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PhD Thesis

Optimizing age at first calving in the Italian Mediterranean buffalo: integrated analysis of productive, reproductive and economic outcomes

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I choose not to celebrate what was still to come,
but what has shaped me along the way.
Not the new beginnings,
but the projects carried forward with patience and perseverance.
Not the promises,
but the commitments that were honored.
Not what was missing,
but what through effort, determination, and resilience was finally achieved.

This work is dedicated to the journey itself, to the challenges overcome, to the lessons learned, and to those who walked beside me
holding my hand on the steep descents and encouraging me through every uphill climb.

From here, I continue.

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AFC	Age at first calving
AI	Artificial insemination
AIA	Italian Breeders' Association
AIC	Akaike information criterion
ANASB	National Association of Buffalo Breeders
ART	Assisted reproductive technologies
BCS	Body condition score
BDN	National Zootechnical Database
BIC	Bayesian information criterion
BW	Body weight
CI	Calving interval
CLAL	Dairy Technology Center
CSN2	Beta-casein gene
CTMBC	Consortium for the Protection of Mozzarella di Bufala Campana PDO
DIM	Days in milk
DMP	Days to milk peak
DO	Days open
EC	European commission
ECM	Energy-corrected milk
EU	European union
FADN	Farm accountancy data network
FAOSTAT	Food and agriculture organisation statistical database
GLIMMIX	Generalized linear mixed models
GLM	General linear model
GLMM	Generalized linear mixed model
GLMSELECT	General linear model selection
GnRH	Gondadotropin-releasing hormone
HSP7	Heat shock protein
ISTAT	Italian National Institute of Statistics
IZS	Experimental Zooprophylactic Institute
LH	Luteinizing hormone
MASAF	Ministry of Agriculture, Food Sovereignty and Forests
MC1R	Melanocortin 1 receptor gene
Nramp1BB	Genotype associated with resistance to Brucella abortus

OEV	Veterinary Epidemiological Observatory
OPU	Ovum pick-up
PDO	Protected designation of origin
PLF	Precision livestock farming
SCC	Somatic cell count
SCS	Somatic cell score
SD	Standard deviation
SNP	Single nucleotide polymorphism
SNPRBb	Buffalo SNP database
VetInfo	National Veterinary Information System
VIF	Variance inflation factor

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Abstract

The water buffalo (*Bubalus bubalis*) is a strategic species for the global dairy sector and is of particular importance in Italy, where the Italian Mediterranean buffalo breed is reared to produce Mozzarella di Bufala Campana PDO. Over the past two decades, the increasing intensification of Italian buffalo dairy systems has heightened the need to optimise herd management to sustain economic competitiveness while ensuring environmental and biological sustainability. Within this framework, age at first calving (AFC) represents a key management and breeding parameter, as it can affect rearing costs, genetic turnover, productive longevity, and overall herd efficiency. In Italian Mediterranean buffalo herds, AFC has progressively reduced and currently averages approximately 34–38 months, a trend largely driven by the widespread adoption of out-of-breeding mating technique aimed at controlling calving seasonality and aligning milk production with consumer demand. While the reduction of AFC reflects advances in reproductive management in buffalo heifer rearing, excessive decreases in AFC in the absence of adequate nutritional, health, and reproductive strategy may negatively compromise lifetime productive and reproductive performance of buffaloes. Therefore, the objectives of this doctoral thesis were to: (i) quantify the effects of AFC on productive and reproductive performance in primiparous Italian Mediterranean buffaloes; and (ii) evaluate the long-term implications of AFC on lifetime productivity, udder health, and economic profitability across the entire productive life of the animal. Chapter 1 provides a broad and integrative overview of buffalo, addressing its taxonomic classification, domestication history, and global and European population distribution, with a specific focus on the Italian production system. Attention is given to the Italian Mediterranean buffalo, highlighting the breed's physiological and productive peculiarities, its adaptation to intensive farming conditions, and its central role in a highly specialized and market-oriented dairy chain. Chapter 2 investigates sources of variation in productive and reproductive performance among primiparous Italian Mediterranean buffaloes using 362,703 first-lactation test-day records from more than 50,000 animals. The results identify an optimal AFC window between 34 and 38 months, associated with the most favorable balance between milk production and fertility. While a delayed AFC (>42 months) was linked to higher milk and energy-corrected milk yields (+0.50 and +0.90 kg/day, respectively), buffaloes calving within the intermediate AFC range exhibited shorter calving interval and days open (449–450 and 137–139 days, respectively). In contrast, excessively lower AFC (≤32 months) was associated with higher somatic cell score (SCS). Chapter 3 extends the analysis to the entire productive life of Italian Mediterranean buffaloes, confirming the biological patterns observed in primiparous animals. An AFC between 34 and 38 months was consistently associated with superior lifetime productive and reproductive performance, including shorter calving interval and days open, together with lower SCS. Conversely, both lower (<30 months) and greater (>42 months) AFC were associated with suboptimal lifetime performance. The economic evaluation, integrating model-based milk yield estimates with cost structures derived from the *Farm Accountancy Data Network (FADN)*, estimated an average milk production cost of €1.38 L^{-1} and a farm-gate milk price of €1.50 L^{-1}. Under these conditions, the average net profit amounted to €314 per lactation, with the highest profitability (€339 per lactation) observed in mid-parity buffaloes calving the first time between 37–38 months. These findings demonstrate that the AFC range identified as biologically optimal also maximizes economic efficiency. Overall, the results of this thesis highlight the multifactorial role of AFC management in determining the biological, welfare-related, and economic sustainability of Italian Mediterranean buffalo farming. Targeting an intermediate AFC window of 34–38 months emerges as the most effective strategy to balance milk production, reproductive efficiency, udder health, and farm profitability. Achieving this objective requires the integration of targeted nutritional strategies during the *pre*-weaning and *pre*-breeding phases with improved reproductive management and a holistic approach to herd longevity, thereby supporting farmers in the transition towards more efficient and resilient production systems.

1. Introduction

1.1. Taxonomic Classification and Evolutionary Background of Buffaloes Species

The water buffalo (*Bubalus bubalis*) belongs to the family *Bovidae*, subfamily *Bovinae*, and the genus *Bubalus*, which also includes several wild species such as *B. arnee* (wild water buffalo), *B. depressicornis* (lowland anoa), and *B. quarlesi* (mountain anoa). Within *B. bubalis*, domesticated buffaloes are conventionally classified into two subspecies, the river type (*B. bubalis*) and the swamp type (*B. carabanensis*), based on differences in cytogenetic profile, morphology, and geographical distribution. These two types differ markedly in body conformation and productive aptitude, as well as in their primary production orientation (Curaudeau et al., 2021). The most relevant biological distinction between river and swamp buffalo lies in their karyotype. River buffaloes, which are widely distributed in the Indian subcontinent and the Mediterranean basin, including the Italian Mediterranean breed, possess 50 chromosomes ($2_n = 50$). In contrast, swamp buffaloes, predominant in Southeast Asia, have 48 chromosomes ($2_n = 48$), resulting from a centric fusion between chromosomes 1 and 9 (Mattapallil and Ali, 1999). Although the two types are interfertile, their hybrids exhibit an intermediate karyotype ($2_n = 49$), which may impair fertility in subsequent generations due to meiotic segregation anomalies (Iannuzzi, 1994; Borghese, 2005). Molecular phylogenetic and archaeological evidence supports the hypothesis that river and swamp buffalo originated from independent domestication events, likely occurring during the *Pleistocene* (Curaudeau et al., 2021). Despite their domesticated status, river buffaloes retain substantial genetic variability at the global level, reflecting long-standing and region-specific breeding practices. This diversity is particularly evident in South and Southeast Asia, where buffaloes have historically been selected for both milk and meat production. For example, microsatellite analyses of Indian river buffalo populations have identified distinct genetic clusters among major breeds such as *Murrah*, *Jaffarabadi*, and *Toda*, highlighting structured genetic differentiation within the species (Vijh et al., 2008). More recent advances in high-throughput sequencing have provided deeper insight into buffalo population structure. Whole-genome and transcriptome studies have revealed pronounced genetic, metabolic, and transcriptional divergence even among geographically proximate populations. In this regard, multi-omics analyses conducted on *Chinese* swamp buffalo breeds demonstrated that genetic differentiation does not necessarily mirror geographic distance but rather reflects complex domestication pathways and breeding histories (Shen et al., 2024). Consistently, phylogenetic reconstructions based on nuclear markers have resolved river and swamp buffaloes into two clearly separated clades (Lei et al., 2007). Mitochondrial DNA analyses further corroborate this divergence and indicate that the *tamaraw* (*B. mindorensis*), a distinct wild species, is more closely related to the swamp lineage than to the river type (Villamor et al., 2021).

In parallel with these evolutionary studies, the buffalo genome has become the focus of extensive investigation through large-scale sequencing and bioinformatic approaches in the past 30 years. The development of dedicated genomic resources, such as the BuffGR database, has enabled open access to more than 7.70 million single nucleotide polymorphisms (SNPs) identified across major buffalo breeds, including the Italian Mediterranean and Murrah (Khan et al., 2022). These tools support marker-assisted selection, genomic traceability, and the conservation of genetic diversity, particularly in numerically limited or locally adapted populations. Complementary transcriptome atlases have also been generated to functionally characterize gene expression patterns and to identify molecular signatures associated with economically relevant traits (Young et al., 2019). Finally, large-scale comparative genomic analyses have identified clear signatures of domestication and positive selection in the buffalo genome. Studies integrating hundreds of river and swamp buffalo genomes have detected selection signals linked to key production and adaptation traits, including milk yield (e.g., CSN2), reproductive function (e.g., GKAP1), and coat color (MC1R), providing further insight into the genetic basis of buffalo productivity and adaptation (Si et al., 2024).

1.2. General Characteristics and Ethology of Buffaloes Species

Both river and swamp buffaloes possess a remarkable ability to cope with hot and humid climates, low-quality forage, parasitic pressure, and infectious challenges, making them a key resource for sustainable livestock production, particularly in developing countries. Their strong adaptation to wetland environments, such as river basins, rice paddies, and mangrove ecosystems, is reflected in both anatomical and physiological traits. The two species are characterized by broad, splayed hooves that provide stability on soft or waterlogged soils, while their digestive physiology enables efficient utilization of highly fibrous diets, allowing them to thrive on low-quality agricultural by-products commonly available in tropical floodplains (Khan and Iqbal, 2009). These adaptive advantages coexist with specific physiological constraints related to thermoregulation. The buffalo's dark, sparsely haired skin and relatively low density of sweat glands, compared with cattle, limit its capacity for evaporative heat dissipation. Consequently, buffaloes rely primarily on behavioral mechanisms to regulate body temperature. Wallowing in water or mud represents a key thermoregulatory strategy, facilitating heat loss while simultaneously providing protection against ectoparasites (Marai and Haeeb, 2010).

From an ethological perspective, buffaloes are highly gregarious animals with a well-defined social structure. This strong sociality makes them particularly sensitive to disruptions in routine, changes in management, or unfamiliar human interactions, which may elicit defensive or aggressive responses (De Rosa et al., 2009). Such behavioral reactivity has important implications for dairy management, as stress can directly inhibit oxytocin release and impair milk ejection (Borghese, 2005), underscoring the need for calm and consistent handling practices.

Clear sexual dimorphism is evident in the species. Adult males are generally larger and more robust than females, exhibiting a more pronounced cervical crest and thicker horn cores. Body weight typically ranges from 600 to 900 kg in adult bulls and from 450 to 650 kg in adult females, although these values are influenced by genotype, nutritional level, and rearing system (Petrocchi Jasinski et al., 2023). Sexual dimorphism is already apparent at birth. Studies on the Italian Mediterranean buffalo indicate that male calves are consistently heavier than females, with birth weights ranging from approximately 40–45 kg in males and 38–42 kg in females (Infascelli et al., 2003; Zicarelli, 2004). These differences, typically amounting to 2.50–4.50 kg, are modulated by dam parity and nutritional status and reflect sex-specific fetal growth trajectories linked to differences in placental function and endocrine environment.

1.3. Physiological Adaptations and Environmental Resilience of Buffaloes Species

As previously outlined, despite its evolutionary origin in tropical and subtropical environments, the river buffalo *(B. bubalis*) exhibits a relatively low physiological tolerance to direct solar radiation and elevated ambient temperatures when compared with dairy cattle. This apparent paradox is largely explained by species-specific anatomical and physiological traits. The dark, sparsely haired skin of buffaloes favors solar heat absorption, while the markedly lower density of sweat glands limits evaporative heat dissipation (Napolitano et al., 2022). Consequently, behavioral thermoregulation, particularly wallowing in water or mud, represents the primary mechanism for heat loss and plays a central role in maintaining welfare and productivity under hot climatic conditions (Khan and Iqbal, 2009). In parallel, buffaloes display metabolic adaptations that partially mitigate heat load. Compared with cattle, they exhibit slower ruminal fermentation rates and lower methane emissions per unit of dry matter intake, contributing to reduced endogenous heat production during thermal stress (Malik et al., 2021; Malik et al., 2023). At the cellular level, transcriptomic studies have shown a stronger activation of stress-response pathways in buffaloes exposed to heat, with increased expression of heat shock proteins such as HSP70 and HSP90, which enhance protein stability and cellular protection (Young et al., 2019).

Despite these adaptive strategies, evidence from both Mediterranean and tropical production systems indicates that buffaloes remain highly susceptible to heat under intensive conditions. In Italian Mediterranean and Murrah breeds, a temperature–humidity index (THI) above 72 is widely recognized as a critical threshold beyond which significant declines in performance occur (Dash et al., 2016; Matera et al., 2022). The initial physiological response to heat stress is a voluntary reduction in dry matter intake, leading to negative energy balance and reduced availability of metabolic substrates for milk synthesis at the mammary gland level. In Italian herds, this translates into seasonal milk yield losses of approximately 10–15% during summer compared with cooler periods (Bernabucci et al., 2010). In addition to quantitative losses, heat stress negatively affects milk quality. Elevated THI is associated with reductions in milk fat and protein content (Matera et al., 2022) and with alterations in mineral composition. Recent studies have documented decreased concentrations of calcium, copper, and selenium in buffalo milk when THI exceeds 72, impairing micellar stability and worsening coagulation properties, including longer rennet coagulation time and reduced curd firmness (Albenzio et al., 2024). Such technological impairments are directly linked to increased fat and protein losses during processing and, ultimately, to lower mozzarella yield and reduced production efficiency (Sales et al., 2017).

Heat stress also exerts profound effects on reproductive function. Prolonged exposure to high THI activates the hypothalamic–pituitary–adrenal axis, increasing circulating cortisol levels, which inhibit the pulsatile release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH). This endocrine disruption results in a higher incidence of silent estrus and anovulatory follicles (Campanile et al., 2010). At the gamete level, oocytes developing under heat stress show increased apoptosis and reduced developmental competence, with negative effects that may persist for several weeks after a heat event (Di Francesco et al., 2012). Moreover, thermoregulatory redistribution of blood flow from the viscera to peripheral tissues reduces uterine perfusion, while concurrent oxidative stress impairs *corpus luteum* (CL) development and progesterone secretion, both of which are critical for early embryo survival. Therefore, THI values exceeding 74 are associated with increased late embryonic mortality and marked declines in fertility. In synchronized buffalo herds, pregnancy rates have been shown to decrease from over 50% in the cold season to below 30% during summer months (Campanile et al., 2005; Neglia et al., 2008). Collectively, these findings highlight heat stress as a major limiting factor for both productive and reproductive efficiency in intensive buffalo dairy systems, reinforcing the need for targeted mitigation strategies.

1.4. Health and Welfare of Buffaloes Species

Buffalo health is central to the productivity and sustainability of dairy and meat production systems. The paradigm of modern livestock farming has shifted significantly towards a holistic approach where animal welfare is not merely considered an ethical obligation but a fundamental aspect for productivity and economic sustainability. In this context, scientific evidence increasingly demonstrates that high welfare standards are intrinsically linked to physiological homeostasis, immune competence, and reproductive efficiency. As noted by De Rosa et al. (2009), the assessment of welfare in buffaloes requires a multidimensional approach that goes beyond the simple absence of disease to include the ability to express natural behaviors and cope with environmental stressors. When welfare is compromised, the resulting stress response triggers the hypothalamic-pituitary-adrenal axis, leading to elevated cortisol levels which can impair immune function and suppress mechanism important for reproduction (Campanile et al., 2010). Consequently, the economic viability of buffalo farming is dependent on maintaining a delicate balance between health management, environmental comfort, and behavioral needs. Recent studies have highlighted that alterations in the metabolic profile, such as elevated non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB), are closely correlated with oxidative stress and impaired immune competence (Lisuzzo et al., 2024).

Researchers have validated specific protocols for buffaloes that integrate physiological parameters with behavioral observations. For instance, the provision of wallowing areas or adequate cooling systems is essential for thermoregulation in this species (Napolitano et al., 2023). Behavioral indicators, such as lying time, social interactions, and human-animal relationship scores, provide immediate feedback on the quality of stockmanship and housing design (De Rosa et al., 2009). Moreover, physical inspections focusing on body condition score (BCS), integument alterations, and lameness offer retrospective evidence of long-term nutritional and environmental management. Recent studies indicate that hard, abrasive concrete surfaces contribute to the prevalence of lameness; therefore, the implementation of appropriate bedding management is increasingly investigated to improve lying comfort and reduce hoof pathologies (Trapanese et al., 2024). The design of housing facilities is a critical determinant of welfare for the Italian Mediterranean buffalo, acting as the interface between the animal's inherent ethological needs and production goals. The deprivation of adequate movement space has been correlated with increased aggressive interactions and elevated biomarkers of stress (Napolitano et al., 2004; Tripaldi et al., 2004). Conversely, ensuring sufficient space allowance, particularly in lying areas and feeding alleys, facilitates natural behaviors such as rumination and socialization, which are now considered non-invasive indicators of positive welfare status (Trapanese et al., 2024).

Consequently, to synergize with improved welfare standards, control of infectious and parasitic diseases is paramount for maintaining longevity and preventing reproductive failure. For instance, in the Southern of Italy, eradication programs for brucellosis (*Brucella abortus*) and tuberculosis (*Mycobacterium tuberculosis*) have been rigorously implemented due to their zoonotic potential and severe economic impact (Ciuca et al., 2020). These chronic diseases are frequently associated with late-term abortion, infertility, and compulsory culling, which disrupts the genetic progress of the herd. Similarly, viral infections such as *Bubaline Herpesvirus 1* (BuHV-1) have been identified as significant contributors to reproductive inefficiency, manifesting in reduced conception rates and increased embryonic mortality (Ferrara et al., 2024).

1.5. Buffalo Production Worldwide

The water buffalo (*B. bubalis*) represents a fundamental component of the global livestock sector, playing a crucial role in the livelihoods of millions of rural households across Asia, South America, and Southern Europe. According to recent estimates, the global buffalo population exceeds 208 million heads, distributed across 77 countries. However, this distribution is highly asymmetrical, with nearly 90% of the population located in Asia, particularly in India, Pakistan and China. In these regions, buffaloes act as a multi-functional asset, providing milk, meat, draught power, and manure, thereby offering resilience to smallholder systems in marginal environments, where other livestock species may struggle to thrive (Minervino et al., 2020). Beyond its agricultural output, buffalo farming reveals distinct gendered dimensions that are critical for socio-economic equity. In many rural economies, women are the primary caretakers of dairy buffaloes, managing daily tasks such as milking and feeding, while men typically handle marketing and herd management. Empirical studies indicate that income derived from buffalo dairying is often controlled by women, who reinvest it into household necessities, including education and healthcare (Khan and Iqbal, 2009).

1.6. India and Pakistan Buffalo Production

India retains most of buffalo population worldwide, with over 110 million buffaloes, and contributes to approximately 55% of the national milk supply. Whereas Pakistan retains the second most important population of buffaloes, relying on the *Nili-Ravi* and *Kundi* breeds to provide over 70% of the country's milk. Both sectors are characterized by a large genetic diversity (e.g., *Murrah*, *Jaffarabadi* breeds) and a dual focus on dairy and meat production. India has built a major export industry around buffalo meat (carabeef), supplying large volumes to countries in the Middle East and South of Asia. For instance, in 2022 India exported 1.44 million tons of carabeef (FAS 2023), and in 2023–24 it exported over 1.29 million tons of buffalo meat destined to Malaysia, Vietnam, Egypt and Iraq (APEDA 2024). Significant proportion of buffalo milk is consumed as raw milk, although there might be some differences according to the considered geographical area and consumer preferences. For instance, in Northern India (e.g., Punjab, Haryana) buffalo milk is historically preferred over cow milk for direct consumption due to its higher total solids and fat content (Minervino et al., 2020). Moreover, the unique composition of buffalo milk, specifically its high fat content (7–8%), large fat globule size, makes it technologically superior to produce specific traditional products. As an example, the *ghee* (Clarified

Butter), a byproduct of buffalo dairy industry, is the most important fat source in the Indian diet. Indeed, buffalo milk is used for *ghee* production because it can lead to a product with a higher melting point and a granular texture, characteristics which are highly valued by the consumers (Mor et al., 2022). In addition, the production of *Khoa* (desiccated milk) usually involves buffalo milk as it offers a favorable soft, granular consistency compared to the drier texture derived from cow milk (Pal et al., 2011). Whereas in cheese production, the elevated casein and calcium levels of buffalo milk facilitate the creation of *Paneer* (Acid-Coagulated Cheese) with a firm, spongy structure that retains integrity during cooking (Kumar et al., 2014).

1.7. Origins of the species and its introduction in Italy

The water buffalo (*B. bubalis*) belongs to a Eurasian lineage whose fossil and subfossil can be referred to the *Pleistocene* (Koenigswald et al., 2019; Vislobokova et al., 2021). Climate oscillations during the late *Pleistocene* and early *Holocene* likely fragmented suitable habitats, contracting ancestral areas toward warmer, humid zones of Asia (Zhang et al., 2020). Therefore, buffalo populations expanded from Asia and were later mobilized along early exchange corridors into Mesopotamia and the Nile valley, with sporadic archaeological signals in the Balkans and the Levant (Wordsworth et al., 2021). However, interpretative caution is warranted regarding ancient records, as morphological ambiguity, taphonomy distortion, and obsolete nomenclature often obscure the distinction between *Bubalus* species and other large bovids (Koenigswald et al., 2019; Vislobokova et al., 2021). Domestication, generally placed in the third millennium BCE in the Indus Valley and slightly later in adjacent cultural spheres (Zhang et al., 2020). As previously introduced, early genetic, morphometric, and historical evidence pointed to two domestication pathways that today correspond to distinct cytotypes and husbandry ecologies, namely the river buffalo and the swamp buffalo (Mcgregor, 1939; Zhang et al., 2020; Iannuzzi et al., 2021).

