relative humidity during the entire summer and winter season; THIavg –THI calculated according to the average values of temperature and relative humidity during the entire summer and winter season; AvT-5daysBC – average temperature values five days before collection; MaT-5daysBC − maximum temperature values five days before collection; MiT-5daysBC − minimum temperature values five days before collection; AvRH-5daysBC – average values of relative humidity five days before collection; RHmax-5daysBC − maximum values of relative humidity five days before collection; THIavg-5daysBC − THI calculated from the average values of temperature and relative humidity five days before collection; THImax-5daysBC − THI calculated with maximum values of temperature and relative humidity five days before collection.

Hematological parameters

Most parameters of the erythrocyte lineage, including RBC, Hb, PCV, MCH and RDW, in all investigated areas were within the reference values, even under conditions of thermal stress. However, during summer, a significant increase in Hb content, MCH, and MCHC were noted only in the Nevesinje area compared to winter (Table 2). A significant increase in the levels of Ne during winter in the Vlasic and Kupres areas compared to summer is shown in Table 3. In contrast, significantly higher WBC count were found in the Podvelezje area compared to the Nevesinje area during summer (Table 2). Basophil counts were significantly higher during summer across all areas, except Vlasic, compared to winter (Tables 2 and 3), while eosinophils levels were significantly higher in Nevesinje compared to Podvelezje during summer. PLT values exhibited a statistically significant elevation during winter compared to summer within the Vlasic area (Table 3), as did MPV, including the Kupres area.

Biochemical parameters

During summer, the serum biochemical analysis of examined sheep revealed a significant increase in TP, ALB and GLO in all investigated areas (Tables 4 and 5). TP values were significantly higher in the Nevesinje area compared to the Podvelezje area during summer (Table 4). AST values exceeded reference levels in both seasons, with statistically higher levels during summer in Kupres compared to Vlasic, and in Nevesinje compared to Podvelezje (Tables 4 and 5). In the central part of the country, a significant increase in CK and GGT was recorded, during summer and winter, respectively (Table 5). Significantly higher values were found in the Vlasic area during winter compared to summer (Table 5), where the Dubska pramenka strain was exposed to severe cold stress. Significantly higher Ca values were found during summer compared to winter in the Nevesinje and Kupres areas (Tables 4 and 5). Changes in electrolyte concentrations were obsereved under both cold and heat conditions. During winter, a significant increase in the value of Mg was recorded in the Vlasic area, while Ca values were significantly higher in Nevesinje and Kupres during summer compared to winter.

Table 2 Seasonal changes in hematological parameters within and between the Nevesinje and Podvelezje areas in the Pramenka sheep breed

Hercegovacka strain								
	Nevesinje (n=24)		**P**	**Podvelezje (n=23)**		**P**	**P_{bl}**	
	S (n=12)	W (n=12)		S (n=12)	W (n=12)		S	W
RBC (10^{12}/l)	9.14 ± 0.25	8.73 ± 0.34	0.391	8.92 ± 0.49	8.79 ± 0.41	0.844	0.985	0.999
Hb (g/l)	107.48 ± 2.70	94.33 ± 3.30	**0.016**	100.00 ± 10.35	94.82 ± 4.40	0.635	0.944	1.000
PCV (%)	28.40 ± 0.85	27.41 ± 0.87	0.471	29.25 ± 1.47	28.75 ± 1.29	0.767	0.970	0.811
MCV (fl)	31.10 ± 0.57	31.58 ± 0.62	0.551	33.02 ± 0.93	32.80 ± 0.74	0.892	0.311	0.543
MCH (pg)	11.80 ± 0.24	10.79 ± 0.11	**0.003**	11.24 ± 1.04	10.74 ± 0.13	0.751	0.971	0.991
MCHC (g/l)	379.66 ± 7.88	344.33 ± 5.67	**0.002**	341.73 ± 32.25	330.64 ± 5.88	0.778	0.762	0.342
RDW (%)	15.47 ± 0.36	15.96 ± 0.32	0.373	15.86 ± 0.21	15.41 ± 0.31	0.123	0.680	0.722
WBC (10^9/l)	9.03 ± 0.60	8.96 ± 0.34	0.940	13.36 ± 1.38	9.48 ± 0.90	**0.027**	**0.005**	0.941
Ne (10^9/l)	1.81 ± 0.39	1.94 ± 0.23	0.788	1.52 ± 0.19	1.59 ± 0.21	0.801	0.908	0.823
Eo (10^9/l)	1.66 ± 0.24	1.22 ± 0.18	0.247	0.99 ± 0.18	0.91 ± 0.17	0.782	0.084	0.552
Ba (10^9/l)	0.36 ± 0.04	0.18 ± 0.02	**0.002**	0.42 ± 0.07	0.24 ± 0.03	**0.048**	0.841	0.151
Mo (10^9/l)	0.69 ± 0.23	0.44 ± 0.08	0.491	1.22 ± 0.27	0.74 ± 0.14	0.174	0.200	0.143
Ly (10^9/l)	4.52 ± 0.40	5.03 ± 0.31	0.366	9.20 ± 1.07	5.78 ± 0.55	**0.011**	**0.000**	0.625
Ne (%)	19.26 ± 3.07	21.63 ± 2.33	0.568	11.74 ± 1.24	17.82 ± 2.36	**0.036**	0.109	0.725
Eo (%)	18.12 ± 2.35	13.76 ± 2.12	0.279	8.23 ± 1.70	9.08 ± 1.12	0.735	**0.003**	0.241
Ba (%)	3.98 ± 0.45	1.94 ± 0.17	**0.009**	3.09 ± 0.48	2.56 ± 0.29	0.398	0.610	0.154
Mo (%)	8.18 ± 3.13	4.93 ± 0.90	0.491	8.06 ± 1.23	7.69 ± 1.01	0.859	1.000	0.139
Ly (%)	49.92 ± 3.78	51.44 ± 4.72	0.830	68.33 ± 2.03	60.86 ± 1.77	**0.028**	**0.000**	0.238
PLT (10^9/l)	594.92 ± 45.83	605.25 ± 39.75	0.893	727.36 ± 150.2	652.18 ± 56.11	0.766	0.522	0.906
MPV (fl)	14.16 ± 0.78	14.70 ± 0.91	0.719	13.56 ± 0.87	17.70 ± 0.88	**0.006**	0.999	0.053

Statistical significance: $P < 0.05$, S – summer, W – winter, P – P value for comparisons within the area between the summer and winter periods, P_{bl} - P value for comparisons between areas within the season - summer and winter periods

Table 3 Seasonal changes in hematological parameters within and between the Vlasic and Kupres areas in the Pramenka sheep breed

Dubska strain								
	Vlasic (n=24)		**P**	**Kupres (n=23)**		**P**	**P_{bl}**	
	S (n=12)	W (n=12)		S (n=12)	W (n=12)		S	W
RBC (10^{12}/l)	10.71 ± 0.56	9.99 ± 0.24	0.182	10.68 ± 0.50	9.81 ± 0.32	0.145	0.992	0.958
Hb (g/l)	99.60 ± 14.82	103.50 ± 3.53	0.883	118.22 ± 5.41	107.11 ± 3.69	0.111	0.474	0.940
PCV (%)	33.29 ± 2.60	28.83 ± 1.11	0.168	31.93 ± 1.58	30.77 ± 1.15	0.544	0.997	0.852
MCV (fl)	29.90 ± 1.02	28.77 ± 0.68	0.378	29.95 ± 0.81	31.33 ± 0.54	0.251	0.928	0.167
MCH (pg)	9.70 ± 1.44	10.27 ± 0.19	0.770	11.06 ± 0.15	10.86 ± 0.11	0.382	0.784	**0.028**
MCHC (g/l)	326.80 ± 49.22	359.40 ± 4.35	0.519	371.22 ± 7.26	349.11 ± 6.93	0.065	0.653	0.954
RDW (%)	16.18 ± 0.37	16.46 ± 0.36	0.622	15.92 ± 0.22	16.53 ± 0.58	0.172	0.942	0.948
WBC (10^9/l)	7.23 ± 0.78	8.93 ± 0.59	0.088	8.83 ± 0.75	8.97 ± 0.83	0.912	0.790	1.000
Ne (10^9/l)	1.65 ± 0.17	2.63 ± 0.40	**0.005**	1.48 ± 0.31	2.61 ± 0.36	0.054	0.857	0.998
Eo (10^9/l)	0.92 ± 0.12	0.81 ± 0.20	0.635	0.88 ± 0.19	0.47 ± 0.07	0.152	1.000	0.840
Ba (10^9/l)	0.17 ± 0.04	0.13 ± 0.02	0.533	0.30 ± 0.08	0.13 ± 0.01	0.075	0.379	0.994
Mo (10^9/l)	0.42 ± 0.08	0.45 ± 0.07	0.831	0.59 ± 0.12	0.44 ± 0.08	0.362	0.977	1.000
Ly (10^9/l)	4.22 ± 0.47	4.76 ± 0.26	0.385	5.57 ± 0.53	5.24 ± 0.66	0.747	0.512	0.943
Ne (%)	22.75 ± 1.81	28.80 ± 2.96	0.094	16.42 ± 3.00	29.07 ± 3.58	**0.023**	0.114	0.999
Eo (%)	12.19 ± 1.11	8.98 ± 1.99	0.173	10.24 ± 2.11	5.56 ± 1.01	0.359	0.955	0.922
Ba (%)	2.15 ± 0.41	1.47 ± 0.23	0.247	3.37 ± 0.73	1.46 ± 0.14	**0.028**	0.260	0.991
Mo (%)	5.64 ± 0.97	5.00 ± 0.77	0.595	6.18 ± 0.98	4.94 ± 0.97	0.418	1.000	0.1000
Ly (%)	55.88 ± 2.45	54.19 ± 2.90	0.591	63.38 ± 3.28	58.00 ± 4.19	0.405	0.198	0.960
PLT (10^9/l)	347.80 ± 40.40	620.60 ± 63.8	**0.005**	410.66 ± 58.71	480.00 ± 47.53	0.180	1.000	0.243
MPV (fl)	16.03 ± 1.28	8.92 ± 0.79	**0.003**	14.63 ± 1.50	8.93 ± 0.76	**0.004**	0.936	0.999

Statistical significance: $P < 0.05$, S – summer, W – winter, P – P value for comparisons within the area between the summer and winter periods, P_{bl} - P value for comparisons between areas within the season - summer and winter periods

Table 4 Seasonal changes in blood biochemical parameters within and between the Nevesinje and Podvelezje areas in the Pramenka sheep breed

	Hercegovacka strain							
	Nevesinje (n=24)		**P**	**Podvelezje (n=23)**		**P**	**P_{bl}**	
	S (n=12)	W (n=12)		S (n=12)	W (n=12)		S	W
TP (g/l)	86.21 ± 1.89	70.63 ± 1.68	**0.001**	76.83 ± 2.32	67.61 ± 1.73	**0.005**	**0.010**	0.536
ALB (g/l)	34.02 ± 0.55	31.65 ± 0.78	**0.033**	33.43 ± 0.84	29.24 ± 0.84	**0.006**	0.966	0.223
GLO (g/l)	52.16 ± 2.19	38.98 ± 1.7^{A}	**0.001**	46.33 ± 3.63	38.34 ± 1.76	**0.037**	0.307	0.992
TB (µmol/l)	2.41 ± 0.46	1.71 ± 0.33	0.199	2.36 ± 0.50	1.57 ± 0.24	0.223	0.996	0.988
TC (mmol/l)	2.41 ± 0.14	2.10 ± 0.15	0.058	2.30 ± 0.16	2.06 ± 0.09	0.218	0.945	0.998
GLU (mmol/l)	2.99 ± 0.13	2.83 ± 0.15	0.336	2.76 ± 0.09	3.20 ± 0.16	0.113	0.362	0.212
UREA (mmol/l)	6.75 ± 0.19	3.28 ± 0.29	**0.001**	3.18 ± 0.15	5.05 ± 0.29	**0.003**	**0.000**	0.100
CRE (µmol/l)	36.60 ± 2.27	54.27 ± 4.27	**0.001**	37.38 ± 1.66	46.88 ± 2.06	**0.005**	0.989	0.406
AST (U/l)	164.89 ± 11.08	142.59 ± 11.99	0.149	128.22 ± 3.82	140.80 ± 7.77	0.123	**0.009**	0.999
GGT (U/l)	30.14 ± 7.70	41.08 ± 5.35	0.348	32.21 ± 4.63	36.85 ± 4.40	0.482	0.993	0.918
CK (U/l)	209.43 ± 23.06	198.22 ± 25.39	0.705	280.15 ± 41.29	546.80 ± 129.5	0.095	0.646	**0.004**
NEFA (mmol/l)	0.71 ± 0.19	0.44 ± 0.06	0.209	1.33 ± 0.27	0.57 ± 0.16	**0.046**	0.467	0.813
BHB (mmol/l)	0.33 ± 0.03	0.44 ± 0.08	0.144	0.82 ± 0.08	0.43 ± 0.06	0.001	0.112	0.997
Ca (mmol/l)	2.60 ± 0.03	2.46 ± 0.03	**0.040**	2.44 ± 0.05	2.42 ± 0.04	0.769	0.103	0.931
P (mmol/l)	1.68 ± 0.08	1.43 ± 0.12	0.102	1.52 ± 0.07	1.91 ± 0.19	0.080	0.706	0.182
Na (mmol/l)	141.44 ± 1.98	144.46 ± 1.01	0.196	145.19 ± 1.79	147.21 ± 1.31	0.317	0.531	0.284
Cl (mmol/l)	104.55 ± 1.03	108.00 ± 0.93	**0.040**	106.01 ± 1.03	107.49 ± 1.21	0.329	0.802	0.988
Mg (mmol/l)	0.98 ± 0.08	1.08 ± 0.03	0.285	1.06 ± 0.09	1.09 ± 0.04	0.728	0.905	0.998

Statistical significance: $P < 0.05$, S – summer, W – winter; P – P value for comparisons within the area between the summer and winter periods; P_{bl} - P value for comparisons between areas within the season - summer and winter periods

Table 5 Seasonal changes in blood biochemical parameters within and between the Vlasic and Kupres area in the Pramenka sheep breed

Dubska strain								
	Vlasic (n=24)		**P**	**Kupres (n=23)**		**P**	**P_{bl}**	
	S (n=12)	W (n=12)		S (n=12)	W (n=12)		S	W
TP (g/l)	78.01 ± 1.88	65.27 ± 1.26	**0.008**	81.08 ± 1.95	64.90 ± 1.57	**0.002**	0.703	0.906
ALB (g/l)	34.65 ± 0.93	26.90 ± 1.16	**0.001**	36.43 ± 1.20	28.00 ± 0.88	**0.006**	0.545	0.921
GLO (g/l)	43.36 ± 1.57	38.37 ± 1.54	**0.028**	44.68 ± 1.14	36.90 ± 1.57	**0.003**	0.981	0.992
TB (µmol/l)	2.21 ± 0.66	2.40 ± 0.33	0.534	3.83 ± 0.62	2.48 ± 0.32	**0.031**	0.311	0.991
TC (mmol/l)	2.04 ± 0.19	2.19 ± 0.16	0.527	2.34 ± 0.08	2.19 ± 0.16	0.454	0.997	1.000
GLU (mmol/l)	3.12 ± 0.10	3.18 ± 0.12	0.672	3.00 ± 0.08	3.00 ± 0.08	0.999	0.846	0.538
UREA (mmol/l)	4.99 ± 0.62	1.45 ± 0.28	**0.003**	3.40 ± 0.19	1.74 ± 0.13	**0.001**	**0.006**	0.666
CRE (µmol/l)	46.18 ± 1.42	58.58 ± 3.64	**0.014**	33.30 ± 1.58	57.16 ± 3.43	**0.001**	**0.000**	1.000
AST (U/l)	124 ± 62 ± 4.07	129.76 ± 8.61	0.643	167.76 ± 9.05	129.90 ± 5.21	**0.004**	**0.005**	1.000
GGT (U/l)	32.42 ± 6.00	64.91 ± 6.35	**0.003**	16.08 ± 2.69	41.79 ± 2.99	**0.001**	0.194	**0.002**
CK (U/l)	286.16 ± 33.14	205.15 ± 20.08	0.054	350.22 ± 62.79	161.61 ± 23.65	**0.021**	0.742	0.988
NEFA (mmol/l)	0.84 ± 0.22	0.26 ± 0.04	**0.033**	1.66 ± 0.44	0.36 ± 0.09	**0.015**	0.255	0.842
BHB (mmol/l)	0.39 ± 0.03	0.31 ± 0.03	0.052	0.56 ± 0.04	0.38 ± 0.03	**0.015**	0.139	0.955
Ca (mmol/l)	2.44 ± 0.05	2.36 ± 0.07	0.423	2.61 ± 0.06	2.37 ± 0.05	**0.013**	0.096	0.888
P (mmol/l)	1.93 ± 0.14	1.81 ± 0.14	0.496	1.35 ± 0.11	1.50 ± 0.06	0.258	**0.002**	0.128
Na (mmol/l)	142.79±1.93	147.57± 1.05	0.055	149.26 ± 2.19	147.57 ± 1.03	0.576	0.133	1.000
Cl (mmol/l)	105.48±1.56	109.51 ± 0.78	0.094	112.69 ± 1.08	110.47 ± 1.53	0.302	**0.001**	0.962
Mg (mmol/l)	0.93 ± 0.06	1.06 ± 0.03	**0.008**	0.93 ± 0.11	1.16 ± 0.03	0.058	1.000	0.524

Statistical significance: $P < 0.05$, S – summer, W – winter; P – P value for comparisons within the area between the summer and winter periods; P_{bl} - P value for comparisons between areas within the season - summer and winter periods

DISCUSSION AND CONCLUSION

Heat stress exerts significant effects on hematological parameters in sheep, reflecting physiological adaptations and potential challenges to health and productivity (Marai et al., 2007). Elevated Hb, MCH, and MCHC may be due to a compensatory mechanism to enhance oxygen delivery to tissues during heat stress. Similarly, Njidda et al. (2014) and Sejian et al. (2013) also observed higher Hb concentrations in heat-stressed sheep. Sejian et al. (2013) also observed a significant increase in the Hb and PCV% values due to short-term heat stress in Malpura sheep. Alam et al. (2011) reported a significant increase in the RBC, PCV%, Hb%, total leukocyte count (TLC) and differential leukocyte count (DLC) in the other ruminant species, i.e., goats. On the other hand, several reports indicated a reduction in the number of RBCs and, thus, PCV and Hb values during heat stress values (Temizel et al., 2009; Sivakumar et al., 2010; Kumar et al., 2011). These variations in the results might be due to the differences in the design of the experiments, long-term (study under natural environment) versus short-term exposure of the animals (either in a climatic chamber for 6 h duration carried out by Sejian et al. (2013), or short 6–8 h exposure by Alam et al. (2011) to heat stress). The higher levels of Hb during summer could be due to hemoconcentration resulting from the loss of water through panting and increased respiratory rate to dissipate heat from the body in hot and humid climates (Reddy et al., 2018). Habibu et al. (2018) stated that increased values of MCHC could be indicators of poor dynamics of body fluids in conditions of heat stress. Karthik et al. (2021) in their study showed that there were no differences in MCV, MCH and MCHC during heat stress compared to thermoneutral conditions in sheep in extensive housing. Examining the erythrocyte parameters, our results suggest that heat stress causes more alterations on a red bloodline than cold stress since no statistically significant changes were observed in the breeding areas where cold stress was observed.

In our study, we observed elevations in Ba and Eo levels in the Hercegovacka and Dubska Pramenka sheep strains during the summer period. It has been reported that Eo and Ba are responsive immune cells to heat stress, but they show differential responses (Park et al., 2021). Consistently, several studies have reported that animals have increased Ba counts following exposure to heat stress (Mitchell et al., 1992; Altan et al., 2000). As a part of type 2 immunity, the Ba counts in blood and mesenteric lymph nodes also may be expanded during parasite infection (Roland et al., 2014, Reitz et al., 2018). In addition, eosinophilia is a reliable diagnostic clue for helminthic and bacterial infections (Ramirez et al., 2018). Based on cellular and biological aspects of Ba and Eo, there is a higher chance that several parasitic infections could occur in a stressful environment. Therefore, we could not rule out the possibility that heat-stressed sheep in Nevesinje and Podvelezje areas suffered parasitic infections during high-temperature conditions in this study. However, our results should be interpreted with caution, given that the significant differences in Eo values found may be the result of allergic reactions in the examined sheep herds. Namely, there are differences in geological composition of the land in the Nevesinje area (Figure 2) compared to the Podvelezje area, i.e., a substantially larger share of alpine forests and a mosaic of agricultural areas. The presence of these geological determinants could also mean the presence of an enormous number of allergens with which the tested animals could come into contact in the summer period, and this could potentially cause elevated values of Eo. Therefore, it seems that the increased values of Eo and Ba during the summer season are not specific indicators of heat stress, and may be the result of different adaptation abilities among breeds, but also the presence of a greater number of parasites and allergens in the summer months.

Heat stress affects both erythrocyte and leukocyte parameters, while cold stress mainly impacts leukocytes (Banerjee et al., 2014). Leukocyte counts may vary during thermal stress, either increasing or decreasing, depending on factors such as species, exposure duration, and adaptation level (Habibu et al., 2018). The results of our study indicate the elevated levels of Ne during the winter season in the areas of Vlasic and Kupres, which confirms the findings of Banerjee et al. (2014), since extremely strong cold stress was established in these areas. While our findings hint at a connection between leukocyte fractions and thermal stress, it is crucial to acknowledge that these changes may not solely stem from the thermal stressors. Nonetheless, the elevated THI values during winter (Table 1) necessitate further exploration and discussion regarding their potential impact on leukocyte dynamics.

Thrombocytes, recognized for their role in clot formation and aggregation, serve as essential mediators of both innate and adaptive immunity (Ferdous et al., 2015; Habibu et al., 2018). In our study, PLT values exhibited a significant elevation during winter compared to summer in the Vlasic area. Correspondingly, Casella et al. (2013) demonstrated that lactating Bruna cows displayed diminished PLT and plateletcrit (PCT) values during periods characterized by elevated THI. Moreover, reduced PLT counts have been documented in cattle subjected to heat stress conditions (Mazzullo et al., 2014), although Wojtas et al. (2014) did not observe significant alterations in sheep. Consistently, several studies have reported decreased PLT counts in cattle with rising environmental temperatures and/or during hot seasons (Casella et al., 2013; Giri et al., 2017). Thrombocytopenia observed during severe weather conditions has been correlated with hemodilution, thrombopoietic suppression, increased platelet aggregation, and augmented consumption and destruction of PLT (Roland et al., 2014; Habibu et al., 2018). Consistent with the findings of Velayudhan et al. (2022), thrombocytic indices, such as high MPV and platelet distribution width (PDW), alongside low PLT and PCT during periods of high THI and/or summer, suggest a megakaryocytic response characterized by accelerated production and release of larger PLT into circulation (Kocatürk et al., 2010; Habibu et al., 2018). These mechanisms may be interpreted as adaptive responses to heat stress exhibited by the investigated crossbred dairy cows.

During thermal stress, animals undergo complex biochemical and metabolic responses to maintain internal stability and cope with the physiological challenges imposed by high or low environmental temperatures (Sejian et al., 2018). In our research, higher values of TP, ALB and GLO were noticed during the summer period compared to the winter period in all investigated areas, emphasizing that TP was higher in the Nevesinje area compared to Podvelezje. This may be the result of increased relative humidity in the area of Nevesinje compared to Podvelezje, since high ambient relative humidity makes thermolysis much more difficult and indirectly leads to dehydration through excessive sweating, loss of electrolytes and difficulty breathing. This occurs due to the negative effects of high humidity on the dissipation of body heat because of the decline in the effectiveness of radiation, conduction and convection and the effectiveness of evaporative cooling (Aggarwal et al., 2013).

AST values exceeded reference levels during both seasons, with significantly higher values at the Kupres and Nevesinje area during the summer season compared to winter. Hrkovic-Porobija et al. (2017) observed significant variations in AST concentration between tissues and blood serum. They noted that when tissues abundant in AST undergo lesions, the enzyme was discharged into circulation, leading to increased AST activity in blood serum. Based on ours and others results, it seems that elevated serum AST values may indicate low heat tolerance in combination with the nutritional status, housing conditions and physiological state of the animal. Among other liver enzymes, elevated levels of CK during the summer period compared to winter in the area of Kupres were found. Chulayo et al. (2014) stated that increased CK activity in sheep might have been caused by physical stress during the summer period. However, our results should be interpreted with caution, given that the significant differences in CK values found may be the result of some individual factors such as age, growth, reproduction, animal breeding system and diet.

Thermal stress can also have effects on electrolyte concentrations. Nazifi et al. (2003) found higher values of Mg in conditions of cold stress compared to heat stress, which is also our finding, since significantly higher values were found in the Vlasic area during winter compared to summer, where the Dubska Pramenka strain was exposed to severe cold stress. According to Hamzaoui et al. (2013), usual reaction to heat stress is a decrease in Na, Ca and P levels and an increase in Cl concentration. However, elevated values of Ca during summer compared to winter in the Nevesinje and Kupres areas were found. The level of minerals in the blood depends on the diet, which indicates that the calcemia may be directly correlated with the category and diet of sheep (Hrkovic et al., 2009).

The findings of this study demonstrated notable variations in the blood parameters of Pramenka sheep exposed to thermal stress, underscoring the significant impact of breeding area characteristics, including geological composition, spatial distribution, and land cover. Key results indicated that while some variations in hematological and biochemical parameters were observed, none of the measured values exceeded established physiological reference ranges. This suggests a remarkable adaptive capacity of the studied Pramenka strains to cope with thermal stress, likely rooted in their long-term evolutionary adaptation to local environmental conditions. These results contribute a valuable insight into the resilience of indigenous breeds and reinforce the importance of preserving such animals within their native habitats, where they have naturally developed tolerance to climatic extremes. Practical applications of this research include breeding and management strategies toward the conservation and sustainable utilization of local genetic resources, particularly in the context of climate change. The study supports the promotion of location-specific livestock breeding practices that leverage the inherent adaptability of native breeds. However, our study was confined to a specific region and a limited number of animals, which may restrict the generalizability of the findings. Future research should expand to include larger populations across diverse geographic areas and incorporate genetic and molecular analyses to further elucidate the mechanisms underlying thermal resilience in indigenous sheep breeds.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTIONS

Conception: HO, AS, VB, AH; Design: HO, AS, VB, LŠ; Supervision: LŠ, AH; Fundings: HO, VB, AH; Materials: HO, KB, VB, IA, MO, LŠ, AH; Data Collection and/or Processing: HO, AS, KB, VB, IA, MO; Analysis and/or Interpretation of the Data: HO, AS, KB, IA, MO, LŠ, AH; Literature Review: AS, IA; Writing: HO, AS, AH; Critical Review: HO, VB, IA, LŠ, AH.

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EVALUACIJA UČINKA SEZONE I PODRUČJA UZGOJA NA KRVNE PARAMETRE PRAMENKE IZLOŽENE UVJETIMA TERMALNOG STRESA

SAŽETAK

Cilj istraživanja je procjena sezonskih varijacija krvnih parametara kod dva soja ovce pramenke, dubskog i hercegovačkog, na četiri područja Bosne i Hercegovine pod uvjetima termalnog stresa. Istraživanje je uključilo 48 ovaca s Vlašića i Kupresa (područja uzgoja dubskog soja) i Nevesinja i Podveležja (područja uzgoja hercegovačkog soja). Istraživanje je provedeno u ljetnoj i zimskoj sezoni, a obuhvatilo je ukupno osam stada (po dva na svakom području). Hematološki parametri su uključili broj eritrocita, leukocita i trombocita, srednji volumen trombocita, ukupni broj granulocitnih i agranulocitnih leukocita, hemoglobin, širinu distribucije eritrocita i parametre eritrocita. Biohemijske analize su obuhvatile bilirubin, ukupni holesterol, glukozu, ureu, kreatinin, kreatin kinazu, neesterificirane masne kiseline, beta-hidroksibutirat, proteine, jetrene enzime i elektrolite. Rezultati su pokazali signifikantno više vrijednosti eritrocitnih parametara u ljetnoj sezoni za područje Nevesinja ($P<0.05$) u usporedbi s Podveležjem. Važno je naglasiti signifikantno povišenu vrijednost bazofila u ljetnoj sezoni za sva područja, osim Vlašića, u usporedbi sa zimskom sezonom. Nadalje, vrijednosti ukupnih proteina, albumina i globulina su bile signifikantno povišene u svim područjima u ljetnoj u usporedbi sa zimskom sezonom. Rezultati istraživanja naglašavaju znatan utjecaj koji područje uzgoja u smislu geološkog sastava, prostorne distribucije i pokrivača imaju na specifične krvne parametre ispitivane kod ovaca u periodima karakteriziranim nepovoljnim temperaturnim uvjetima.

Ključne riječi: Krvni parametri, ovca, pramenka, sezona, termalni stres

RESEARCH ARTICLE

PREDICTION OF BODY WEIGHT FROM MORPHOMETRIC TRAITS OF WHITE LEGHORN USING DATA MINING ALGORITHM

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ABSTRACT

The study aimed to establish a predictive model for body weight in White Leghorn using morphometric traits through different data mining algorithms. Data was collected from 100 chickens, including body weight (BW), beak length (BKL), body length (BL), keel length (KL), chest girth (CG), body girth (BG), shank length (SL), back length (BCL), shank circumference (SC) and wing length (WL). Chi-Squared Automatic Interaction Detection (CHAID), Classification and Regression Trees (CART) and Exhaustive chi-squared Automatic Interaction detection (EX-CHAID) were used for data analysis. Based on goodness of fit criteria, CART model was the best model for prediction of body weight in White Leghorn chickens with higher values of correlation coefficient (r = 0.84) and coefficient of determination (R^2 = 0.71), and lower root mean square error (RMSE = 0.18), Akaike information criterion (AIC = -341.77) and Bayesian information criterion (BIC = -339.16). CART model identified CG, BL, and WL as key contributors to BW variation, suggesting that focusing on these traits can assist in BW prediction and support farmers in improving their chickens.

Keywords: CHAID, CART, Exhaustive CHAID, goodness of fit

INTRODUCTION

White Leghorn is a white feathered chicken breed, which is also known as an egg layer. It originates from Egypt, and they are valued for their egg-laying abilities and adaptability (Ewonetu, 2017). Morphometric traits have emerged as effective tools for predicting the body weight, that is critical for livestock management and breeding (Ebong et al., 2023). Data mining algorithms offer robust models for body weight prediction by leveraging morphometric traits (Tyasi et al., 2020). Body weight and morphometric traits have a significant relationship in

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measuring the growth of the domesticated chickens (Nosike et al., 2017). However, accurate animal weighing and assessment may be hampered by a lack of technical knowledge; small-scale farmers in rural areas are facing difficulty on accessing weighing scales, which makes this method challenging (Negash, 2021). Morphometric traits have been used to determine body weight in poultry and livestock species (Assan, 2013). Several studies focus on using data mining algorithms to predict body weight in chickens. Ogunshola et al. (2017) reported that morphometric traits of a chicken could be used to predict the ration of body weight at any period of age from 17-25 weeks old of Fulani ecotype chicken. Dalal et al. (2020) concluded that morphometric traits had an excessive effective and crucial correlation with 40 weeks body weight, indicating the effectiveness of the use of morphometric traits in predicting body weight in synthetic White Leghorn strain.

However, according to the author's knowledge, there is limited information on the prediction of body weight from morphometric traits of White Leghorn using data mining algorithms. Therefore, the objectives of the study were as follows: 1) To determine the relationship between the body weight and morphometric traits of the White Leghorn chicken breed, 2) To establish a model for prediction of body weight from morphometric traits of White Leghorn chicken breed using data mining algorithm. This study will help local farmers to gain knowledge about the relationship between the body weight and morphometric traits of White Leghorn and understand which morphometric traits can be used as a selection criterion to estimate body weight of White Leghorn chickens.

MATERIALS AND METHODS

Study area

The study was carried out at the University of Limpopo Experimental Farm, Limpopo province, South Africa. The farm is situated 10 kilometres north-west of the University of Limpopo (23°49' S; 29°41' E). The area has a semi-arid climate with an average temperature ranging from 10° C to 36° C in summer and 5° C to 28° C in winter, the farm receives an annual rainfall of less than 400 mm (Molabe et al., 2024).

Experimental animals, management and study design

The study used a total of 100 White Leghorn chickens. The chickens were raised following the ordinary husbandry practices of feeding systems, housing, vaccination, and health care as described by Alabi (2012). The chickens were housed under intensive production conditions. The chicken house was cleaned seven days before the chickens arrived and disinfected with Virokill disinfectants to avoid transmission of pathogenic diseases to the chickens. The biosecurity protocols were followed in the area, where the footbaths with disinfectant were placed at the door for disinfecting before entering the chicken house. The study used a cross-sectional study design with one-time measurements per bird.

Data collection

The data was collected through measurements of the body weight (BW) and morphometric traits. Morphometric traits such as beak length (BKL), body length (BL), keel length (KL), back length (BCL), chest girth (CG), shank length (SL), shank circumference (SC) and wing length (WL) of White Leghorn chickens were measured using a measuring tape calibrated in centimetres (cm), while the body weight of each chicken was measured in kilograms (kg) using a weighing scale as described by Tyasi et al. (2021). The measurements were collected by one person throughout to avoid individual variation on measuring.

Statistical analysis

The data collected was analysed using Statistical Package for Social Sciences (IMB SPSS, 2023) version 29.0 software. Pearson's correlation was used to examine the association between the body weight and morphometric traits, while CHAID, CART and Ex-CHAID were used to develop the models to predict body weight from morphometric traits. The predictive performance of CHAID, CART and Ex-CHAID was done using the goodness of fit criteria.

The following goodness of fit criteria were used in the study:

Correlation coefficient

$$R=\frac{cov\ (Yi\ ,Yip\ )}{Syi\ SYip}$$

Coefficient of determination

$$R^2=\left[1-\frac{\sum_{i=1}^{n}(Y_i-\hat{Y}_i)^2}{\sum_{i=1}^{n}(Y_i-\bar{Y})^2}\right]\times 100$$

Adjusted coefficient of determination

$$R^2_{Adj}=\left[1-\frac{\frac{1}{n-k-1}\sum_{i=1}^{n}(Y_i-\hat{Y}_i)^2}{\frac{1}{n-1}\sum_{i=1}^{n}(Y_i-\bar{Y})^2}\right]\times 100$$

Root mean square error

$$RMSE=\sqrt{\frac{\sum_{i=1}^{n}(Y_i-\hat{Y}_i)^2}{n}}$$

Akaike information criterion

$$AIC= NLn\ (\frac{SSE}{N}) + 2p$$

Bayesian information criterion

$$BIC= NLn\ (\frac{SSE}{N}) + pLnN$$

Where:

Y_i, the actual body weight (g) of the White Leghorn;

$\hat{Y}_i$, the predicted body weight value of White Leghorn;

$\bar{Y}$, average of the actual body weight of the White Leghorn;

k, number of significant independent variables in the model;

and n, the total number of White Leghorns.

The residual value of each White Leghorn is expressed as $Ɛ_i = Y_i - \hat{Y}_i$.

RESULTS

Table 1 below represents descriptive statistics of BW and morphometric traits of White Leghorn chicken breed. The findings displayed that the mean values of the morphometric traits ranged from 3.17 to 42.64 cm, with the chicken height having the highest mean value. The minimum and maximum values range from 1.20 to 50.00.

Table 1 Descriptive statistics for body weight and morphometric traits

Traits	Mean	SE	SD	Minimum	Maximum
BW	2.09	0.03	0.27	1.20	2.57
BL	24.78	0.22	2.16	20.00	29.00
WL	20.07	0.17	1.66	16.00	23.00
CG	37.46	0.27	2.74	31.00	43.00
BKL	3.17	0.07	0.72	2.00	5.00
BCL	6.44	0.13	1.28	4.00	10.00
CH	42.64	0.35	3.51	36.00	50.00
TBL	26.52	0.22	2.16	13.00	31.00

SE: stand. error, SD: stand. deviation, BW (kg): body weight, BL (cm): body length, WL (cm): wing length, CG (cm): chest girth, BKL (cm): beak length, BCL (cm): back length, CH (cm): chicken height, TBL (cm): tail-back length.

Table 2 below shows the correlation between body weight and morphometric traits of the White Leghorn chicken breed. The results showed that BW had a positive highly significant correlation ($P < 0.01$) with the BL, but positive significant correlation ($P < 0.05$) with CG, WL and CH. It was also found that the BKL, BCL, and TBL had a non-significant correlation ($P > 0.05$) with the BW.

Table 2 Correlation between body weight and morphometric traits

Traits	BW	BL	WL	CG	BKL	BCL	CH	TBL
BW (kg)								
BL (cm)	0.49**							
WL (cm)	0.27*	0.30**						
CG (cm)	0.18*	0.06^{ns}	-0.00^{ns}					
BKL (cm)	0.05^{ns}	-0.02^{ns}	0.06^{ns}	-0.19*				
BCL (cm)	0.12^{ns}	-0.04^{ns}	0.15^{ns}	-0.01^{ns}	0.21*			
CH (cm)	0.21*	-0.09^{ns}	0.16^{ns}	0.05^{ns}	0.04^{ns}	0.04^{ns}		
TBL (cm)	0.14^{ns}	-0.02^{ns}	0.25**	0.00^{ns}	0.08^{ns}	0.19*	0.27**	

BW: body weight, BL: body length, CG: chest girth, BKL: beak length, BCL: back length, CH: chicken height, TBL: tail-back length. **highly significant (P<0.01). *Significant (P<0.05). ns: non-significant.

Figure 1 below represents the CART model. CART model revealed that CG, BL and WL were found to be highly contributing to the variation of the BW of White Leghorn. This data mining algorithm consists of 8 nodes, with node 0 as the root node. Node 0 shows the average mean BW as 2.03 kg. Node 0 was split through CG into two subgroups, node 1 (CG ≤ 42.50 cm) with mean BW of 1.99 kg, and node 2 (CG > 42.50 cm) with mean BW of 3.00 kg. Node 1 was subdivided according to BL into two subgroups, node 3 (BL ≤ 21.50 cm) with mean BW of 1.43 kg, and node 4 (BL > 21.50 cm) with mean BW of 2.03 kg. Node 4 was further subdivided through WL into two subgroups, node 5 (WL ≤ 21.50 cm) with mean BW of 2.00 kg, and node 6 (WL > 21.50 cm) with mean BW of 2.17 kg. Node 6 was further split according to BL into two subgroups, node 7 (BL ≤ 25.50 cm) with mean BW of 2.50 kg, and node 8 (BL > 25.50 cm) with mean BW of 2.00 kg. The predicted values among the all nodes range from 1.43 to 3.00.

Figure 2 below, represents the CHAID model. The findings displayed that BL and BKL were significant variables for prediction of BW of White Leghorn. The CHAID model consists of 7 nodes with node 0 as the root node. The root node showed the mean BW of 2.03 kg. The root node was subdivided through BL into five subgroups, node 1(BL ≤ 21.00 cm) with mean BW of 1.43 kg, node 2 (BL, 21.00-22.00 cm) with mean BW of 2.00 kg, node 3 (BL, 22.00-23.00 cm) with mean BW of 2.00 kg, node 4 (BL, 23.00-24.00 cm) with mean BW of 2.50 kg, and node 5 (BL >24.00 cm) with mean BW of 2.05 kg. Node 5 was subdivided through BKL into two subgroups, node 6 (BKL ≤4.00 cm) with mean BW of 2.03 kg, and node 7 (BKL >4.00 cm) with mean BW of 2.50 kg. All these terminal nodes, node 4 and node 7 produced the maximum predicted value compared to other nodes observed.

Figure 3 below display of Ex-CHAID model. This model revealed BL and WL as significant morphometric traits, which highly contributed to the body weight variation of White Leghorn chicken breed. 11 nodes were obtained with node 0 as the root node. Node 0 as the root node was subdivided through BL into 5 subgroups, node 1(BL ≤21.00 cm) with mean BW of 1.43 kg, node 2 (BL, 21.00-22.00 cm) with mean BW of 2.00 kg, node 3 (BL, 22.00-23.00 cm) with mean BW of 2.00 kg, node 4 (BL, 23.00-24.00 cm) with mean BW of 2.50 kg, node 5 (BL >24.00 cm) with mean BW of 2.05 kg. Node 5 was also subdivided through WL into 6 subgroups, node 6(WL ≤18.00 cm) with mean BW of 2.00 kg, node 7 (WL, 18.00-19.00 cm) with mean BW of 2.00 kg, node 8 (WL, 19.00-20.00 cm) with mean BW of 2.00 kg, node 9 (WL, 20.00-21.00 cm) with mean BW of 2.00 kg, node 10 (WL, 21.00-22.00 cm) with mean BW of 2.33 kg, and node 11 (WL >22.00 cm) with mean

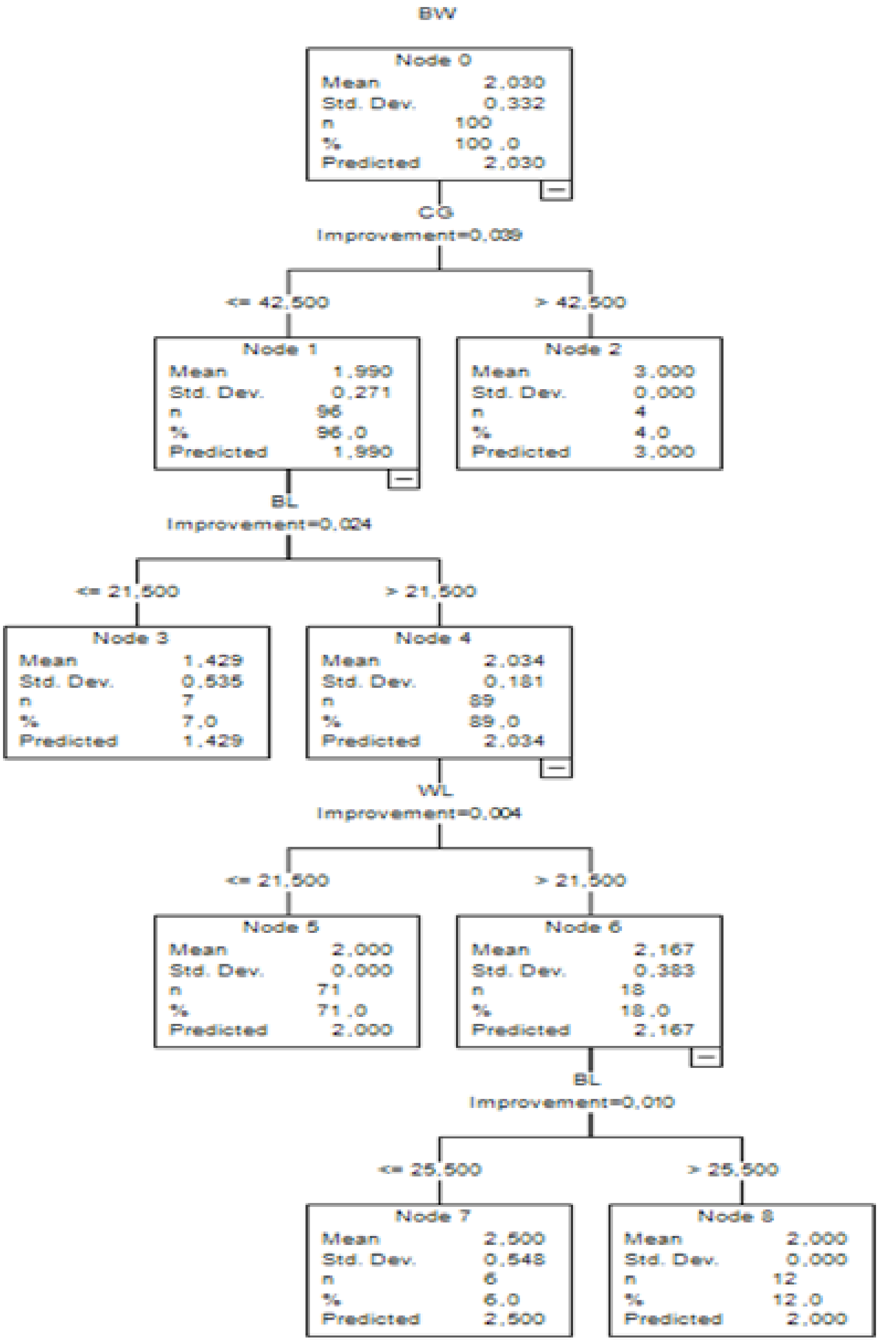

Figure 1 CART model

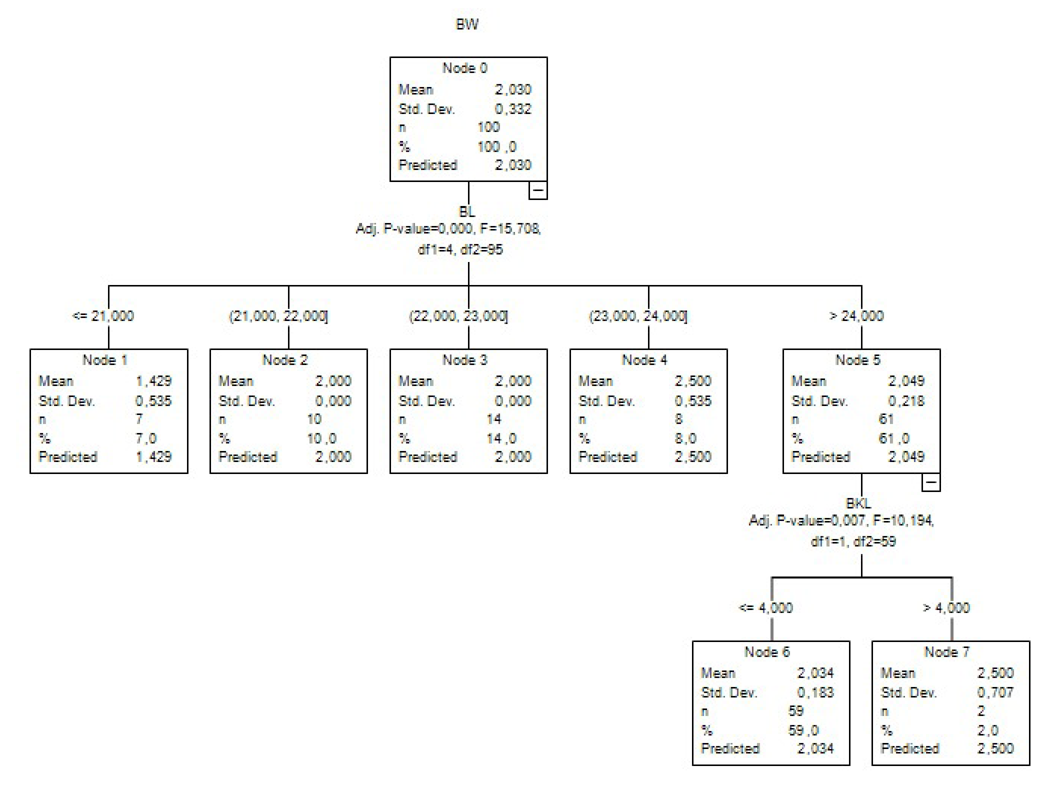

Figure 2 CHAID model

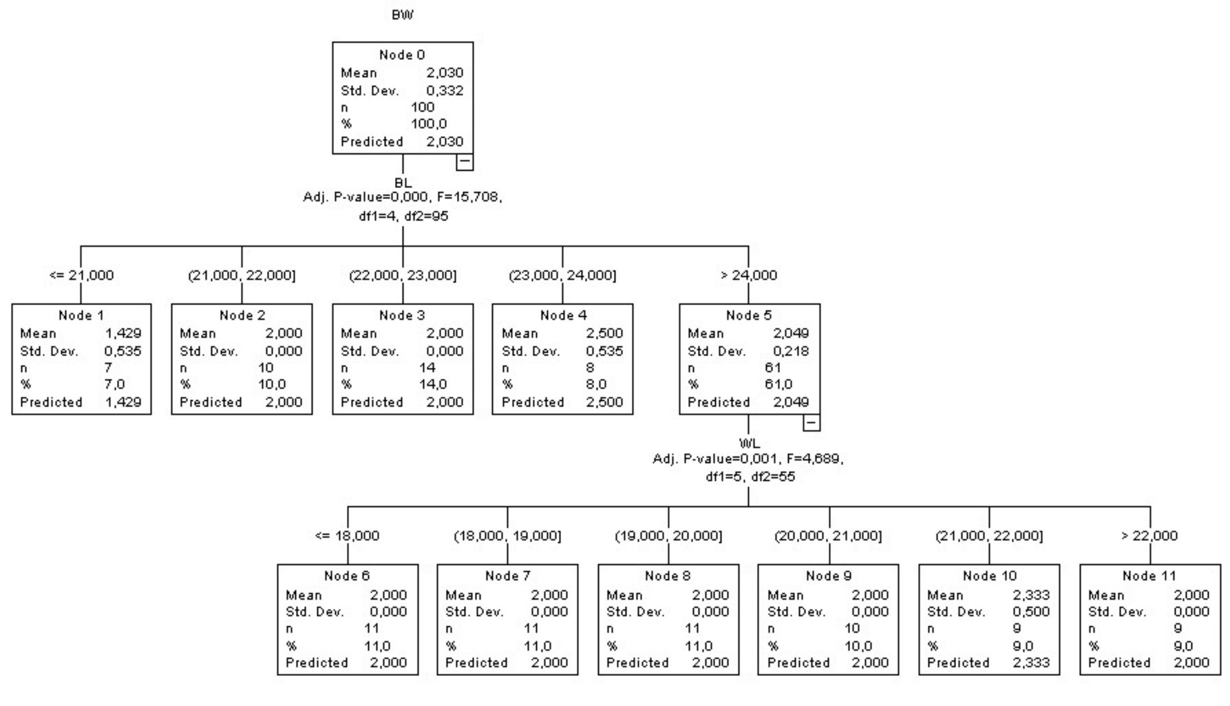

Figure 3 Exhaustive-CHAID model

BW of 2.00 kg. Among all these terminal nodes, node 4 shared a maximum predicted value than other nodes.