How and when buffaloes reached the Italian peninsula remains a debated question. Renaissance naturalists such as Ulisse Aldrovandi (1642,) in his *Quadrupedum omnium bisulcorum historia*, already noted the confusion surrounding *B. Bubalis* in classical sources, where the word could denote wild cattle broadly. Later, the English misnomer buffalo to identify the American bison further increase the presence of popular translations. Three non-exclusive hypotheses were generated. The first one is that buffaloes are an autochthonous relict species. Indeed, *Quaternary* fossils from Lazio region and the island of Pianosa have been interpreted as evidence for a long European presence of buffalo species. A biochemical study on fibrinopeptides (A/B) in twentieth century on Italian Mediterranean buffaloes suggested distinct primary structures relative to Indian samples (Maymone, 1945). While intriguing, these molecular differences are hard to interpret as *post*-domestication founder effects, drift, and selection can produce sequence divergence without implying independent domestication in Italy (Zicarelli et al., 1977). The second hypothesis concerns the Lombard introduction of buffaloes. Narratives tied to the Lombard population migrations may explain the arrival of buffaloes described in Paolo Diacono's *Historia Longobardorum* sometimes confused with the origins of rustic taurine breeds (e.g., Podolica, Maremmana). Yet the *Leges Langobardorum* (Rothari's Edict) omits buffalo from detailed schedules of damage in their regulations, an omission that some legal historians take as indirect evidence that the species was unfamiliar to lawmakers (Cantalupo, 1990). The third hypothesis consists in the Arab–Norman introduction. Given the documented presence of buffalo in Egypt and the centrality of maritime circuits in the early second millennium, an introduction from North Africa through Sicily and Campania regions during the Arab or later Norman periods may be plausible (Zicarelli et al., 1977). The earliest unambiguous attestations in Italy cluster between the twelfth and thirteenth centuries, after which references become more frequent and practical, pointing to traction, dairy use, and integration into local economies (Wordsworth et al., 2021).

In any case, regardless of the precise circumstances of its introduction, buffalo husbandry took firm root in the lowlands of Campania and neighboring regions, where the combination of heavy soils, seasonal waterlogging, and malaria-prone wetlands discouraged the expansion of specialized bovine breeding. In Bourbon and *pre*-Bourbon periods, buffalo herds were routinely employed for hydraulic labor, dragging nets, hauling loads across fords, and, crucially, treading and disturbing water vegetation to maintain drainage capacity (Zicarelli and Campanile, 2001). Their by-products fed local industries: hides (thick, low-stretched) for harness and military leather, horns for cutlery handles and ornaments and milk for fresh and stretched-curd cheeses. Italian toponyms (e.g., *bufalara, masserie bufaline*) and estate records preserve this agro-hydraulic imprint. Accounts by travelers, including Goethe's 1786 description of the *Pæstum* plain, corroborate the visual dominance of buffalo in these liminal agrarian settings (Cantalupo, 1990).

At the end of the twentieth century, national estimates indicated several thousand buffaloes reared in Campania region, with a numerical increase after World War II under the combined pressures of accelerated industrialization, urban labor pull, and the *Pontine Marshes*. Unlike segments of the bovine sector that pursued rapid breed substitution, buffaloes persisted in the Southern regions of Italy where the demand for fresh cheese (Mozzarella) warranted continued investment. Therefore, the introduction of mechanized milking, the establishment of the national buffalo herd book gradually shifted the producing system from marginal toward highly specialized dairy production. The contemporary river-type population in Italy, thus, represents the confluence of ancient biological plasticity and modern institutional supports (Zicarelli, 2021).

1.8. Buffalo Production System in Italy

According to the most recent census data provided by the National Zootechnical Database (BDN), the Italian Mediterranean buffalo population reached approximately 437,500 heads in 2025, marking a consistent demographic expansion compared to the previous decade. The geographical distribution remains strongly polarized, with most of the population located in the Southern regions of Italy as previously indicated. Campania region represents the epicenter of buffalo dairy industry, hosting over 70% of the national herd, mostly in the provinces of Caserta (190,000 heads) and Salerno (115,000 heads), followed by Lazio (approx. 18-20%) and Puglia regions. This localized density reflects the rigid boundaries of the Mozzarella PDO cheese production area (BDN, 2024; IZS, 2025). Indeed, most of the buffalo milk is processed into the Mozzarella di Bufala Campana supply chain, which was awarded the Protected Designation of Origin (PDO) status in 1996 (Commission Regulation EC No 1107/96) and represents the most important food-brand in Central and Southern Italian agribusiness. In 2024, Mozzarella sector involved roughly 1,600 certified farms, delivering a total output of 55,718 tons of certified product (+0.23% vs. 2023). In economic terms, this volume generates an estimated turnover of approximately €750 million for producers, rising to over €1.20 billion once sold to consumers (CTMBC, 2024). Moreover, the recent market dynamics indicate a strong export orientation for Mozzarella: while the domestic market absorbs about 60% of production, exports have increased up to 40%, with France, Germany, and the USA representing the primary international importers.

The economic sustainability of this system is largely underpinned by the significant market value of raw buffalo milk. While standard bovine milk is typically traded between €0.52–0.58/L, buffalo milk price can fluctuate between 1.00 and 2.00 €/kg depending on the seasonal demand in Italy (ISMEA, 2024; CLAL, 2025). This high profitability per liter has been a key driver for the expansion of buffalo farming beyond its historical boundaries. Indeed, buffalo breeding has been introduced also in Central and Northern regions of Italy, where numerous bovine dairy facilities have been converted into buffalo operations to capitalize on this price differential, during the milk quota crisis (Tiezzi et al. 2009).

1.9. Comparison of Productive and Reproductive Performance Between Dairy Buffaloes and Cattle in the Italian context

In modern dairy systems, buffaloes represent a distinct biological model that requires management strategies substantially different from those applied to dairy cattle, particularly with respect to nutrition and reproduction (Minervino et al., 2020). These differences arise from species-specific physiological, anatomical, and behavioral traits that shape productive performance and management responses.

A direct comparison with specialized dairy cattle breeds highlights the divergent productive potential of the Italian Mediterranean buffalo. While high-genetic-merit Holstein cows routinely exceed 10,000 kg of milk per lactation, phenotypic analyses of the Italian Mediterranean buffalo report an average yield of approximately 2,350 ± 580 kg over a standard 270-day lactation, with elite animals reaching peak production in the third parity order (Costa et al., 2020; Matera et al., 2023). Daily milk yield generally ranges from 7 to 12 kg in buffaloes. Notably, milk yield per lactation has increased markedly over the past two decades (ANASB, 2025), reflecting a production trajectory that differs from that of dairy cattle, which have largely approached physiological plateaus for milk yield. In contrast, buffalo populations still exhibit substantial additive genetic variance, indicating considerable scope for further genetic improvement (Cesarani et al., 2021).

A defining physiological feature of the Italian Mediterranean buffalo is the internal organization of the mammary gland. Unlike dairy cattle, in which up to 20% of milk is stored in the cisternal compartment and is immediately available for removal, buffaloes store approximately 90–95% of milk in the *alveoli*, with a minimal cisternal fraction (Thomas et al., 2004; Boselli et al., 2004; Neglia et al., 2008). Therefore, milk ejection in buffaloes is highly dependent on neuroendocrine oxytocin release, resulting in longer milk let-down times and a higher incidence of bimodal milk flow patterns when *pre*-stimulation is inadequate. Despite growing research aimed at optimizing milking management according to buffalo-specific morphology (Pierro et al., 2025), milking equipment and settings are still frequently adapted from bovine models (Bava et al., 2007). However, the buffalo mammary gland displays a greater intrinsic resistance to mastitis than that of dairy cattle, due to a combination of anatomical and immunological factors, including a thicker teat sphincter, higher keratin and melanin content, and a richer presence of antibacterial peptides and immune cells in milk (De Araujo and Gheller, 2005). Nevertheless, the intensification of buffalo farming systems has led to an increased prevalence of subclinical mastitis (Costa et al., 2020), which represents a sensitive indicator of both hygiene standards and animal welfare (Moroni et al., 2006). Mastitis, most caused by *Staphylococcus aureus* and environmental *streptococci*, induces marked alterations in milk composition, such as reduced lactose and casein content and increased somatic cell count, with direct consequences for cheese yield and quality (Cerón-Muñoz et al., 2002; Tripaldi et al., 2010).

From a technological perspective, buffalo milk exhibits a markedly superior compositional profile compared with bovine milk, with average fat and protein contents ranging from 7.72–8.10% and 4.65–4.70%, respectively (Costa et al., 2020). Buffalo milk is characterized by a distinct micellar structure, with almost fully micellized caseins, larger micelles, and higher concentrations of colloidal calcium and phosphate, which underpin its excellent coagulation properties (Zicarelli, 2004; Ahmad et al., 2009). Approximately 4 kg of buffalo milk is required to produce 1 kg of mozzarella, compared with about 8 kg of bovine milk. In addition, buffalo milk shows shorter rennet coagulation times and greater curd firmness, leading to improved syneresis dynamics and the characteristic elastic and chewy texture of mozzarella cheese (Radovanovic et al., 2021; Fenga et al., 2025).

Reproductive physiology further distinguishes the Italian Mediterranean buffalo from specialized dairy cattle breeds. Intrinsic anatomical constraints affect advanced reproductive technologies in buffaloes. Histomorphology studies show that the buffalo ovary has a markedly smaller primordial and antral follicle reserve than that of dairy cattle, with follicle populations estimated to be 30–50% lower (Gasparrini, 2002). Moreover, while Holstein heifers typically reach puberty between 8 and 12 months of age, buffaloes are late-maturing and attain sexual maturity between 15 and 24 months, resulting in an age at first calving (AFC) commonly exceeding 32–38 months. Extensive experimental and longitudinal studies indicate that this delay is largely driven by suboptimal *pre*-pubertal nutrition and management rather than intrinsic genetic limitations (Campanile et al., 2001; Barile, 2005; Sabia et al., 2014; D'Occhio et al., 2020). Puberty is generally triggered when body weight reaches approximately 360–380 kg (Campanile et al., 2009). At temperate latitudes, the Italian Mediterranean buffalo is a short-day seasonal breeder. As day length decreases from late summer to autumn, ovarian cyclicity intensifies and reproductive efficiency improves, reflecting an evolutionary adaptation to monsoon climates in which calving at the end of the rainy season maximized offspring survival (Zicarelli, 1997; Minervino et al., 2022). This seasonality is mediated by melatonin-driven modulation of gonadotropin-releasing hormone secretion (Terzano et al., 2012). Consequently, herds relying on natural mating tend to concentrate calvings between late summer and winter, generating a mismatch between milk supply and market demand. To overcome this limitation, Italian producers have widely adopted out-of-breeding season mating strategies, often combined with estrus synchronization and fixed-time artificial insemination, to align calving and milk production with market requirements (Zicarelli et al., 1997; Carvalho et al., 2016; Napolitano et al., 2023).

1.10. Genetic Selection and Improvement Programs in Italy

The progressive increase in productivity observed in recent decades reflects a synergistic interaction between genetic improvement and advances in herd management. From a genetic perspective, structured breeding programs implemented by the Italian Buffalo Breeder Association (ANASB) have effectively exploited additive genetic variance for milk yield and protein content, while also improving teat morphology to facilitate the widespread adoption of automatic milking systems. In parallel, improvements in nutritional and environmental management have enhanced the expression of the species' productive potential (Matera et al., 2025). For example, the introduction of total mixed ration feeding systems has led to measurable gains in feed efficiency and milk yield in buffalo herds (Evangelista et al., 2024).

In 2024, ANASB recorded 121,342 selected animals distributed across 1,024 farms. Genetic progress in buffalo has historically been slower than in dairy cattle, largely due to longer generation intervals and the limited adoption of artificial insemination in some production contexts. Early selection strategies relied primarily on phenotypic milk yield; however, the increasing intensification of the sector has required a more comprehensive breeding approach that integrates productivity with functional efficiency and longevity. In response, ANASB developed a composite selection index, the Italian Mediterranean Buffalo Index (IBMI), designed to balance production and functional traits. The IBMI assigns major weight to milk production, with particular emphasis on protein percentage, while also incorporating linear udder morphological scores. These focus primarily on udder depth and attachment, teat placement, and feet and legs, traits that are critical for animal longevity and adaptability to mechanical milking systems, now standard in Italian buffalo farms.

Moreover, to support on-farm selection decisions, ANASB provides breeders with computerized breeding plans based on estimated breeding values (EBVs). More recently, the integration of high-density SNP genotyping platforms, such as the 90K Axiom buffalo array, has enabled the development of a genomic reference population and the estimation of genomic breeding values (GEBVs). This approach allows selection decisions to be made on young bulls shortly after birth, substantially reducing the generation interval and increasing the efficiency of genetic gain. Genome-wide analyses have identified selection signatures associated with key traits, including milk yield (CSN2), reproductive performance (GKAP1), and coat pigmentation (MC1R) (Si et al., 2024). In this context, the SNPRBb database represents a major resource, cataloguing SNPs linked to economically relevant traits such as milk production, feed efficiency, and postpartum fertility, thereby enabling large-scale implementation of genomic selection in buffalo breeding (Mishra et al., 2021). In parallel, genetic markers related to health and reproduction are increasingly being explored. For instance, the Nramp1BB genotype has been associated with increased resistance to Brucella abortus, offering the possibility of reducing *brucellosis* prevalence through targeted selection strategies (Borriello et al., 2006). Collectively, these developments indicate that the integration of genomic tools with traditional breeding programs is reshaping buffalo genetic improvement, with clear implications for productivity, health, and long-term sustainability.

1.10.1. Advanced Reproductive Technologies in Italian Mediterranean Buffalo: From Hormonal Synchronization to In Vitro Embryo Production

Although artificial insemination (AI) is routinely and efficiently applied in dairy cattle, the reproductive physiology of the Italian Mediterranean buffalo has required the development of species-specific fixed-time AI (FTAI) protocols. Conventional bovine-based synchronization schemes, such as ov-synch, show inconsistent efficacy in buffaloes during the out-of-breeding season. This limitation arises because ov-synch assumes the presence of a functional ovarian environment, including active follicular waves and a responsive *corpus luteum* (CL). During the long-day period (spring–summer), many buffaloes exhibit reduced ovarian cyclicity or anestrus, impaired follicular dynamics, and absence of a functional CL, resulting in poor responsiveness to GnRH and frequent synchronization failure (Neglia et al., 2003; De Rensis and López-Gatius, 2007). To overcome these constraints, progesterone-based FTAI protocols using intravaginal devices have become the reference strategy in buffalo reproduction. Exogenous progesterone mimics the luteal phase, suppresses follicular dominance, and re-sensitizes the hypothalamic–pituitary axis, thereby promoting the emergence of a synchronized follicular wave after device removal (Campanile et al., 2010). During anestrous and transitional periods, these protocols are typically supplemented with equine chorionic gonadotropin (eCG), which stimulates final follicular growth, increases *pre*-ovulatory follicle diameter, and enhances ovulation rate, traits consistently associated with improved conception outcomes (Baruselli et al., 2020). Importantly, progesterone-based regimens also ensure an adequate *post*-ovulatory rise in progesterone, reducing late embryonic mortality, which in buffaloes can exceed 40% and is often linked to luteal insufficiency (Campanile et al., 2016).

In intensive production systems, reproductive efficiency is further improved through early resynchronization strategies. The application of color doppler ultrasonography allows functional assessment of CL vascularization and blood perfusion as early as 20–22 days post-AI, enabling discrimination between functional CL compatible with pregnancy and regressing CL in non-pregnant animals (Esposito et al., 2020). This early diagnosis permits rapid re-enrolment of non-pregnant buffaloes into synchronization protocols, reducing days open by approximately 10–15 days compared with conventional pregnancy diagnosis and significantly improving herd reproductive efficiency.

Parallel to advances in hormonal control, the use of sex-sorted semen in buffaloes has revealed species-specific constraints. Compared with cattle, buffalo spermatozoa exhibit a flatter head morphology and a smaller DNA content difference between X- and Y-bearing cells, making flow cytometric sorting technically more demanding and increasing cellular stress during the process (Andrei et al., 2024). As a result, the use of sexed semen in conventional AI has often been associated with reduced conception rates relative to unsorted semen. These limitations have driven a strategic shift toward integrating sexed semen with ovum pick-up (OPU) and in vitro fertilization (IVF) technologies, where sperm longevity and motility constraints are less penalizing than in vivo.

Ovum pick-up enables repeated recovery of oocytes by transvaginal ultrasound-guided aspiration, regardless of the reproductive status of the donor, and allows embryo production to continue during the non-breeding season. Nevertheless, seasonal variation in oocyte competence has been documented, with oocytes collected during short-day periods often exhibiting superior developmental potential (Di Francesco et al., 2012). Within IVF systems, fertilization can be achieved using markedly lower sperm concentrations than those required for AI, improving the efficiency and economic sustainability of sex-sorted semen use in buffalo breeding programs. Moreover, optimization of fertilization media, particularly through the inclusion of antioxidants, has been shown to mitigate oxidative stress induced by the sorting process, resulting in blastocyst yields and quality comparable to those obtained with conventional semen (Liang et al., 2008; Mishra et al., 2024). Consequently, for high-genetic-merit donors, OPU–IVF is increasingly considered a strategic alternative to FTAI with sexed semen, enabling targeted production of female embryos and accelerating genetic gain (Baruselli, 2018).

Despite these advances, *in vitro* embryo production in buffaloes remains biologically challenging due to intrinsic oocyte characteristics. Buffalo oocytes are characterized by high cytoplasmic lipid content and increased sensitivity to metabolic and oxidative stress, which negatively affects developmental competence and cryotolerance under standard culture conditions (Mondadori et al., 2010; Xu et al., 2019; Kosior et al., 2022). To address these limitations, culture systems have been progressively refined through the inclusion of compounds that support redox balance and cytoplasmic maturation, such as cysteamine and polyphenols (Gasparrini et al., 2006; Salzano et al., 2014). Under optimized laboratory conditions, blastocyst development rates of approximately 40–50% of cleaved oocytes have been consistently achieved in high-performing laboratories.

Finally, embryo cryopreservation techniques have evolved substantially with the adoption of vitrification-based methods, such as Cryotop, which minimizes chilling injury associated with the high lipid content of buffalo embryos. Compared with traditional slow-freezing, vitrification has markedly improved post-thaw survival and hatching rates, enabling the establishment of frozen embryo banks for the dissemination and conservation of elite buffalo genetics (Attanasio et al., 2010; Boccia et al., 2013).

1.11. Reproductive Physiology and Genetic Improvement in Italian Mediterranean Buffalo

The reproductive physiology of water buffalo (*B. bubalis*) has long been recognized as one of the major limiting factors for the productivity of this species. Unlike cattle, where reproductive efficiency has been extensively optimized through decades of breeding programs and advanced reproductive technologies, buffaloes is characterized by lower fertility, delayed puberty, and longer calving intervals as previously introduced. These features, when combined with their relatively poor estrus expression and marked reproductive seasonality, can significantly reduce their reproductive efficiency.

1.11.1. Female Reproductive Physiology and Major Inefficiency Factors in Italian Buffaloes

As previously introduced, female buffaloes generally reach puberty later than dairy cattle, with substantial variability driven by breed, nutritional level, and management conditions. This delayed sexual maturation has important implications for breeding programs, as it prolongs the generation interval and slows genetic progress. A clear contrast emerges between production systems: river-type buffalo heifers reared under intensive, high-energy feeding regimes, typical of the Italian dairy buffalo sector, often attain puberty between 18 and 24 months of age, whereas swamp-type buffaloes and river-type animals managed in extensive tropical systems, where forage availability and quality fluctuate seasonally, may not reach puberty until 30–36 months of age or later. The onset of puberty reflects the functional maturation of the hypothalamic–pituitary–gonadal axis, marked by the establishment of pulsatile GnRH secretion and the initiation of regular ovarian activity.

In Italian Mediterranean buffaloes, the estrous cycle generally lasts 21–24 days, although marked seasonal variability is observed. Ovarian activity is characterized by follicular growth occurring in discrete waves, with most buffaloes exhibiting a three-wave follicular pattern (Neglia et al., 2003). Within this physiological framework, effective reproductive management is challenged by the weak expression of estrus behavior. Sexual receptivity is brief, typically lasting only 6–18 hours, and behavioral signs are often subtle. Silent estrus is particularly frequent during transition periods and under heat stress, while visual indicators such as vulvar swelling may be obscured by the pigmented skin of the species, further complicating heat detection (Baruselli et al., 2020). Against this background, the timing of puberty attainment and the resulting AFC emerge as central control points for both biological efficiency and farm sustainability. Optimizing AFC represents a strategic lever to mitigate inherent reproductive constraints of the species and to improve long-term herd performance. Rather than a purely zootechnical target, AFC optimization affects multiple dimensions of farm operation, generating cascading effects on productivity, animal welfare, and economic viability.

From an economic standpoint, the heifer rearing phase constitutes a prolonged period of investment without direct revenue. In dairy ruminants, delays in AFC are well known to substantially increase rearing costs due to extended expenditures on feed, housing, and labor. Estimates in cattle indicate additional costs of approximately €1.50–3.00 per day of delayed calving, a burden that similarly applies to buffalo systems (Mohd Nor et al., 2013; Boulton et al., 2017; Overton and Dhuyvetter, 2020).

Reducing AFC lowers the number of replacement heifers required to maintain herd size, thereby decreasing stocking density. This reduction has direct welfare implications, as high stocking density is associated with reduced lying time, increased competition for resources, elevated stress, and higher disease transmission risk. Conversely, lower stocking density improves comfort, social stability, and ease of individual monitoring, all of which support welfare and health (Fregonesi et al., 2007; Huzzey et al., 2012; Tucker et al., 2021).

Physiologically, optimizing AFC is also essential to ensure adequate somatic development at first calving. Achieving appropriate body weight and pelvic growth reduces the risk of dystocia, which is more likely in underdeveloped heifers or in over-conditioned animals calving at excessively advanced ages (Purohit et al., 2011; Sary et al., 2022). Lower dystocia incidence translates into reduced veterinary interventions, decreased pharmaceutical use, and lower maternal morbidity and mortality (Purohit et al., 2012). In addition, the benefits of optimal AFC extend beyond the dam to the next generation. Heifers calving at appropriate physiological maturity tend to produce colostrum with higher immunoglobulin G concentrations (Lotito et al., 2023). Calves born from uncomplicated parturitions are more vigorous and exhibit a stronger suckling reflex, improving colostrum intake and passive immune transfer (Barrier et al., 2012). Thus, AFC optimization contributes to improved neonatal survival and lays the foundation for the productive life of replacement animals.