Table 3 below shows the predictive performance of CART, CHAID and EX-CHAID models. The results from the Table showed that CART model had a high value of r, R^2, AdjR^2, and a lower RMSE, AIC and BIC. The findings further displayed that the CHAID model had the lowest r, R^2, AdjR^2, and the highest RMSE, AIC and BIC. In this case, CART was the best predictive model for the prediction of body weight of the White Leghorn chicken breed.

Table 3 Predictive performance of CART, CHAID and Ex-CHAID

Criteria	CART	CHAID	EXHAUSTIVE-CHAID	Decision
r	0.84	0.66	0.72	Greater is better
R^2	0.71	0.44	0.52	Greater is better
Adj R^2	0.70	0.43	0.52	Greater is better
RMSE	0.18	0.25	0.23	Smaller is better
AIC	-341.77	-276.94	-293.38	Smaller is better
BIC	-339.16	-274.33	-290.78	Smaller is better

r: correlation coefficient, R^2: coefficient of determination, Adj R^2: adjusted coefficient of determination, RMSE: root mean square error, AIC: Akaike information criterion, BIC: Bayesian information criterion.

DISCUSSION AND CONCLUSION

Due to the lack of weighing scales, rural farmers with limited resources can predict livestock body weight using morphometric traits (Adhianto and Harris, 2020). Firstly, the present study examined the relationship between body weight and morphometric traits of White Leghorn using Pearson's correlation. The results on morphometric traits revealed that body length had a positive highly significant correlation with body weight, with chest girth, wing length and chicken height having a positive significant correlation with body weight. The findings of the current study were in accordance with those of Tyasi et al. (2020), who found that beak length, wing length and back length played an important role in the body weight of Potchefstroom Koekoek laying hens. Similarly, Dzungwe et al. (2018) found that morphological traits such as wing length had a significant role in the body weight of French Broiler Guinea Fowl. Ojo et al. (2014) also indicated that wing length had a significant correlation to the body weight of Japanese Quail. However, the findings of the current study contradict with the findings of Tyasi et al. (2017) who reported non-significant correlation between body weight and studied morphometric traits of Chinese Dagu chickens. The differences observed might be due to the genetic variations of the breed. Yunusa and Adeoti (2014) reported similar results to the current study, where body length and breast length were crucial morphometric traits, which helped to establish body weight of Yoruba and Fulani ecotype chickens. The correlation results of the current study imply that increasing body length, wing length, chest girth and chicken height might improve the body weight of White Leghorn chicken breed. The study further developed the best model for prediction of body weight from morphometric traits of White Leghorn chicken breed using data mining algorithms. The current study revealed that CART model explained 71% of the variance in White Leghorn body weight and demonstrated superior predictive accuracy. The CART model outperformed the CHAID and Ex-CHAID models. CART model showed chest girth as the best predictor trait of body weight of White Leghorn chicken breed. The findings of the current study were in accordance with those of Tyasi et al. (2024), who found that CART is the most effective model in the prediction of body weight in chickens as compared to CHAID. The findings of the current study were in disagreement with the findings of Gevrekçi and Takma (2018) who showed that CHAID model was the best in the prediction of body weight in poultry species. However, the variations in the findings might be due to breed and environment differences, and data characteristics. More studies need to be conducted on the prediction of body weight using data mining algorithms in chickens. CHAID performed poorly, and this might be caused by small sample size within the nodes, which resulted in overfitting of the model. The findings of the current study on CHAID performance were supported by Lemke et al. (2009).

The current study concludes that there is a positive relationship that exists between body weight and chest girth, body length and wing length. These traits can be used to enhance body weight of White Leghorn chickens. CART model proved to be the best-fitting model for accurately predicting body weight, indicating that chest girth is a key predictor of body weight in the White Leghorn chicken breed. The findings from the current study might help researchers, chicken breeders or farmers on which morphometric traits to use when they want to predict the BW of their chickens. It is recommended that further studies can be conducted on prediction of the body weight of chickens using different data mining algorithms.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTION

Conception: TLT; Design: TLT; Supervision: TLT, MCM; Materials: HLM, RLM, VRH, HJP, LTR, PXR; Collection/or Processing: HLM, RLM, VRH, HJP, LTR, PXR; Analysis and/or Interpretation: MCM, HLM, RLM, VRH, HJP, LTR, PXR; Literature Search: HLM, RLM, VRH, HJP, LTR, PXR; Writing – Original Draft: HLM, RLM, VRH, HJP, LTR, PXR; Critical Review: TLT, MCM

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PREDVIĐANJE TJELESNE TEŽINE NA OSNOVI MORFOMETRIJSKIH KARAKTERISTIKA BIJELOG LEGHORNA KORIŠTENJEM ALGORITMA ZA RUDARENJE PODATAKA

SAŽETAK

Cilj istraživanja je kreirati prediktivni model za određivanje tjelesne težine Bijelog leghorna na osnovi morfometrijskih karakteristika korištenjem algoritama za rudarenje podataka. Podaci su prikupljeni na 100 pilića, a odnose se na tjelesnu težinu (BW), dužinu kljuna (BKL), dužinu grebena (KL), obim prsa (CG), obim tijela (BG), dužinu goljenice (SL), dužinu leđa (BCL), obim golenjače (SC) i dužinu krila (WL). Za analizu su korišteni: *Chi-Squared Automatic Interaction Detection* (CHAID), *Classification and Regression Trees* (CART) i *Exhaustive chi-squared Automatic Interaction detection* (EX-CHAID). Na osnovu usklađenosti, CART model je bio najbolji model za predviđanje tjelesne težine kod Bijelog leghorna sa višim vrijednostima koeficijenta korelacije (r = 0.84) i koeficijenta determinacije (R^2 = 0.71) i nižim korijenom srednje kvadratne pogreške (RMSE = 0.18), *Akaike* informacijskim kriterijem (AIC = -341.77) i Bayesovim informacijskim kriterijem (BIC = -339.16). CART model je identificirao CG, BL i WL kao ključne faktore varijacije BW, ukazujući kako fokusiranje na ove karakteristike može olakšati predviđanje tjelesne težine i biti od pomoći uzgajivačima u poboljšanju karakteristika pilića.

Ključne riječi: CART, CHAID, Exhaustive CHAID, usklađenost

RESEARCH ARTICLE

THE EFFECTS OF ORAL SERRATIOPEPTIDASE ADMINISTRATION ON MOTOR ACTIVITY, EXPLORATORY BEHAVIOR, AND SEIZURE SUSCEPTIBILITY IN MICE

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ABSTRACT

This study investigated the effects of orally administered serratiopeptidase on motor activity, coordination, exploratory behavior and seizure susceptibility in mice. Male Swiss albino mice (n=8 per group) at doses of 5, 10, and 20 mg/kg via oral gavage for a period of 15 days. A control group received an equivalent volume of distilled water. Motor activity was assessed using the open field test, with horizontal activity (number of squares crossed) and vertical activity (number of rearing) recorded. Motor coordination and exploratory behavior were evaluated using negative geotaxis and a head-pocking test. Then, mice were intraperitoneally administered with 200mg/kg of pilocarpine. The onset of convulsion, number of convulsions and survival rate were assessed. Diazepam (1mg/kg) served as positive control. Serratiopeptidase significantly increased both horizontal and vertical activity in the open field test and improved exploratory behavior, particularly at the 20 mg/kg dose. Motor coordination improvements were also observed, though with a biphasic dose-response pattern. Notably, serratiopeptidase significantly delayed the onset of seizures, reduced seizure frequency, and improved survival rates - effects that were comparable to diazepam at the 10 and 20 mg/kg doses. These findings suggest that oral administration of serratiopeptidase may positively modulate motor function in mice, furthermore, serratiopeptidase possesses anticonvulsant properties and may be a potential therapeutic agent for seizure disorder. Further investigation is warranted to elucidate the exact mechanism of action and to explore the translational relevance of these findings.

Keywords: Convulsion, open field test, mice, pilocarpine, serratiopeptidase

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INTRODUCTION

Seizures result from abnormal or excessive neuronal discharges and are a hallmark of epilepsy, the most common neurological disorder globally (Abed, 2023; Chauhan et al., 2022; Pedley, 2020). Serious medical emergencies can result from certain seizure types, leading to status epilepticus and the possibility of brain injury or death (Ascoli et al., 2021). However, the physical resiliency in the body enabled by neural plasticity can also facilitate quick recovery with support, such as anti-seizure medication and/or hospital management. There are many different theories about the potential causes of seizures (Alavi et al., 2024).

Approximately two to eight percent of the American population will experience at least one seizure event in their lifetime (Bensken et al., 2021). The main therapeutic strategy for the management of seizures is the use of antiepileptic drugs (AEDs), which are effective in most types of idiopathic and symptomatic epilepsy. In general, treatment is much more complex for the so-called difficult-to-control forms as compared to non-chronic focal forms (Ghosh et al., 2021). The enzyme is also known as Serratia E15, S-E15, Serratia Peptidase, and Serrapeptase. Its name is systematically named “Peptidase S3” and the Enzyme Commission number (EC number) is 3.4.24 (Calogero et al., 2017). However, commercially available products are usually called “Serrapeptase.” Serrapeptidase was discovered in the intestinal microflora of silkworms and was isolated and identified in the 1960s (Ahmed et al., 2023). In the 1960s, there were reports of tissue-dissolving action in the case of inflammation and other injuries in the silkworms (Ahmed et al., 2023).

Pilocarpine is an agonist on muscarinic receptor, it’s tertiary amine and can cross blood brain barrier and produce central nervous impacts, it’s widely used in preclinical studies to induce seizure in animals models (Duffy, 2014; Yang et al., 2022). Temporal lobe epilepsy models, especially those induced by pilocarpine, replicate many clinical and neuropathological features of human epilepsy (Henkel et al., 2021). Serratiopeptidase is a proteolytic enzyme produced by the Gram-positive bacterium Serratia sp. E-15 through expressing the gene encoding Serratiopeptidase (Curia et al., 2008; Kumar et al., 2023; Lévesque et al., 2021; Narayanan, 2025). To our knowledge, this is among the few studies exploring the potential anticonvulsant properties of serratiopeptidase, a proteolytic enzyme, in a pilocarpine-induced seizure model. Given the role of inflammation and oxidative stress in seizure pathophysiology, we hypothesized that serratiopeptidase could attenuate seizure severity and improve behavior in a rodent model. The present study aimed to assess its effects on motor activity, coordination, and seizure susceptibility in pilocarpine-induced seizures. We hypothesize that repeated oral administration of serratiopeptidase would improve motor activity and reduce seizure severity in a pilocarpine-induced mouse model through anti-inflammatory and neuromodulatory mechanisms. Additionally, we have specified that the primary outcomes are seizure onset, seizure frequency, and survival, and the secondary outcomes are motor activity, coordination, and exploratory behavior.

MATERIAL AND METHODS

Animals

Male Swiss albino mice aged 6-8 weeks at the start of the experiment were used. Mice were housed in standard laboratory conditions. Mice were housed in groups of 4-5 per cage to standard rodent chow and water *ad libitum.* Randomization was performed using computer-generated sequences, and all behavioral assessments and seizure observations were conducted by blinded experimenters.

Ethical Consideration

The study adhered to the ARRIVE 2.0 guidelines and was approved by the Institutional Animal Care and Use Committee (UM.VET.2024.006) by the University of Mosul/College of Veterinary Medicine.

Drugs and Treatments

Serratiopeptidase was dissolved in distilled water to prepare the required concentrations (5,10 and 20 mg/kg). Pilocarpine hydrochloride (eye drop 2%) was diluted with distilled water to prepare the required concentration (200 mg/kg) for intraperitoneal injection. Diazepam was diluted in sterile saline and administered i.p at a dose of 1 mg/kg. Doses were selected based on prior studies reporting anti-inflammatory and behavioral effects of serratiopeptidase in rodents (Ahmed et al., 2013; Naser, 2024).

Control Groups

Vehicle Control: Mice received an equivalent volume of distilled water (the vehicle for serratiopeptidase) via oral gavage.

Positive Control: Mice received diazepam (1mg/kg, i.p.).

Experimental Design

Mice were randomly divided into five groups (n=8 per group):

Vehicle Control (distilled water, oral gavage).

Serratiopeptidase 5 mg/kg (oral gavage).

Serratiopeptidase 10 mg/kg (oral gavage).

Serratiopeptidase 20 mg/kg (oral gavage).

Positive Control (Diazepam 1mg/kg, i.p.)

Behavioral Assessments

Serratiopeptidase and vehicle were administered daily for 15 days. On the Day 16 the mice in all groups except positive control underwent for behavioral assessments.

1. Open Field Test (for Motor Activity): The open field apparatus consisted of square arena (40x40x30cm) with the floor divided into 225 equal squares. Mice were placed individually in the center of the open field and allowed for a defined period (5minutes) (Sturman et al., 2018). The following parameters were recorded:

Horizontal activity: Number of squares crossed with all four paws.

Vertical activity: Number of rearing (standing on hind limbs).

2. Negative Geotaxis Test: This test was performed to assess coordination and balance. Each mouse was placed head-downwards on a 45° inclined plane. The time taken for the mouse to turn itself 180° (so that its head was pointing upwards) was recorded in seconds. A maximum cutoff time (not specified) was likely used (Motz and Alberts, 2005).

3. Head-Poking Test: This test was used to evaluate exploratory behavior. This apparatus likely consisted of circular arena diameter of 30cm and contained 8 holes (2 cm diameter each). Each mouse was placed in the arena, and the number of times the mouse poked its head into the holes was recorded over a 5-minute period (Al-Shalchi and Mohammad, 2024; Soni and Parle, 2017).

Seizure Assessment

On Day 17, all groups except the positive control were administered pilocarpine (200 mg/kg, i.p.) to induce seizures. The positive control group received diazepam 30 minutes before pilocarpine administered. Following pilocarpine administration, mice were continuously observed for 1 hour for seizure activity (Bröer and Löscher, 2015).

The following parameters were recorded:

Latency to the first convulsion (onset of convulsion): Time elapsed from pilocarpine injection to the first observed seizure.

Numbers of convulsions: The total number of seizures observed during the observation period.

Survival rate: The number of mice surviving the observation period.

Statistical Analysis Data are expressed as mean± standard error of the mean (SEM). Statistical analysis was performed using on-way analysis of variance (ANOVA) followed by appropriate *post hoc* tests for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Open Field Test

Horizontal activity (Locomotion): The control group exhibited a baseline horizontal activity of 76.75±7.74 squares crossed. The 5mg/kg serratiopeptidase group showed a moderate increase in horizontal activity (91.25±8.22 squares crossed) compared to the control group. The 10mg/kg serratiopeptidase group displayed a slight decrease in horizontal activity (83.25± 8.52 squares crossed) compared to the 5mg/kg group, but still higher than the control group. The 20 mg/kg serratiopeptidase group demonstrated the highest horizontal activity (96.13±6.65 squares crossed).

Vertical activity (Exploration/ Rearing): The 5mg/kg serratiopeptidase group exhibited a moderate increase in vertical activity (20.75±2.50 rearing) compared to the control. Both the 10 mg/kg (22.38±4.56 rearing) serratiopeptidase groups displayed further increases in vertical activity.

Negative Geotaxis Test

The results of the 15-day oral administration of serratiopeptidase on motor coordination and exploratory behavior in mice are summarized in Table 2. In the Negative Geotaxis test, control mice demonstrated a mean latency of 8.00 ± 4.27 seconds. Treatment with serratiopeptidase at 5 mg/kg and 10 mg/kg resulted in slight, non-significant increases in latency to 10.40 ± 2.61 and 11.60 ± 6.60 seconds, respectively, suggesting a mild delay in motor response. However, at the highest dose of 20 mg/kg, the latency was markedly reduced to 4.80 ± 1.20 seconds, indicating an apparent improvement in motor coordination compared to the control, although this difference was not statistically significant.

Head Poking Test

In the Head Poking test, which reflects exploratory behavior and general activity, the control group showed 10.20 ± 2.71 pokes in 3 minutes. Serratiopeptidase treatment at increasing doses induced a dose-dependent rise in head poking behavior: 11.40 ± 2.63 (5 mg/kg), 13.40 ± 1.40 (10 mg/kg), and significantly up to 17.40 ± 2.10 pokes at 20 mg/kg ($p \leq 0.05$). This statistically significant increase at the highest dose indicates a stimulatory effect of serratiopeptidase on exploratory activity, which may reflect enhanced cognitive engagement or reduced anxiety-like behavior in mice.

Pilocarpine- induced Seizure Model

Negative control Mice in this group experienced rapid onset of convulsion (8 minutes), a high number of seizures (10.3), and a survival rate of 6/8 (75%). This reveals the baseline effect of pilocarpine.

Positive control (Diazepam) this group significantly delayed the onset of convulsions (32.75 minutes) and reduced the number of seizures (5.8). All mice in this group survived (8/8, 100%), revealing the effectiveness of diazepam as an anticonvulsant.

Serratiopeptidase

5 mg: delayed the onset of convulsion (14.5 minutes) and reduced the number of seizures (4.5) compared to the negative control. However, the survival rate was lower (4/8, 5%).

10 mg and 20 mg; Both doses significantly delayed the onset of convulsion (24.5 and 24.25 minutes, respectively) and reduced the number of seizures (3.5 in both cases). The survival rate in both groups was 8/8 (100%), comparable to the positive control.

Table 1 The influence of oral serratiopeptidase (15 day of administration) on motor activity and exploratory behavior in mice

Parameters / Groups	Horizontal Activity (Squares Crossed)	Vertical Activity (Rearings)
Control Group	76.75 ±7.74	16.00 ±2.67
Serratiopeptidase (5mg/kg)	91.25 ±8.22	20.75 ± 2.50
Serratiopeptidase (10mg/kg)	83.25 ±8.52	22.38 ± 4.56
Serratiopeptidase (20mg/kg)	96.13 ±6.65	24.25 ±1.68

10 mg and 20 mg: Both doses significantly delayed the onset of convulsions (24.5 and 24.25 minutes, respectively) and reduced the number of seizures (3.5 in both cases). The survival rate in both groups was 8/8 (100%), comparable to the positive control.

The data are mean ± SE of 8 mice/group.

Table 2 The effect of 15-day oral administration of serratiopeptidase on negative geotaxis test and gead poking test

Parameters / Groups	Negative Geotaxis\60 sec	Head Poking \3 min
Control	8.00 ± 4.27	10.20± 2.71
Serratiopeptidase (5 mg/kg)	10.40 ± 2.61	11.40 ± 2.63
Serratiopeptidase (10 mg/kg)	11.60 ± 6.60	13.40± 1.40
Serratiopeptidase (20 mg/kg)	4.80 ± 1.20	17.40± 2.10*

The data are mean ± SE of 8 mice/group
* Significantly different from the data of control group at ($p \leq 0.05$).

Table 3 The effect of serratiopeptidase (15 day of administration) on the convulsion induced by pilocarpine 200 mg/kg

	Onset of Convulsion (minute)	Number of Convulsions	Survival Percentage
Negative Control (Distilled Water)	8.00±2.0	10.3±2.1	6/8
Positive Control (Diazepam 1mg/kg IP)	32.75±3.2*	5.8±1.2*	8/8
Serratiopeptidase (5 mg)	14.50±2.7*A	4.5±0.6*	4/8
Serratiopeptidase (10 mg)	24.50±2.4 *AB	3.5±0.4*	8/8
Serratiopeptidase (20 mg)	24.25±0.5*A B	3.5±0.3*	8/8

The data are mean ± SE of 8 mice/group.

* Significantly different from the data of control group at ($p \leq 0.05$).
A Significantly different from the data of positive control at ($p \leq 0.05$).
B Significantly different from the data of group of 5 mg at ($p \leq 0.05$).

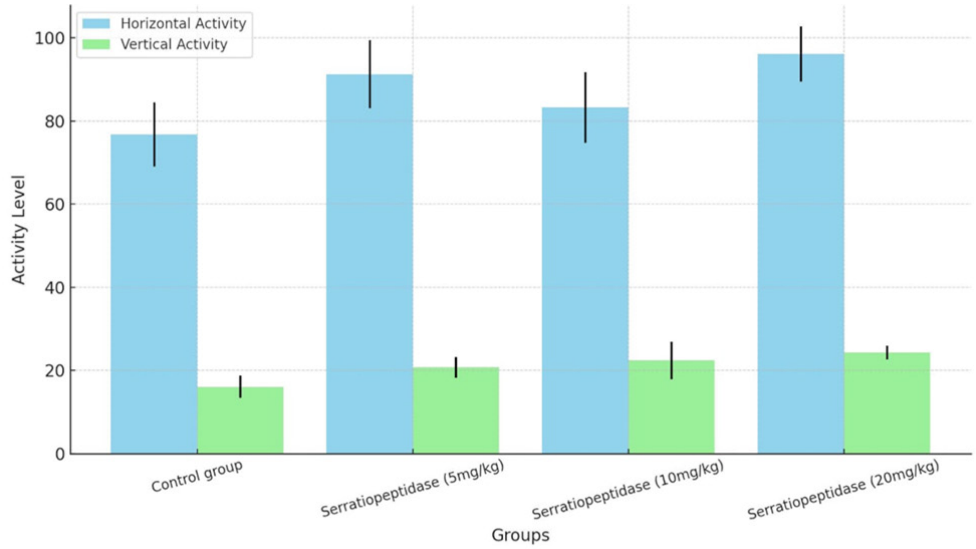

Figure 1 Effect of serratiopeptidase on motor activity in mice

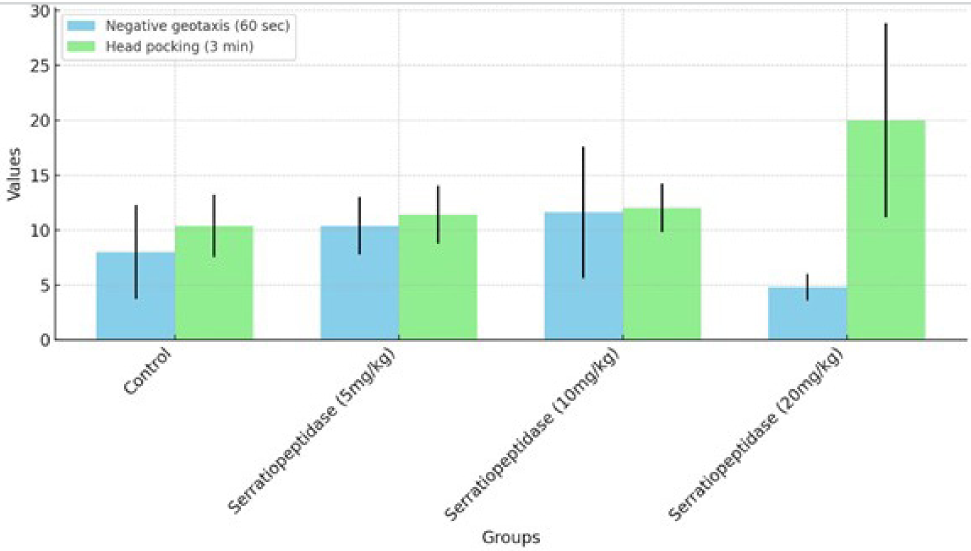

Figure 2 Effect of serratiopeptidase on negative geotaxis and head poking tests

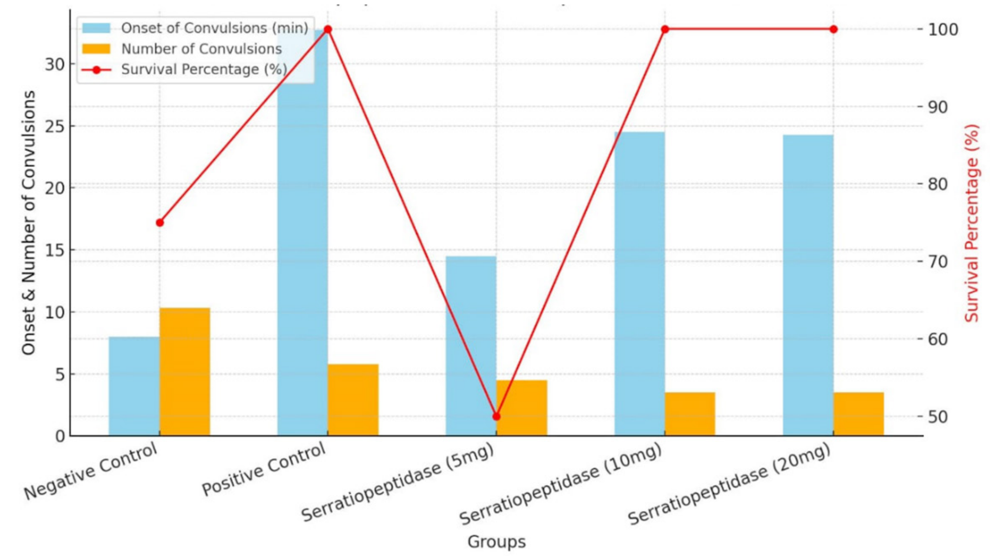

Figure 3 Effect of serratiopeptidase on pilocarpine-induced convulsions

DISCUSSION AND CONCLUSION

This study investigated the impacts of repeatedly orally administered serratiopeptidase on motor activity, exploration behavior and its possible anticonvulsant efficacy in a pilocarpine-induced seizures model in mice. The findings provide evidence that serratiopeptidase can modulate motor function, exploration and exert protective effects against pilocarpine-induced seizures.