Beyond heifer management, reproductive inefficiency in adult buffaloes is multifactorial, arising from interactions among physiological, environmental, and pathological factors. Embryonic mortality represents the major source of reproductive loss, with reported rates ranging from 20% to 45% depending on season and herd conditions. Most losses occur within the first 45 days of gestation and are commonly linked to luteal insufficiency. Compared with cattle, buffaloes exhibit lower circulating progesterone concentrations, which may fall below the threshold required to sustain endometrial receptivity under heat stress or metabolic imbalance, leading to early embryo loss (Campanile et al., 2005). In contrast, pregnancy losses occurring later in gestation are more frequently associated with infectious agents. Intracellular pathogens such as *Chlamydiaceae* and *Neospora caninum* are recognized as major threats, with abortion rates reaching up to 36% in affected herds in southern Italy, significantly impacting calf crop size and involuntary culling rates (Greco et al., 2007; Greco et al., 2008).

1.11.2. Male Reproductive Physiology and Major Inefficiency Factors in Italian Buffaloes

Male reproduction in the Italian Mediterranean buffalo has historically been less investigated than female physiology, despite its crucial role in breeding programs based on artificial insemination. This gap is particularly relevant because buffalo semen displays biological characteristics that differ markedly from bovine semen, creating specific challenges for semen processing, cryopreservation, and field fertility. Seasonal variation in semen quality traits such as motility and morphology has been reported in buffalo bulls; however, under Mediterranean photoperiod conditions, consistent evidence of a marked seasonal decline in *libido*, testicular volume, or overall semen quality in the Italian Mediterranean breed is lacking. Nevertheless, reproductive performance generally peaks during autumn and winter, in line with the natural breeding season of females, with practical implications for semen collection management (Isnaini et al., 2009; Castro et al., 2017).

The main limitations to AI efficiency in buffaloes arise from semen composition. Buffalo seminal plasma is more viscous, sperm concentration per ejaculate is lower, and sperm membranes contain a higher proportion of polyunsaturated fatty acids, which increases susceptibility to oxidative damage during cryopreservation. Consequently, post-thaw sperm viability and motility are often lower than those achieved in cattle (Gasparrini et al., 2013). To mitigate these constraints, extender formulations enriched with antioxidants such as resveratrol or melatonin have been developed, significantly reducing DNA fragmentation and improving acrosomal integrity after thawing (Salzano et al., 2014). In parallel, Computer-Assisted Sperm Analysis has identified kinematic parameters, such as curvilinear velocity and linearity, as more reliable predictors of field fertility in buffaloes than conventional subjective motility scoring (Singh et al., 2017). At the testicular level, buffalo spermatogenesis also exhibits species-specific features. Ultrastructural studies have described distinctive Sertoli cell morphologies, suggesting specialized mechanisms that may support efficient sperm production during favorable reproductive periods (Rutland et al., 2017).

Overall, these characteristics highlight the need for buffalo-specific strategies in semen evaluation and preservation, rather than the direct application of cattle-derived protocols, to fully exploit male genetic potential in Italian Mediterranean buffalo breeding programs.

1.12. Nutrition–Reproduction Interactions in Italian Mediterranean Buffaloes

Nutritional management constitutes the primary environmental factor modulating reproductive performance in the Italian Mediterranean Buffalo. Inadequate energy or protein intake is the leading cause of reproductive inefficiencies, manifesting as delayed puberty in heifers, prolonged *post-partum* anestrus, and low conception rates in multiparous buffaloes. While buffaloes can efficiently use high-fiber, low-energy diets, such rations are insufficient to support the demands of rapid growth and high lactation yields. Reliance on poor-quality forage leads to a chronic energy deficit that suppresses the secretion of metabolic hormones, specifically insulin and insulin-like growth factor 1 (IGF-1), which is a critical signal for ovarian follicular growth and ovulation (Campanile et al., 2010). The onset of puberty in buffalo heifer is not merely a function of chronological age but is a physiological milestone determined by achieving a specific somatic development and metabolic status. Research indicates that the nutritional management of female calf during the *pre*-weaning and *post*-weaning phases exerts a long-term metabolic imprinting which may directly influences the timing of the first estrus and the quality of its lifetime fertility (Campanile et al., 2001).

In the Italian intensive system, the traditional practice of restricted milk feeding to encourage early starter intake must be carefully balanced. While early solid feed intake is necessary to stimulate the development of ruminal *papillae* and enzymatic activity, severe nutrient restriction before 3 months of age can lead to weaning issues. Studies suggest that calves achieving a weaning weight of approximately 90–100 kg (typically around 90 days), through a balanced intake of milk replacer and high-quality starter concentrate, exhibit a smoother transition to functioning ruminants, maintaining the growth curve required for early puberty (Napolitano et al., 2013; Mota-Rojas et al., 2024;).

After weaning the objective is to maintain a steady average daily gain of approximately 550-700 g/day (Terzano et al. 2012). This growth rate allows the heifer to reach 60–65% of her mature body weight (approx. 350–380 kg) by 18–20 months of age. Campanile et al. (2010) established that heifers fed with high-energy diets exhibit significantly higher plasma levels of IGF-1. This hormone acts synergistically with gonadotropins on the ovary, promoting the proliferation of granulosa cells and the maturation of the dominant follicle. Low IGF-1 levels, typical of low-energy diets, are a primary cause of ovarian inactivity and delayed puberty. As the heifer accumulates adipose tissue, circulating levels of leptin rise, signaling to the hypothalamus that somatic energy reserves are sufficient to support a pregnancy. Once a threshold concentration is reached, leptin permits the activation of the GnRH pulse generator, triggering the first fertile ovulation. Nutritional errors in this phase have irreversible consequences. Under-nutrition, heifers fed with poor-quality forage, fail to secrete sufficient IGF-1, leading to a delayed puberty (30–36 months). This dramatically increases rearing costs and delays the return on genetic investment. Moreover, over-nutrition and excessive energy intake lead to over-conditioning and insulin resistance, resulting in a profound alteration of metabolic homeostasis. High circulating insulin and NEFA concentrations negatively affect ovarian function and oocyte competence, impairing follicular development and early embryonic survival. High-energy diets have been shown to induce metabolic imbalance and to compromise ovarian activity in buffalo heifers, while elevated BCS is consistently associated with reduced reproductive performance (Neglia et al., 2008; De Rensis et al., 2008; Campanile et al., 2010).

In addition, the Italian Mediterranean Buffalo often experience negative energy balance *post*-calving, which is characterized by the mobilization of body fat reserves releases NEFAs and BHB. Previous studies have shown that low-energy diets and calculated negative energy provision alter metabolic and hormonal profiles and tend to reduce oocyte quality in Italian Mediterranean buffaloes

(Campanile et al., 2001; Campanile et al., 2010;). These findings, integrated with evidence on seasonal changes in follicular environment (Kosior et al., 2022), support the concept that metabolic stress can compromise oocyte competence and subsequent fertility in this species. An excess of degradable protein (high urea), often resulting from unbalanced total mixed rations, may alter the uterine pH, creating a hostile environment for the embryo. In addition, a recent study emphasizes that maintaining a BCS between 3.00 and 3.50 is optimal for minimizing the days open (Neglia et al., 2008). Moreover, deficiencies in phosphorus, selenium, and zinc are common in buffalo diets and have been linked to silent estrus, repeat breeding, and embryonic mortality. Vitamin A and E supplementation improves ovarian function and reduces *post-partum* anestrus duration. Tailored mineral mixes and fortified feed blocks are now being used in smallholder systems to counteract these deficiencies (Sarwar et al., 2009).

1.11.3. Economic Trade-offs of AFC: Global Perspectives and Knowledge Gaps

While the economic impact of AFC has been extensively modelled in livestock systems worldwide, a critical knowledge gap persists for the Italian Mediterranean buffalo population. In intensive dairy cattle systems, particularly in Holstein cows, the bio-economic framework is well established, with a clear consensus identifying an optimal AFC, typically between 22 and 24 months. This range is routinely adopted as a management target to minimize heifer rearing costs, which represent the second largest production expense after feeding lactating cows (Boulton et al., 2015). Within this context, the literature consistently shows that deviations from the optimal AFC lead to measurable economic losses, either through increased rearing investments associated with delayed calving or through reduced first-lactation performance when calving occurs too early (Wathes et al., 2008; Eastham et al., 2018). Empirical evidence from Italian Holstein herds further supports this paradigm, demonstrating that a reduction in AFC from 26 to 24 months results in an increase in net returns of approximately $41.5 per heifer (Pirlo et al., 2000), with economic efficiency largely driven by savings in rearing costs rather than by marginal gains in milk production. In contrast, comparable bio-economic and physiological evidence remains scarce for Mediterranean buffaloes, despite their growing relevance within the Italian dairy sector. Consequently, the lack of robust, parity-specific evaluations of AFC in Mediterranean buffaloes represents a major limitation for evidence-based herd management and economic optimization. For instance, significant economic insights have emerged from the Brazilian buffalo sector, where extensive production systems often coexist with varying management levels. In this context, recent genetic analyses on *Murrah* breed have demonstrated that incorporating AFC into selection indices can generate substantial economic gains, estimated between $102 and $147 per animal (Aspilcueta-Borquis et al., 2022). These gains are primarily driven by the favorable genetic correlation between earlier calving and functional longevity, suggesting that reducing the non-productive phase not only lowers immediate rearing investments but also supports a longer and more profitable productive life. Similarly, in other buffalo populations such as the *Murrah* and *Egyptian* breeds, recent studies have begun to quantify these trade-offs. Research indicates that reducing AFC in these breeds is generally correlated with improved lifetime profitability and functional longevity, if heifer growth targets are met (El-Awady et al., 2021; Rautela et al., 2024). Conversely, data on *Anatolian* buffaloes suggest that while delayed calving may maximize individual lactation yields, it fails to optimize net farm income due to the burden of prolonged non-productive periods (Yılmaz et al., 2025). However, these international models cannot be directly transposed to the Italian context. The profitability of Italian herds is inextricably linked to milk quality (protein and fat content for cheese yield) and specific seasonal production quotas (Rosati and Van Vleck, 2002; Otava et al., 2021).

To address this lack, the present thesis aims to provide the first extensive evaluation of AFC effect on productive and reproductive performances in the Italian Mediterranean buffalo population. The primary objective was to quantify the biological outcomes of several AFC thresholds, analyzing their impact on milk yield, composition, and reproductive efficiency in primiparous buffaloes. Concurrently, the thesis seeks to determine economic sustainability by performing a bio-economic analysis through the Farm Accountancy Data Network indicators (FADN, 2010), which was used to obtain the net return per lactation.

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2. Chapter 2

The role of age at first calving in shaping production and reproductive outcomes in Italian buffaloes

Calanni Macchio, A., M. Santinello, G. Bifulco, R. Matera, S. Biffani, M. Gómez-Carpio, G. Campanile, and G. Neglia. 2025. The role of age at first calving in shaping production and reproductive outcomes in Italian buffaloes. J. Dairy Sci. 108:7235–7247. https://doi.org/10.3168/jds.2025-26369.

Abstract

The AFC is a critical parameter in dairy herd management due to its impact on feeding costs, genetic progress, longevity, and thus farm profitability. With the growing global demand for buffalo milk products, recent advancements in reproductive practices and feeding techniques have contributed to a reduction in AFC in Italy. This study investigates the impact of AFC on the productive and reproductive performance of primiparous Italian Mediterranean buffaloes. Data was collected from 50,661 animals across 115 herds, covering 362,703 first lactation test-day records from 2013 to 2023. The study analyzed productive traits, including milk yield (kg/d), days to milk peak (DMP, d), energy-corrected milk (ECM; kg/d), fat and protein contents (%), and somatic cell score (SCS). Whereas reproductive traits, such as calving interval (CI, d) and days open (DO, d), were analyzed from 2013 to 2021. Repeated mixed linear models were used to assess the effects of AFC on the afore-mentioned traits, accounting for days in milk (DIM), calving year and season, and, when applicable, their interactions. Results highlighted a significant reduction in AFC (from 36 to 35 months), CI (from 457 to 447 d), and DO (from 147 to 136 d) over the studied period. Buffaloes with AFC > 42 months produced 0.52 kg/d more milk and 0.90 kg/d more ECM, with slightly higher fat content (+0.04%) compared to those with AFC ≤ 32 months. This trend was consistent throughout DIM categories. Somatic cell score was significantly lower in buffaloes with AFC between 32-35 months (2.78), whereas higher SCS was observed in those with AFC ≤ 32 months (2.81) or > 35 months (2.80). Buffaloes with AFC comprised between 34 and 37 months had significantly lower CI and DO (449-450 and 137-139 d, respectively) compared to those with both lower or greater AFC (454 and 143 d, respectively). Whereas DMP was not significantly affected by AFC. As expected, likely due to the out-of-breeding technique, the highest values for both CI and DO were observed in buffaloes calving the first time in winter and spring (on average 455 and 145 d, respectively), and lower values were recorded for those calving in autumn and summer (on average 449 and 138 d, respectively). Buffaloes with AFC ≤ 30 months that calved during winter exhibited significantly greater DO (> 150 d) compared to those calving with AFC > 42 months during the same period (140 d). This is likely due to the lower capacity to restore ovarian activity that may reduce fertility in younger buffaloes compared to older ones.
Conversely, buffaloes with AFC > 42 months that calved in summer tended to have extended DO (139 d) in respect to those with lower AFC (≤ 30 months) in the same period (135 d). This may be due to the interaction between the increased photoperiod and the reduced feed intake caused by summer heat stress, which may have an impact on older buffaloes' fertility. Therefore, AFC between 34 and 37 months represents the optimal window for Italian Mediterranean buffaloes, combining shorter CI and DO with sustained milk production. The reduction of AFC could significantly enhance farm profitability and may be achievable through the integration of breeding programs, better reproductive

technologies across seasons, and targeted nutritional strategies during the *pre*-weaning and *pre-partum* phases.

Key words: Days open, Fertility, Heifers, Reproduction management.

2.1. INTRODUCTION

Buffalo milk production has increased from 67 to 144 million tons over the last two decades, emerging as an important and growing market within the global dairy industry (FAOSTAT, 2024). Italy plays a key role in buffalo milk production in Europe, hosting approximately 3% of the world's buffalo population (FAOSTAT, 2024). Around 131,000 lactating buffaloes are monitored annually in Italy (Italian National Association of Buffalo Breeders, 2024), where most of the population is reared in the southern regions to produce mozzarella di Bufala Campana PDO (CLAL, 2023). From 2012 to 2021, mozzarella production increased by 40% (CLAL, 2023). This growth was driven by the rising market value of mozzarella as well as improved farming practices, such as optimized nutrition, reproductive strategies, and enhanced animal welfare, which in turn contributed to greater production efficiency (Neglia et al., 2023).

Among the key factors influencing buffalo and dairy production, heifer management is crucial to ensure long-term productivity at herd level, optimize resource use, and improve overall farm efficiency and profitability (Gómez-Carpio et al., 2024; Ferrari, 2024). Heifer rearing, accounting for 15–20% of total dairy farm costs (Boulton et al., 2015), constitutes a long-term investment, as animals generate returns only after first calving and the onset of lactation. In Italian buffaloes' population, fertility peaks in autumn and winter due to the reduced photoperiod (D'Occhio et al., 2020), while milk demand is higher in spring and summer due to increased need for mozzarella cheese production (Otava et al., 2021). Consequently, this mismatch has led to increased interest in reproductive management in buffaloes to improve fertility in late winter and spring (Baruselli et al., 2003; D'Occhio et al., 2020), leading to the development of the out-of-breeding season mating technique. This technique consists in the interruption of reproduction in some periods of the year to avoid milk production when is not desired (Costa et al., 2020a). However, this can lead to extended calving interval (CI) and AFC, highlighting the need to optimize reproductive strategies. Reducing AFC can help lower feeding costs, shorten the CI, accelerate genetic progress, and improve the overall reproductive efficiency of the herd (Zicarelli, 2007; El-Awady et al., 2021; Gómez-Carpio et al., 2023). Moreover, as in dairy cows, managing AFC can influence the retention of productive livestock in the herd, thereby affecting animal productive career (Strapáková et al., 2013; Boulton et al., 2017). Gómez-Carpio et al. (2024) reported that delaying the AFC significantly increased the risk of culling in Italian Mediterranean buffalo population. The same study also reported that a reduction in AFC could be associated with insufficient body condition score (BCS), which, in turn, might negatively affect *post-partum* reproductive and productive performance due to negative energy balance. Inadequate dietary energy intake, leading to either excessive or insufficient BCS, is associated with an increased number of inseminations per conception, prolonged calving to conception intervals, and extended calving intervals in younger heifers, ultimately impairing ovarian function and oocyte quality (Campanile et al., 2006; Hassan Ali and Abdel-Raheem, 2013).

Over the past 30 years, breeding programs and husbandry improvements in the Italian Mediterranean buffalo had successfully reduced the average AFC to 35–36 months (Gómez-Carpio et al., 2024). It is generally well known that the assessment of specific criteria, such as AFC, is a common approach to evaluate the efficiency and profitability of dairy farms (Lopes et al., 2009). However, lower AFC is also associated with potential trade-offs, including reduced milk production during the first lactation, a factor that also contributes to higher culling rates (Rostellato et al., 2021). Although interest in AFC as a management tool in dairy herds is increasing, its longitudinal impact on fertility, milk production and quality traits during the first lactation in buffaloes, particularly under commercial farming conditions, remains unexplored. Existing studies on buffaloes are often constrained by limited observation periods, further reducing the generalizability of their findings. Moreover, the interaction between AFC and seasonal reproductive dynamics is still poorly understood, hindering the development of targeted reproductive strategies throughout the year. Thus, the present study, based on ten years of data collection, aims to investigate the relationship between AFC and both milk production and reproductive performance in a large cohort of primiparous Italian Mediterranean buffaloes to identify the optimal AFC window.

2.2. MATERIALS AND METHODS

Ethical approval from an animal welfare and use committee was not required for this study, as data was obtained from routine animal recording practices conducted by the Italian National Association of Buffalo Breeders (ANASB).

2.2.1. Data and editing

Data included information on routine test-day records of Italian Mediterranean buffaloes from January 2013 to December 2023, including individual data on milk yield (kg/d), stage of lactation (DIM, d), birth and calving dates. Test-day records were taken once a month at fixed intervals within the same herd. Fat and protein contents (%) were estimated from milk spectra using buffalo-specific prediction models in the MilkoScan™ FT6000 (Foss Electric A/S, Hillerød, Denmark) at Italian Breeder's Associations (AIA) milk laboratory in Benevento (Italy). Whereas somatic cell count (SCC, cells/mL) were determined using Fossomatic™ (Foss Electric A/S, Hillerød, Denmark). Based on this information, energy-corrected milk (ECM, kg/d) was calculated using the buffalo-specific formula from Campanile et al. (2003):

$$ECM = \{[Fat\,(\frac{g}{kg}) - 40 + Protein\,(\frac{g}{kg}) - 31] \times 0.01155\} + 1) \times Milk\ yield\ \text{(kg)}.$$

Data was checked for outliers using the productive trait limits outlined in the phenotypic characterization by Costa et al. (2020a). Specifically, milk yield, ECM, fat, and protein content were set as missing values if outside the range of mean ± 3 SD and excluded from the analysis, while SCC was retained within 1,000–999,000 cells/mL and converted into a linear score (SCS) using the following formula:

$$SCS = 3 + log_2(SCC/100{,}000).$$

Only primiparous buffaloes from herds with continuous monitoring throughout the study period were included. Moreover, to increase the robustness of the subsequent analysis, only primiparous with at least 3 test-day records in the first lactation and a minimum of 5 monitored buffaloes per herd-test-day (HTD) were retained, resulting in 362,703 test-day records from 50,661 buffaloes in 115 farms, primarily reared in southern regions of Italy (90%). Descriptive statistics of productive traits after editing are summarized in Table 2.1.

Table 2.1. *Descriptive statistics of milk yield (kg/d), composition traits (%), energy corrected milk (ECM[2], kg/d), and somatic cell score (SCS) based on individual test-day records of primiparous buffaloes from 2013 to 2023 (n = 362,703).*

Traits	N.	Mean	SD	Median	IQR[1]
Milk (kg/d)	50630	8.87	3.18	8.70	4.30
Fat (%)	49114	8.06	1.76	8.05	2.23
Protein (%)	49151	4.72	0.43	4.70	0.56
ECM[2] (kg/d)	48812	14.4	4.69	14.3	6.17
SCS	48371	2.81	1.66	2.58	2.14

[1]*IQR: Inter quartile range.*

[2]*ECM = Energy corrected milk, calculated using the following formula adapted for buffaloes (Campanile et al., 2003): {[Fat (g/kg) – 40 + Protein (g/kg)– 31] × 0.01155} +1) × Milk yield (kg).*

Other reproductive traits were derived from previously recorded data: AFC (d) was calculated as the difference between the date of first calving and date of birth; CI (d) was calculated as the difference between the dates of the second and first calving; conception date was estimated assuming a standard gestation length of 310 d for Italian Mediterranean buffaloes; days open (DO, d) were calculated as the difference between the estimated conception date and the first calving date. Values of AFC and CI were excluded based on the following criteria: AFC <23 or >47 months, and CI <350 or

>800 days as specified by Gómez-Carpio et al. (2023). These thresholds were applied to remove any possible confounder factors since fertility traits are highly influenced by non-genetic factors such as out-of-breeding season technique (Gómez-Carpio et al., 2023). Values of DO falling outside the mean ± 1/2 SD were treated as missing, as they were calculated based on a standard gestation length (310 d) to estimate conception dates, rather than using actual conception dates which were not available. Moreover, since data collection ended in 2023, CI and DO records from animals that calved after 2022 were excluded from the analysis, as these values could be underestimated due to data truncation. Days to milk peak (DMP, d) were determined by identifying the test-day with the highest recorded milk yield value during the first lactation, including only buffaloes with at least one test-day record between 30 and 70 DIM. The DMP was calculated as the interval (d), between the first calving date and the determined peak milk yield test-day.