Effect on motor activity: The results revealed a significant increase in both horizontal and vertical motor activity in mice treated with serratiopeptidase compared to the control group. Several potential mechanisms could underlie these observed effects. Serratiopeptidase is known for its anti-inflammatory properties (Nair, 2022). It is possible that subtle, subclinical inflammation could be affecting motor function and exploratory drive (Cordone, 2024; Jia, 2022), and the anti-inflammatory effect of serratiopeptidase could be mitigating this. Alternatively, serratiopeptidase may influence neurotransmitter system involved in motor control and behavior, such as dopaminergic or serotonergic systems (Ahmed et al., 2013; Naser, 2024). Many studies demonstrated serratiopeptidase having an inhibitory effect on brain cholinesterase, which is responsible for degradation of excitatory neurotransmitter acetylcholine (Jadhav et al., 2020; Naser and Albadrany, 2024). Future studies exploring these mechanisms are necessary to fully elucidate the underlying processes.

The results of Negative Geotaxis and Head Poking test present a complex picture of serratiopeptidase's effects on motor coordination and exploratory behavior. The Negative Geotaxis effect test results suggest a biphasic dose response. While the lower doses (5 mg and 10 mg) seemed to impair motor coordination (though not statistically significant), the highest dose (20 mg) appeared to improve it. This non-linear dose response warrants further investigation to understand the underlying mechanism. At the lower doses of 5 mg and 10 mg, the slight increase in time suggests a possible negative effect on motor coordination or muscle strength at these doses. This could be to various reasons such as mild muscle relaxation; serratiopeptidase has some anti-inflammatory effects, which might lead to mild muscle relaxation at doses, slightly hindering the mice's ability to quickly turn over (Malanga and Wolff, 2008; Malm and Borisch, 2015). Another reason, interference with nerve signaling; it's possible that doses subtly interfere with nerve impulses involved in motor control. High dose of 20mg effect, the significant decrease in time, is intriguing. It suggests a potential improvement in motor function. This could be due to:enhancement of muscle strength or coordination or stimulatory effect on the nervous system (Sheffler and Chae, 2007). The head poking reveals a different pattern. The highest dose of serratiopeptidase lead to a significant increase in head pokes, suggesting increase exploratory and/or potentially anxiety-related behavior. This finding raises concerns about potential behavioral side effects of higher doses of serratiopeptidase. High dose of 20 mg's effect of the substantial increase to 17.4 poks is particularly noteworthy, which may be due to exacerbations of underlying neurological processes. If these mice have an underlying predisposition to repetitive movements (which is common in some mouse strains) (Barr and Barbe, 2002; Moy et al., 2008), the serratiopeptidase might be exacerbating those tendencies. Other interpretation relatesto inducing or unmasking repetitive behavior; it's possible that the high dose directly induces these repetitive movements, or unmasks them by affecting certain brain circuits (Rossini and Pauri, 2000; Shadmehr, 2017).

Several factors could contribute to those observed effects. The anti-inflammatory properties of serratiopeptidase is one of them: The varying effects at different doses could be related to the modulation of inflammatory pathways at different concentrations. Furthermore, it's possible that serratiopeptidase interacts with the neurotransmitter system like dopamine, serotonin, or acetylcholine. Different doses could differentially affect these systems. Although the behavioral data at 20 mg/kg suggest functional enhancement, the exaggerated head-poking behavior raises concerns regarding potential neurotoxicity. High doses of proteolytic enzymes may exert off-target effects, such as degradation of extracellular matrix proteins or interference with synaptic structure and function (Rudmann, 2013). Furthermore, overactivation of microglia or astrocytes in response to enzymatic stress could paradoxically induce neuroinflammation, disrupting neural circuitry and promoting abnormal behavior (Barr and Barbe, 2002; Moy et al., 2008).

The study also revealed that serratiopeptidase possessed significant anticonvulsant activity in the pilocarpine-induced seizure model. Pretreatment with serratiopeptidase resulted in a significant delay in the onset of convulsions and a reduction in the number of seizures compared with negative control group. Notably, the 10 and 20 mg/kg doses of serratiopeptidase exhibited comparable efficacy to diazepam, a commonly used benzodiazepine anticonvulsant, in reducing seizure severity and improving survival rates. These findings suggest that serratiopeptidase may offer a therapeutic benefit in the management of seizure. The potential mechanism underlying these anticonvulsant impacts may include modulation of neurotransmitter systems (Sills & Rogawski, 2020). Pilocarpine induces seizure by activating cholinergic muscarinic receptors, and serratiopeptidase may interfere with this process by modulating cholinergic neurotransmission, or by affecting other neurotransmitter systems involved in seizure generation and propagation, such as GABAergic or glutamatergic systems (Akyuz et al., 2021). Neuroinflammation and oxidative stress play a crucial role in the pathophysiology of seizures (Parsons et al., 2022). Serratiopeptidase's known anti-inflammatory and antioxidant properties could contribute to its anticonvulsant effects by mitigating these pathological processes. Concerning the blood- brain barrier modulation, some studies suggest that serratiopeptidase may enhance drug delivery across the blood-brain barrier (Tiwari, 2017). This potentially facilitates the access to endogenous anticonvulsant substances or improves the clearance of pro-convulsant substances from the brain.

This study has several limitations, including the use of only male mice, which necessitates further research in humans for broader applicability, as well as the lack of formal seizure-scoring and pharmacokinetic data. Additionally, the precise mechanism by which serratiopeptidase influences motor activity, coordination, exploratory behavior, and seizures, remains unclear. Future studies should address these gaps by incorporating sham groups, biochemical analyses, and mechanistic investigations.

In conclusion, this study provides evidence that orally administered serratiopeptidase can modulate motor function. Also, serratiopeptidase has dose-dependent effects on motor coordination and exploratory behavior in mice. While the highest tested dose of 20mg appeared to improve motor coordination, it also significantly increased exploratory/repetitive behavior. These findings highlighted the need for careful dose selection and further investigation into the mechanism of action and potential behavioral side effects of serratiopeptidase. Finally, serratiopeptidase exerts anticonvulsant activity in a pilocarpine-induced seizure model in mice. These findings suggest that serratiopeptidase may have a potential for management of seizure disorders.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CONTRIBUTIONS

Authors: YMAh and ASN contributed equally in all aspects.

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EFEKTI ORALNE APLIKACIJE SERATIOPEPTIDAZE NA MOTORNU AKTIVNOST, EKSPLORATIVNO PONAŠANJE I SKLONOST KONVULZIJAMA KOD MIŠEVA

SAŽETAK

U ovom istraživanju ispitivani su efekti oralno aplicirane seratiopeptidaze na motornu aktivnost, koordinaciju, eksplorativno ponašanje i sklonost konvulzijama kod miševa. Mužjacima švicarskih albino miševa (n=8 po grupi) su oralnom sondom aplicirane doze od 5, 10 i 20 mg/kg u trajanju od 15 dana. Kontrolnoj grupi je apliciran isti volumen destilirane vode. Motorna aktivnost je procijenjena testom otvorenog polja, pri čemu su zabilježene horizontalna (broj pređenih kvadratića) i vertikalna (broj podizanja) aktivnost. Motorna koordinacija i eksplorativno ponašanje su procijenjeni korištenjem negativne geotaksije i testom guranja glave. Potom je miševima aplicirano 200 mg/kg pilokarpina. Zabilježeni su početak konvulzija, broj konvulzija i stopa preživljenja. Dijazepam (1mg/kg) je služio kao pozitivna kontrola. Seratiopeptidaza je signifikantno povećala horizontalnu i vertikalnu aktivnost na testu otvorenog polja i poboljšala eksplorativno ponašanje, posebno u dozi od 20 mg/kg. Zabilježena su i poboljšanja u motornoj koordinaciji, iako je postojao bifazni odgovor na dozu. Značajno je da je seratiopeptidaza signifikantno odložila početak konvulzija, smanjila njihovu učestalost i poboljšala stope preživljenja - efekti koji su bili komparabilni s dijazepamom u dozama od 10 i 20 mg/kg. Ovakvi rezultati sugeriraju da oralna primjena seratiopeptidaze može pozitivno modulirati motornu funkciju kod miševa, štoviše, seratiopeptidaza posjeduje antikonvulzantna svojstva i može imati ulogu potencijalnog terapijskog agensa za kontrolu konvulzija. Potrebna su daljnja istraživanja kako bi se razjasnio tačan mehanizam djelovanja i istražila translacijska relevantnost ovih nalaza.

Ključne riječi: Konvulzija, miševi, pilokarpin, seratiopeptidaza, test otvorenog polja

RESEARCH ARTICLE

CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE OF VAGINAL MICROBIOTA IN MARES FROM LAGOS AND OYO STATES, NIGERIA

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ABSTRACT

Despite the importance of microbiota in mammalian reproductive health, research on equine vaginal microbiota remains limited, particularly in tropical regions. The composition of mares' vaginal microbiota in Lagos and Oyo States, Nigeria is undocumented. Microbiota profiles are essential for developing effective reproductive health management strategies. We aim to characterize vaginal microbiota composition and determine antimicrobial resistance profiles of isolated bacteria in mares from Lagos and Oyo States, Nigeria. Vaginal swabs from 81 apparently healthy mares, comprising Nigerian indigenous breeds, Sudanese breeds, and Argentine Polo Ponies, were collected using sterile guarded culture swabs. Samples underwent bacterial isolation on selective and differential media including Trypticase soy, Mannitol salt, Eosine methylene blue, Muller-Hinton, Salmonella-Shigella, and MacConkey agar. Bacterial identification involved morphological and biochemical tests, including oxidase, catalase, motility, Gram staining, and sugar fermentation. Antimicrobial susceptibility was tested against 18 antimicrobials using the Kirby-Bauer disc diffusion method following CLSI standards. Analysis revealed distinct bacterial distribution patterns between locations. Of 81 samples, *Staphylococcus aureus* dominated (66.7%), while *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* each represented 1.2%, 30.9% showed no growth. Biochemical characterization demonstrated typical profiles for each species. *S. aureus* showed complete sensitivity to imipenem and streptomycin, but resistance to cefuroxime, penicillin-streptomycin, enrofloxacin, ciprofloxacin, and metronidazole. *P. aeruginosa* was sensitive to clindamycin and imipenem, while resistant to most other antibiotics. Given the high presence of drug-resistant *S. aureus*, veterinarians should prioritize aminoglycosides and carbapenems for treating mare vaginal infections, using other antibiotics only with susceptibility testing guidance.

Keywords: Antimicrobial resistance, equine reproduction, Nigerian mares, *Staphylococcus aureus*, vaginal microbiota

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INTRODUCTION

The reproductive well-being of mammals is closely connected to various microbial communities that inhabit their reproductive systems. These microorganisms, referred to as the microbiota, are essential for sustaining reproductive health and fitness (Malaluang et al., 2024).

The lower female genital area contains a dynamic microbial ecosystem, featuring numerous microorganisms that can inhabit both the vagina and uterus in healthy animals (Adekunle et al., 2024). Various factors affect the composition and stability of equine vaginal microbiota. These consist of physiological elements, like the phase of the oestrous cycle, age, and reproductive condition; environmental elements such as geographical area, climate, and seasonal changes; and management aspects like nutrition, veterinary care, and previous use of antimicrobial drugs (Gil-Miranda et al., 2024; Malaluang et al., 2024). The dynamic characteristics of the bacterial community are also highlighted by its reactions to interventions like antibiotic therapy or breeding practices, along with pathological states such as uterine infections. This complexity highlights the significance of comprehending these interactions, since dysbiosis—an imbalance in bacterial populations—can result in pathogen growth and ensuing reproductive health problems (Gil-Miranda et al., 2024).

Earlier research has recognized various prevalent bacterial species within mares' vaginal microbiota, such as *Escherichia coli, Staphylococcus capitis, Streptococcus equisimilis*, *Streptococcus thoraltensis*, and *Streptococcus zooepidemicus* (Malaluang et al., 2024). Nonetheless, these results mainly originate from research conducted in a different area, and the bacterial composition of mares in Lagos and Oyo States, Nigeria, is still not documented.

This research aims to characterize the vaginal microbiota composition and determine the antimicrobial resistance profile of isolated bacteria in mares from Lagos and Oyo States, Nigeria. This information is especially important for veterinary professionals and researchers aiming to tackle infertility and subfertility challenges in mares in tropical settings. Moreover, comprehending the patterns of dysbiosis and microbial interactions will aid in creating more efficient treatment approaches for reproductive tract infections.

This research hypothesizes that the vaginal microbiota of mares in the studied regions possesses unique characteristics, shaped by the tropical climate, nutritional resources, and management practices. These factors are expected to influence bacterial diversity and the prevalence and distribution of bacterial species from mare vagina.

MATERIALS AND METHODS

Ethical Approval

The study was conducted in strict accordance with ethical guidelines for animal research and was approved by the Animal Care, Use and Research Ethics Committee of the University of Ibadan, Nigeria (Approval No.: NHREC/UIACUREC/07/11/2025B). Informed consent was also obtained from all animal owners.

Animal and Sample Collection

A total of 81 adult mares comprising Nigerian indigenous breed, Sudanese, and Argentine polo ponies were used for this study. All mares were apparently healthy at the time of sampling. Vaginal swabs were collected from these mares in Lagos and Oyo States, Nigeria. Prior to sample collection, each mare was properly restrained with minimal stress to ensure safety and proper sample collection. The tail was wrapped and secured away from the perineal area using a clean tail bandage. The perineal area was thoroughly cleaned with mild soap and water, rinsed with clean water, and dried using sterile gauze to prevent contamination.

A sterile, long-handled guarded culture swab was used to collect samples from the vaginal wall, approximately 10-15 cm cranial to the vestibulo-vaginal junction. The swab was rotated against the vaginal mucosa with gentle pressure to ensure adequate sample collection. Care was taken to avoid contamination from the vestibule during withdrawal of the swab.

Immediately after collection, each sample was properly labelled with a unique identification number, the date and time of collection, and the mare's details. The samples were maintained at 4°C in a cold chain and transported to the laboratory within 2 hours of collection for microbiological analysis.

Aseptic techniques were maintained throughout the sampling process to prevent cross-contamination between samples and from the environment. All personnel involved in sample collection wore clean disposable gloves, which were changed between animals.

Bacterial isolation and characterisation

The sterile swabs were placed in 10 ml of buffered peptone water for pre-enrichment, then streaked on selective and differential media, including Trypticase soy agar (HiMEDIA®, USA), Mannitol salt (MSA), Eosine methylene blue (EMB), Muller-Hinton, Salmonella-Shigella, and MacConkey (MAC) agar. The samples were then incubated at 37°C for 24 hours. The main colonies on selective and differential agar were continuously transferred to Trypticase soy agar (TSA) to analyse their colonial morphology and purity (Anifowose et al., 2023).

Bacterial isolates were identified based on morphological and biochemical tests, including oxidase, catalase, motility, Gram staining, simmon citrate, indole, glucose (gas), hydrogen sulfide production, lysine, ornithine and urease, methyl red, vogues proskauer, triple sugar Iron agar slant culture as well as sugar fermentation tests (Anifowose et al., 2023)

Antimicrobial Susceptibility Testing

Each isolate was analyzed for antibiotic susceptibility. By using the agar disc diffusion method, which was carried out in accordance with CLSI standards Mo2, pure isolates of the identified bacteria were evaluated for their sensitivity and resistance to antibiotics (CLSI, 2020). The susceptibility was evaluated against 18 antimicrobials (micrograms) from the following ten classes: penicillin (amoxicillin– clavulanic acid 30 µg (AC); ampicillin 10 µg (AP, amplicox 25 µg (AM)), penicillin (penstrep 25 µg), cephalosporin (third-generation) ceftriaxone 30 µg (CEF), cefuroxime 30 µg (CEX), tetracycline (doxycycline 30 µg (D), tetracycline 30 µg (T), chloramphenicol 30 µg (CH), aminoglycoside: gentamicin 10 µg (G)) streptomycin 10 µg (S), metronidazole 5 µg (M), lincomycin (clindamycin 2 µg (CLI), carbapenem (imipenem 10 µg (IPM), fluoroquinolone (ciprofloxacin 5 µg (CIP), (ofloxacin 5 µg (OF)), enrofloxacin 5 µg (ENR)), and glycopeptide: vancomycin 30 µg (V) based on the Kirby-Bauer disc diffusion method (CLSI, 2020; Gbanamou et al., 2024).

Preparation of Antibiotic Disc

The disc was constructed in accordance with the CLSI Mo2 disc diffusion guide; six 6-mm sterilized Whatman filter paper discs were placed on a 100-mm plate, and the discs were impregnated with the known antimicrobial concentrations stated above. Each isolate on a sterile Mueller-Hinton agar plate was made and appropriately labelled with an indelible marker (CLSI, 2020).

Preparation of Inoculums

By adjusting the suspension of each isolate colony in sterile normal saline to the density of a McFarland 0.5 turbidity standard, the direct colony suspension method was employed. Each isolate was grown overnight on Nutrient agar, and pure cultures were harvested using a sterile loop. The colonies were suspended in sterile normal saline that had been blended to a consistent turbidity. Saline or additional bacteria were added in order to get the density of each isolate suspension to McFarland 0.5. After that, Muller-Hinton agar was streaked with the suspended each isolate colony. Quality control was performed using the previously identified *Escherichia coli* ATCC (CLSI, 2020).

Inoculation of Agar Plates

The sterile pointed forceps were used to pick one antibiotic disc (for each bacterial isolate) and applied to the surface of Mueller-Hinton agar plates. This allowed the discs to effectively touch the medium. Afterward, the plates were incubated for 18 hours at 37^0C (CLSI, 2020). For the purpose of identifying which antibiotics the isolate was sensitive to or resistant to, the diameter of the visible zone of growth inhibition surrounding the several discs was measured in millimetres. Each isolate's resistance pattern was created using the antibiogram (Anifowose et al., 2024).

Data Analysis

The data was analyzed using IBM SPSS Statistics software version 26. Descriptive statistics were employed to analyze and present the data. Frequency distributions and percentages were calculated for distribution of bacterial isolates by location (Oyo State and Lagos State), prevalence of different bacterial species (*S. aureus*, *P. aeruginosa*, and *S. epidermidis*) and antimicrobial susceptibility patterns.

RESULTS

Distribution of Bacterial Isolates by Location

Based on Table 1, a total of 81 vaginal swab samples were collected from apparently healthy mares across two locations in Southwestern Nigeria, with 56 bacterial isolates successfully obtained overall. Lagos State contributed the majority of samples (n=60) and yielded 48 bacterial isolates, while Oyo State provided 21 samples with 8 bacterial isolates recovered. The chi-square analysis ($\chi^2 = 12.85$, df = 1, $p < 0.001$) reveals a statistically significant association between sampling location and bacterial isolation patterns from the vaginal microflora of these mares.

Prevalence of Isolated Bacterial Species

The prevalence data showed that *S. aureus* was the dominant organism, representing 66.7% of all samples from the vaginal swabs, while *P. aeruginosa* and *S. epidermidis* each represented 1.2% of the isolates. Notably, 25 samples (30.9%) showed no bacterial growth (Table 2).

Biochemical Characteristics of Bacterial Isolates

The biochemical characterization revealed distinct profiles for each bacterial species isolated from the mare vaginal swabs. Both *Staphylococcus* species demonstrated typical Gram-positive cocci characteristics, showing positive results for catalase and negative for oxidase. *S. aureus* and *S. epidermidis* could be differentiated by their behaviour on Mannitol salt agar, with *S. aureus* producing yellowish colonies and *S. epidermidis* forming pinkish colonies. Both species showed similar sugar fermentation patterns, being positive for glucose, maltose, lactose, mannitol, xylose, sucrose, and arabinose. *P. aeruginosa* displayed characteristic Gram-negative rod morphology with unique biochemical features including positive oxidase and motility tests. It showed limited sugar fermentation capabilities compared to the *Staphylococcus* species, being positive only for glucose and mannitol, while producing a distinctive greenish-blue pigment on Mueller-Hinton agar (Table 3).

Antimicrobial Susceptibility Patterns

The antimicrobial susceptibility testing revealed varying patterns of resistance and sensitivity among the isolated bacteria. For *S. aureus* isolates (Table 4), complete sensitivity was observed to imipenem and streptomycin, while gentamicin showed moderate effectiveness with over half of the isolates being sensitive. However, concerning resistance patterns were noted against multiple antibiotics including cefuroxime, penicillin-streptomycin, enrofloxacin, ciprofloxacin, and metronidazole. The single *S. epidermidis* isolate (Table 5) demonstrated complete sensitivity to gentamicin and streptomycin but showed resistance to several antibiotics, including cefuroxime, ampicillin, and various fluoroquinolones. *P. aeruginosa* isolate (Table 6) showed a distinct susceptibility pattern, being completely sensitive to clindamycin and imipenem, while demonstrating resistance to most other tested antibiotics. This comprehensive resistance profile suggests the need for careful antibiotic selection in treating vaginal infections in mares, with aminoglycosides and carbapenems appearing to be the most reliable treatment options across all isolated species.