2.2.2. Statistical Analyses

Descriptive statistics for reproductive traits were calculated. Days in milk were categorized into 10 classes of 30 DIM each, except for the first and last categories which encompassed 5–30 DIM and >271 DIM, respectively. Each category accounted for approximately 12% of the observations. For the same purpose AFC has been converted into categories. The AFC was categorized into 8 categories: ≤30 months, >30 to ≤32 months, >32 to ≤34 months, >34 to ≤35 months, >35 to ≤37 months, >37 to ≤38 months, >38 to ≤42 months, and >42 months. These categories were designed to group animals into intervals that reflect both a uniform number of observations and meaningful biological thresholds for reproductive management. The effect of AFC on productive traits was investigated through a mixed linear model with repeated measurements implemented through GLIMMIX procedure in SAS 9.4 software (SAS Institute Inc., Cary, NC, USA):

$$y_{ijklmn} = \mu + AFC1_i + DIM1_j + \text{Season}_k + Year_l + (AFC1 \times DIM1)_{ij} + (AFC1 \times Season)_{ik} + (DIM1 \times Season)_{jk} + Animal\_ID_m + HTD_n + e_{ijklmn},$$

where, μ is the population mean for the evaluated traits: milk yield, fat and protein content, ECM and SCS; $AFC1_i$ is the fixed effect of the i^{th} category of buffalo AFC (i = from 1 to 8 categories); $DIM1_j$ is the fixed effect of the j^{th} category of DIM (j = from 1 to 10 categories); $Season_k$ is the fixed effect of the k^{th} calving season (k = Autumn: September, October, November; Winter: December, January, February; Spring: March, April, May; Summer: June, July, August); $Year_l$ is the fixed effect of the l^{th} calving year (l = from 2013 to 2022); $(AFC1 \times DIM1)_{ij}$ is the fixed interaction effect between AFC and

DIM categories; *(AFC1×Season)*$_{ik}$ is the fixed interaction effect between AFC categories and calving season; *(DIM1×Season)*$_{jk}$ is the fixed interaction effect between DIM categories and calving season; *Animal_ID*$_m$ is the random effect of the m^{th} Animal ID~ $N(0,\sigma^2{}_a)$, where $\sigma^2{}_a$ is the animal variance; *HTD*$_n$ is the random effect of the n^{th} Herd-Test-Day ~ $N(0,\sigma^2{}_a)$, where $\sigma^2{}_a$ is the HTD variance; and e_{ijklmn} is the random residual $\sim N(0,\sigma^2{}_e)$, where $\sigma^2{}_e$ is the error variance. Residual normality and homoscedasticity were assessed using diagnostic plots generated with the PROC GLIMMIX procedure. Multicollinearity among fixed effects was evaluated prior to model fitting, using the PROC GLMSELECT. All predictors showed variance inflation factors values below 2, indicating the substantial absence of collinearity.

A similar model was applied to reproductive traits, such as CI, DO and DMP excluding the effect DIM categories and their interactions. In this case, only the random effect of herd was included, as these variables were not measured repeatedly within each individual animal, but rather within individuals of the same herds. Additionally, because of the data skewness, a generalized linear mixed model with a gamma distribution and log link function was used for the analysis of CI and DO (Diana et al., 2020), implemented through the GLIMMIX procedure in SAS:

$$y_{ijkl} = \mu + AFC1_i + \text{Season}_j + Year_k + (AFC1 \times Season)_{ij} + Herd_l + e_{ijkl},$$

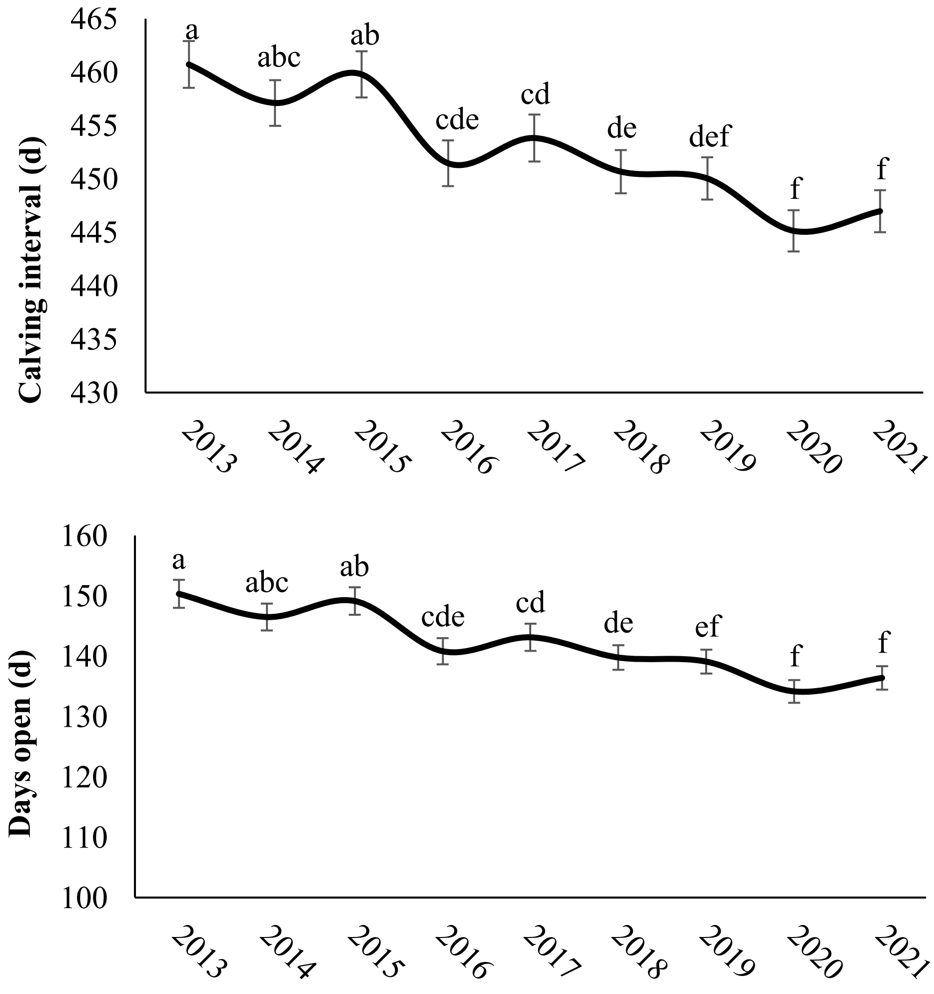
where, μ is the population mean for the evaluated traits: CI, DO; $AFC1_i$ is the fixed effect of the i^{th} ategory of buffalo AFC (i = from 1 to 8 categories); *Season*$_j$ is the fixed effect of the j^{th} calving season (j = Autumn: September, October, November; Winter: December, January, February; Spring: March, April, May; Summer: June, July, August); *Year*$_k$ is the fixed effect of the k^{th} calving year (k = from 2013 to 2021); *(AFC1×Season)*$_{ij}$ is the fixed interaction effect between AFC categories and calving season; *Herd*$_l$ is the random effect of the l^{th} Herd~ $N(0,\sigma^2{}_a)$, where $\sigma^2{}_a$ is the herd variance; and e_{ijklmn} is the random residual $\sim N(0,\sigma^2{}_e)$, where $\sigma^2{}_e$ is the error variance. In addition, DMP was analyzed using the same model incorporating the test-day records effect to adjust for the randomness of the sampling date. The goodness of fit of each model was evaluated lowering Akaike's Information Criterion and Bayesian Information Criterion. Results were presented as least squares means and associated pooled standard error, and Bonferroni *post-hoc* test ($P < 0.05$) has been conducted for multiple comparisons.

2.3. RESULTS AND DISCUSSIONS

2.3.1. Descriptive statistics of productive traits

Descriptive statistics and phenotypic annual trend of reproductive traits based on least squares means are summarized in Supplementary Figures 2.1 and 2.2.

Supplementary Figure 2.1. *Annual trend and descriptive statistics of calving interval (d) and days open (d) of primiparous buffaloes from 2013 to 2021.*

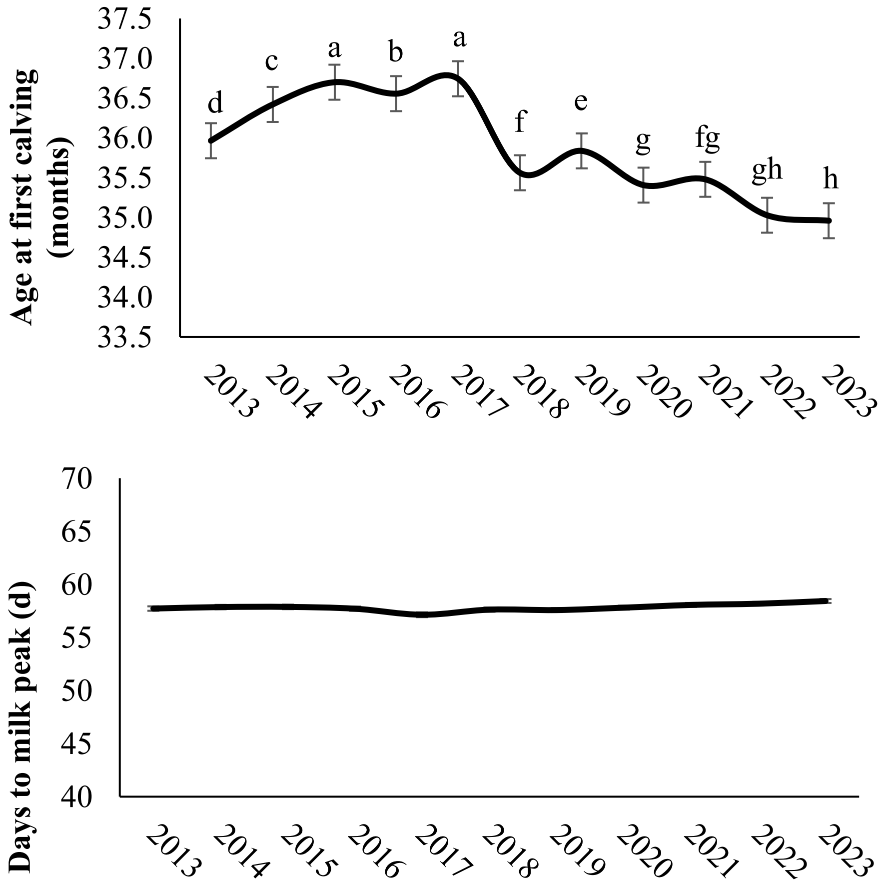


Reproductive traits	N.	Mean	SD	Minimum	Maximum	Median	IQR[1]
Calving interval (d)	29987	452	72.3	350	634	434	115
Days open (d)	29987	141	72.3	40	324	125	115

[1]*IQR = Inter quartile range.*

[a–f] *Different superscript letters indicate significant differences according to Bonferroni post-hoc multiple comparison adjustment (P < 0.05).*

Supplementary Figure 2.2. *Annual trend and descriptive statistics of age at first calving and days to milk peak[1] of primiparous buffaloes from 2013 to 2023.*



Reproductive traits	N.	Mean	SD	Minimum	Maximum	Median	IQR[2]
Age at first calving	50661	35.52	5.01	24.0	48.0	35.3	6.27
Days to peak (d)	31792	56.3	17.5	25.0	93.0	55.0	26.0

[1]Days to milk peak = number of days from calving to the highest milk yield during the first lactation, calculated as the difference between the date of milk peak and the date of first calving, based on individual test-day records;
[2]IQR = Inter quartile range.

Calving interval and DO denote moderate variability, averaging 452 ± 72 and 141 ± 72 d, respectively. Literature reported that reproductive traits can be affected by herd management (Abdalla, 2003; Gomez-Carpio et al., 2023) in relation to the out-of-breeding season mating technique. Heifers that reach puberty in autumn or winter may experience a delayed age at first conception due to the increased demand for buffalo milk at the onset of spring (Campanile et al., 2009), which may lead farmers to postpone breeding to the following spring to align calving with market needs and increase their variability. The results showed a significant decrease in both CI and DO between 2013 and 2021, with CI declining from 461 to 447 d and DO from 150 to 136 d (Supplementary Figure 2.1). These improvements can be attributed to the advancements in feeding strategies and welfare conditions implemented over time in Italian Mediterranean buffalo farms (Campanile et al., 2001). Moreover, the adoption of artificial insemination, as well as advancements in synchronization protocols (Presicce et al., 2022), have contributed to improved reproduction efficiency (Baruselli, 2018). Otava et al. (2021), in a study conducted on a single farm between 2015 and 2017, reported an average DO of 113 d for primiparous buffaloes managed with an estrus synchronization protocol. While their study applied artificial insemination continuously throughout the year, buffaloes in the present study may be influenced by the out-of-breeding season mating technique adopted on-farm. However, a gradual reduction in DO was achieved in our population, making our results comparable to those of Otava et al. (2021).

The average AFC was 35.5 months, showing a significant reduction from 36 months in 2013 to 35 months in 2023 (Supplementary Figure 2.2), in line with the findings of Gómez-Carpio et al. (2023) in the same population (35 months). Whereas DMP were stable and did not significantly vary across the years ($P > 0.05$; Supplementary Figure 2.2). The age of puberty in buffaloes, and thus AFC, may vary according to breed and latitude. For instance, the average AFC reported for Murrah breed buffaloes in India can range between 37 and 54 months (Rautela et al., 2024). In Italian Mediterranean buffalo, puberty is reported to occur between 17 and 19 months, with an average AFC of 35 months at a body weight (BW) ranging from 340 to 360 kg (Campanile et al., 2009; Gómez-Carpio et al., 2023). Advancements in herd management have led to a gradual reduction in AFC, decreasing by approximately one month every five years in Italy, as reported by Zicarelli (2016). Balanced and optimized nutritional management of heifers represents a key factor in this reduction, as it has been demonstrated to be crucial in reproductive management of both cows (Heinrichs et al., 2005) and buffaloes (Campanile et al., 2001). For example, in the study of Campanile et al. (2010) on Italian Mediterranean buffaloes, heifers fed a high-energy diet exhibited higher BCS compared to those receiving a low-energy diet (4.20 vs. 3.40, respectively). The same study reported that the oocytes produced by heifers fed with the low-energy diet were of lower quality. In line with these findings, Baruselli et al. (2001) reported that poor BCS is associated with higher incidence of anestrus in buffaloes, while Kumar et al. (2020) observed that low BCS was linked with reduced pregnancy rates. As observed in dairy cows, primiparous buffaloes with low BCS, and consequently lower BW at calving, combined with a relatively high calf weight, may face an increased inflammatory response and slower *post-partum* recovery (Mammi et al., 2021). Medium BCS has been linked to greater stayability beyond the third lactation in dairy cows (Bonaiuto et al., 2023), however excessive BCS can lead to calving difficulties. These findings suggest that applying appropriate nutritional strategies to ensure an adequate BCS during heifer rearing could positively impact the future reproductive performance and longevity of buffaloes (Barile, 2005; Sarwar et al., 2009).

2.3.2. Effect of AFC on productive performance

F-values and significance (*P-value*) of fixed effects and interactions influencing milk yield, ECM, fat and protein contents and SCS are presented in Supplementary Table 2.1.

Supplementary Table 2.1. *Selected models based on P-value and F-value explaining the observed variation in milk yield (kg/d), energy corrected milk (ECM[1], kg/d), composition traits (%), and somatic cell score (SCS) based on individual test-day records of primiparous buffaloes from 2013 to 2023 (n = 362,703).*

Effect	Milk (kg/d)		ECM[1] (kg/d)		Fat (%)		Protein (%)		SCS	
	F value	*P-value*	*F value*	*P-value*	*F value*	*P-value*	*F value*	*P-value*	*F value*	*P-value*
AFC[2]	210	<.0001	211	<.0001	3.21	0.002	2.31	0.050	4.85	<.0001
DIM[3]	9825	<.0001	4236	<.0001	6348	<.0001	5767	<.0001	599	<.0001
Calving season	137	<.0001	119	<.0001	8.73	<.0001	8.50	<.0001	6.73	0.0002
Calving year	42.2	<.0001	34.4	<.0001	45.6	<.0001	32.05	<.0001	4.92	<.0001
AFC[1] * DIM[3]	2.42	<.0001	2.58	<.0001	3.07	<.0001	2.16	0.234	2.06	<.0001
AFC[1] * Calving season	9.84	<.0001	8.27	0.0003	1.72	0.021	2.51	0.135	3.11	0.657
DIM[2] * Calving season	9.42	<.0001	12.8	<.0001	12.5	<.0001	9.18	<.0001	3.38	<.0001

[1]*ECM = Energy corrected milk, calculated using the following formula adapted for buffaloes (Campanile et al., 2003): {[Fat (g/kg) – 40 + Protein (g/kg)– 31] × 0.01155} +1) × Milk yield (kg).*

[2]*AFC: age at first calving (months) was categorized into 8 categories: first: ≤30 months; second: 30-32 months; third: 32-34 months; fourth: 34-35 months; fifth: 35-37 months; sixth: 37-38 months; seventh: 38-42 months; and eight: >42 months;*
[3]*DIM: days in milk (d) was categorized into 10 intervals of 30 DIM each, except for the first and last categories which encompassed 5–30 DIM and >271 DIM, respectively.*

All the fixed effects significantly explained the variability of the analyzed traits ($P < 0.05$), except for AFC on protein content ($P = 0.05$). The interaction between AFC categories and calving season did not have an impact on protein content, SCS (respectively $P = 0.13$ and $P = 0.66$, Supplementary Table 2.1) and DMP ($P = 0.62$; Supplementary Table 2.2).

Supplementary Table 2.2. *Selected models (based on P-value and F-value) for days to milk peak[1] (d), calving interval (d) and days open (d) applied to primiparous Italian Mediterranean Buffaloes monitored from 2013-2021.*

	Days to milk peak[1]		Calving interval		Days open	
Effect	*F-values*	*P-value*	*F-values*	*P-value*	*F-values*	*P-value*
AFC[2]	2.14	0.050	2.24	0.025	2.56	0.011
Calving season	24.3	<.0001	22.4	<.0001	21.3	<.0001
Test-day	71276	<.0001	-	-	-	
Calving year	3.32	<.0001	35.4	<.0001	34.5	<.0001
AFC[2] * Calving season	0.88	0.615	1.46	0.070	1.78	0.012

[1]*Days to milk peak = number of days from calving to the highest milk yield during the first lactation, calculated as the difference between the date of milk peak and the date of first calving, based on individual test-day records;*

[2]AFC: age at first calving (months) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 months; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;

Table 2.2 provides the least squares means of productive traits for the effect of AFC categories. An increase in AFC was associated with significant increases in milk yield, ECM, and fat content ($P < 0.01$).

***Table 2.2** Least squares means and associated pooled standard error of the mean (SEM[3]) for milk yield (kg/d), days to milk peak (d)[1], energy corrected milk (ECM[4], kg/d), composition traits (%), and somatic cell score (SCS) according to the effects of AFC[2] based on individual test-day records of primiparous buffaloes from 2013 to 2023 (n = 362,703).*

	AFC[2] (mo)								
Traits	≤30	30-32	32-34	34-35	35-37	37-38	38-42	>42	*SEM[3]*
Milk (kg/d)	7.97^{e}	8.17^{d}	8.27^{c}	8.30^{bc}	8.35^{b}	8.45^{a}	8.46^{a}	8.49^{a}	0.03
Days to milk peak[1] (d)	58	58	58	58	58	58	58	58	0.20
ECM[4] (kg)	13.1^{e}	13.4^{d}	13.6^{c}	13.7^{bc}	13.8^{b}	13.9^{a}	14.0^{a}	14.0^{a}	0.04
Fat (%)	8.18^{b}	8.18^{b}	8.19^{ab}	8.19^{ab}	8.19^{ab}	8.20^{ab}	8.22^{a}	8.22^{a}	0.01
Protein (%)	4.76	4.76	4.76	4.76	4.76	4.77	4.77	4.76	0.004
SCS	2.81^{abc}	2.79^{bc}	2.78^{c}	2.78^{c}	2.81^{ab}	2.83^{a}	2.80^{ab}	2.80^{ab}	0.01

[1]Days to milk peak = number of days from calving to the highest milk yield during the first lactation, calculated as the difference between the date of milk peak and the date of first calving, based on individual test-day records;

[2]AFC: (months) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;

[3]SEM = Standard error of the mean;

[4]ECM = Energy corrected milk, calculated using the following formula adapted for buffaloes (Campanile et al., 2003): {[Fat (g/kg) – 40 + Protein (g/kg)– 31] × 0.01155} +1) × Milk yield (kg).

[a–e]Least squares means with different superscript letters within a row differ significantly according to Bonferroni post-hoc multiple comparison adjustment (P < 0.05).

Specifically, buffaloes with AFC > 42 months produced +0.52 kg/d more milk in their first lactation compared to those with AFC ≤ 30 months. Zicarelli et al. (2007), in a study of 953 primiparous buffaloes, reported a positive correlation between AFC and milk production during the first lactation, although this effect diminishes in subsequent lactations. Whereas, in a study on 314 Murrah buffaloes, Rautela et al. (2024) found that primiparous animals with an AFC between 60–65 months produced more milk (+ 182 kg/lactation) and had higher milk peak yields (+2.10 kg/d) compared to those calving between 30–35 months. Similarly to milk yield, ECM was 0.90 kg/d higher in buffaloes calving at an

AFC > 42 months compared to those with an AFC ≤ 30 months. This increase was likely driven by the slightly greater fat content (+0.04%) observed in the > 42 month AFC category. The observed increase in milk production may be attributed to the enhanced physiological maturity, including more advanced mammary gland development, typically associated with primiparous buffaloes calving at greater AFC (Macias and Hinck, 2012; Challana et al., 2014). Primiparous animals with lower AFC may experience reduced development of their mammary secretory tissue, limiting milk production in their first lactation (Sejrsen et al., 2000). Catillo et al. (2002) reported no effect of AFC on either protein or fat contents in a population of 534 Italian Mediterranean buffaloes. In contrast, Ettema and Santos (2004), in a study involving 1,933 Holstein heifers, observed lower protein content in animals with lower AFC. These discrepancies may depend on the population studied and management factors affecting heifers *post*-calving period (Atashi et al., 2021). Regarding SCS, the lowest values were observed in buffaloes with an AFC between 32–35 months, while higher SCS levels were recorded in animals that calved either earlier or later. On average, the SCS values observed in this study are consistent with those reported by Costa et al. (2020b) for Italian Mediterranean buffaloes, who documented a range between 2.71 to 3.11 for SCS. Previous studies in dairy cattle supported the association between AFC and udder health. Eastham et al. (2018) observed that higher AFC in dairy cows was associated with increased SCC, likely due to prolonged exposure to mastitis pathogens during the *pre*-calving period in older heifers, as also suggested by De Vliegher et al. (2012). Similarly, Atashi et al. (2021) reported higher SCS at both 100 and 305 d in Holstein heifers with greater AFC. According to their findings, healthier heifers, which tend to calve earlier, may exhibit greater resilience to mastitis. Mastitis can compromise udder health during the first lactation and this in turn can be reflected throughout their productive life. Indeed, Costa et al. (2019) found that buffalo heifers with high SCS during the first lactation tended to maintain elevated SCS and lower milk yields in subsequent lactations. Furthermore, Eastham et al. (2018) highlighted that higher AFC in dairy cows was associated with reduced average daily lifetime milk production and a lower likelihood of consecutive calvings. Therefore, while higher AFC in buffaloes may be associated with increased milk and fat yields during the first lactation, it could have detrimental effects on udder health and future lactations, suggesting that AFC may be anticipated within 32–35 months.