Table 1 Distribution of bacterial isolates from vaginal swabs of apparently healthy mares in different locations of Southwestern Nigeria

Location	No. of Samples	No. of Bacterial Isolates	Chi-square (χ^2)	df	P-value
Oyo State	21	8	12.85	1	< 0.001
Lagos State	60	48			
Total	81	56			

Table 2 Prevalence of bacterial species isolated from mare vaginal swabs

Bacterial isolates	Number	Prevalence (%)
Staphylococcus aureus	54	66.7
Pseudomonas aeruginosa	1	1.2
Staphylococcus epidermidis	1	1.2
No growth	25	30.9
Total	81	100

Table 3 Biochemical characteristics of *Staphylococcus aureus* isolates

Biochemical Characteristics	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa*
Gram Staining	Gram-positive cocci	Gram-positive cocci	Gram-negative rods
Catalase	Positive	Positive	Positive
Oxidase	Negative	Negative	Positive
Motility	Negative	Negative	Positive
Methyl-red	Positive	Positive	Negative
Hydrogen sulphide production	Negative	Negative	Negative
Indole production	Negative	Negative	Negative
Citrate	Positive	Positive	Positive
Voges-proskauer	Negative	Negative	Negative
Urea Hydrolysis Test	Negative	Negative	Negative
Mannitol Salt agar	Yellowish colonies	Pinkish colonies	-
Haemolysis	Positive	Positive	-
OF (Oxidative-Fermentative)	-	-	Facultative anaerobes
TSI Agar	-	-	Alkaline slant, alkali butt
Mueller-Hinton agar			Greenish-blue pigment
Sugar Fermentation Test			
Glucose	Positive	Positive	Positive

Biochemical Characteristics	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa*
Maltose	Positive	Positive	Negative
Lactose	Positive	Positive	Negative
Mannitol	Positive	Positive	Positive
Xylose	Positive	Positive	Negative
Sucrose	Positive	Positive	Negative
Arabinose	Positive	Positive	Negative

Table 4 Frequency of antimicrobial susceptibility for *Staphylococcus aureus*

Number of isolates n = 12

Antimicrobial Class	Antimicrobial Agent	Disk Potency	Sensitive n (%)	Intermediate n (%)	Resistance n (%)
Aminoglycoside	Gentamicin	10 µg	7 (58.33)	5 (41.67)	0 (0.00)
Cephalosporin (3rd generation)	Ceftriaxone	30 µg	0 (0.00)	8 (66.67)	4 (33.33)
Cephalosporin (3rd generation)	Cefuroxime	30 µg	0 (0.00)	0 (0.00)	12 (100.00)
Lincomycin	Clindamycin	2 µg	1 (8.33)	11 (91.67)	0 (0.00)
Penicillins	Ampicillin	10 µg	0 (0.00)	12 (0.00)	0 (0.00)
Penicillins	Amoxicillin	25 µg	0 (0.00)	12 (100.00)	0 (0.00)
Penicillins	Amoxicillin Cloxacillin	30 µg	3 (25.00)	9 (75.00)	0 (0.00)
Penicillins	Penstrep	25 µg	0 (0.00)	0 (0.00)	12 (100.00)
Carbapenem	Imipenem	10 µg	12 (100.00)	0 (0.00)	0 (0.00)
Fluoroquinolones	Enrofloxacin	5 µg	0 (0.00)	0 (0.00)	12 (100.00)
Fluoroquinolones	Ciprofloxacin	5 µg	0 (0.00)	0 (0.00)	12 (100.00)
Fluoroquinolones	Ofloxacin	5 µg	0 (0.00)	12 (100.00)	0 (0.00)
Glycopeptide	Vancomycin	30 µg	2 (16.66)	10 (83.33)	0 (0.00)
Tetracyclines	Oxytetracycline	30 µg	0 (0.00)	11 (91.67)	1 (8.33)
Tetracyclines	Doxycycline	30 µg	0 (0.00)	10 (83.33)	2 (16.66)
Phenicols	Chloramphenicol	30 µg	0 (0.00)	12 (100.00)	0 (0.00)
Metronidazole	Metronidazole	5 µg	0 (0.00)	0 (0.00)	12 (100.00)
Aminoglycoside	Streptomycin	10 µg	12 (100.00)	0 (0.00)	0 (0.00)

Table 5 Frequency of antimicrobial susceptibility for *Staphylococcus epidermidis*

Number of isolates n = 1

Antimicrobial Class	Antimicrobial agent	Disc Potency	Sensitive n (%)	Intermediate n (%)	Resistance n (%)
Aminoglycoside	Gentamicin	10 µg	1 (100.00)	0 (0.0)	0 (0.00)
Cephalosporin (3rd generation)	Ceftriaxone	30 µg	0 (0.00)	1 (100.00)	0 (0.0)
Cephalosporin (3rd generation)	Cefuroxime	30 µg	0 (0.00)	0 (0.00)	12 (100.00)
Lincomycin	Clindamycin	2 µg	0 (0.00)	1 (100.0)	0 (0.00)
Penicillins	Ampicillin	10 µg	0 (0.00)	0 (0.00)	1 (100.00)
Penicillins	Amoxicillin	25 µg	0 (0.00)	1 (100.00)	0 (0.00)
Penicillins	Amoxicillin Cloxacillin	30 µg	0 (0.00)	1 (100.00)	0 (0.00)
Penicillins	Penstrep	25 µg	0 (0.00)	0 (0.00)	1 (100.00)
Carbapenem	Imipenem	10 µg	0 (100.00)	1 (100.00)	0 (0.00)
Fluoroquinolones	Enrofloxacin	5 µg	0 (0.00)	0 (0.00)	1 (100.00)
Fluoroquinolones	Ciprofloxacin	5 µg	0 (0.00)	0 (0.00)	1 (100.00)
Fluoroquinolones	Ofloxacin	5 µg	0 (0.00)	1 (100.00)	0 (0.00)
Glycopeptide	Vancomycin	30 µg	0 (0.0)	1 (100.0)	0 (0.00)
Tetracyclines	Oxytetracycline	30 µg	0 (0.00)	1 (100.0)	0 (0.0)
Tetracyclines	Doxycycline	30 µg	0 (0.00)	1 (100.0)	0 (0.0)
Phenicols	Chloramphenicol	30 µg	0 (0.00)	1 (100.00)	0 (0.00)
Metronidazole	Metronidazole	5 µg	0 (0.00)	0 (0.00)	1 (100.00)
Aminoglycoside	Streptomycin	10 µg	1 (100.00)	0 (0.00)	0 (0.00)

Table 6 Frequency of antimicrobial susceptibility for *Pseudomonas aeruginosa*

Number of isolates n = 1

Antimicrobial Class	Antimicrobial Agent	Disc Potency	Sensitive n (%)	Intermediate n (%)	Resistance n (%)
Aminoglycoside	Gentamicin	10 µg	0 (0.00)	1 (100.0)	0 (0.00)
Cephalosporin (3rd generation)	Ceftriaxone	30 µg	0 (0.00)	0 (0.00)	1 (100.00)
Cephalosporin (3rd generation)	Cefuroxime	30 µg	0 (0.00)	0 (0.00)	1 (100.00)
Lincomycin	Clindamycin	2 µg	1 (100.00)	0 (0.00)	0 (0.00)

Antimicrobial Class	Antimicrobial Agent	Disc Potency	Sensitive n (%)	Intermediate n (%)	Resistance n (%)
Penicillins	Ampicillin	10 µg	0 (0.00)	1 (100.00)	0 (0.00)
Penicillins	Amoxicillin	25 µg	0 (0.00)	0 (0.00)	1 (100.00)
Penicillins	Amoxicillin Cloxacillin	30 µg	0 (0.00)	1 (100.00)	0 (0.00)
Penicillins	Penstrep	25 µg	0 (0.00)	0 (0.00)	1 (100.00)
Carbapenem	Imipenem	10 µg	1 (100.00)	0 (0.00)	0 (0.00)
Fluoroquinolones	Enrofloxacin	5 µg	0 (0.00)	0 (0.00)	1 (100.00)
Fluoroquinolones	Ciprofloxacin	5 µg	0 (0.00)	0 (0.00)	1 (100.00)
Fluoroquinolones	Ofloxacin	5 µg	0 (0.00)	0 (0.00)	1 (100.00)
Glycopeptide	Vancomycin	30 µg	0 (0.00)	0 (0.00)	1 (100.00)
Tetracyclines	Oxytetracycline	30 µg	0 (0.00)	0 (0.00)	1 (100.00)
Tetracyclines	Doxycycline	30 µg	0 (0.00)	0 (0.0)	1 (100.00)
Phenicols	Chloramphenicol	30 µg	0 (0.00)	1 (100.00)	0 (0.00)
Metronidazole	Metronidazole	5 µg	0 (0.0)	1 (100.00)	0 (0.00)
Aminoglycoside	Streptomycin	10 µg	0 (0.00)	1 (100.00)	0 (0.00)

DISCUSSION AND CONCLUSION

The investigation of vaginal microbiota in mares and other animals serves as a guide in understanding different conditions that may affect the normal flora and possible intervention to resolve it (Malaluang et al., 2024). In this study, only 3 bacteria organisms were isolated from the vagina of apparently healthy breeding mares as follows: *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. *Staphylococcus aureus* was the most prevalent bacteria organism isolated from the vagina of breeding mares from the two sampled location. The reason for the high prevalence of *Staphylococcus aureus* in the vagina of sampled mares is unknown but may be connected to its commensal nature (Nwobi et al., 2023). Earlier report by Hinrichs et al. (1988) observed the presence of *Staphylococcus* (n = 4), *Streptococcus* (n = 4) and *Arcanobacterium* (n = 10) in investigated vagina samples of clinically normal mares. The report by Hinrichs et al. (1988), is slightly similar to our observation of *Staphylococcus* (n=55) but differs in the area of its abundance without species specification. The present finding is in contrast with an early report on vaginal microbiome of mares from slaughterhouse, in which coliforms and β-haemolytic streptococci were the most abundant (Scott et al., 1971), while a more recent study on vagina microbiota of maiden mares and mares with no history of breeding for over 10 years observed the presence of the following: *Escherichia coli* (40% prevalence in the vaginal samples investigated), *Streptococcus zooepidemicus, Streptococcus equisimilis, Streptococcus thoraltensis* and *Staphylococcus capitis* (Malaluang et al., 2022). Also, Malaluang et al. (2024) reported *Escherichia coli* and *Streptococcus zooepidemicus* as predominate microbes during Days 3, 7 and 14 post-ovulations of the oestrous cycle of healthy mares. These findings by Malaluang et al. (2022) and Malaluang et al. (2024) are in contrast with the vaginal microbes isolated from the breeding mares in this study. Again, Fraga et al. (2007) observed *Enterococcus faecalis*, *E. faecium*, *Lactobacillus equi*, *L. mucosae* and *L. pantheris* in the vagina, with more *Lactobacillus* identification in 18 out of the 26 sampled healthy mares. Barba et al. (2020) observed the presence of the following genera in the vagina of Arabian mares during oestrous cycle: *Kiritimatiaellae spp*, *Porphyromonas spp, Corynebacterium spp, Streptococcus spp, Campylobacter spp, Arcanobacterium spp, Fusobacterium spp, Lactobacillus spp* and *Akkermansia spp*.

A very low percentage of *Pseudomonas aeruginosa* was identified as part of vagina microbiome of breeding mare in this study. Although, there is a dearth of information on its presence in the mares' vagina in previous reports, Hinrichs et al. (1988) observed *Pseudomonas aeruginosa* in clitoral fossa swab samples of mares. Also, Gil-Miranda et al. (2024) reported it as one of the organisms previously identified in uterine microbiome in mares.

This study also reports a very low composition (1.2%) of *Staphylococcus epidermidis* in the vagina of breeding mare. Similar report by Malaluang et al. (2024) observed a low percentage (0.7%) of *Staphylococcus epidermidis* from the vagina of sampled mares during the oestrous cycle. Though *Staphylococcus epidermidis* belongs to the genus *Staphylococcus,* its role in equine reproduction is not fully known. It had previously been reported that *Staphylococcus epidermidis* plays a symbiotic role in mice embryo implantation (Ono et al., 2015). Iwase et al. (2010) also reported that *Staphylococcus epidermidis* was of a beneficial role in displacing the pathogenic *Staphylococcus aureus* from nasal cavities in humans.

The variations in the organisms detected may be due to several factors that influence the vagina microbiome, such as diet, health status, hormonal status and geographical location. Also, the method of sampling and sample processing may have contributed to the differences observed with our present findings and other studies (Gil-Miranda et al., 2024).

Staphylococcus aureus and *Pseudomonas aeruginosa* had been implicated as part of the causative agents of equine endometritis (LeBlanc, 2010; Canisso et al., 2015). Therefore, their presence in the vagina of breeding mares should be of great concern because of their pathogenic property under unfavourable conditions. A recent study identified multiple drug- and heavy metal-resistant as well as methillicin-resistant *Staphylococcus aureus* isolated from nasal and groin skin swabs of horses slaughtered for meat in Obollo-Afor Southeastern Nigeria (Nwobi et al., 2023). This study by Nwobi et al. (2023) is similar to our present finding of *Staphylococcus aureus* in breeding mare and supports our concern for more vigilance on the reproductive health of breeding mares. Also, *Pseudomonas aeruginosa* has been associated with infertility in inseminated mares (Malaluang et al., 2021). Although these bacteria isolates are located in the vagina, they can invade the uterus during gynaecological evaluation, obstetrical intervention, mating or artificial insemination, despite strict aseptic protocol leading to varying reproductive problems under unfavourable conditions (Malaluang et al., 2021).

Development of antimicrobial resistance by bacteria occurs from either therapeutic or low dose exposure (Malaluang et al., 2021). The three bacteria organisms in this study were all highly resistant to cefuroxime, penicillin-streptomycin, enrofloxacin and ciprofloxacin. Additionally, *Staphylococcus aureus* was also highly resistant to metronidazole and showed very low resistance to ceftriaxone, oxytetracycline and doxycycline, while *Staphylococcus epidermidis* was also highly resistant to ampicillin and metronidazole. Malaluang et al. (2022) observed resistance of isolated *Staphylococcus spp to* erythromycin, oxacillin, penicillin and gentamicin in inseminated mares in Sweden in contrast with present findings. Same report stated that *Staphylococcus epidermidis* was highly resistant to erythromycin, fusidic acid and penicillin.

Pseudomonas aeruginosa showed high level of resistance to most of the antimicrobials (10 out of 18) tested when compared with other bacteria isolates. It was also highly resistant to ceftriaxone, ofloxacin, amoxicillin, oxytetracycline, vancomycin and doxycycline. The multiple drug resistance exhibited by *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa* should be of great concern because it limits the spectra of antimicrobials available for treating infection-dependent reproductive problems in mare. Differences in age, previous treatment, location, grouping and personnel may have contributed to the varying resistance to antimicrobials tested in this study. The development of antimicrobial resistance may be acquired or natural through exposure to resistant genes (Malaluang et al., 2022).

Antimicrobial sensitivity for *Staphylococcus aureus* isolates revealed 100% susceptibility to imipenem and streptomycin, below 60% susceptibility to gentamicin and less than 25% to amoxicillin cloxacillin, clindamycin and vancomycin, while *Staphylococcus epidermidis* was highly susceptible to streptomycin and gentamicin, and *Pseudomonas aeruginosa* was highly susceptible to clindamycin and imipenem with intermediate susceptibility to gentamicin, ampicillin, amoxicillin cloxacillin, chloramphenicol, metronidazole and streptomycin. The observation of a few antimicrobial susceptibilities suggests that previous treatment, location, grouping and personnel may have influenced the bacteria presence in the vagina (Malaluang et al., 2022).

A significant limitation of this study is the reliance solely on conventional morphological and biochemical identification methods for bacterial characterization. Molecular techniques such as 16S rRNA gene sequencing, PCR-based identification, or metagenomics approaches are absent in the present study. Future studies should incorporate molecular identification techniques alongside traditional culture methods to provide a more comprehensive understanding of the mare vaginal microbiome and its clinical implications. This study is also limited by unequal sampling between locations (60 mares from Lagos vs. 21 from Oyo) and the absence of critical demographic data, including estrous cycle phase, mare age, and previous antimicrobial exposure history. These factors are known to significantly influence vaginal microbiota composition and antimicrobial resistance patterns, potentially affecting result interpretation. Future studies should employ stratified sampling with equal geographical representation and comprehensive metadata collection to enable more robust analyses.

In conclusion, the study found a high presence of *Staphylococcus aureus* in mares' vaginal microbiota, with significant multi-drug resistance. However, aminoglycosides and carbapenems showed consistent effectiveness against the isolated bacteria. It is strongly recommended that veterinarians in these regions prioritize the use of aminoglycosides and carbapenems as first-line treatments for vaginal infections in mares, while avoiding the use of antibiotics showing high resistance patterns, unless guided by susceptibility testing.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTIONS

Concept – DS; Design – DS, ORA; Supervision – OOA[2]; Resources – OGB, TRL; Materials – OGB, LCO; Data Collection and Processing – OOA[1], TRL, DS; Analysis and Interpretation – OOA[1], ORA; Literature Search – ORA, TAO, DS; Writing Manuscript – LCO, MJO, ORA; Critical Review – MJO, DS, OOA[1],

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KARAKTERIZACIJA I ANTIMIKROBNA REZISTENCIJA VAGINALNOG MIKROBIOMA KOD KOBILA IZ DRŽAVA LAGOS I OYO U NIGERIJI

SAŽETAK

Uprkos značaju mikrobioma u reproduktivnom zdravlju sisara, istraživanja o vaginalnom mikrobiomu kod konja ostaju ograničena, posebno u tropskim područjima. Sastav vaginalnog mikrobioma kobila u državama Lagos i Oyo u Nigeriji još nije zabilježen. Profili mikrobioma su neophodni za razvoj učinkovitih strategija upravljanja reproduktivnim zdravljem. Naš cilj je karakterizacija sastava vaginalnog mikrobioma i određivanje profila antimikrobne rezistencije izoliranih bakterija kod kobila u državama Lagos i Oyo u Nigeriji. Korištenjem sterilnih briseva za kulture su prikupljeni vaginalni brisevi od 81 naizgled zdrave kobile, uključujući nigerijske domaće vrste, sudanske pasmine i argentinske Polo ponije. Iz uzoraka su izdvojeni bakterijski izolati na selektivne i diferencijalne podloge, uključujući triptozu sojin agar, manitol slani agar, eozin metilen plavo, Muller-Hinton, salmonelu-šigelu i MacConkey agar. Bakterijska identifikacija je uključivala morfološke i biohemijske testove, uključujući oksidaze, katalaze, motilitet, bojenje po Gramu i fermentaciju šećera. Osjetljivost na antimikrobna sredstva je testirana za 18 antimikrobika korištenjem Kirby-Bauer disk difuzijske metode, prema CLSI standardima. Analiza je pokazala različite obrasce bakterijske distribucije za različite lokacije. U uzorku od 81 kobile je dominirao *Staphylococcus aureus* (66.7%), dok su *Pseudomonas aeruginosa* i *Staphylococcus epidermidis* pojedinačno bili zastupljeni s 1.2%, dok 30.9% uzoraka nije pokazalo nikakav rast. Biohemijska karakterizacija je pokazala tipične profile za svaku od vrsta. *S. aureus* je pokazao potpunu osjetljivost na imipenem i streptomicin, a rezistenciju na cefuroksim, penicilin-streptomicin, enrofloksacin, ciprofloksacin i metronidazol. *P. aeruginosa* je bila osjetljiva na klindamicin i imipenem, dok je na većinu drugih antibiotika bila rezistentna. Imajući u vidu veliku zastupljenost *S. aureusa* rezistentnog na antibiotike, veterinari bi trebali davati prednost aminoglikozidima i karbapenemima kod liječenja vaginalnih infekcija kobila, a koristiti druge antibiotike samo kada se testiranjem dokaže osjetljivost.

Ključne riječi: Antimikrobna rezistencija, nigerijske kobile, reprodukcija konja, *Staphylococcus aureus*, vaginalni mikrobiom

SHORT COMMUNICATION

A PRELIMINARY SURVEY OF THE NUTRITION AND HEALTH OF GERMAN PET RABBITS

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ABSTRACT

This survey aimed to investigate the nutrition and health of German pet rabbits with a special emphasis on dental diseases and the impact of diet on dental health. The questionnaire yielded 82 responses (n=57 online and n=25 in-person interviews). Most rabbits were ≥7 years old (32.9%), followed by 3-4 years old (30.5%). The sex ratio was almost 50-50%, and most rabbits were neutered (65.8%). Complete pelleted feed was the most fed concentrate (71.9%) followed by muesli-type mixed feed (11.0%). The majority of rabbits (95.1%) had *ad libitum* access to hay, and 45.1% received fresh vegetables daily. Dental disorders were reported in 26 rabbits (31.7%), primarily affecting the cheek teeth (20.6%), compared to the incisors (9.5%) or both (3.2%). Of the other diseases and symptoms, epiphora (n=12, 13.3%), obesity (n=8, 9.7%), and weight loss (n=7, 7.8%) were the most common. Fisher's exact test did not show a significant association between age (≤ 4 years old, > 4 years old), breed (dwarf or other), sex (male or female) and the number of diagnosed health problems (≤ 3 or more) and forms of diseases or symptoms. Also, the incidence of obesity was not higher in neutered rabbits. The reported feeding practices suggest a relatively high standard of nutrition, though objective nutrient analysis was not performed. As another limitation, survey data might show a bias towards committed rabbit owners.

Keywords: Feeding, hay, incisor, malocclusion

INTRODUCTION

Improper nutrition of pet rabbits leads to common disorders, such as dental diseases, diarrhoea, or other gastrointestinal problems (Huynh et al., 2014; Oneill et al., 2020). All teeth are open-rooted and grow continuously lifelong. For maintaining the shape of the continually growing teeth tooth-to-tooth contact plays a major role (Harcourt-Brown, 2003; Reiter, 2008). Because of too short maxilla in combination with a normal mandible, brachycephalic breeds are 3.19 times at risk of dental disease compared to normal breeds (Harcourt-Brown, 2009; Siriporn and Weerakhun, 2014). Numerous studies have

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shown that diet influences dental growth and wear, especially hay promoting adequate movement and wear during chewing, while rabbits without access to hay develop dental disease more frequently (Palma-Medel et al., 2023). Rabbits consuming muesli-type mixes also have a higher incidence of dental problems (Harcourt-Brown, 2003; Reiter, 2008; Martin et al., 2022). The survey aimed to investigate the nutrition and health of German pet rabbits, with a special emphasis on dental diseases and the impact of diet on dental health. The survey was part of Melina Berger's thesis.

MATERIAL AND METHODS

The questionnaire was shared in German in Facebook groups "Veterinary Exotic Small Talk" (23.853 members), "Pet rabbit keepers and friends" (20.010 members) and "Pet rabbit advice" (40.406 members). Beside that, responses were collected through personal interviews with rabbit owners. Respondents were told to answer rabbit-related questions about their oldest owned rabbit. Ethical approval was not required as data collection was anonymized, and participation was voluntary. Data were collected from April 30, 2023, to August 31, 2023.

The questionnaire consisted of 24 questions that were presented in both single-choice, multiple-choice, and free-text formats. The questionnaire was divided into 4 sections:

Section 1. Age and gender of the respondent.

Section 2. The number of currently owned rabbits. Breed, sex age and neutering of the rabbit.

Section 3. Access to/type of concentrates, vegetables, and treats. Acces to hay.

Section 4. Past diseases. Reasons for veterinary visits. Details of dental diseases.

Fisher's exact test was used to evaluate the association between the age (≤ 4 years old, > 4 years old), breed (dwarf or other), or sex (male or female) and the number of diagnosed health problems (< 3 or more) and diseases. The same test was used to check the association between neutering status and obesity. R statistical program (version 4.3.1, 2023) was used for the calculations; p-values lower than 0.05 were significant. Beside that – because of the number of records – we only made descriptive statistics.

RESULTS

The questionnaire yielded 82 responses (n=57 Facebook and n=25 personal interviews). Female pet rabbit owners formed the majority in this study (n=70, 85%) and most of the participants owned two rabbits (n=27, 32.9%).Twenty owners (24.4%) had one rabbit, seven (8.5%) had three rabbits and seven owned more than three animals (8.5%). The majority of the pet rabbit owners were between 25 and 34 years old (n=36, 45.0%), 21.9% (n=18) were <18–24 years, 25.6% (n=21) were 35–54 years and five (6.1%) >55 years old. Figure 1 shows the rabbit breeds.

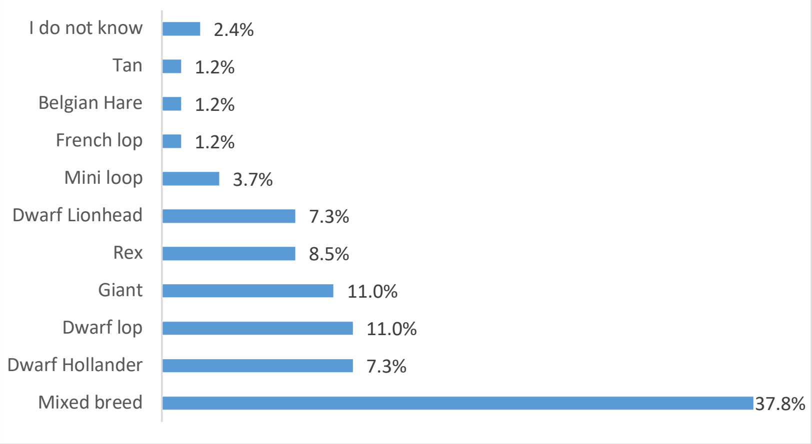

Figure 1 Breed of the rabbits (n=82)

Most rabbits in this study were ≥7 years old (n=27, 32.9%), followed by 3-4 years old (n=25, 30.5%), 5-6 years old (n=16, 19.5%) and ≤2 years old (n=9, 11%). Five owners (4.9%) were unsure about the age of the rabbit. The sex ratio was close to 50-50% (female n=36, 43.9% vs. male n=40, 48.8%; six owners were unsure). The majority of the rabbits were neutered (n=54, 65.8%), twenty (24.4%) were intact and not planning to be neutered, and 2.4% (n=2) were intact, but planning to be neutered. Six owners (7.3%) did not respond. There was no association between neutering status and obesity (p>0.05).

Table 1 shows the frequency of access to feeds. Most of the owners fed their rabbits with concentrates (n=68, 82.9%). Complete pelleted feed (extruded pellets with homogenous appearance) was the most fed concentrate (n=59, 71.9%), followed by muesli-type mixed feed (a mixture of pellets and other ingredients, non-homogenous appearance; n=9, 11.0%) and 12.2% (n=10) did not feed concentrates. Figure 2 shows the most and least preferred ingredients of mixed feeds. The fed vegetables and treats are shown in Figure 3 and Figure 4.

Table 1 The frequency of access to feeds

	Hay		Concentrate		Vegetables		Treats	
	n	%	n	%	n	%	n	%
Ad libitum	78	95.1%	7	8.5%	7	8.5%	0	0%
Once a day	1	1.2%	44	53.6%	37	45.1%	19	23.2%
More than once a day	1	1.2%	8	9.7%	22	26.8%	3	3.6%
Less than once a day	1	1.2%	13	15.8%	12	14.6%	46	56.1%
Never	0	0%	10	12.2%	0	0%	7	8.5%
Non-respondents	1	1.2%	0	0%	4	4.9%	7	8.5%

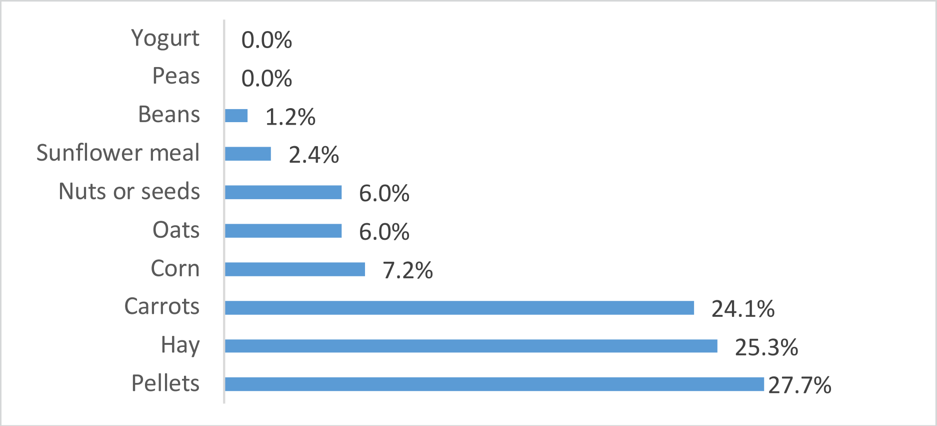

Figure 2 Preferred ingredients of mixed feed (multiple-choice question, n=83)

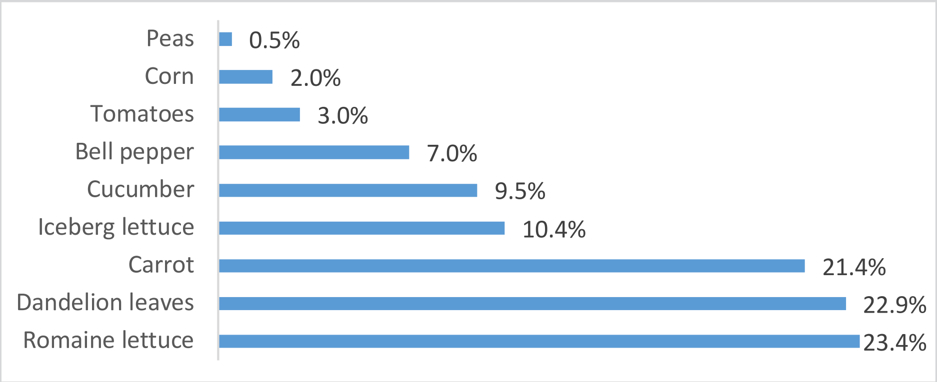

Figure 3 The most commonly fed vegetables (multiple-choice question, n=201)

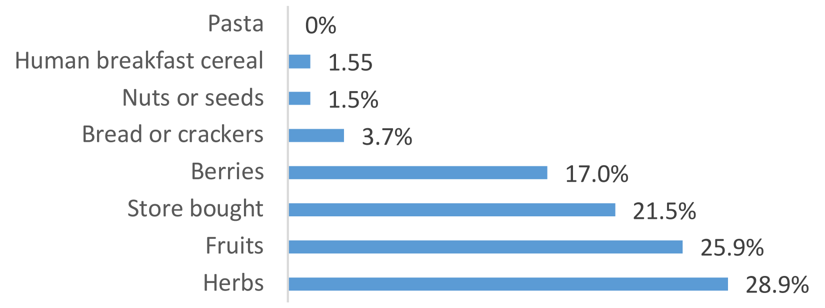

Figure 4 The most given treats (multiple-choice question, n=135)

Most of the owners considered their rabbits healthy at the current moment (n=64, 78.0%). Dental disorders were reported in 26 rabbits (Figure 5). Overgrown teeth (n=10 vs. n=4), tooth root abscesses (n=4 vs. n=0) and other dental diseases (n=7 vs. n=1) were more common in rabbits over 4 years of age, but it was not significant. Except for other dental diseases (n_{male}=5, n_{female}=3), dental disorders were more common in males (overgrown teeth: n_{male}=8, n_{female}=6; tooth root abscess: n_{male}=4, n_{female}=0). However, none of these differences were significant. Eleven rabbits (13.4%) had dental surgery in the past, and in 90.9% (n=10) the surgery was successful.

Figure 6 shows the other diseases and symptoms. Figure 7 shows the reasons for visiting a veterinarian.

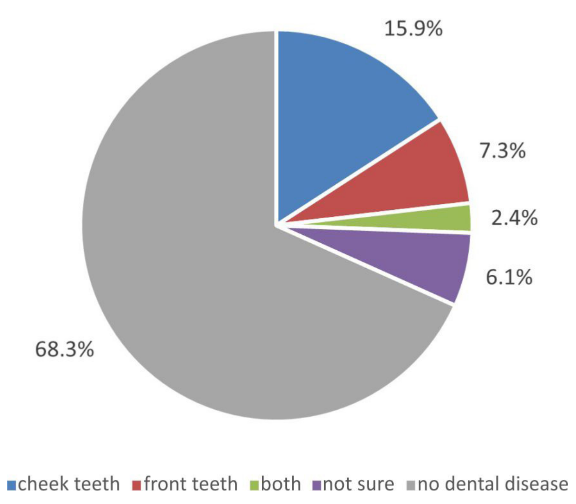

Figure 5 Teeth affected by dental diseases

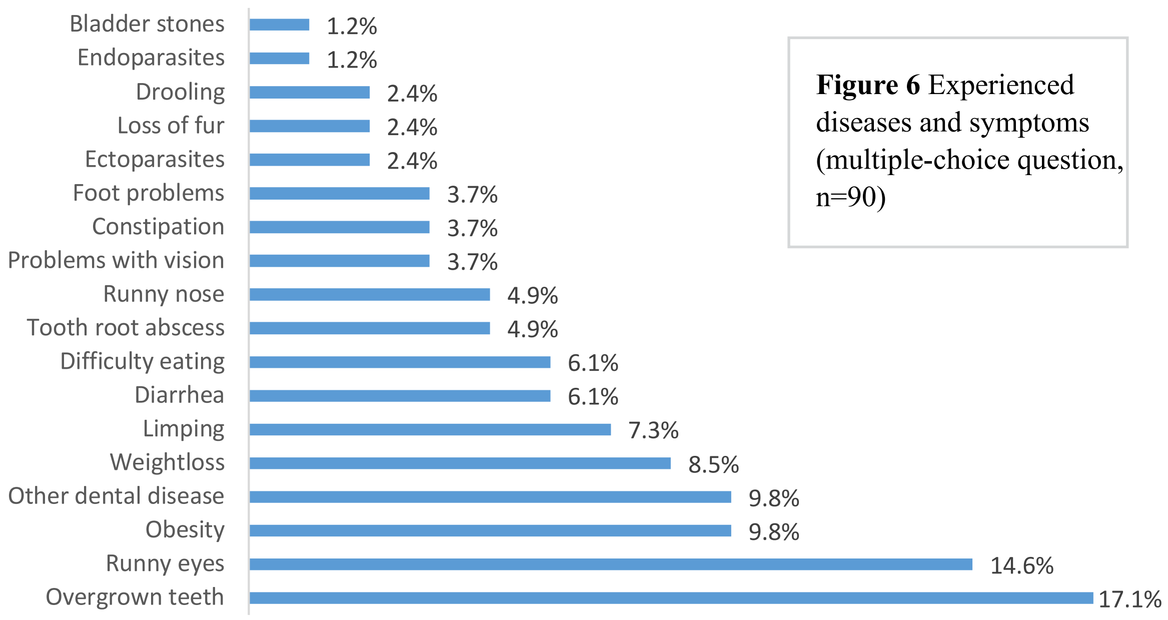

Figure 6 Experienced diseases and symptoms (multiple-choice question, n=90)

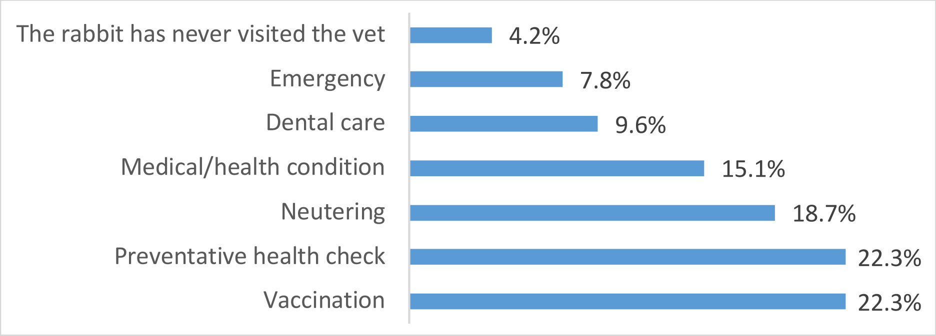

Figure 7 Reasons for visiting a veterinarian (multiple-choice question, n=166)

DISCUSSION AND CONCLUSION

Like in other surveys, most of the owners in the present study were female (Rooney et al., 2014; Welch et al., 2017; Kristensen, et al., 2023). As in the study of Kristensen et al. (2023), rabbits were mostly mixed breeds (39.2%). Among the purebred rabbits, the dwarf loop was the most common breed, as in other surveys (Mullan and Main, 2006; Edgar and Mullan, 2011; Mäkitaipale et al., 2015; Hetényi and Sátorhelyi, 2021). Likewise, the dispersal of female and male rabbits was approximately 50-50 in this study, which is also shown in the previous data collections (Mullan and Main, 2006; Mäkitaipale et al., 2015; Hetényi and Sátorhelyi, 2021; Kristensen, et al., 2023).

Only nine (11%) owners gave muesli-type mixed feed, contrary to the survey of Edgar and Mullan (2011) with 45% and Harcourt-Brown (1996) with 99%. Beside the type of concentrate (complete extruded pellet), another sign of a relatively high standard of nutrition in this survey is the controlled access to it. Typically, the muesli-type mixed feed is higher in carbohydrate and lower in fibre than the complete extruded pellets. Perhaps the owners taking part in this present study were aware of the health risks (selective feeding and dental problems) connected to this kind of feed (Harcourt-Brown, 1996; Prebble and Meredith, 2014). Selective feeding was observed, but except for carrots, the energy and carbohydrate-rich components were not preferred by the rabbits. The most and least preferred ingredients of the mixed feed as well as the provided treats and fruits,were similar to the findings of Kristensen et al. (2023). Among the most commonly fed vegetables, Romaine lettuce and dandelion are adequate, but carrots should be limited because of their high carbohydrate content. Starch-rich human foods and energy-rich nuts and seeds are not recommended for rabbits, and these were not commonly fed by the owners. The most adequate treats are herbs that were used by the majority. It was closely followed by fruits, which are also not optimal because of their high carbohydrate content. The fact that hay was a regular part of the diet of almost all rabbits corresponds with other studies (Mullan and Main, 2006; Rooney et al., 2014; Hetényi and Sátorhelyi, 2021; Kristensen et al., 2023).

Neutering procedures and routine health assessments were the most commonly cited reasons for veterinary consultations in the present study, similar to a previous study by Kristensen et al. (2023). Epiphora was a common problem that corresponded with the literature, as in the study by Artiles et al. (2020) and Mäkitaipale et al. (2015). Increased ocular discharge is caused by the elongation of the maxillary incisor, which constricts the nasolacrimal duct (Harcourt-Brown, 2009). Poor teeth cause insufficient grooming; consequently, fur balls can develop and small intestinal blockage (Mosallanejad et al., 2010). Gastrointestinal problems were less common than in some other surveys (Mullan and Main, 2006; Hetényi and Sátorhelyi, 2021) and similar to the results of Rooney et al. (2014) and Kristensen et al. (2023). Other health issues were also less common (Mullan and Main, 2006; Rooney et al., 2014; Mäkitaipale et al., 2015; Hetényi and Sátorhelyi, 2021; Kristensen et al., 2023).

The prevalence of dental disease correlates with previous studies (Mullan and Main, 2006; Mäkitaipale et al., 2015; Böhmer and Böhmer, 2017; Hetényi and Sátorhelyi, 2021). Considering these results, most of the rabbits having dental disease corresponded to the older age group (>4 years). At older ages, there is a longer exposure time for factors like diet or genetic predisposition, resulting in dental alterations (Artiles, et al., 2020; Mosallanejad et al. 2010). In contrast, Palma-Medel et al. (2023) described the dental disease in young rabbits. This may be explained by the fact that juveniles have a higher demand for calcium for growth. Thus, suboptimal calcium intake can lead to dental alterations, such as enamel defects (Harcourt-Brown, 1996). In this survey, no female was presented with the tooth root abscess. Several studies demonstrated that male rabbits showed an increased risk of dental disease compared to females (Siriporn and Weerakhun., 2014; O'Neill et al., 2020; Palma-Medel et al., 2023). This can be related to the hormone estrogen, which increases the serum calcium concentration, consequently facilitating the formation of the enamel and dentin (Palma-Medel et al., 2023). No rabbits under 4 years of age developed dental abscesses in this survey. This might be explained by the lower incidence of dental diseases in this age group.

The association between concentrate feeding and dental disease remains a subject of debate in the current literature. Pellets - in contrast to the natural diets - are crushed between the teeth, followed by much higher axial strain on the cheek teeth. Lateral gliding movements as well as chewing duration are decreased, resulting in insufficient tooth wear and a tendency to retrograde elongation (Reiter, 2008; Böhmer and Böhmer, 2017). The tougher and stiffer hay requires more chewing, which results in longer chewing periods compared to pellets (Böhmer and Böhmer, 2017). On the other hand, a study by Müller et al. (2014) showed that pellets with high fiber content did not increase the risk of dental disease in the short term. For the prevention of dental disease, a diet of grass and/or good quality hay with plenty of leafy green plants is recommended. The present study could not evaluate the quality of each ingredient in the diet, which may answer the discrepancy in the results, according to the literature. A more common involvement of the cheek teeth compared with the front teeth was also observed by the other studies (Artiles et al., 2020; Palma-Medel et al., 2023).

Further studies involving a higher number of participants would be important to evaluate the health and nutrition of pet rabbits.The reported feeding practices suggest a relatively high standard of nutrition, though objective nutrient analysis was not performed. As another limitation, data might show a bias towards committed rabbit owners. A more detailed survey recording the type of hay, the manufacturer of the concentrate and its portion size would be necessary to see the potential connection between diet and dental health.

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PRELIMINARNO ISTRAŽIVANJE O ISHRANI I ZDRAVLJU NJEMAČKIH KUNIĆA KAO KUĆNIH LJUBIMACA

SAŽETAK

Cilj ovog istraživanja je ispitati ishranu i zdravlje njemačkih kunića kao kućnih ljubimaca s posebnim naglaskom na dentalna oboljenja i djelovanje ishrane na dentalno zdravlje. Upitnik je sadržavao 82 odgovora (n=57 elektronska i n=25 intervju uživo). Većina kunića je bila starija od ≥7 godina (32.9%), potom 3-4 godine (30.5%). Omjer spolova je iznosio skoro 50-50%, a većina kunića je prethodno sterilizirana (65.8%). Kompletna čvrsta hrana je predstavljena koncentratom (71.9%), potom miješanom hranom tipa žitarica (11.0%). Većina kunića (95.1%) je imala ad libitum pristup sijenu, a 45.1% je dobivalo svježe povrće svaki dan. Dentalni poremećaji su zabilježeni kod 26 kunića (31.7%), zahvatajući prvenstveno obrazne zube (20.6%), potom sjekutiće (9.5%) ili oboje (3.2%). Od ostalih bolesti i simptoma, najčešći su bili epifora (n=12, 13.3%), gojaznost (n=8, 9.7%) i gubitak težine (n=7, 7.8%). Fisherov egzaktni test nije dokazao statistički signifikantnu povezanost između starosti (≤ 4 godine, > 4 godine), pasmine (patuljasta ili druga), spola (mužjaci ili ženke), broja dijagnosticiranih zdravstvenih problema (≤ 3 ili više) i oblika bolesti ili simptoma. Također, incidenca gojaznosti nije bila viša kod steriliziranih kunića. Zabilježeni načini ishrane sugeriraju relativno visok standard, iako nije izvedena objektivna nutritivna analiza. Drugo ograničenje istraživanja je potencijalna pristrasnost s vlasnicima kunića kao uzrokom.

Ključne riječi: Malokluzija, prehrana, sijeno, sjekutić

CASE REPORT

FIRST REPORT OF BOVINE TRYPANOSOMIASIS IN THE KASHMIR VALLEY: THERAPEUTIC APPROACH

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ABSTRACT

Trypanosomiasis is a protozoan disease of major economic importance in livestock worldwide, but it has not been documented in the high-altitude, temperate climate of Kashmir, India. This article reports the first confirmed case of trypanosomiasis in non-descript cattle from the Kashmir Valley. A two-year old female non-descript cow in the first trimester of pregnancy was presented with a history of weakness, infestation of ticks, anorexia, haematochezia, unilateral epistaxis and pica to the Division of Veterinary Clinical complex, for the treatment. Clinical examination revealed hypothermia, white mucous membrane, enlarged prescapular lymph nodes, tachycardia, respiratory distress and hypersalivation. Haematological analysis revealed very low haemoglobin content and on blood smear examination, *Trypanosoma evansi* organisms were identified, which was further confirmed through presence of *Trypanosoma evansi* tryps in the lymph node aspirate. Blood transfusion was done with whole blood immediately, and then, Inj. diminazeneaceturate 7% RTU@ 5 mg/kg intramuscularly was given, which was repeated on 5th day. After 5 days, haemantinics were also given. The animal showed clear signs of recovery after blood transfusion. This constitutes the first case of surra in the Kashmir Valley, highlighting the parasite's expansion into the temperate region of the country and marking a critical observation for veterinary practitioners in the region. Future work is needed to identify the vector and possible reservoirs to prevent the spread of the disease.

Keywords: Trypanosomiasis, surra, *Trypanosoma evansi*

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INTRODUCTION

Trypanosomiasis, also known as "surra," or "*mal de las caderas*" is a hemoprotozoan disease caused primarily by *Trypanosoma evansi* in cattle (Aregawi et al., 2019). It is transmitted through mechanical vectors, such as *Tabanus* and *Stomoxys* flies. In Africa, the vector of the disease is the *Glossina spp*., commonly known as the tsetse fly, while in America, Asia, and Europe, where tsetse flies are absent, transmission occurs through mechanical means by tabanids and stomoxys (Bello et al., 2024; Desquesnes et al., 2013). Additionally, in parts of Central and South America, it can be transmitted by the vampire bat *Desmodus rotundus*, which serves as a vector and a reservoir and does not involve a specific biological relationship between the parasite and its vector, enabling the parasite to spread beyond Africa (Desquesnes et al., 2013). The disease has severe economic consequences, resulting in huge losses to the livestock owners (Swallow, 2000). While the disease is endemic in many tropical and subtropical regions, its occurrence in the temperate Kashmir Valley is previously undocumented. This study represents the first documented incidence of bovine trypanosomiasis in Kashmir and highlights the clinical implications, diagnosis, and management strategies adopted.

CASE DESCRIPTION

A two-year old female non-descript cow in the first trimester of pregnancy was presented with a history of weakness, infestation of ticks, anorexia, haematochezia, unilateral epistaxis and pica to the Division of Veterinary Clinical complex, Faculty of Veterinary Sciences and Animal Husbandry (FVSc & AH) Shuhama for the treatment. Clinical examination revealed a body temperature of 98.7^{o}F, white conjunctival mucus membranes, enlargement of prescapular lymph nodes, heart rate of 98 bpm and respiratory rate of 49 bpm and hypersalivation. No history of recent travel outside the region was reported, but the presence of biting flies in the locality was noted.

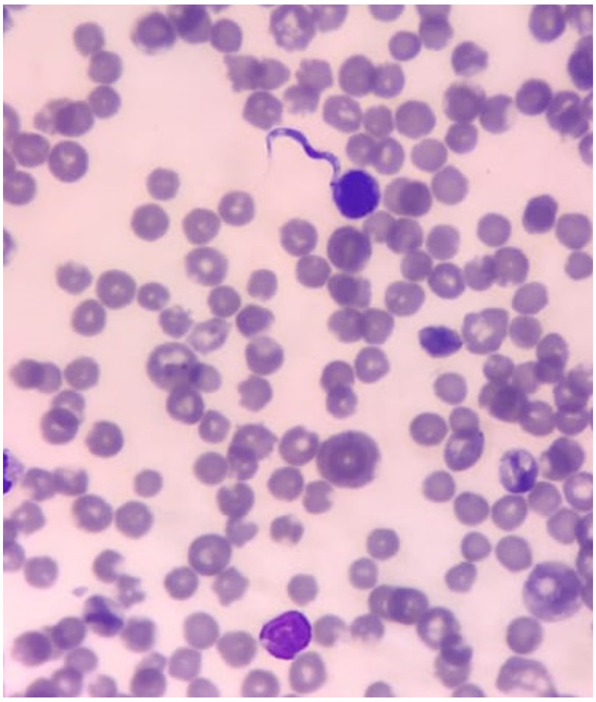

Figure 1 Trypanosoma within blood smear stained with Field stain

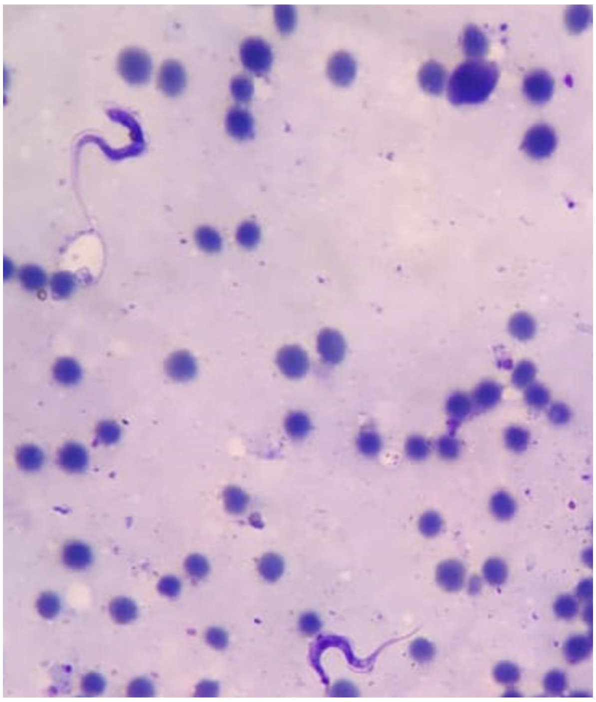

Figure 2 Trypanosoma within lymph node aspirate stained with Field stain

Diagnostic Evaluation

Blood samples collected in EDTA vials were examined after preparing thin and thick blood smears with Field stain (Amer et al., 2024). Lymph node aspirates were also collected in sterile EDTA vials and stained with Field stain. Microscopy revealed motile trypanosomes in field stained smears, and characteristic *Trypanosoma evansi* morphology (undulating membrane, kinetoplast, flagellum) is shown in Figure 1 and Figure 2.