2.3.3. Effect of season on productive performances

As previously indicated, calving season is a crucial factor in Italian Mediterranean buffalo's husbandry management. Table 2.3 presents the least squares means of productive traits according to calving season.

Table 2.3 *Least squares means and associated pooled standard error of the mean (SEM) for milk yield (kg/d), days to milk peak1 (d)*[1]*, energy corrected milk (ECM*[2]*, kg/d), composition traits (%), and somatic cell score (SCS) according to the effect of calving season based on individual test-day records of primiparous buffaloes from 2013 to 2023 (n = 362,703).*

Traits	Calving season				
	Autumn	Winter	Spring	Summer	*SEM*
Milk (kg/d)	8.13^{c}	8.46^{a}	8.43^{a}	8.20^{b}	0.03
Days to milk peak (d)[1]	58.0^{b}	58.0^{b}	57.0^{c}	59.0^{a}	0.14
ECM[2] (kg)	13.5^{c}	13.9^{a}	13.9^{b}	13.5^{c}	0.03
Fat (%)	8.23^{a}	8.19^{bc}	8.20^{ab}	8.17^{c}	0.01
Protein (%)	4.77^{a}	4.76^{b}	4.76^{b}	4.76^{b}	0.003
SCS	2.81^{ab}	2.77^{c}	2.79^{bc}	2.82^{a}	0.01

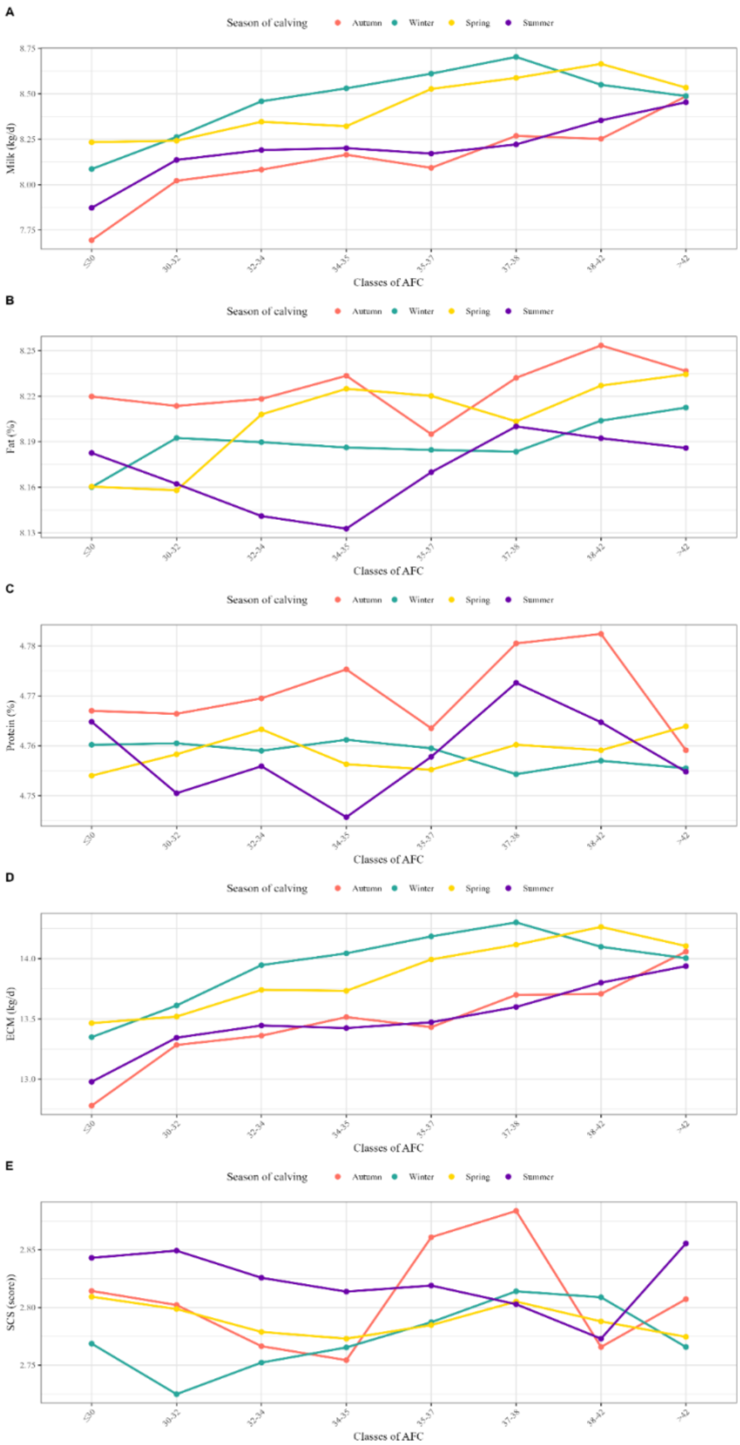
[1]*Days to milk peak = number of days from calving to the highest milk yield during the first lactation, calculated as the difference between the date of milk peak and the date of first calving, based on individual test-day records;*

[2]*ECM = Energy corrected milk, calculated using the following formula adapted for buffaloes (Campanile et al., 2003): {[Fat (g/kg) – 40 + Protein (g/kg)– 31] × 0.01155} +1) × Milk yield (kg).*

[a–c]*Least squares means with different superscript letters within a row differ significantly according to Bonferroni post-hoc multiple comparison adjustment (P < 0.05).*

Buffaloes that calved in winter and spring exhibited significantly higher milk production and ECM compared to those that calved in summer and autumn, consistent with previous findings ($P < 0.01$; Pawar et al., 2012; Zicarelli, 2017). Similarly, Carvalho et al. (2016) reported that buffaloes calving in winter produced significantly higher total milk yield in respect to summer (8.47 vs 7.66 kg/d). Consistently, Gomez-Carpio et al. (2023) observed that the calving season effect was particularly evident during the first phase of lactation, with the lowest milk yield recorded in buffaloes that calved in summer. Seasonal variations in DMP were observed as well, with buffaloes calving in spring reaching milk peak earlier (57 d) compared to the other seasons ($P < 0.01$). Notably, DMP significantly increased during summer (59 d), possibly due to the associated heat stress. This seasonal effect, particularly pronounced in southern regions of Italy, may be more evident in primiparous buffaloes, as heat stress can impair feed intake and in turn their energy balance, delaying DMP (Zicarelli, 2017). Furthermore, fat and protein contents in primiparous buffaloes were significantly influenced by calving season, with the highest values observed in autumn ($P < 0.01$; respectively, 8.23% and 4.77%). Since primiparous buffaloes that calved in autumn had the lowest significant milk yield, the increase in fat and protein content may partly result from a reduction in milk volume and a corresponding increase in concentration due to the possible dilution effect (Boro et al., 2018). Moreover, summer heat stress can adversely affect milk production by reducing feed intake, which in turn diminishes milk yield as well as fat and protein contents (Catillo et al., 2002). Additionally, SCS was significantly higher in summer and autumn, aligning with findings in the literature (Costa et al., 2021). This pattern parallels that observed in dairy cows, where Wu et al. (2021) documented higher SCS during summer. Supplementary Figure 2.3 showed the interaction between AFC categories and calving seasons, highlighting that buffalo with higher AFC had greater milk yield, ECM, and fat content across all seasons (*P < 0.01*). Among the possible combinations, buffaloes that calved between 34 and 37 months achieved a favorable balance of milk production and fat content across seasons.

Supplementary Figure 2.3. *Least square means of milk yield (kg/day), energy corrected milk (ECM[1], kg/d), composition traits (%) and somatic cell score (SCS) for the interaction effect of age at first calving (AFC)[2] and calving season. Associated standard errors of the means were within the following ranges: 0.033–0.035 (milk yield), 0.030–0.045 (ECM[1]), 0.011–0.015 (fat content), 0.003–0.006 (protein content), and 0.008–0.012 (SCS).*



[1]ECM = Energy corrected milk, calculated using the following formula adapted for buffaloes (Campanile et al., 2003): {[Fat (g/kg) – 40 + Protein (g/kg)– 31] × 0.01155} +1) × Milk yield (kg).

[2]AFC: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo.

Whereas DMP, protein and SCS, were not significantly affected by the interaction between calving season and AFC categories (Figure 2.1). These findings confirm that an AFC between 34 and 37 months is preferable to optimize production traits across seasons.

2.3.4. Effect of DIM on productive performance

The effects of DIM on productive traits are presented in Table 2.4.

Table 2.4*. Least squares means and associated pooled standard error of the mean (SEM) for milk yield (kg/d), energy corrected milk (ECM[2], kg/d), composition traits (%), and somatic cell score (SCS) according to the effects of days in milk effect (DIM)[1] based on individual test-day records of primiparous buffaloes from 2013 to 2023 (n = 362,703).*

Traits	DIM (d)[1]										
	5-30	31-60	61-90	91-120	121-150	151-180	181-210	211-240	241-270	271-305	*SEM*
Milk (kg/d)	9.55[d]	10.8[a]	10.5[b]	9.65[c]	8.84[e]	8.10[f]	7.42[g]	6.71[h]	6.06[i]	5.50[j]	0.03
ECM[2] (kg/d)	14.6[d]	16.2[a]	16.1[b]	15.4[c]	14.5[e]	13.8[f]	13.0[g]	12.0[h]	11.1[i]	10.3[j]	0.03
Fat (%)	7.04[j]	7.09[i]	7.4[h]	7.78[g]	8.11[f]	8.47[e]	8.76[d]	8.97[c]	9.11[b]	9.25[a]	0.01
Protein (%)	4.74[f]	4.50[i]	4.50[i]	4.58[h]	4.67[g]	4.77[e]	4.86[d]	4.94[c]	5.00[b]	5.05[a]	0.003
SCS	2.47[i]	2.34[j]	2.52[h]	2.71[g]	2.82[f]	2.88[e]	2.94[d]	3.00[c]	3.10[b]	3.19[a]	0.01

[1]DIM: days in milk (d) was categorized into 10 intervals of 30 DIM each, except for the first and last categories which encompassed 5–30 DIM and >271 DIM, respectively.

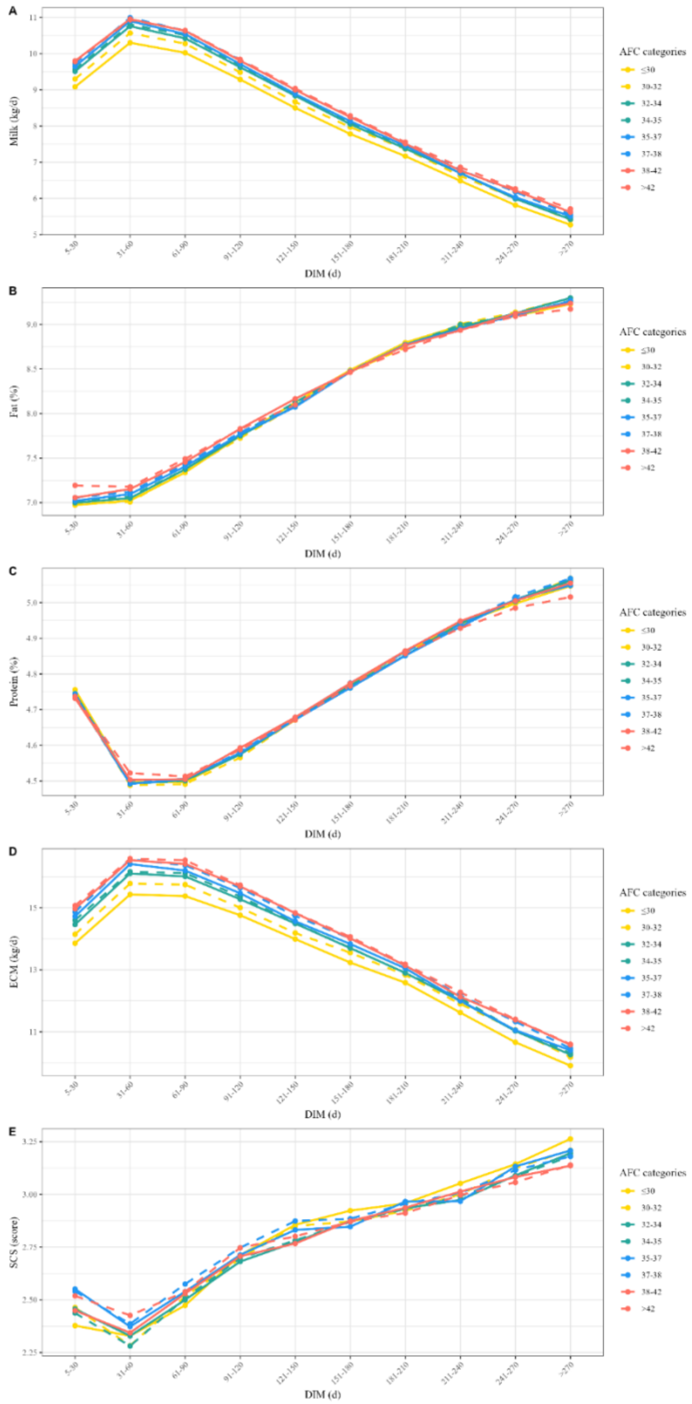
[2]ECM = Energy corrected milk, calculated using the following formula adapted for buffaloes (Campanile et al., 2003): {[Fat (g/kg) – 40 + Protein (g/kg)– 31] × 0.01155} +1) × Milk yield (kg).

[a–j]Least squares means with different superscript letters within a row differ significantly according to Bonferroni post-hoc multiple comparison adjustment ($P < 0.05$).

Consistent with previous studies on Italian Mediterranean (Costa et al., 2020a, b) and Murrah breeds (Cerón-Muñoz et al., 2002), milk production and ECM peaked between 31 and 60 d of lactation and progressively declined thereafter ($P < 0.01$) with an average of 56 d (Supplementary Figure 2.2). In contrast, fat and protein contents had a significant negative peak in correspondence with milk and ECM positive production peaks ($P < 0.01$) and increased throughout lactation, reaching their highest values in late lactation. Whereas SCS was significantly lower in early lactation but increased as lactation progressed ($P < 0.01$). This was more evident in buffaloes with lower AFC (Supplementary

Figure 2.4), likely due to their lower ability to produce milk and manage subsequent pregnancy at the same time, potentially lowering their immune defenses and increasing the risk of mastitis.

Supplementary Figure 2.4. *Least square means of milk yield (kg/day), energy corrected milk (ECM[1], kg/d), composition traits (%) and somatic cell score (SCS) for the interaction effect of age at first calving (AFC[2]) and days in milk (DIM[3]). Associated standard errors of the means were within the following ranges: 0.035–0.040 (milk yield), 0.030–0.044 (ECM[1]), 0.012–0.016 (fat content), 0.003–0.007 (protein content), and 0.009–0.0013 (SCS).*



[1]*ECM = Energy corrected milk, calculated using the following formula adapted for buffaloes (Campanile et al., 2003): {[Fat (g/kg) – 40 + Protein (g/kg)– 31] × 0.01155} +1) × Milk yield (kg).*

[2]*AFC: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;*

[3]*Days to milk peak = number of days from calving to the highest milk yield during the first lactation, calculated as the difference between the date of milk peak and the date of first calving, based on individual test-day records.*

It has been observed that different AFC in relation to DIM had an impact on milk yield, ECM and fat content ($P < 0.01$; Supplementary Figure 2.4). Buffaloes' heifers with greater AFC exhibited greater milk yield, fat content, and ECM at peak compared to those that calved with lower AFC. This trend is likely attributable to the higher dry matter intake potential of older heifers, coupled with their more advanced mammary gland development, that both enhance their milk production and metabolic mobilization. Additionally, older heifers, having completed most of their growth, can allocate more energy toward lactation. Similar patterns have been observed in dairy cows, where animals with lower AFC tend to prioritize energy allocation toward growth rather than milk production, resulting in reduced milk yields (Ettema and Santos, 2004; Atashi et al., 2021). Therefore, achieving an appropriate BW at calving may help younger buffaloes sustain milk production, particularly during early lactation. In dairy cattle, BW at calving is strongly influenced by *pre*-weaning management; for example, higher milk intake during *pre*-weaning period accelerates weight gain, while sufficient starter feed intake supports consistent *post*-weaning growth (Diaz et al., 2001; Bach, 2012). Hu et al. (2020) reported that excessive reliance on milk replacers during the weaning transition can negate these growth benefits, leading to reduced BW gain and impaired structural development due to lower nutrient digestibility. In the case of Italian Mediterranean buffaloes, Vecchio et al. (2013) found that a single daily administration of milk replacer during weaning did not adversely affect calf growth or health. Nevertheless, discrepancies in the literature (Omidi-Mirzaei et al., 2015) suggest that outcomes are highly dependent on the quantity and quality of milk replacers used in individual trials, underlining the need for further research to determine optimal feeding strategies for buffalo calves weaning phase. However, *pre*-weaning management is often neglected in Italian Mediterranean buffalo farms, primarily due to the high cost of milk replacers, and the labor-intensive requirements of calf rearing.
Replacement buffalo calves are frequently exposed to cold winter temperatures and overcrowded housing conditions, a consequence of seasonal calving concentration, which can compromise their welfare and stayability in the herd (Vecchio et al., 2007a). Moreover, also effective *pre*-calving management strategies are crucial to optimize AFC in buffaloes (Sarwar et al., 2009). Improving these conditions fosters the development of healthier heifers, better equipped to meet the metabolic demands associated with their first lactation. Indeed, Vecchio et al. (2007a) reported that increasing the level of concentrate supplementation in heifer diets enhanced both fertility and milk production in primiparous

buffaloes. Nutritional imbalances during early lactation, such as those leading to sub-acute ruminal acidosis in dairy cows, can trigger systemic inflammation and reduce productivity (Plaizier et al., 2012). Although specific evidence in buffaloes remains limited, it is reasonable to assume that comparable mechanisms may underline *post-partum* challenges in this species, underscoring the need for balanced nutritional management and targeted interventions during early lactation to safeguard metabolic health and overall animal welfare. In this context, as suggested by Cavallini et al. (2022) for dairy cows. immunomodulatory feed additives, such as yeast cell wall derivatives, may help mitigate stress by supporting immune function, red blood cell regeneration, and energy balance. Additionally, innovative approaches such as mid-infrared spectroscopy applied to milk analysis have emerged as effective, non-invasive tools for herd-level screening of cows at risk of negative energy balance and subclinical ketosis, thereby supporting more informed decision-making and management strategies at farm level (Magro et al., 2024).

2.3.5. Effect of AFC and calving season on reproductive performances

Both AFC and calving season had a significant effect on CI and DO (Supplementary Table 2.2; $P = 0.03$ and $P = 0.01$, respectively). While the interaction between AFC categories and calving season did not significantly influence CI ($P = 0.07$), it had a significant effect on DO ($P = 0.01$). Optimizing AFC is crucial for effective herd management, as studies in cattle have demonstrated that higher AFC is associated with shorter productive lifespan and reduced fertility, ultimately compromising animals' stayability in the herd (Vukasinovic et al., 2001; Strapáková et al., 2013). In the present study, primiparous buffaloes that calved with AFC between 34-37 months had the shortest CI and DO (on average 449 and 138 d, respectively; Table 2.5), whereas both higher (>37 months) and lower (≤34 months) AFC groups recorded extended CI and DO.

Table 2.5. *Least squares means and associated pooled standard error of the mean (SEM) for calving interval (d) and days open (d) according to the effect of AFC[1] for primiparous Mediterranean Buffaloes monitored from 2013-2021.*

	AFC categories[1]								
Traits	≤30	30 - 32	32 - 34	34 - 35	35 - 37	37 - 38	38 - 42	>42	*SEM*
Calving interval (d)	454^a	453^a	453^a	449^b	450^b	451^a	453^a	454^a	2.07
Days open (d)	143^a	141^a	141^a	137^b	139^b	139^a	139^a	143^a	2.09

[1]AFC: (months) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo.

[a–b]Least squares means with different superscript letters within a row differ significantly according to Bonferroni post-hoc multiple comparison adjustment ($P < 0.05$).

Rautela et al. (2024), in their study on Murrah breed, have reported significantly shorter CI in buffaloes that calved with AFC between 36 and 47 months (approximately 470 d). Although calving to first service, services per conception, and calving to conception interval were not affected, these parameters exhibited numerically lower values within the same AFC category in the same study. These differences could be due to the generally greater AFC observed in Murrah breed when compared with Italian Mediterranean buffaloes. Additionally, variations in feeding strategies and management practices between production systems may further explain those increased AFC values. Nevertheless, in both production systems, enhancing reproductive performance can significantly improve farm profitability, primarily by enabling an earlier onset of milk production and consequently shortening the non-productive rearing period. In the present study, reducing AFC between 34 and 37 months lead to better reproductive performance, thereby supporting higher lifetime productivity and enhancing the economic sustainability of buffalo farming. In dairy cows, reducing AFC from 26 to 24 and 22 months increased the net return by $41.5 and $24 per heifer, respectively, whereas delaying AFC beyond 26 months led to reduced profitability due to increased rearing costs (Pirlo et al., 2000).

Typically, primiparous buffaloes show longer DO, CI, and requires more services per conception, with these parameters decreasing in subsequent parities (Carvalho et al., 2016). However, CI in buffaloes is also influenced by reproductive management strategies, synchronization protocols, and seasonal variations (Rossi et al., 2014). In this study, both CI and DO were significantly influenced by calving season (Table 2.6), with the longest intervals recorded in buffaloes that calved during winter and spring (on average 455 and 145 d, respectively), compared to those calving in autumn and summer (on average 449 and 138 d, respectively; $P < 0.01$).