The haematological and biochemical changes before and after treatment are shown in Table 1.

Table 1 Haematological and biochemical parameters at Day 0 and Day 9 of the treatment

Parameters	Unit	Value at Day 0	Value at Day 9
WBC	$\times10^3/\mu l$	10.55	9.52
RBC	$\times10^6/\mu l$	1.41	5.32
Hb	g/dl	3.3	7.8
HCT	%	7.9	23.11
MCV	Fl	56.02	46.22
MCH	Pg	23.40	14.66
MCHC	g/dl	41.77	33.75
Platelet	$\times10^3/\mu l$	10	189
Glucose	mg/dl	43	68
ALT	IU/L	112	94
AST	IU/L	92	72
TP	g/dl	7.1	6.9
Albumin	g/dl	3.0	3.6
Globulin	g/dl	4.1	3.3

Treatment and Outcome

The case was treated with Inj. diminazeneaceturate 7% RTU@ 5 mg/kg intramuscularly at Day Zero and repeated after 5th day of the treatment. Blood transfusion was done with whole blood after proper cross matching through the CPDA containing bags. After 5 days, oral haemantinics were also given. The animal started to respond well to the treatment after the completion of blood transfusion with improved appetite and diminished clinical signs. The peripheral blood sample was found to be negative for the organism on the 5thday of the treatment. The prescapular lymph node swelling had subsided, hence lymph node aspirate was not collected. Complete recovery of the animal was observed on the 9th day, with much improvement in the haematological and biochemical parameters.

DISCUSSION AND CONCLUSION

Trypanosomosis (Surra) caused by the haemo-flagellate protozoan *Trypanosoma evansi*, is a significant constraint on the health and productivity of domestic animals throughout the tropics and subtropics (Singla et al., 2004; Da Silva et al., 2010; Tariq et al., 2024). In India, *T. evansi* is the most common and most prevalent trypanosome of livestock, although isolated cases of *Trypanosoma theileri* have also been encountered (Sood et al., 2011). The organism is transmitted mechanically by tabanid flies (Singh et al., 1993; Desquesnes et al., 2013), and the disease is more common in areas where the environment for the breeding of the fly vectors is most suitable (Bhatia et. al., 2006). The presence of *Trypanosoma evansi* in Kashmir Valley is a significant epidemiological finding. The detection in non-descript cattle, which are typically hardy and resistant to many endemic diseases, suggests a possible expansion of vector habitats due to climate shifts or increased movement of animals and vectors. Early diagnosis and prompt treatment are crucial for recovery. This case underscores the need for regular surveillance, awareness among veterinarians and farmers, and vector control initiatives in emerging areas.

The present investigation deals with an acute trypanosomosis in a non-descriptive cattle. Significant results from various studies of *T. evansi* seroprevalence in India have been published by many authors (Juyal et al., 2005; Chandu et al., 2021). These studies demonstrated the importance of environmental factors in the epidemiology of Surra by highlighting the climatic risk factors linked to the disease's prevalence in cattle.

Among clinical signs, viz., fever, respiratory distress, pale mucous membrane, anorexia, swollen lymph nodes, unilateral epistaxis (which might be due to thrombocytopenia, formation of microthrombi, and hemorrhage suggestive of disseminated intravascular coagulation) have been also previously reported (Parragimenez and Reyna-Bello, 2019; Magez and Radwanska, 2014).

A significant decrease in the haematological variables was seen in the present case, especially in RBC count, PCV and haemoglobin. This alteration can be credited to the extravascular annihilation of the red blood cells, resulting in anaemia and a drop in PCV (Lelisa and Meharenet, 2021). Blood glucose was decreased in this case, which may be due to utilization of glucose by parasite (Bal et al., 2014; Fiorin et al., 2025).

The present study revealed a substantial increase in liver enzymes like ALT and AST in initial days of the infection following inflammatory cascade and tissue necrosis due to the infection. Additionally, the trypanosomal infestation leads to the increase in immunoglobulin concentration following antibody production to muddle through the antigens, resulting in hyperproteinaemia. Our findings were supported by the work of Pandya et al., (2018). Moreover, the albumin and globulin in initial infection were decreased and increased respectively, which is in agreement with the study of Bal et al. (2014) and Pandya et al., (2018).

Improvements have also been made to *T. evansi's* diagnostic capabilities. A multiplex PCR assay was developed by Charaya et al. (2021) to detect several hemoprotozoan illnesses, including *T. evansi,* simultaneously. This technique enhances the speed and specificity of diagnosis, essential for prompt intervention in affected cattle populations. The incidence of *T. evansi* in cattle has also been the subject of investigations by Kizza et al. (2021) and Ogolla et al. (2023), which employed molecular techniques to identify the parasite and evaluate related risk factors in various locations, including Kenya and Uganda.

This landmark case of bovine trypanosomiasis in the Kashmir Valley highlights the expanding geographical range of *Trypanosoma evansi* and reinforces the importance of clinical vigilance. Timely diagnosis and effective therapeutic intervention led to full recovery, demonstrating that with proper management, even emerging diseases can be effectively controlled.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTIONS

RAB: Data collection and processing, literature review, writing. AM: Conception, design, writing. MIY: Supervision, writing, critical review. SM: Conception, design, writing. AS: Supervision, writing, critical review.

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PRVI IZVJEŠTAJ O BOVINOJ TRIPANOSOMIJAZI U KAŠMIRSKOJ DOLINI: TERAPIJSKI PRISTUP

SAŽETAK

Tripanosomijaza je protozoarna bolest koja ima veliki ekonomski značaj u uzgoju stoke širom svijeta koja nije dokumentirana na višim nadmorskim visinama umjerene klime Kašmira u Indiji. Ovaj članak opisuje prvi potvrđeni slučaj tripanosomijaze kod stoke bez primjerene pasmine u Kašmirskoj dolini. Dvogodišnja krava bez pasmine u prvom trimestru graviditeta dovedena je na liječenje u Veterinarski klinički odjel. Anamnestički je bila prisutna slabost, infestacija krpeljima, anoreksija, hematohezija, unilateralna epistaksa i pika. Klinički pregled je pokazao hipotermiju, bjelilo mukoznih membrana, uvećane preskapularne limfne čvorove, tahikardiju, respiratorni distres i hipersalivaciju. Hematološka analiza je pokazala veoma nizak hemoglobin, dok je na krvnom razmazu identificirana *Trypanosoma evansi* koja je kasnije potvrđena prisustvom tripomastigotnih oblika u aspiratu limfnog čvora. Uključena je transfuzija cijelom krvi, a potom je intramuskularno aplicirana injekcija diminazeneaceturate 7% RTU@ 5 mg/kg, što je ponovljeno nakon pet dana. Petog dana su aplicirani i hemantinici. Životinja je pokazala jasne znakove oporavka nakon transfuzije krvi. Ovo je prvi slučaj sure u Kašmirskoj dolini koji dokazuje širenje parazita u područja umjerene klime u državi. Ovo opažanje je od presudnog značaja za područne veterinare. U budućnosti je neophodno identificirati vektore i moguće rezervoare kako bi spriječilo širenje bolesti.

Ključne riječi: Sura, tripanosomijaza, *Trypanosoma evansi*

PROFESSIONAL PAPER

RELATIONSHIPS BETWEEN SOME FERTILITY AND MILK PRODUCTION CHARACTERISTICS IN HOLSTEIN FRIESIAN CATTLE

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ABSTRACT

This study examined how key reproductive and milk-production traits interacted in 582 Holstein Friesian cows on a commercial farm in Malkara, Thrace, over five years. After screening 1,305 lactation and breeding records for consistency, researchers recorded milk-yield parameters—lactation length, total and standardized 305-day yield, dry period, day of peak production, and peak yield—alongside fertility traits: age at first insemination, conception and calving, calving interval, inseminations per conception, days open, and parity. Traits were grouped by lactation number, calving year and season, and production level, and persistency indices for Days 0–100, 100–200, and 200–300 were calculated. Using General Linear Models and Duncan's test, least-squares means were compared, while Pearson correlations and simple regressions evaluated interdependencies. Cows averaged 2.29 inseminations per conception and a 395.1-day calving interval; mean lactation yield was 8,508.8 kg. Lactation length ($r = 0.783$) and calving interval ($r = 0.649$) showed the strongest positive correlations with milk yield (all $P < 0.001$), whereas peak day and age at first calving were weakly associated. These results highlight the close link between reproductive efficiency and milk production, underscoring the importance of integrated genetic and management strategies for optimizing herd performance.

Keywords: Calving interval, insemination frequency, lactation milk yield, persistency indices, reproductive efficiency

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INTRODUCTION

The primary objective of cattle farming is to produce meat and milk, and considerable efforts over the past several decades have led to the development and widespread adoption of high-yielding breeds. Initially, breeding programs focused on maximizing the quantity of output per production cycle; more recently, however, the attention has shifted toward ensuring animal health and product quality.

Enhancements in production performance hinge on two principal factors: the genetic potential of the herd and the prevailing environmental conditions, especially management and nutrition. Crossbreeding and traditional selection methods have been employed to increase the frequency of favourable traits within populations (Alpan, 1993; Evrim and Güneş, 2000). While crossbreeding can introduce genetic improvement, sustained progress across generations requires systematic selection. Technological advances have further refined selection methodologies, notably through the integration of genomic evaluations. However, possessing superior genetics alone does not guarantee performance. The phenotypic expression of high-yield traits is profoundly influenced by the environment, including the consistency with which optimal management practices are applied across successive generations (Aritürk and Yalçın, 1966; Evrim and Güneş, 2000). Thus, even genetically elite animals will underperform if subjected to suboptimal feeding, care, or herd management.

Although nutrition and veterinary care are traditionally addressed within the domain of animal nutrition, this study centres on herd management strategies that directly influence reproductive efficiency -the foundation of sustained milk production. Successful lactation depends on timely parturition, making reproductive milestones such as age at first insemination, conception, and calving critical for initiating productive life without compromising physiological development (Harrison et al., 1990; Noakes et al., 2001; Heinrichs et al., 2013). Reproductive efficiency is further characterized by metrics, such as the number of inseminations per conception, days open, and overall calving interval, all of which must remain within optimal thresholds to ensure both productivity and sustainability.

Key milk yield parameters include lactation duration, total and standardized 305-day yields, dry period length, peak production day, and peak yield. Given the intrinsic interplay between fertility and milk production traits, this study aims to elucidate the relationships between selected reproductive and lactation characteristics, thereby providing actionable insights for producers and a robust foundation for future academic inquiry.

MATERIAL AND METHODS

This study drew upon five years of systematically maintained records from Holstein Friesian cattle on a commercial farm in Malkara, Thrace - an area of strategic importance to Turkish cattle production. Animals were managed under standard husbandry and feeding protocols, with no additional experimental treatments.

Data Collection and Trait Definitions

Milk production traits included lactation duration, milk yield over the full lactation and standardized 305-day period, dry period length, peak milk yield day, and peak yield amount. The 305-day yield was calculated from completed lactations, or, for cows dried off early, from raw unadjusted data. Records from animals culled before the lactation completions were excluded. Reproductive traits comprised age at first insemination, age at first conception and calving, calving interval, inseminations per conception, interval from calving to first insemination, and days open.

Data Quality and Classification

All digital records were screened for consistency, and entries with missing or invalid values were removed, yielding a final dataset of 1.305 records from 582 cows. Selected traits were then categorized based on key factors -lactation number, year and season of calving, and overall production level- using the criteria detailed in Tables 1 and 2. Records marginally outside predefined thresholds were reassigned to the nearest group. While the interval ranges were generally uniform, lactation duration categories were adjusted to reflect the actual data distribution (one-, two-, or three-month groupings).

Table 1 Study groups I: Insemination number for pregnancy, age at first calving, days open, and calving interval

Insemination number for pregnancy (number)	First calving age (month)	Days open (day)	Calving interval (day)
1	24	-60	-355
2	25	61-90	356-385
3	26	91-120	386-415
4	27	121-150	416-445
5+	28	151+	446+

Table 2 Study groups II: Lactation duration, lactation number, lactation milk yield, and parity

Lactation duration (day)	Lactation number	Lactation milk yield (kg)	Parity
-270	1	-5000	1
271-300	2	5001-7000	2
301-360	3	7001-9000	3
361-450	4	9001-11000	4
451+	5+	11001+	5+

Peak Yield and Persistency Measures

Peak yield was defined as the highest daily milk production during lactation; the corresponding day was noted as the peak day. Peak duration spanned the period in which daily yields remained within ±10 percent of that maximum, with both total and average yields calculated for this interval.

To assess persistency, total milk yields were computed for Days 0–100, 100–200, and 200–300, and persistency indices ($P_{2:1}$, $P_{3:1}$, $P_{3:2}$) were calculated, according to Johansson and Hansson (1940).

$$P_{2:1} = \frac{\text{Milk yield between days } 101 - 200 \text{ of lactation}}{\text{Milk yield in the first } 100 \text{ days of lactation}} * 100$$

$$P_{3:1} = \frac{\text{Milk yield between days } 201 - 300 \text{ of lactation}}{\text{Milk yield in the first } 100 \text{ days of lactation}} * 100$$

$$P_{3:2} = \frac{\text{Milk yield between days } 201 - 300 \text{ of lactation}}{\text{Milk yield between days } 101 - 200 \text{ of lactation}} * 100$$

The study evaluated the effects of environmental factors, namely, year and season of production, lactation and parity order, insemination number, production level, and age at first calving on both milk yield and fertility traits. Milk yield parameters included lactation duration, total and 305-day yields, dry period length, persistency indices, peak period characteristics, and daily yield during the peak. Fertility traits comprised age at first conception and first calving, service period (days open), calving interval, number of inseminations per conception, and interval from calving to subsequent insemination.

Statistical Analysis

Fixed-effect models were constructed to analyze calving interval and lactation yield, incorporating environmental factors, such as year and season of lactation, parity, gestation order, number of inseminations, production level, and age at first calving. Least Squares Means (LSM) were estimated via the General Linear Models (GLM) procedure (Harvey, 1975), and group differences were tested with Duncan's multiple range test (Duncan, 1955; Searle, 1971). Pearson correlations and linear regressions explored inter-trait relationships (Evrim and Güneş, 1994), under the assumption of no significant factor interactions. Multivariate equation systems were solved by using SPSS (Goodnight and Harvey, 1978; Searle et al., 1980; Welsch, 1977; Einot and Gabriel, 1975).

The statistical model used to analyze calving interval (CI) incorporated the following fixed effects: parity order (G), lactation milk-yield class (L_s), lactation duration (P_t), days-open (D_o), age at first calving (F_p), and inseminations-per-conception (T_i) (Equation 1)

Equation 1: $Y_{ilstopa} = \bar{\mu} + G_l + L_s + P_t + D_o + F_p + T_i + e_{ilstopa}$

For lactation milk yield (LMY), the fixed-effects model incorporated the following factors: lactation order (N_l), lactation duration (P_t), age at first calving class (Fp), number of inseminations per conception class (T_i), days-open (D_o), and calving-interval (C_l). The model can be expressed as in Equation 2.

Equation 2: $Y_{iltopna} = \bar{\mu} + N_l + P_t + T_i + D_o + F_p + C_n + e_{iltopna}$

Where:

$Y_{iltopna}$: Observed value of the trait under study for an individual.

μ: Overall mean of the trait across all records.

C_n: Effect of calving-interval class, where n = <356, 356–385, 386–415, 416–445, ≥446 days.

D_o: Effect of days-open class, where o = <61, 61–90, 91–120, 121–150, ≥151 days.

F_p: Effect of age-at-first-calving class, where p = 24, 25, 26, 27, 28 months.

G_l: Effect of parity (gestation order), where l = 1, 2, 3, 4, ≥5.

L_s: Effect of lactation-milk-yield class, where s = <5,000, 5,001–7,000, …, 9,001–11,000, ≥11,001 kg.

N_m: Effect of lactation number, where m = 1, 2, 3, 4, ≥5.

P_t: Effect of lactation-duration class, where t = <270, 271–300, 301–360, 361–450, ≥451 days.

T_i: Effect of insemination-number class, where i = 1, 2, 3, 4, ≥5 per conception.

$e_{iltopna}$: Random residual error term for each observation.

RESULTS

A total of 1,305 records from 582 Holstein Friesian cows were analyzed over a five-year period.

Descriptive statistics for reproductive and milk yield traits are summarized in Table 3.

Cows on the study farm required an average of 2.29 inseminations per conception (n = 1,302).

Table 3 General production traits of the cattle

Yield characteristics	n	$\bar{x}$	$s_{\bar{x}}$
Reproduction traits			
First insemination age (month)	573	14.46	0.053
First pregnancy age (month)	573	16.99	0.094
First calving age (month)	573	25.20	0.091
First service period (day)	515	69.48	1.275
Days open (day)	515	107.03	1.966
Inseminations number for pregnancy	1302	2.29	0.046
Gestation period (day)	1302	279.54	0.192
Calving interval (day)	743	395.06	2.632
Milk production traits			
Lactation duration (day)	1094	326.67	2.342
Dry period (day)	715	67.70	1.073
Lactation milk yield (kg)	1094	8508.84	83.418
305-day milk yield (kg)	1094	7720.73	58.168
Lactation period daily milk yield (kg)	1094	25.96	0.168
305-day period daily milk yield (kg)	1094	25.31	0.191
$P_{2:1}$ (%)	1094	71.12	0.420
$P_{3:1}$ (%)	1094	48.94	0.615
$P_{3:2}$ (%)	1094	67.29	0.683
Milk yield peak day	1094	57.66	0.578
Peak milk yield (kg)	1094	37.91	0.191
Peak period (day)	1094	19.01	0.200
Peak period daily milk yield (kg)	1094	36.39	0.191
Peak period total milk yield (kg)	1094	695.88	8.586

Table 4 presents the mean calving intervals (days) stratified by lactation milk yield, lactation duration, age at first calving, days open, number of inseminations per conception, and parity. The statistical significance of these subgroup differences was evaluated via analysis of variance (ANOVA), with sources of variation and detailed results reported in Table 5.

The mean calving interval, based on 743 records, was 395.06 days (range: 337.53–508.40 days; Table 4). When grouped by lactation milk yield and lactation duration (n = 727), the mean interval was 395.49 days, while classification by age at first calving, insemination number, and parity (n = 743) yielded the same average of 395.06 days. Grouping by days open (n = 341) produced a slightly higher mean calving interval of 406.26 days. All subgroup differences in calving interval were statistically significant ($P < 0.05$).

Table 4 Mean calving intervals (days) by lactation milk yield, lactation duration, age at first calving, days open, number of inseminations per conception, and parity, with significance of group differences assessed by Duncan's multiple range test

Factors	Groups	Calving interval (day)		
		n	$\bar{x}$	$s_{\bar{x}}$
Lactation milk yield (kg)	-5000	35	352.83^{c}	7.457
	5001-7000	146	352.82^{c}	3.581
	7001-9000	210	361.56^{c}	3.004
	9001-11000	186	406.46^{b}	4.355
	11001+	150	480.89^{a}	5.499
	overall	727	395.49	2.665
Lactation duration (day)	-270	115	337.53^{d}	3.294
	271-300	181	344.11^{d}	2.016
	301-360	216	387.49^{c}	2.349
	361-450	122	454.47^{b}	4.889
	451+	93	508.40^{a}	6.650
	overall	727	395.49	2.665
First calving age (month)	24	377	395.51^{a}	3.549
	25	169	394.25^{a}	5.744
	26	100	402.97^{a}	7.944
	27	44	367.70^{b}	8.447
	28	53	402.23^{a}	10.317
	overall	743	395.06	2.632
Days open (day)	-60	62	351.18^{d}	9.129
	61-90	72	394.96^{c}	9.005
	91-120	87	$409.47^{b,c}$	7.383
	121-150	58	423.86^{b}	8.046
	151+	62	453.50^{a}	6.956
	overall	341	406.26	4.037
Insemination number	1	321	371.18^{c}	3.481
	2	209	388.08^{c}	4.267
	3	96	417.17^{b}	7.921
	4	55	449.20^{a}	10.310
	5+	62	460.03^{a}	7.817
	overall	743	395.06	2.632
Gestation number	1	402	385.56^{b}	3.387
	2	200	$403.62^{a,b}$	5.199
	3	90	$407.60^{a,b}$	8.089
	4	30	417.63^{a}	13.229
	5+	21	$409.48^{a,b}$	17.686
	overall	743	395.06	2.632

^{a,b,c,d,e} : Differences between groups with different letters are statistically significant ($P<0.05$).

Lactation milk yield, lactation duration, days open, and insemination number each exerted highly significant effects on calving interval (P<0.001), and parity was also significant (P<0.01). In contrast, age at first calving had no statistically significant effect (P>0.05) (Table 5).

Cattle play a central role in livestock production, and within this industry, milk yield stands out as a fundamental performance metric. Accordingly, this study evaluated a comprehensive suite of lactation-related traits, including lactation duration, dry period length, total lactation yield, 305-day yield, persistency indices, and both daily and cumulative yields during the peak production period. Moreover, the statistical effects of several derived factors, such as lactation duration class, age at first calving, insemination count, days open, and calving interval, were examined individually. Consistent with most of the dairy research, analyses of lactation milk yield here accounted not only for management, nutrition, and husbandry practices but also for key environmental variables, namely, lactation order, year and season of lactation onset, and month of calving. Additionally, two specialized classification schemes were employed. The first scheme grouped cows by fertility-related characteristics (lactation duration, age at first calving, insemination count, days open, and calving interval) to assess their impact on milk yield. The second scheme, though not detailed in this section, categorizes cows by peak production parameters (peak day, peak yield, peak duration, and total yield during peak).

Table 5 Analysis of variance for calving interval, with fixed effects of lactation milk yield, lactation duration, age at first calving, days open, number of inseminations per conception, and parity

Factors	Source	Degrees of freedom	Type III Sum of squares	Mean square	*F*-value
			Calving interval		
Lactation milk yield	Between groups	4	1,687,752.6	421,938.15	147.730***
	Within groups	722	2,062,141.1	2,856.15	
	Total	726	3,749,893.7		
Lactation duration	Between groups	4	2,487,906.7	621,976.67	355.841***
	Within groups	722	1,261,987.0	1,747.90	
	Total	726	3,749,893.7		
First calving age	Between groups	4	42,092.4	10,523.11	2.057$^{n.s.}$
	Within groups	738	3,775,733.6	5,116.17	
	Total	742	3,817,826.0		
Days open	Between groups	4	354,537.8	88,634.44	19.406***
	Within groups	336	1,534,620.0	4,567.32	
	Total	340	1,889,157.8		
Insemination number	Between groups	4	663,136.3	165,784.08	38.783***
	Within groups	738	3,154,689.7	4,274.65	
	Total	742	3,817,826.0		
Parity	Between groups	4	84,686.0	21,171.51	4.185**
	Within groups	738	3,733,140.0	5,058.46	
	Total	742	3,817,826.0		

$^{n.s.}$: P>0.05 ** : P<0.01 *** : P<0.001

In this section, in addition to primary factors such as lactation order, year, season, and month of lactation onset, milk yields were evaluated according to secondary factors derived from other performance metrics, namely, calving interval, lactation duration, age at first calving, insemination count, days open, and parity. The resulting subgroup means are presented in Table 6, and the corresponding ANOVA results for these classifications are detailed in Table 7.