Table 2.6. *Least squares means and associated pooled standard error of the mean (SEM) for calving interval (d) and days open (d) according to the effect of calving season.*

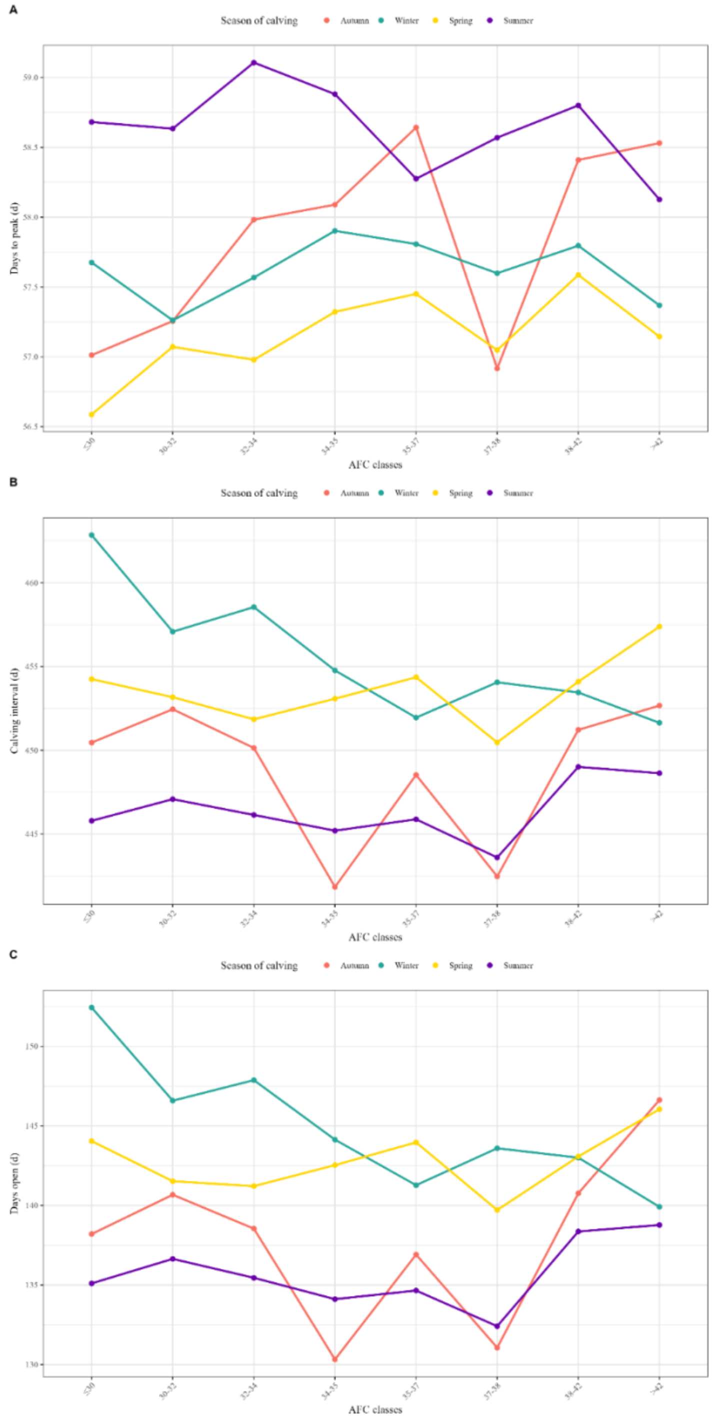
	Calving season				
Traits	Autumn	Winter	Spring	Summer	*SEM*
Calving interval (d)	450^{b}	456^{a}	455^{a}	447^{b}	1.84
Days open (d)	139^{b}	146^{a}	144^{a}	137^{b}	1.84

$^{a–b}$*Least squares means with different superscript letters within a row differ significantly according to Bonferroni post-hoc multiple comparison adjustment (P < 0.05).*

Similarly, Zicarelli (2007) reported seasonal variations in CI, observing longer intervals for buffaloes calving between late autumn and early spring (478–488 d), and shorter intervals for those calving from late spring to early autumn (460–468 d). These higher values for CI could be partly explained considering that their study was conducted more than 10 years ago, and reproductive performance traits have since improved due to advancements in management and breeding strategies (Supplementary Figure 2.1). Mating during the out-of-breeding season period reduces fertility in buffaloes, as calving is confined to 7-8 months of the year, corresponding to the least favorable reproductive period for the species. To enhance reproductive efficiency, natural mating is recommended during the transition and non-breeding season, given the reduced success of assisted reproductive techniques during these periods (Vecchio et al., 2007b). Moreover, younger buffaloes (AFC ≤ 30 months) that calved in winter tended to exhibit longer CI and significantly prolonged DO (> 150 d) compared to older buffaloes (AFC > 42 months) calving in the same season (140 d; Figure 2.1). This may be attributed, at least in part, to their lower BW and reduced BW reserves, which can delay the resumption of ovarian activity (Dash et al., 2016), particularly in late winter when increasing daylight begins to negatively influence reproductive physiology. Indeed, photoperiod is a well-documented factor affecting reproduction in buffaloes, especially at higher latitudes (Campanile et al., 2010). Accordingly, buffaloes calving in winter and spring may experience delayed ovarian reactivation and reduced conception rates due to seasonal anestrus, with the duration of anestrus closely linked to increasing day length (Campanile et al., 2009). Moreover, extended photoperiods have also been associated with higher rates of embryonic loss and prolonged anestrus in Italian Mediterranean buffaloes (Vecchio et al., 2007b).

Buffaloes with greater AFC (> 42 months) calving in summer tended to exhibit longer CI and had significantly prolonged DO (respectively 449 and 139 d) compared to their younger counterparts (≤ 30 months) calving in the same season (respectively 446 and 135 d; Figure 2.1).

Figure 2.1. *Least square means of days to milk peak (d)[1], calving interval (d) and days open (d) for the interaction effect of AFC[2] and calving season. Associated standard errors of the means were within the following ranges: 0.11–0.23 (days to milk peak), 1.70–2.40 (calving interval) and 1.72–2.44 (days open).*



[1]Days to milk peak = number of days from calving to the highest milk yield during the first lactation, calculated as the difference between the date of milk peak and the date of first calving, based on individual test-day records;

[2]AFC: (months) was categorized into 8 categories: first: ≤30 months; second: 30-32 months; third: 32-34 months; fourth: 34-35 months; fifth: 35-37 months; sixth: 37-38 months; seventh: 38-42 months; and eight: >42 months.

During summer, elevated temperatures can compromise feed intake, metabolic efficiency, and overall energy balance, particularly in older buffaloes (Marai and Haeeb, 2010). Although buffaloes are generally more heat-tolerant than cattle (Dash et al., 2016), prolonged exposure to heat stress, combined with seasonal nutritional challenges such as lower forage quality and availability, can exacerbate negative energy balance and metabolic stress. These conditions can affect reproduction, impairing ovarian activity and delaying the resumption of reproductive cycles, ultimately extending CI and DO (D'Occhio et al., 2020). This highlights the complex interplay between environmental stressors and physiological status, indicating that seasonal impacts on productive and reproductive performance are multifactorial. For instance, Salzano et al. (2019) demonstrated that increasing day length, another seasonal factor, negatively affects ovarian function in buffalo heifers by reducing both the number of follicles and cumulus-oocyte complexes, whereas an improved metabolic status is associated with better oocyte quality and reproductive outcomes. Taken together, these findings emphasize the need for integrated approaches that combine effective management practices with genetic selection to mitigate the seasonal challenges affecting reproductive performance.

In this regard, selective breeding strategies offer promising avenues to reduce AFC, considering the moderate to low heritability estimates reported for this trait in both Murrah buffaloes (0.15–0.48; Tamboli et al., 2022; Dos Santos et al., 2025) and Italian Mediterranean buffaloes (0.07–0.13; Gomez-Carpio et al., 2023). Notably, Gomez-Carpio et al. (2023) identified a favorable positive genetic correlation between AFC and first CI (0.10 and 0.41), indicating that selection for earlier AFC would have a favorable effect on reduction of CI. In this context, the adoption of precision livestock farming (PLF) technologies represents a valuable complementary approach during season with lower fertility. Tools such as wearable collars with integrated heat detection sensors enable more accurate and timely estrus identification, improving insemination timing and conception rates (Lamanna et al., 2025). Moreover, PLF technologies may enhance phenotype recording accuracy through continuous data collection, thereby accelerating at the same time the progress in breeding programs.

2.4. CONCLUSIONS

This study provides new insights into the optimal management of AFC in Italian Mediterranean buffaloes, identifying the range of 34–37 months as the most favorable balance between productive and reproductive performance. Our findings highlight that both early and delayed AFC can compromise reproductive efficiency, underscoring the critical role of heifer nutrition, BCS, and breeding seasonality in achieving reproductive outcomes. Further research should focus on strategies to further reduce AFC through improved feeding regimes and management strategies, while also exploring season-specific reproductive technologies to enhance conception rates. Finally, integrating reproductive traits into breeding programs, alongside traditional milk yield criteria, could accelerate genetic progress and strengthen the resilience of buffalo farming systems.

2.5. NOTES

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Nonstandard abbreviations used: AFC = Age at first calving; SCS = Somatic cell score; CI = Calving interval; DO = Days open; DMP = Days to milk peak.

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3. Chapter 3

Age at first calving in dairy buffaloes: impacts and profits

The cost of being early or late: biological and economic outcomes of age at first calving in dairy buffaloes

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ABSTRACT

Age at first calving (AFC) is a key determinant of lifetime productivity and profitability in dairy herds, yet its long-term effects in buffaloes remain poorly documented. This study evaluated the impact of AFC on milk yield, reproductive performance, and economic outcomes in Italian Mediterranean buffaloes under commercial conditions. The dataset included 576,028 test-day records from 27,744 buffaloes across 110 herds, collected over a 10-year period (2013–2023). Animals were grouped into eight AFC classes and stratified by parity: primiparous, mid-parity (second–third), and greater-parity (≥4). Productive traits comprised daily milk yield, fat and protein contents, somatic cell score (SCS), and cumulative yields of milk, fat, and protein per lactation, while reproductive traits included calving interval (CI) and days open (DO). Mixed-model analysis indicated that multiparous buffaloes calving between 35-38 mo achieved superior productive and reproductive performance ($P<0.05$), whereas in primiparous buffaloes, daily milk yield increased progressively with AFC ($P<0.05$). Milk fat content was unaffected by AFC, whereas the highest protein percentages were observed in first-, second- and third-parity buffaloes calving at an AFC between 32 and 34 months, however primiparous buffaloes maintained stable protein content across AFC classes. SCS significantly decreased as AFC increased across all parity groups ($P<0.05$), except in primiparous buffaloes, where a temporary increase in SCS was observed at 37–38 months of AFC ($P<0.05$). Moreover, CI and DO were shortest in buffaloes with AFC between 32 and 37 mo ($P<0.05$). The economic analysis integrated model-based estimates of milk yield with farm-level cost data from the farm accountancy data network (FADN) covering the same 10-year period. Production costs were organized into three categories: direct costs, overheads, and imputed costs. Based on this structure, the average production cost was estimated at €1.38/L of milk. Total production costs per lactation were calculated as the unit cost multiplied by the estimated cumulative milk yield for each AFC class and parity group. Revenues were obtained in the same way, by applying the actual farm-gate milk price (€1.50/L) to the estimated yields, thereby providing a standardized framework for comparison across AFC classes. Net profit was then derived as the difference between revenues and costs, resulting in an overall average of €314 per lactation across AFC and parity groups. The highest profitability, reaching €339 per lactation, was observed in buffaloes calving between 37-38 mo in mid-parity buffaloes. Conversely, both early (<30 mo) and late (>42 mo) AFC values were associated with reduced economic returns, driven by lower milk yields. Overall, these results indicate that targeting an AFC of 35-38 mo provides the most favorable balance between biological performance, udder health, reproductive efficiency, and farm profitability.

Keywords: Calving interval, Culling risk, Economic return, Fertility.

3.1. Introduction

Over the last two decades, global buffalo milk production has more than doubled, rising from 67 to 144 million tons, thereby reinforcing its significance as a dynamic and expanding segment of the dairy industry (FAOSTAT, 2024). Renowned for its nutritional value and suitability for high quality dairy products (Basilicata et al., 2018), buffalo milk has gained increasing consumer appeal, stimulated rural economies, and promoted the adoption of more sustainable livestock practices (Kumar et al., 2020; Matera et al., 2025). These developments have coincided with growing attention to advances in husbandry, particularly in feeding strategies, reproductive efficiency, and animal welfare (Serrapica et al., 2022; Javed et al., 2022).

Italy is the leading producer of buffalo milk in Europe, accounting for approximately 87% of the entire European buffalo population (FAOSTAT, 2024). The national buffalo population includes over 400,000 animals (BDN, 2025), of which around 121,000 lactating females are annually monitored by the Italian Buffalo Breeders Association (ANASB, 2025). Dairy buffalo farming in Italy comprises about 2,400 herds, with an average size of 170-180 animals per farm (BDN, 2025). Buffaloes are known for their longevity and adaptability to Mediterranean conditions, with productive lifespan reaching up to 10 lactations. Approximately 70% of the national buffalo population is concentrated in southern regions, where most milk is processed into Mozzarella di Bufala Campana PDO, which obtained the denomination of origin label in 1996. Notably, the production of this PDO cheese has grown by 40% over the past decade, reaching 55.7 million kg in 2024 (Napolitano et al., 2021; Consorzio di tutela mozzarella di bufala campana PDO, 2024).

Despite this steady growth and the increasing economic importance of the sector, reproductive efficiency continues to represent a critical challenge for Italian buffalo farming. Seasonal fertility remains a major constraint in buffalo dairy systems, even with recent advancements in reproductive management (Neglia et al 2020). As short-day breeders, buffaloes reach peak fertility in autumn and winter (Gasparrini, 2018; D'Occhio et al., 2020), while consumer demand for Mozzarella typically peaks in spring and summer (Otava et al., 2021). To address this mismatch and shift calving towards periods of higher milk demand, out-of-breeding-season mating technique has been commonly applied in Italian Mediterranean buffaloes (Zicarelli, 1997). Within this framework, optimizing age at first calving (AFC) is crucial to maximize lifetime productivity and economic returns. Nutritional management of prepubertal body weight (BW) plays a pivotal role in determining the timing of reproductive onset (Krpálková et al., 2014a,b). Early-life nutritional strategies critically influence growth rates, metabolic development, and pubertal onset in both cattle (Bruinjé et al., 2021) and buffaloes (Terzano et al., 2007a,b). In Italian Mediterranean buffaloes, the average AFC is approximately 35–36 mo (Gomez-Carpio et al., 2025). However, this value may vary across buffalo population. For example, in Murrah buffaloes, it can range from 37 to 54 mo (Rautela et al., 2024). Although reducing AFC is often pursued to lower rearing costs (Campanile et al., 2006), early calving without adequate BW and physiological maturity can compromise body condition at parturition, impair reproductive performance, and reduce milk yields in subsequent lactations. Recent findings from Calanni Macchio et al. (2025) showed that buffaloes calving the first time before 30 mo had longer calving intervals (CI) and days open (DO), as well as lower milk and fat percentages during first lactation. Comparable trends have been observed in dairy cattle, where insufficient BW at calving is associated with suboptimal lactation and reproductive outcomes (Eastham et al., 2018; Kusaka et al., 2023). Conversely, higher AFC may enhance physiological development and improve first-lactation performance, but it may prolong the non-productive period, delays the onset of milk production, increases the risk of involuntary culling, postponing economic returns. While the short-term effects of AFC on lactation performance and fertility are relatively well established, its long-term impact on lifetime productivity in buffaloes remains underexplored. In this context, Aspilcueta-Borquis et al. (2022) provided valuable insights, reporting favorable genetic correlations between AFC and functional longevity in a population of 3,431 buffaloes across six herds. Furthermore, the authors highlighted the economic relevance of this trait, estimating potential gains of +$102 to $147 per animal when AFC is used as a selection criterion, thus supporting its integration into breeding programs aimed at enhancing productive lifespan and economic sustainability.

The present study investigates the impact of AFC on the lifetime performance of Italian Mediterranean buffaloes, with a focus on milk production, reproductive efficiency, and overall economic returns. By analyzing data from a large commercial population over 10 years of observation, the study offers robust, field-based evidence to inform management strategies and enhance the sustainability of buffalo dairy systems.

3.2. Materials and methods

Ethical approval from an animal welfare and use committee was not required for this study, as the data were obtained from routine animal recording practices conducted by the Italian Buffalo Breeders Association (ANASB).

3.2.1. Data and editing

Data used in this study consisted of routine test-day records collected by the Italian Buffalo Breeders Association (ANASB) between January 2013 and December 2023, using data from farms located in the four main provinces of the Mozzarella di Bufala Campana PDO area (Caserta, Salerno, Latina, and Foggia), which together account for approximately 90% of total PDO-certified output. Each test-day record included information on fat and protein contents (%), somatic cell count (SCC), daily milk yield (kg) and animal characteristics such as parity order and days in milk (DIM). To determine milk composition, milk samples were analyzed using mid-infrared spectroscopy (MilkoScan FT6000, Foss Electric A/S, Hillerød, Denmark) at the official AIA-certified laboratory in Benevento (Italy). Additionally, cumulative yields (kg) for milk, fat, and protein were available for each individual lactation. Reproductive data included complete birth and calving dates for each animal.

To ensure data reliability, a series of data quality control procedures were applied. Lactation data were retained only for buffaloes reared in herds with continuous and complete data reporting throughout the study period, restricted to farms with at least 40 lactating buffaloes. Records were further limited to buffaloes between the first and twelfth parity. Only lactations with a minimum of three test-day records between 5 and 305 DIM were included. To reduce variability due to small group sizes, herd-test-day (HTD) contemporary groups were considered only when comprised at least five lactating animals. Outliers' values for milk yield, fat and protein contents, and cumulative lactation yields exceeding ±3 SD from the mean were set to missing, following the method described by Calanni Macchio et al. (2025), to minimize the influence of extreme or biologically implausible records that could bias model estimates. Somatic cell count was log-transformed into somatic cell score (SCS) using the standard formula (Ali and Schook, 1980). AFC was calculated as the number of days between birth and first calving. Since AFC was normally distributed (Supplementary Figure 3.1), it was categorized into eight biologically and balanced classes, each comprising approximately 12% of the records: ≤30, >30–≤32, >32–≤34, >34–≤35, >35–≤37, >37–≤38, >38–≤42, and >42 mo. Implausible AFC values (<23 or >47 mo) were excluded following Gómez-Carpio et al. (2023)., to reduce potential bias related to voluntary delays in first insemination often adopted by farmers applying out-of-breeding mating techniques.

Supplementary Figure 3.1. *Frequency distribution of age at first calving in the studied buffalo population.*

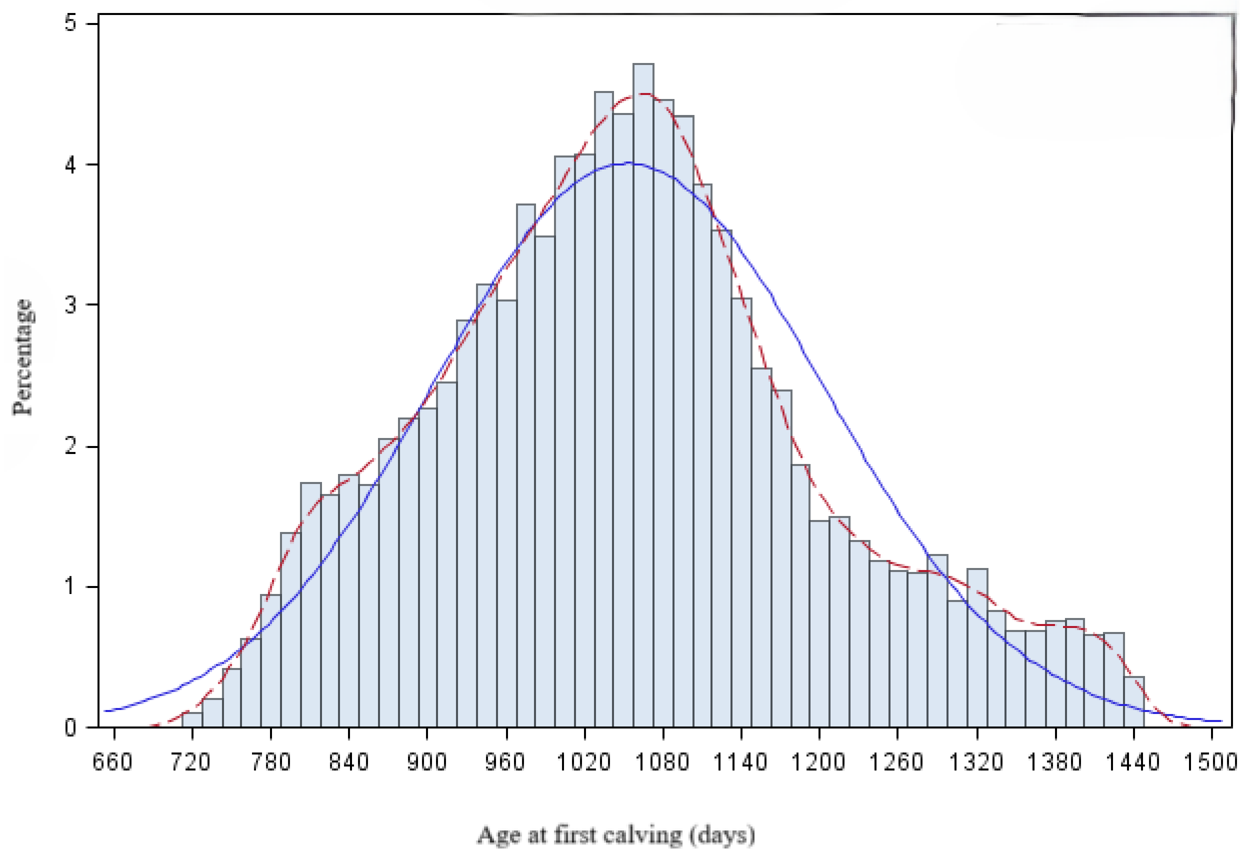
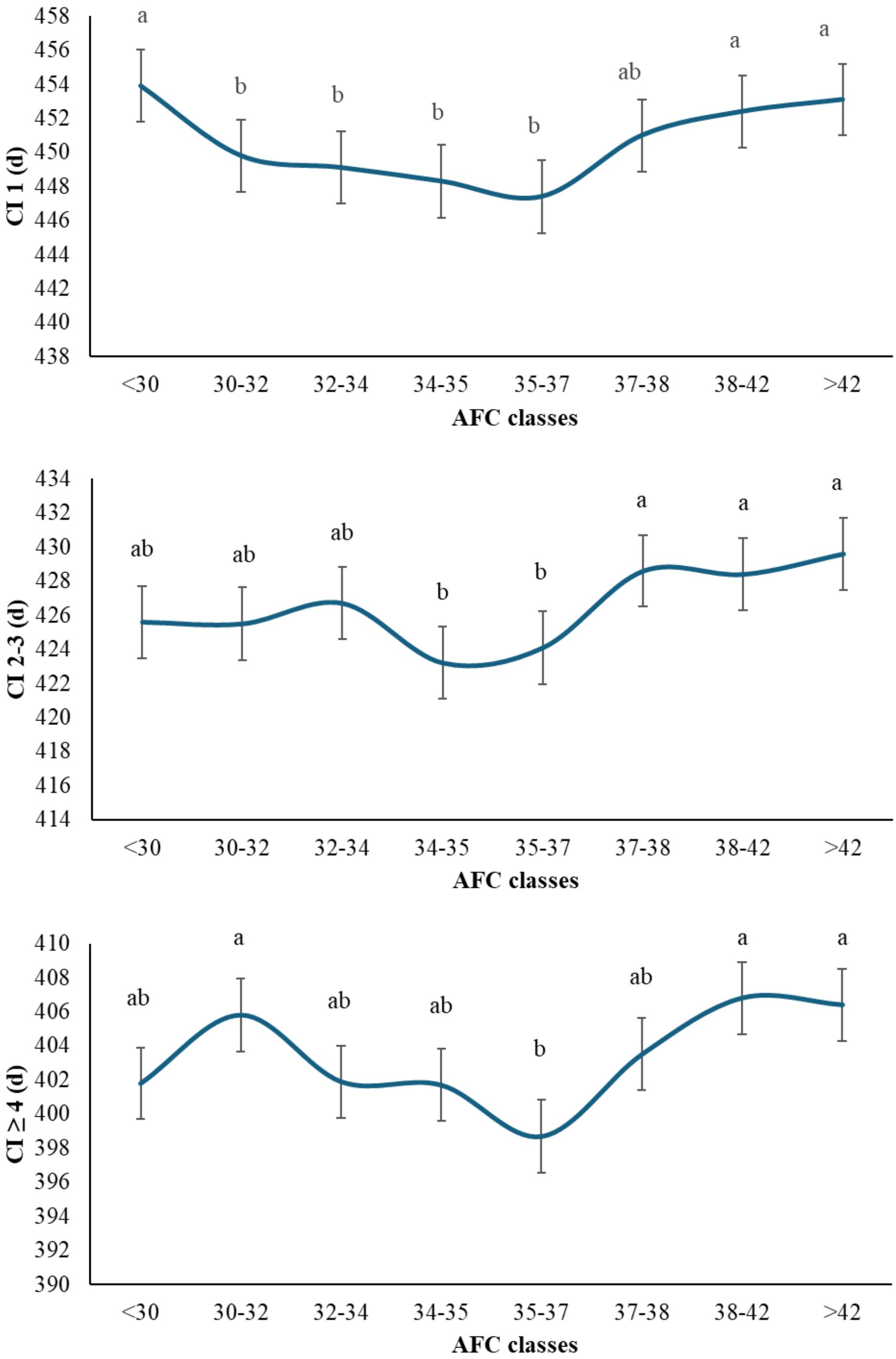


Figure 3.1. *Least squares means of calving intervals1* (days) in relation to classes AFC*[2].



[1]*CI1 represents to the calving interval between the first and second calvings; CI2–3 represents the calving intervals between the second and third, and third and fourth calvings; CI≥4 represents the calving intervals for all calvings beyond the fourth;* [*]*CI1 estimates were calculated based on 9.599 animals; CI2-3 estimates were calculated based on 11.181 animals; CI≥4 estimates were calculated based on 6.964 animals;*

[2]*AFC classes: (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo.*

Calving interval was defined as the number of days between two consecutive calvings, while the date of conception was estimated by subtracting a fixed gestation length of 310 days from the date of the subsequent calving. Days open were then calculated as the interval between calving and conception dates. To further minimize the influence of extreme reproductive records and ensure consistency with the assumed gestation duration, DO values falling outside the mean ±0.50 SD were excluded. This approach aligns with standard outlier-handling practices in reproductive studies on buffaloes (Gómez-Carpio et al. 2025; Calanni Macchio et al., 2025) and was designed to control for the effect of management decision, such as seasonal breeding or voluntary waiting periods, that can influence reproductive intervals independently of the animal's physiological performance. After the editing procedure, the final dataset comprised 576,028 test-day records from 82,290 lactations from 27,744 buffaloes across 110 herds. Each buffalo contributed, on average, three lactations, with approximately seven test-day records per lactation.

3.3.2. Economic calculations: cost analysis

The cost analysis was conducted using microeconomic data collected from 2013 and 2023 through the farm accountancy data network (FADN), which annually compiles harmonized economic information from over 80,000 farms across Europe (FADN, 2010; Ciaian et al., 2013). The analysis focused on the production cost per liter of buffalo milk (€/L). A total of 517 farm-year observations were included and primarily engaged in buffalo milk production. Production costs were classified into three hierarchical levels to reflect their nature and allocation in buffalo milk production. First-level costs comprised variable expenses directly associated with production activities, including feed, veterinary services, reproduction management, energy, and other consumables (Supplementary Table 3.1).

Supplementary Table 3.1. *Summary of first-level variable costs (€/L of milk) in buffalo milk production retrieved from farm accountancy data network (FADN).*

Items	Mean	Median	STD	Minimum	Maximum
Feedstuff, forage and bedding materials	0.40	0.37	0.30	0.00	1.46
Energy and water	0.04	0.03	0.02	0.00	0.12
Medication and veterinary expenses	0.02	0.02	0.03	0.00	0.59
Replacements	0.22	0.21	0.11	0.01	0.63
Other variable costs	0.03	0.01	0.06	0.00	0.52

Second-level costs represented general overheads that, while essential to farm operations, could be directly attributed to a specific production activity. These included administration, maintenance, and insurance (Supplementary Table 3.2).

Supplementary Table 3.2. *Summary of second-level variable costs (€/L of milk) in buffalo milk production retrieved from farm accountancy data network (FADN).*

Items	Mean	Median	STD	Minimum	Maximum
Taxes and other overhead expenses[1]	0.13	0.10	0.18	0.00	2.56
Mechanization[2]	0.08	0.07	0.05	0.01	0.46
Depreciation[3]	0.14	0.13	0.09	0.00	0.52
Salaries and social security contributions	0.13	0.12	0.08	0.00	0.62
Land Rent[4]	0.03	0.02	0.04	0.00	0.26

[1]*Composed of land-related overhead expenses, financial charges, losses, receivables, and other liabilities;*

[2]*Includes costs related to farm machinery, such as fuel and maintenance expenses;*

[3]*Comprises both the depreciation allowances for owned assets and the provisions for liabilities, including the annual allocation for employee severance indemnities;*

[4]*Refers to expenditures for the lease of land, buildings, or other agricultural structures.*

Third-level costs referred to imputed values for internal production factors, specifically unpaid family labor and the notional rent of owned land. Labor costs were estimated using net hourly wage rates from comparable dairy farms, whereas land rent was derived from average regional values per hectare of utilized agricultural area reported in FADN. In accordance with standard managerial accounting practices, opportunity costs for non-land capital were excluded, given their marginal impact under prevailing economic conditions. Depreciation was calculated using the historical cost method to ensure comparability among farms with different investment timelines. For both second- and third-level costs, an allocation index was applied to proportionally assign expenses to buffalo milk production. This index was calculated as the ratio between the gross output from buffalo farming and the total gross output of the farm, thereby ensuring a consistent and economically sound cost allocation framework:

$$Allocation\ index\ (I) = \frac{Total\ gross\ output\ Buffalo}{Total\ farm\ revenue}$$

3.2.3. Statistical Analyses

Descriptive statistics stratified by parity groups were computed for milk composition traits derived from test-day records, cumulative milk yields (Table 3.1), and reproductive traits with the aim of identifying and highlighting potential differences in both productive and reproductive performance.

Table 3.1*. Descriptive statistics of milk yield and composition according to parity groups.*

Parity	Percentage (%)	Traits	Mean	STD	Minimum	Maximum
1	34.6	Total milk yield (kg)	2496	764	229	4933
		Fat (%)	7.98	0.92	3.94	11.8
		Protein (%)	4.69	0.24	3.37	5.88
		SCS (unit)	2.96	1.68	-2.06	10.7
2-3	40.3	Total milk yield (kg)	2735	823	189	5336
		Fat (%)	7.92	0.96	4.05	11.6
		Protein (%)	4.66	0.25	3.46	5.82
		SCS (unit)	3.37	1.70	-2.06	10.7
≥4	25.1	Total milk yield (kg)	2563	826	40.0	5188
		Fat (%)	7.72	1.00	4.06	11.3
		Protein (%)	4.62	0.26	3.65	5.61
		SCS (unit)	3.63	1.74	-2.06	10.7

Based on descriptive statistics, animals were grouped into three parity classes: primiparous (1^{st} parity), mid-parity (2^{nd}–3^{rd} parities), and greater-parity (≥4^{th} parity), reflecting the distinct patterns observed in early lactations and the stabilization of performance from the fourth parity onward. For analytical purposes, DIM was grouped into ten categories of 30 days each, with the first class covering 5–30 DIM and the last one including records beyond 271 DIM. To assess the effects of AFC on test-day traits, separate linear mixed models with repeated measures were implemented for each parity group using Proc GLIMMIX in SAS 9.4 (SAS Institute Inc., Cary, NC, USA):

$$y_{ijklmn} = \mu + AFC_i + DIM_j + Month_k + Year_l + (AFC \times DIM)_{ij} + (AFC \times Month)_{ik} + (DIM \times Month)_{jk} + Animal\_ID_m + HTD_n + e_{ijklmn},$$

where μ is the population mean for daily milk yield, fat and protein contents, and SCS; AFC_i, DIM_j, $Month_k$, and $Year_l$ represent the fixed effects of AFC (8 classes), DIM (10 classes), calving month and calving year (from 2013 to 2023) respectively; $Animal\_ID_n$ and HTD_o (herd-test-day) were included as independent random effects, assuming normal distribution with mean zero and variance σ^2_o. Only the significant two-way interactions were retained in the final model: *AFC×DIM*, *AFC×Month*, and *DIM×Month.* Specifically, for each analyzed trait, separate models were applied to primiparous buffaloes, to those in their second or third parity, and to animals in their fourth parity or beyond. This stratified modeling approach enabled a more precise and biologically relevant assessment of AFC effects within each parity group, while avoiding overly complex interaction terms and improving the overall interpretability of the results. Normality and homoscedasticity of residuals were assessed using visual diagnostics and the Shapiro-Wilk test. An autoregressive covariance structure was specified in the repeated statement using the TYPE=AR(1) option of SAS. This structure was selected as it yielded the lowest Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) values among the tested models. Moreover, proc GLMSELECT in SAS has been used to check for the absence of collinearity among fixed effects.

A similar approach was applied to cumulative yields for milk, fat, and protein, and reproductive traits evaluated separately within the same parity groups. For these traits, the model excluded DIM and related interactions:

$$y_{ijkl} = \mu + AFC_i + \mathrm{Month}_j + Year_k + (AFC \times Month)_{ij} + Herd_l + e_{ijkl},$$

where μ represent the population mean for cumulative yields for milk, fat and protein, CI_1, $CI_{2\text{-}3}$, $CI_{\geq 4}$, DO_1, $DO_{2\text{-}3}$, $DO_{\geq 4}$: CI_1 and DO_1 refer to reproductive performance between the first and second lactation; $CI_{2–3}$ and $DO_{2–3}$ correspond to the intervals between the second–third and third–fourth lactations; while $CI_{\geq 4}$ and $DO_{\geq 4}$ indicate performance from the fourth to fifth lactation and beyond; AFC_i, $Month_k$, and $Year_l$ represent the fixed effects of AFC (8 classes), calving month and calving year (from 2013 to 2023); $Herd_l$ was modeled as a random effect only for reproductive and cumulative yields of first parity order model. In contrast, random effect of animal nested within herd was included to account for repeated measures within animals from the same herd in mid- and ≥4 parity groups. Due to their right skewed distribution, generalized linear mixed models with gamma distribution and log-link function were used for all reproductive traits (Diana et al., 2021). Results are reported as least squares mean ± standard error, and multiple comparisons were adjusted using the Bonferroni *post-hoc* correction ($P<0.05$).

To evaluate the economic impact of AFC on farm profitability, the cost structure described above was integrated with model-based estimates of milk yield per lactation. The analysis was stratified by AFC classes and parity group to capture differences in productive and economic performance. Revenues were calculated by multiplying the predicted lactation total milk yield by the average market price of buffalo milk. As historical farm-gate prices for the full ten-year period were not available, the current average price (€1.50/L;(CLAL, 2025) was applied uniformly across all scenarios. This approach allowed us to simulate the economic returns of the observed production patterns under present market conditions, providing results that are both directly interpretable and relevant to contemporary decision-making. While this method does not account for historical price fluctuations, it offers a standardized framework for comparing AFC classes under the same economic assumptions, thereby isolating the effect of AFC on profitability from confounding variations in milk prices over time. Net profit for each AFC class was obtained by subtracting the estimated production costs from the calculated revenues, enabling an AFC-specific assessment of economic return for the sampled farms.

3.3. RESULTS

3.3.1. Descriptive statistics.

Buffaloes, in their second and third parity produced the highest cumulative milk yield, averaging 2,735 kg, outperforming all other parity groups (Table 3.1). In contrast, first-parity buffaloes exhibited the highest milk quality, with fat and protein contents of 7.98% and 4.69%, respectively. Somatic cell score increased progressively with parity, rising from 2.96 in primiparous animals to 3.63 in those beyond the fourth parity. Table 3.2 presents the descriptive statistics of reproductive traits across parities. The greatest CI and DO were recorded in first-parity buffaloes, averaging 452 and 142 days, respectively. These values decreased progressively with the advancing parity. Buffaloes in their second and third parity exhibited moderate reproductive intervals (CI_{2-3}: 424; DO_{2-3}:114 days), which decreased in animals in their fourth-or-greater parity ($CI_{\geq 4}$: 415; $DO_{\geq 4}$: 105 days).

Table 3.2. *Descriptive statistics of reproductive intervals[1] (days) according to parity groups[2].*

Variables	Mean	STD	Median	Q1	Q3
DO_1	142	74.1	126	78.0	197
DO_{2-3}	114	62.0	94.1	63.2	155
$DO_{\geq4}$	105	57.3	88.0	60.1	142
CI_1	452	74.1	436	388	507
CI_{2-3}	424	62.0	404	373	464
$CI_{\geq4}$	415	57.1	398	371	453

[1] DO_1 = average days open between the second and the first calvings; DO_{2-3} = average days open between the second and third, and between the third and fourth calvings; $DO_{\geq4}$ = average days open calculated across all calvings from the fourth onward; CI_1 = average interval between the second and the first calvings; CI_{2-3} = average interval between the second and third, and between the third and fourth calvings; $CI_{\geq4}$ = average interval calculated across all calvings from the fourth onward;

[2] CI_1 and DO_1 descriptive statistics were calculated based on 9.599 animals; CI_{2-3} and DO_{2-3} descriptive statistics were calculated based on 11.181 animals; $CI_{\geq4}$ and $CI_{\geq4}$ descriptive statistics were calculated based on 6.964 animals.

3.3.2. Effect of AFC on milk production and composition traits.

Supplementary Table 3.3 showed the significance values for productive traits.

Supplementary Table 3.3. *P-values of minimum selected models explaining the observed variation in daily milk yield (kg), composition traits (%), and somatic cell score (SCS) based on individual test-day records (n = 576,028), with analyses performed separately for each parity group[1].*

Trait	Parity Group[1]	AFC class[2]	DIM class[3]	Calving month	Calving year	AFC class[2]*DIM class[2]	AFC class[2]*Calving month	DIM class[3]*Calving month
Daily milk yield, kg/d	1	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	2-3	<0.001	<0.001	<0.001	<0.001	0.007	<0.001	<0.001
	≥4	<0.001	<0.001	<0.001	<0.001	0.075	<0.001	<0.001
Fat, %	1	0.050	<0.001	0.024	<0.001	0.006	0.006	<0.001
	2-3	0.063	<0.001	<0.001	<0.001	0.245	0.073	<0.001
	≥4	0.073	<0.001	0.849	<0.001	<0.001	<0.001	<0.001
Protein, %	1	0.550	<0.001	0.013	<0.001	0.058	<0.001	<0.001
	2-3	<0.001	<0.001	<0.001	<0.001	0.913	<0.001	<0.001
	≥4	<0.001	<0.001	<0.001	<0.001	0.097	<0.001	<0.001
SCS	1	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	2-3	<0.001	<0.001	<0.001	<0.001	0.011	<0.001	<0.001
	≥4	<0.001	<0.001	<0.001	<0.001	0.111	<0.001	0.545

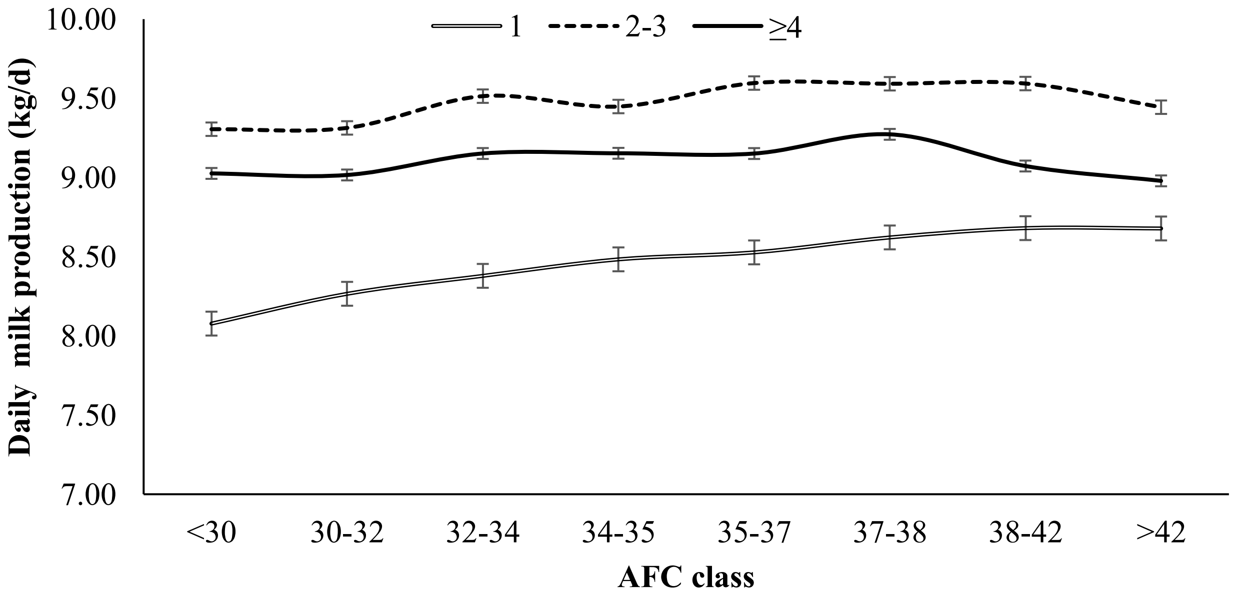
[1]Parity group 1 included 62,394 test-day records from 9,599 lactations; parity group 2-3 included 196,227 test-day records from 22,362 lactations; parity ≥4 included 317,407 test-day records from 56,659 lactations;

[2]AFC classes: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;

[3]DIM classes: Days in milk were grouped into ten categories of 30 days each, with the first class covering 5–30 DIM and the last one including records beyond 271 DIM.

Age at first calving class significantly affected all the analyzed traits (*P<0.05*), except protein content in primiparous buffaloes (*P=0.550*) and fat content (*P>0.05*). The association between AFC groups and productive traits is illustrated in Supplementary Figures 3.2 to 3.5. Multiparous buffaloes (Parity group 2-3 and ≥4) that calved between 35 and 38 mo exhibited the highest daily milk yield (Supplementary Figure 3.2; *P<0.05*), whereas in primiparous buffaloes, daily milk yield increased progressively with AFC (*P<0.05*).

Supplementary Figure 3.2. *Least squares means of daily milk yield according to age at first calving classes (AFC)[1] across parity groups[2].*



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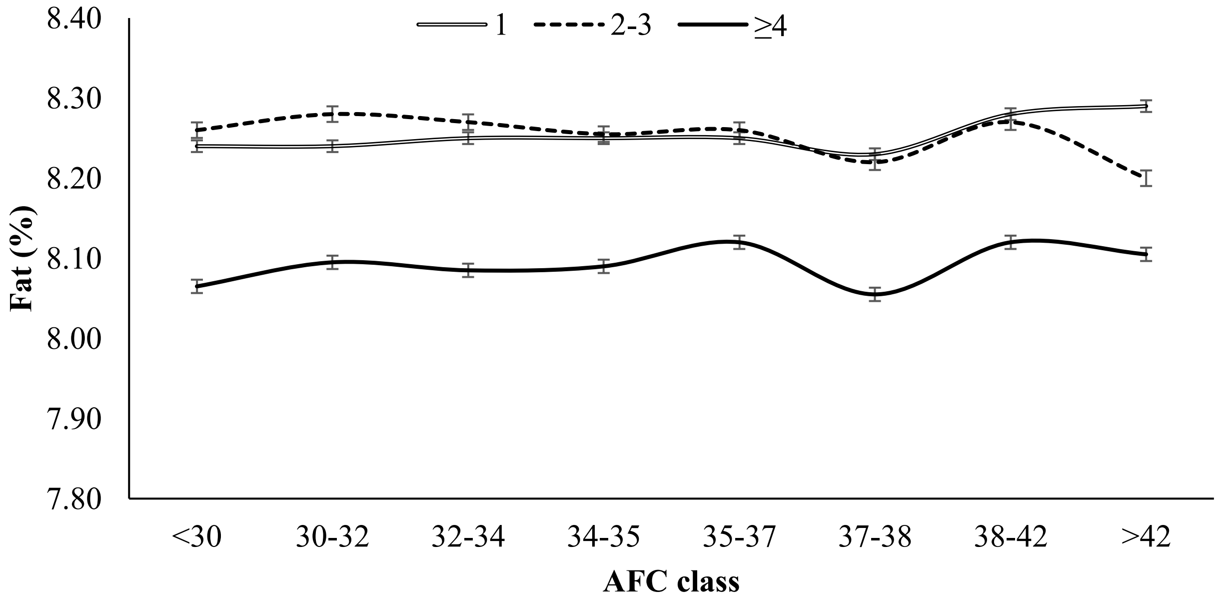
[2]Parity group 1 included 62,394 test-day records from 9,599 lactations; parity group 2-3 included 196,227 test-day records from 22,362 lactations; parity ≥4 included 317,407 test-day records from 56,659 lactations.

Conversely, multiparous buffaloes (Parity order 2-3 and ≥4) that calved either too early (<30 mo) or too late (>38-42 mo) showed a significant reduction in daily milk yield within each parity group (*P<0.05*). Although fat content was not significantly affected by AFC class in all parity groups

($P>0.05$; Supplementary Figure 3.3), protein content showed a clear dependence on both parity group and AFC class ($P<0.05$; Supplementary Figure 3.4).