Table 6 Mean lactation milk yields (kg), stratified by calving interval, lactation duration, age at first calving, days open, number of inseminations per conception, and parity, with subgroup differences assessed for significance using Duncan's multiple range test

Factors	**Groups**	**Lactation milk yield (kg)**		
		n	$\bar{x}$	$s_{\bar{x}}$
Calving interval (day)	-355	264	7291.95^{e}	110.766
	356-385	136	8128.13^{d}	158.848
	386-415	92	9131.39^{c}	185.298
	416-445	71	9685.80^{b}	300.724
	446+	164	11774.35^{a}	188.831
	overall	727	8926.10	98.992
Lactation duration (day)	-270	239	5942.46^{e}	106.400
	271-300	261	7271.61^{d}	98.723
	301-360	317	8819.50^{c}	103.973
	361-450	171	10600.96^{b}	135.345
	451+	106	13037.57^{a}	206.187
	overall	1094	8508.84	83.418
First calving age (month)	24	545	8687.14^{a,b}	119.505
	25	233	8471.76^{a,b}	170.177
	26	152	8171.13^{b}	219.470
	27	78	7417.55^{c}	275.606
	28	86	9066.00^{a}	331.783
	overall	1094	8508.84	83.418
Days open (day)	-60	80	8291.68^{c}	261.380
	61-90	87	8590.66^{b,c}	304.386
	91-120	114	8997.02^{b,c}	281.476
	121-150	90	9221.27^{b}	274.952
	151+	95	10521.53^{a}	325.776
	overall	466	9154.16	135.302
Insemination number	1	435	8035.43^{d}	108.765
	2	300	8219.99^{c,d}	144.023
	3	156	8770.87^{b,c}	245.713
	4	89	9345.67^{b}	318.076
	5+	114	10063.47^{a}	341.614
	overall	1094	8508.84	83.418

Factors	**Groups**	**Lactation milk yield (kg)**		
		n	$\bar{x}$	$s_{\bar{x}}$
Lactation number	1	542	8226.28^{b}	105.633
	2	280	8877.35^{a}	183.706
	3	173	8804.01^{a}	227.761
	4	58	8582.98^{a}	387.783
	5+	41	8377.07^{a}	408.895
	overall	1094	8508.84	83.418

^{a,b,c,d,e} : Differences between groups with different letters are statistically significant (P<0.05).

Mean lactation milk yields (n = 1,094) are presented in Table 6. The overall average was 8,508.84 kg. When grouped by days open (n = 466), mean yield increased to 9,154.16 kg; grouping by calving interval (n = 727) yielded 8,926.10 kg. Depending on the grouping factor, average yields ranged from 5,942.46 kg to 13,037.57 kg.

It is important to note that when subgroup sample sizes were equal across different classification schemes, identical mean values occasionally appeared in multiple tables. However, where subgroup distributions varied, differences in mean values became more pronounced, underscoring the influence of classification criteria on the observed averages.

Analysis of variance (Table 7) demonstrated that calving interval, lactation duration, age at first calving, days open, and insemination number each had highly significant effects on lactation milk yield (P<0.001), whereas lactation number was significant at P<0.01. These findings underscore the critical interplay between reproductive efficiency and milk production.

Table 7 Analysis of variance for lactation milk yield, with fixed effects of calving interval, lactation duration, age at first calving, days open, number of inseminations per conception, and lactation order

Factors	**Source**	**Degrees of freedom**	**Type III Sum of squares**	**Mean square**	***F*-value**
		Lactation milk yield			
Calving interval	Between groups	4	2,166,900,952.2	541,725,238.05	130.148***
	Within groups	722	3,005,238,426.7	4,162,380.09	
	Total	726	5,172,139,378.9		
Lactation duration	Between groups	4	4,926,702,317.0	1,231,675,579.24	395.206***
	Within groups	1089	3,393,915,257.4	3,116,542.94	
	Total	1093	8,320,617,574.4		
First calving age	Between groups	4	154,568,527.2	38,642,131.81	5.153***
	Within groups	1089	8,166,049,047.2	7,498,667.63	
	Total	1093	8,320,617,574.4		
Days open	Between groups	4	267,977,205.2	66,994,301.29	8.350***
	Within groups	461	3,698,905,702.5	8,023,656.62	
	Total	465	3,966,882,907.6		

Factors	Source	Degrees of freedom	Type III Sum of squares	Mean square	*F*-value
Lactation milk yield					
Insemination number	Between groups	4	471,083,610.5	117,770,902.63	16.339***
	Within groups	1089	7,849,533,963.9	7,208,020.17	
	Total	1093	8,320,617,574.4		
Lactation number	Between groups	4	97,399,897.3	24,349,974.32	3.224**
	Within groups	1089	8,223,217,677.1	7,551,164.07	
	Total	1093	8,320,617,574.4		

** : P<0.01 *** : P<0.001

Key milk production parameters include lactation duration, total lactation yield, length of the dry period, time to peak yield, and daily yield during the peak phase. Fertility and milk production traits are inherently interdependent and cannot be viewed in isolation; rather, they continuously interact and influence each other.

In the preceding sections, we examined how groups defined by reproductive traits influenced milk production parameters and vice versa. In this section, we focus specifically on the interrelationships, quantified by correlation and regression, between selected fertility and milk yield traits. Although all reproductive and lactation parameters were evaluated throughout the study, here we concentrate on lactation milk yield and calving interval, as these two metrics encapsulate the core influences on overall productivity. Correlation and regression coefficients between lactation milk yield, calving interval, and their associated traits are presented in Table 8.

Table 8 Correlation (*r*) and regression (*b*) coefficients between lactation milk yield, calving interval, and selected reproductive and milk production traits

	Lactation milk yield			**Calving interval**		
	n	***r***	***b***	***N***	***r***	***b***
Milk yield parameters						
Lactation milk yield	1094	-	-	727	64.9***	0.000$^{\text{n.s.}}$
Lactation number	1094	6.0*	472.98***	727	13.3**	5.235***
Lactation duration	1094	78.3***	26.45***	727	80.6***	0.709***
Dry period	715	5.8$^{\text{n.s.}}$	-8.97***	687	39.4***	1.067***
Daily milk yield	1094	67.3***	396.83***	727	26.3**	5.063***
Peak day	1094	2.1$^{\text{n.s.}}$	2.13$^{\text{n.s.}}$	727	0.2$^{\text{n.s.}}$	0.148$^{\text{n.s.}}$
Peak milk yield	1094	39.6***	-20.05**	727	18.0***	0.518$^{\text{n.s.}}$
Peak duration	1094	25.4***	-214.13***	727	23.2***	0.207$^{\text{n.s.}}$
Peak total milk yield	1094	39.4***	8.30***	727	27.1***	0.057$^{\text{n.s.}}$
Reproduction parameters						
Calving interval	727	64.9***	21.87***	727	-	-
Gestation number	1094	6.0*	-45.27$^{\text{n.s.}}$	727	13.5**	1.889$^{\text{n.s.}}$
Days open	466	23.6***	5.79*	341	41.9***	0.726***

	Lactation milk yield			Calving interval		
	n	*r*	*b*	*N*	*r*	*b*
Insemination number	1094	23.4***	421.55***	743	39.0***	16.390***
First service period	466	$8.2^{n.s.}$	12.75**	341	25.3***	0.764***
First calving age	543	$2.6^{n.s.}$	$28.81^{n.s.}$	402	$-1.9^{n.s.}$	$-0.645^{n.s.}$

$^{n.s.}$: P>0.05 * : P<0.05 ** : P<0.01 *** : P<0.001

Correlation and regression analyses (Table 8) further elucidated these relationships. The strongest correlations with lactation milk yield were lactation duration (*r* = 0.783, P<0.001) and calving interval (*r* = 0.649, P<0.001), whereas peak day (*r* = 0.021) and age at first calving (*r* = 0.026) showed no significant associations. Calving interval exhibited its strongest correlations with lactation duration (*r* = 0.806, P<0.001) and days open (*r* = 0.419, P<0.001), with negligible correlations for peak day (r = 0.002) and age at first calving (*r* = –0.019). Regression coefficients indicated that lactation number (*b* = 472.98 kg) and insemination count (*b* = 421.55 kg) exerted the greatest effects on milk yield, while these same factors influenced calving interval by 5.235 days and 16.390 days, respectively.

Regression analyses (Table 8) revealed that the magnitude of effects on both lactation milk yield and calving interval varied with the nature and levels of the traits examined. Lactation number emerged as the most influential factor on milk yield, increasing output by 472.98 kg per additional lactation, followed by the number of inseminations, which contributed 421.55 kg. These same variables also significantly affected calving interval, extending it by 5.235 days per additional lactation and by 16.390 days per extra insemination, respectively. Together, these results highlight the interdependence of fertility and milk production traits and underscore the importance of optimizing both genetic and management factors to enhance overall herd performance.

DISCUSSION AND CONCLUSION

Based on the lactation milk yield categories, calving intervals ranged from 352.82 to 480.89 days (Table 4). Cows producing 5.001–7.000 kg exhibited the shortest intervals and required the fewest inseminations, whereas those yielding over 11,001 kg had the longest intervals and highest insemination frequencies (P<0.05). These findings suggest that higher-yielding cows with extended lactations tend to need more inseminations and experience longer calving intervals, an interdependence that herd managers must carefully navigate.

When grouped by lactation duration, intervals varied from 337.53 to 508.40 days (P<0.05), with the shortest in cows milked fewer than 270 days and the longest in those milked beyond 451 days. This mirrors the milk yield results: extended milking periods correlate with increased insemination requirements and prolonged calving intervals, particularly in cows exceeding the standard 305-day lactation.

Classification by age at first calving yielded intervals between 367.70 and 402.97 days. Cows calving first at 27 months had the briefest intervals, whereas those calving at 28 months had the longest (P<0.05), highlighting the impact of delayed reproductive onset on subsequent calving rhythm.

Analysis by days open revealed intervals of 351.18 to 453.50 days (P<0.05). Cows conceiving within 60 days post-calving had the shortest intervals, while those requiring more than 151 days to reconceive had the longest, underscoring the importance of prompt conception. When grouped by days open, calving intervals increased progressively with longer open periods, reaching their maximum in cows with days-open of 151 days or more. This extension of the open period, reflecting additional insemination efforts, demonstrated a concomitant increase in both insemination count and calving interval.

Grouping by number of inseminations per conception showed intervals ranging from 351.18 days (one insemination) to 460.03 days (five or more inseminations) (P<0.05), confirming that reproductive inefficiency directly extends the calving interval. Cows that conceived on a single insemination exhibited the shortest calving intervals, whereas those requiring five or more inseminations had the longest (P<0.05). Calving interval increased steadily with each additional service, peaking in the group with five or more inseminations. These findings confirm that higher insemination counts adversely affect the calving interval.

Parity-based classification produced intervals of 385.56 to 417.63 days, with the shortest intervals after first parity and progressive increases in later parities (P<0.05). ANOVA confirmed that lactation milk yield, lactation duration, days open, and insemination count all exerted highly significant effects on calving interval (P<0.001), whereas age at first calving did not (P>0.05), emphasizing the dominant roles of service period and insemination frequency in reproductive efficiency.

Analysis of variance revealed highly significant differences (P<0.001) in calving intervals when cows were grouped by lactation milk yield, lactation duration, and days open. In contrast, grouping by age at first calving did not produce statistically significant differences in calving intervals (P>0.05). Additionally, insemination count was identified as a highly significant factor (P<0.001). The pronounced effects of days open and service number, alongside their interactions with other key factors, underscore their central role in determining calving interval.

Lactation milk yields varied from 7,291.95 kg to 11,774.35 kg across calving-interval classes. A clear positive trend was observed, with longer calving intervals corresponding to higher milk yields and increased cumulative production. Because calving interval is inherently influenced by service number and days open, this relationship is unsurprising: cows with intervals under 355 days produced the least milk, whereas those with intervals exceeding 446 days yielded the most (P<0.05). Despite most intervals clustering below 385 days, a substantial proportion of cows fell into the >446-day category—a finding of potential concern for farm efficiency. Analysis confirmed that differences in milk yield among calving-interval groups were highly significant (P<0.001).

In a study conducted across four Danish herds, each having between 87 and 151 Holstein, Jersey, and crossbred cows, calving intervals were classified into five groups (<13, 14–15, 16–17, 18–19, and >19 months). Results indicated that milk yield increased both with longer calving intervals and higher parity (Lehmann et al., 1996), mirroring our findings. Conversely, an investigation of 1,509 lactations from Holstein Friesians on a private farm near Mansoura, Egypt, categorized calving intervals as <13, 13–19, and >19 months; this study reported no significant effect of calving interval on lactation yield (Shalan et al., 2022). These divergent results highlight the influence of herd-specific management and environmental conditions on the interval–yield relationship.

Lactation milk yield varied from 5,942.46 to 13,037.57 kg across lactation-duration categories, primarily reflecting the cumulative increase in milk production with longer lactations (P<0.05). When grouped into intervals of <270, 271–300, 301–360, 361–450, and >451 days, the highest yield occurred in the >451-day class, while the lowest was in the <270-day group. Notably, most lactations fell within the 301–360-day range. Differences in milk yield among these duration classes were highly significant (P<0.001).

Lactation milk yield ranged from 7,417.55 to 9,066.00 kg across the first-calving–age categories, with older age at first calving associated with significantly higher yields (P<0.05). In this herd, most heifers calved for the first time at 24 or 25 months. Physiologically, delaying first calving allows for more complete mammary gland development, greater metabolic maturity, increased live-weight gain, and enhanced uterine growth, all of which can contribute to higher milk production and reduced postpartum health complications. Consequently, optimal age at first calving not only influences lifetime productivity but also represents a critical economic milestone for the farm. While excessively early calving may compromise both milk and reproductive performance, calving that is too late can similarly incur production losses. Our findings underscore the importance of re-evaluating the practice of targeting first insemination at 13 months; in addition to body weight, heifer management should consider reproductive tract maturity. Notably, a separate study of 2.233 lactations from 1.579 cows in four farms in the Aegean and Marmara regions (2013–2018) reported the highest yields in heifers calved at 24 months (İlhan, 2023), a result that contrasts with our observations ($P<0.001$).

Lactation milk yield ranged from 8,035.43 to 10,063.47 kg across service-number groups, with a significant positive trend observed as the number of inseminations increased ($P<0.05$). This pattern likely reflects extended lactation and delayed dry-off associated with additional insemination attempts. Cows conceiving on the first insemination exhibited the lowest yields, whereas those requiring five or more inseminations achieved the highest yields. As service number increased, both the milking period and the subsequent dry period were prolonged, cumulatively extending total lactation length. These findings underscore service number as a key fertility parameter influencing milk yield. From a management perspective, the goal should be to achieve prompt conception, thereby minimizing the number of inseminations, while maintaining annual calving and optimal milk production. Animals that calve once per year and sustain high yields represent critical assets for both farm profitability and genetic improvement. ANOVA confirmed that service number exerted a highly significant effect on lactation milk yield ($P<0.001$).

Lactation milk yield ranged from 8,291.68 to 10,521.53 kg across the days-open categories, with longer days open positively associated with higher cumulative yields ($P<0.05$). Cows conceiving within 60 days of calving exhibited the lowest yields, whereas those with days open exceeding 151 days achieved the highest yields. Variations in the length of the days-open period may reflect factors such as postpartum uterine infections, metabolic disorders, nutrition management, seasonal influences, heat stress, abortion, embryonic loss, silent heats, and overlooked oestrus signs. Analysis confirmed that days open exerted a highly significant effect on lactation milk yield ($P<0.001$).

Several regional studies have similarly demonstrated significant effects of the days-open interval on lactation performance. In a commercial herd in Konya, 480 lactations from 307 Holstein-Friesian cows were grouped by service period, revealing the lowest milk yields in cows with days-open under 60 and the highest yields in those with 151–180 days open. The authors recommended extending the interval beyond 90 days to optimize yield and reported significant impacts on 305-day yield, peak yield, and persistency (Güler, 2023). Similarly, Bayrıl and Yılmaz (2017) found that classifying days open into 50–75, 76–100, and ≥101 days in 106 Holsteins at the Kazova Vasfi Diren farm produced significant differences in milk yield. Long-term records from the Koçaş farm in Aksaray (362 cows; 1988–1995) identified an average service period of 93.3 days (Duru and Tuncel, 2002). A subsequent analysis of 959 lactations at the same site, classifying days open as <40, 40–60, 61–80, 81–100, 101–120, 121–140, and >140 days, indicated that intervals exceeding 81 days enhanced 305-day yield and persistency, with an optimal range of 61–100 days (Duru and Tuncel, 2004). Data from four government farms in Balıkesir, Muğla, and Kırklareli (1,259 cows; 1980–1992) further supported these findings, showing significant yield differences across days-open classes of ≤60, 61–100, 101–140, 141–180, and ≥181 days, and endorsing 61–141 days, and ideally around 100 days, as most favourable (Kaya et al., 2003). Kino et al. (2019) analyzed 7.083 lactations from 2,000 Holsteins in Japan (2012–2016), classifying days open as ≤52, 53–

65, 66–110, and ≥111 days, and likewise observed significant yield effects. In the UK, Taylor et al. (2003) recommended a 135-day service period, while Esslemont and Kossaibati (2000) reviewed national databases and identified optimal intervals of 86–109 days, acceptable intervals of 110–120 days, and increasing fertility issues beyond 133 days. Collectively, these studies corroborate our findings that moderate days-open intervals are critical to maximizing milk yield and reproductive efficiency.

Lactation milk yield varied from 8,226.28 to 8,877.35 kg across lactation orders, with differences between orders reaching statistical significance ($P<0.05$). Contrary to expectations, cows in their second and third lactations produced more milk than those in later lactations. This likely reflects the numerical distribution of cows by lactation order in our dataset and temporal changes in herd structure over the study period, rather than a biological decline in yield after peak lactations.

During the study, it became apparent that variations in milk yield traits were largely influenced by calving interval, and conversely, that fertility traits impacted lactation performance. Consequently, we evaluated the strength of the relationship between lactation milk yield and calving interval. The resulting correlation coefficients for both reproductive and lactation traits underscore that fertility and milk production are inseparable characteristics in dairy management.

The strongest associations with lactation milk yield were observed for lactation duration ($r = 0.783$) and daily milk yield ($r = 0.673$). Among fertility traits, calving interval exhibited the highest correlation with milk yield ($r = 0.649$). In contrast, correlations between milk yield and dry period length, time to peak yield, first service interval, and age at first calving were negligible and statistically non-significant. Although the correlation coefficients for lactation order ($r = 0.060$) and parity order ($r = 0.060$) were similarly low, they reached statistical significance, reflecting their predictable impact on production and reproductive performance.

The strongest fertility-related correlations mirrored those for milk yield, with calving interval showing high associations with both lactation milk yield ($r = 0.649$) and lactation duration ($r = 0.806$). Among reproductive traits, days open exhibited the highest correlation with calving interval ($r = 0.419$). In contrast, correlations between calving interval and peak day or age at first calving were negligible and non-significant. Notably, whereas first service interval had no significant association with milk yield, it emerged as a meaningful predictor of calving interval.

Milk secreted by cows originally evolved to nourish their calves, yet today the volume produced per animal far exceeds the requirements of calf rearing. Consequently, both production- and economic-driven efforts continue to target further increases in milk yield, alongside occasional shifts toward enhancing milk quality rather than quantity. High lactational performance remains a primary goal, and while genetic improvement plays a major role, management practices, particularly nutrition, housing, and herd monitoring, offer critical, actionable avenues for optimization.

When interpreting the interrelationships between lactation milk yield and calving interval, it is essential to consider not only the magnitude of correlation and regression coefficients but also the class-interval widths for each trait. Moreover, future analyses may benefit from larger and more uniformly distributed datasets to strengthen the robustness of these parameter estimates across all production and fertility characteristics.

One of the most pressing challenges in modern cattle production is the failure to integrate advanced technologies with traditional husbandry practices. Even in Thrace, where some farms exceed optimal herd sizes and possess near-standard facilities, expected performance standards are often unmet. This discrepancy reflects a lack of technical proficiency among many producers, making it unrealistic to expect high-level management from individuals without specialized training. In contrast, family-run enterprises, driven not solely by profit but by a sense of social responsibility and genuine commitment to animal welfare, often provide more conscientious stewardship. Consequently, strategically supporting and developing these family enterprises, which serve as the true engines of livestock progress, should be a priority for the industry.

The farm in Thrace boasts advanced infrastructure-integrated automation systems, an automatic milking station, on-site feed formulation, synchronized breeding and calving monitoring, separate rearing facilities for male and female calves, multiple age-group paddocks, and its own nucleus herd. Despite this capacity to generate and analyze daily, weekly, and monthly performance data, these management tools remain underutilized. To drive productivity gains, the farm should prioritize the effective deployment of its herd-management automation, with particular focus on optimizing nutritional strategies and ration formulations based on real-time data insights.

In conclusion, the farm's management practices fall short of the performance standards expected for a facility of its calibre in the region. Although the infrastructure could be classified as elite, actual productivity parameters have not met their potential. It is, therefore, recommended that herd-management protocols be systematically revised and that the existing record-keeping systems be leveraged more effectively. By conducting frequent, short-interval reviews of daily, weekly, and monthly data, and by applying the same analytical rigor demonstrated in this study to drive continuous improvements, farm managers can optimize decision-making and ultimately enhance both reproductive and milk-production performance.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

CONTRIBUTION

SÇK: Funding, Materials, Data Collection, Literature review, Writing; NÖ: Literature review, HİK: Data Processing, Data Analysis; TB: Data Processing, ZT: Data Processing, OK: Data Analysis, Data Interpretation, Writing, Literature Review, HG: Conception, Design, Supervision, Writing, Literature Review, Critical Review.

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ODNOSI IZMEĐU FERTILITETA I PROIZVODNIH KARAKTERISTIKA MLIJEKA KOD HOLŠTAJN-FRIZIJSKOG GOVEDA

SAŽETAK

U ovom radu smo istražili povezanost ključnih karakteristika reprodukcije i mliječnosti kod 582 krave holštajn-frizijske pasmine na komercijalnoj farmi u Malkari, u Trakiji u petogodišnjem periodu. Pregledano je 1305 izvještaja o mliječnosti i uzgoju u smislu konzistentnosti, pri čemu su zabilježeni podaci o parametrima prinosa mlijeka - dužini laktacije, ukupni i standardizirani prinos mlijeka u 305 dana; suhi period, dan najvećeg prinosa mlijeka i najveći prinos mlijeka - zajedno s karakteristikama fertiliteta: starosti na prvoj inseminaciji, začeću i teljenju, dane otvorenosti i paritet. Karakteristike su potom grupirane po broju laktacija, godini i sezoni teljenja i razini proizvodnje, nakon čega su izračunati indeksi perzistentnosti za dane 0–100, 100–200 i 200–300. Korištenjem Generalnog linearnog modela i Duncanovog testa su komparirane najniže srednje kvadratne vrijednosti, a Pearsonovom korelacijom i jednostavnim regresijama su ispitane međuovisnosti. Krave su u prosjeku imale 2.29 inseminacije po začeću uz interval teljenja od 395.1 dana; srednja vrijednost prinosa mlijeka je iznosila 8.508,8 kg. Dužina laktacije ($r = 0.783$) i interval teljenja ($r = 0.649$) su pokazali najjaču pozitivnu korelaciju sa prinosom mlijeka (svi $P < 0.001$), dok su dan najvećeg prinosa i starost pri prvom teljenju bili veoma slabo povezani. Ovi rezultati naglašavaju čvrstu vezu između reproduktivne učinkovitosti i proizvodnje mlijeka, naglašavajući važnost integriranih genetskih i upravljačkih strategija u optimizaciji performansi stada.

Ključne riječi: Indeksi perzistentnosti, interval teljenja, prinos mlijeka u laktaciji, reproduktivna učinkovitost, učestalost inseminacije

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Books:

Brown C, Laland K, Krause J. 2011. Fish Cognition and Behavior. 2nd ed. Oxford, UK: Wiley-Blackwell.

Chapters in books:

King AS. 1993. Apparatus urogenitalis. In Baumel JJ, King AS, Breazile JE, et al (Eds), Handbook of Avian Anatomy: Nomina Anatomica Avium

(2nd ed., pp. 329-90). Cambridge, USA: Nuttall Ornithological Club.

Conference proceedings:

Bregoli M, Dediasi K, Pasolli C. 2006. Antibiotic resistant E. Coli in free-ranging alpine wild ruminants, in: VII Conference of the European Section of the Wildlife Diseases Association. p. 78.

Web Adresses:

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Theses:

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