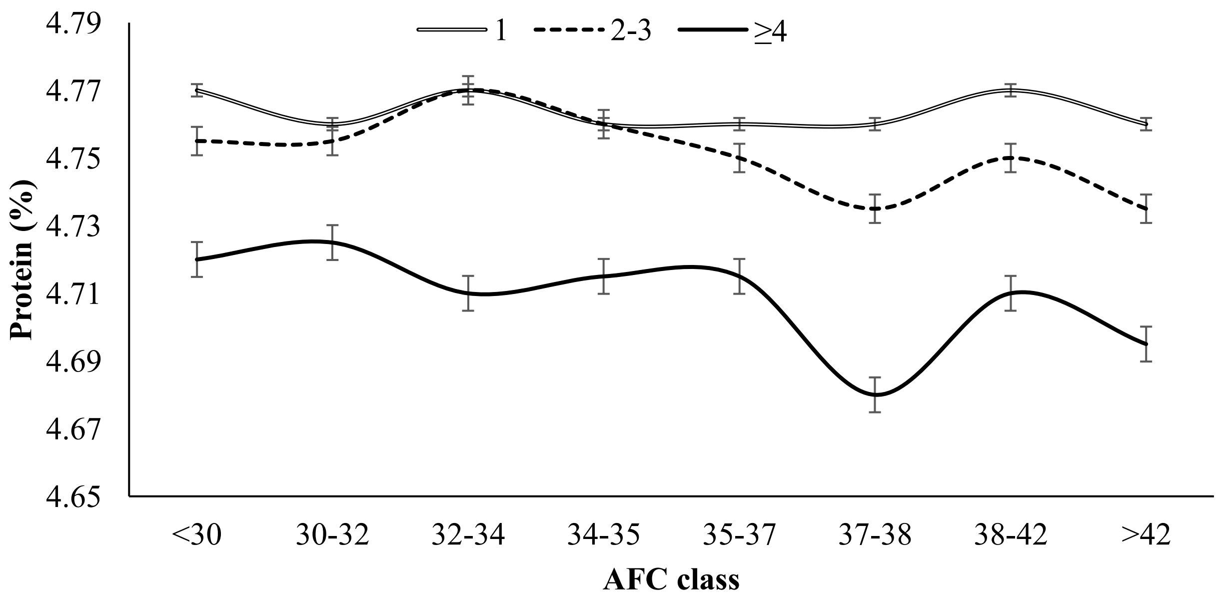
Supplementary Figure 3.3. *Least squares means of fat content according to age at first calving classes (AFC)[1] across parity groups[2].*



[1]AFC classes: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;

[2]Parity group 1 included 62,394 test-day records from 9,599 lactations; parity group 2-3 included 196,227 test-day records from 22,362 lactations; parity ≥4 included 317,407 test-day records from 56,659 lactations.

Supplementary Figure 3.4. *Least squares means of protein content according to age at first calving classes (AFC[1]) across parity groups[2].*

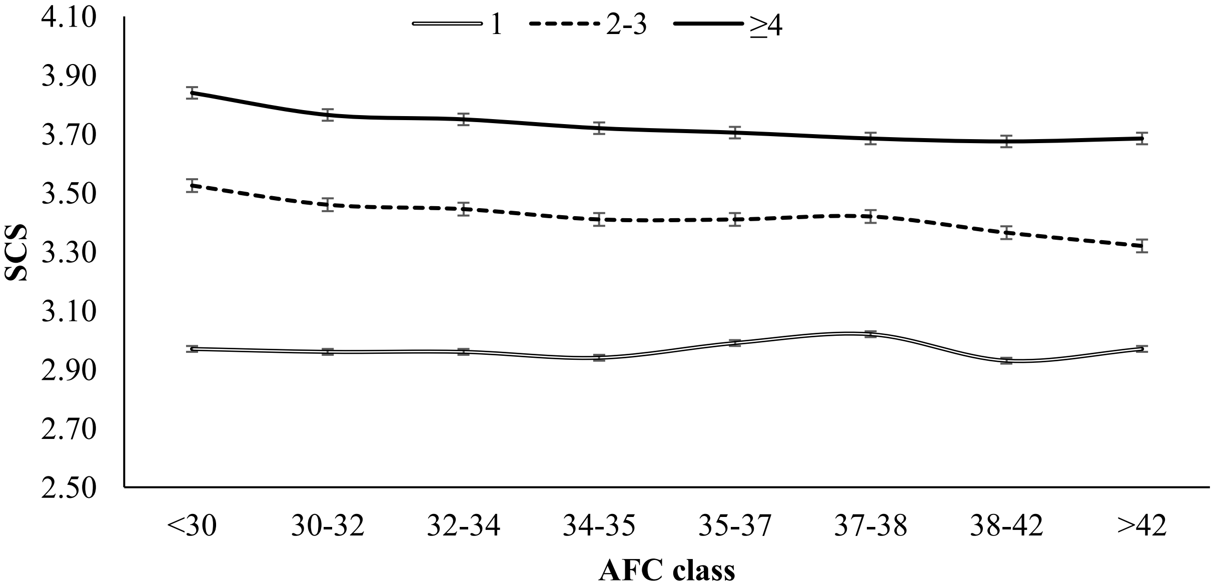


[1]*AFC classes: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;*

[2]*Parity group 1 included 62,394 test-day records from 9,599 lactations; parity group 2-3 included 196,227 test-day records from 22,362 lactations; parity ≥4 included 317,407 test-day records from 56,659 lactations.*

The highest protein percentages were observed in first-, second- and third-parity buffaloes calving at an AFC between 32 and 34 mo, however primiparous buffaloes maintained stable protein content across AFC classes. Whereas multiparous buffaloes (2-3 and ≥4 parity groups) that calved at AFC >37 mo had significantly lower protein content compared with those calving earlier (<37 mo). The effect of AFC on SCS is shown in Supplementary Figure 3.5. Regardless of AFC, SCS increased consistently as parity order increased (*P<0.05*). Moreover, SCS significantly decreased as AFC increased across all parity groups (*P<0.05*), except in primiparous buffaloes, where a temporary increase in SCS was observed for buffaloes calving with an AFC between 37–38 mo (*P<0.05*).

Supplementary Figure 3.5. *Least squares means of somatic cell score (SCS) according to age at first calving classes (AFC[1]) across parity groups[2].*



[1]*AFC classes: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;*

[2]*Parity group 1 included 62,394 test-day records from 9,599 lactations; parity group 2-3 included 196,227 test-day records from 22,362 lactations; parity ≥4 included 317,407 test-day records from 56,659 lactations.*

3.3.3. Effect of AFC on reproductive traits.

Supplementary Table 3.4 showed the significance of fixed effect of minimum selected models for reproductive traits. AFC class significantly explains the variability of all the analyzed traits (*P<0.05*).

Supplementary Table 3.4. *P-values of minimum selected models explaining the observed variation in calving interval (CI, days) and days open (DO, days), with analyses performed separately for each parity group.*

Trait[1*]	AFC class[2]	Calving month	Calving year	AFC class[2]*Calving month
CI_1	0.004	<0.001	<0.001	0.006
CI_{2-3}	0.019	0.074	<0.001	0.187
$CI_{\geq 4}$	0.002	0.019	<0.001	0.186
DO_1	0.022	<0.001	<0.001	0.004
DO_{2-3}	0.005	0.037	<0.001	0.149
$DO_{\geq 4}$	0.039	0.011	<0.001	0.107

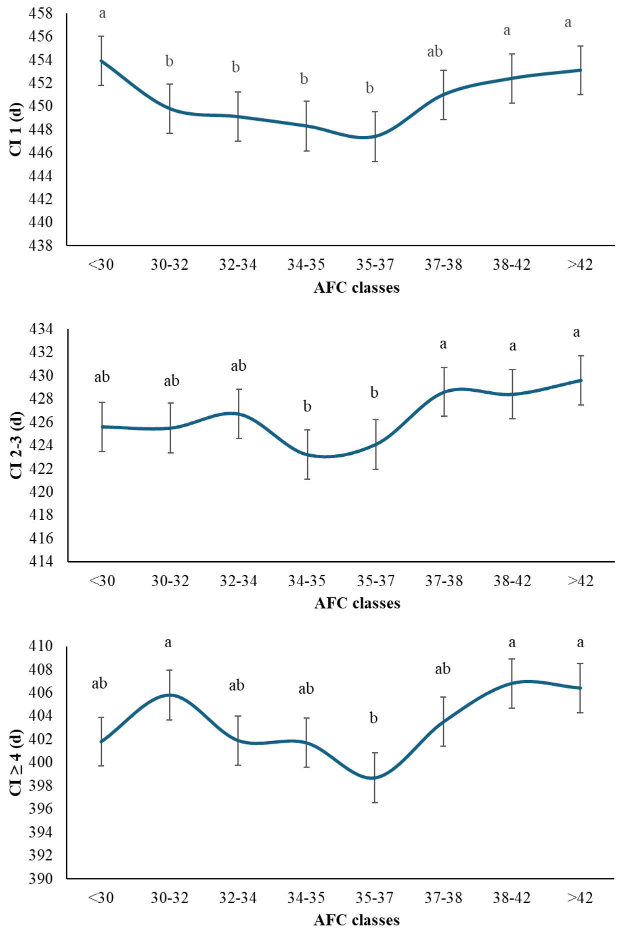
[1]*Traits: CI_1 and DO_1 refer to reproductive performance between the first and second calvings; CI_{2-3} and DO_{2-3} correspond to the intervals between the second–third and third–fourth calvings; while $CI_{\geq 4}$ and $DO_{\geq 4}$ indicate performance from the fourth calvings and beyond;*

[*]*CI_1 and DO_1 were based on 9,599 buffaloes, each contributing one calving; CI_{2-3} and DO_{2-3} were based on 11,181 buffaloes contributing a total of 22,362 calvings; $CI_{\geq 4}$ and $DO_{\geq 4}$ were based on 6,964 buffaloes contributing 56,659 calvings;*

[2]*AFC classes: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 months; and eight: >42 mo.*

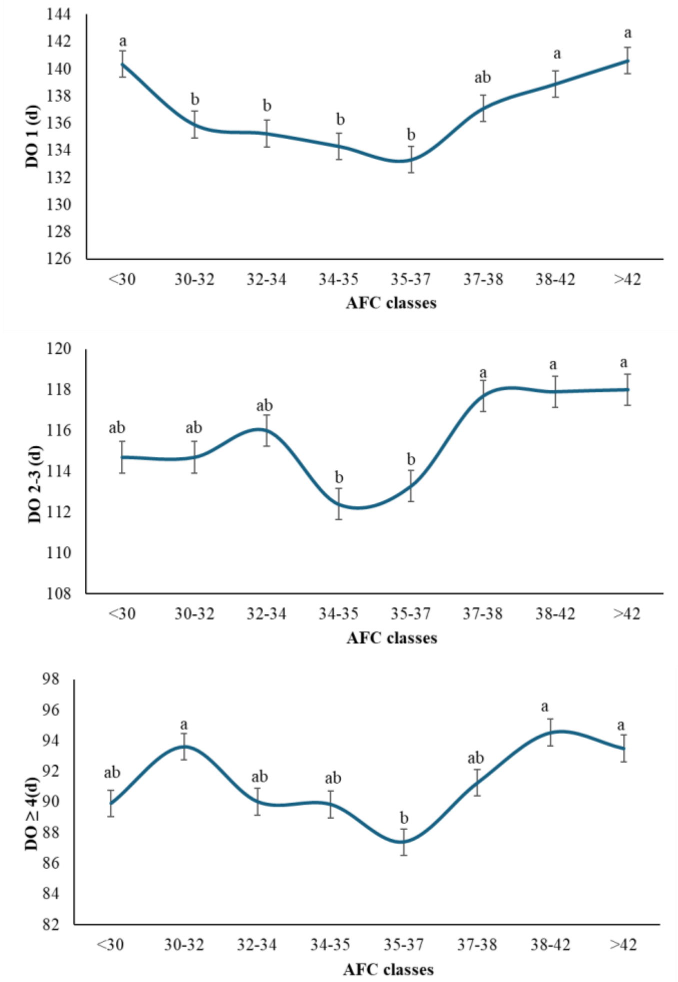
Figures 3.1 and 3.2 illustrate the relationship between AFC and reproductive intervals. The most favorable reproductive intervals, shorter CI and DO, were observed in buffaloes with AFC between 32 and 37 mo (*P<0.05*). Within this range, primiparous buffaloes recorded a CI_1 of 447 days and DO_1 of 133 days. Second- and third-parity animals had a CI_{2-3} of 424 days and DO_{2-3} of 113 days, while those in parity order ≥4 showed the shortest reproductive intervals (*P<0.05*; $CI_{\geq 4}$: 399 days; $DO_{\geq 4}$: 87 days). In contrast, buffaloes calving at either younger or older AFC exhibited significantly extended reproductive intervals in all parity groups (*P<0.05*).

Figure 3.1. *Least squares means of calving intervals[1*] (days) in relation to age at first calving classes (AFC)[2].*



[1]CI_1 represents to the calving interval between the first and second calvings; $CI_{2\text{–}3}$ represents the calving intervals between the second and third, and third and fourth calvings; $CI_{\geq 4}$ represents the calving intervals for all calvings beyond the fourth;
[*]CI_1 estimates were based 9,599 buffaloes, each contributing one calving; $CI_{2\text{-}3}$ estimates were based on 11,181 buffaloes contributing a total of 22,362 calvings; $CI_{\geq 4}$ estimates were based on 6,964 buffaloes contributing 56,659 calvings;
[2]AFC classes: age at first calving (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo.

Figure 3.2 *Least squares means of days open[1*] (days) in relation to classes AFC[2].*



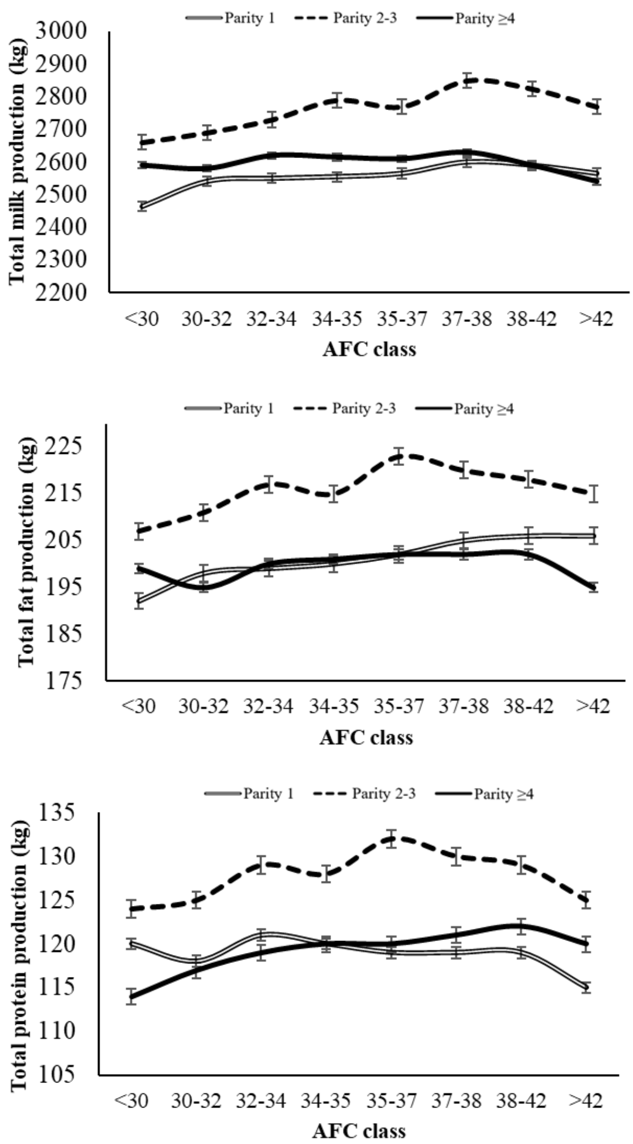
[1]DO_1 represents to the days open between the first and second calvings; $DO_{2\text{–}3}$ represents the days open between the second and third, and third and fourth calvings; $DO_{\geq 4}$ represents the days open for all calvings beyond the fourth;

**DO_1 estimates were calculated based on 9.599 animals; $DO_{2\text{-}3}$ estimates were calculated based on 11.181 animals; $DO_{\geq 4}$ estimates were calculated based on 6.964 animals;*

[2]AFC classes: (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo.

Cumulative yields of milk, fat, and protein across AFC classes and parity groups are presented in Figure 3.3. Buffaloes with an intermediate AFC, particularly between 34 and 37 mo, consistently achieved the highest cumulative production across all traits (*P<0.05*). This effect was most pronounced in second- and third-parity animals, which recorded peak values of approximately 2,850 kg of milk, 225 kg of fat, and 130 kg of protein within this AFC range (*P<0.05*). In contrast, both primiparous and multiparous buffaloes outside this range exhibited lower cumulative yields (*P<0.05*). Overall, the lowest production was observed in animals with an AFC below 30 or above 38 mo.

Figure 3.3 *Least squares means of cumulative milk, protein and fat yields (kg) according to classes AFC[1] and parity groups[2].*



[1]*AFC classes: (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo.*

[2]*Parity group $_1$ included 9.599 animals; Parity group $_{2\text{-}3}$ included 11.181 animals; Parity group $_{\geq 4}$ included 6.964 animals.*

3.3.4. Effect of AFC on economic return

The analysis showed that the average first-level cost amounted to €0.72/L of milk, with a median of €0.73/L (Supplementary Table 3.1). The allocation index, used to apportion second and third-level costs to buffalo milk, averaged 0.95 ± 0.09, highlighting the predominant contribution of buffalo milk to overall farm revenues. Based on this allocation, second-level costs averaged €0.51/L (median of €0.49/L), while third-level costs averaged €0.15/L (median: €0.13/L). The cost structure underlying these results was characterized by first-level direct costs representing 52.2% of the total milk production cost, second-level general overheads accounting for 37.0%, and third-level imputed costs contributing the remaining 10.9%. Summing, the average values from all three cost levels resulted in an estimated total production cost of €1.38/L of buffalo milk. Across the entire dataset, the mean net return was +314 € per lactation (Table 3.3).

Supplementary Table 3.1 *Summary of first-level variable costs (€/L of milk) in buffalo milk production retrieved from farm accountancy data network (FADN).*

Items	Mean	Median	STD	Minimum	Maximum
Feedstuff, forage and bedding materials	0.40	0.37	0.30	0.00	1.46
Energy and water	0.04	0.03	0.02	0.00	0.12
Medication and veterinary expenses	0.02	0.02	0.03	0.00	0.59
Replacements	0.22	0.21	0.11	0.01	0.63
Other variable costs	0.03	0.01	0.06	0.00	0.52

Table 3.3. *Economic performance of buffalo milk yield by classes AFC$_1$ and parity groups. For each AFC class[1], the table shows average total milk yield per lactation (L)[2], total production cost (€)[3], total revenue (€)[4], and net profit (€)[5], calculated using a fixed milk price of €1.50/L and an average production cost of €1.38/L.*

Parity	N.	AFC class[1]	Total milk yield[2]	Average total costs[3]	Average total Revenue[4]	Net profit[5]	95% CI
1	9.599	<30	2364	3262	3546	284[b]	[279-289]
		30-32	2480	3422	3720	298[ab]	[291-303]
		32-34	2475	3416	3713	297[ab]	[292-302]
		34-35	2508	3461	3762	301[a]	[296-306]
		35-37	2502	3453	3753	300[a]	[295-305]
		37-38	2546	3513	3819	306[a]	[300-311]
		38-42	2589	3573	3884	311[a]	[306-316]
		>42	2478	3420	3717	297[ab]	[292-303]
2-3	11.181	<30	2741	3783	4112	329[b]	[324-334]
		30-32	2742	3784	4113	329[b]	[324-334]
		32-34	2760	3809	4140	331[ab]	[326-336]
		34-35	2780	3836	4170	334[a]	[328-339]
		35-37	2779	3835	4169	333[a]	[328-339]
		37-38	2822	3894	4233	339[a]	[333-344]
		38-42	2793	3854	4190	335[a]	[330-340]
		>42	2682	3701	4023	322[b]	[317-327]
≥4	6.964	<30	2616	3610	3924	314[a]	[307-321]
		30-32	2615	3609	3923	314[a]	[307-320]
		32-34	2602	3591	3903	312[ab]	[306-319]
		34-35	2640	3643	3960	317[a]	[310-323]
		35-37	2596	3582	3894	312[ab]	[305-318]
		37-38	2614	3607	3921	314[a]	[307-320]
		38-42	2626	3624	3939	315[a]	[309-322]
		>42	2467	3404	3701	296[b]	[289-303]

[1]*AFC classes: (mo) was categorized into 8 categories: first: ≤30 mo; second: 30-32 mo; third: 32-34 mo; fourth: 34-35 mo; fifth: 35-37 mo; sixth: 37-38 mo; seventh: 38-42 mo; and eight: >42 mo;*

[2]*Total milk yield: Cumulative individual milk yield;*

[3]*Average total costs: calculated by multiplying predicted milk yields by the production cost of €1.38/L, including variable, overhead, and imputed costs, allocated using FADN data;*

[4]*Average total revenue: revenue per entire lactation, calculated by multiplying predicted milk yields by the average milk market price (€1.50/L);*

[5]*Net profit: difference between average total revenue and average total costs;*

[6] *95% CI: 95% confidence intervals.*

[a–b]*Net profit values with different superscript letters within parity group differ significantly according to Bonferroni post-hoc multiple comparison adjustment (P<0.05).*

When stratified by AFC class and parity group, the highest average net returns were consistently associated with an AFC comprised between 34–42-mo. Among primiparous buffaloes, those calving for the first time between 37 and 42 mo achieved the greatest returns (+302 €), while in second- and third-parity animals, the optimal range remained 34–42 mo range, with an average net return of +315. Multiparous buffaloes (parity group ≥ 4) within this same AFC range achieved +€335, matching the overall dataset average.

3.4. Discussions

Milk yield peaked between second and third parity (2,735 kg), confirming this physiological stage as the apex of production performance in buffaloes (Costa et al., 2020). In contrast, the highest fat (7.98 %) and protein (4.69 %) contents were recorded in primiparous animals, with values progressively declining in subsequent parity groups, likely reflecting the increasing metabolic demands associated with higher milk output. Concurrently, SCS rose from 2.96 in primiparous to 3.63 in older animals, indicating a gradual deterioration of udder health and cumulative stress on mammary tissue with advancing parity (Moroni et al., 2006; Matera et al., 2025), a pattern also reported in dairy cattle (Dürr et al., 2008; Fonseca et al., 2024). These results support the classification of animals into three biologically coherent parity groups. Primiparous buffaloes, still completing somatic growth and experiencing their first lactation, face unique metabolic challenges, including a heightened risk of negative energy balance (Campanile et al., 2010). Second- and third-parity animals generally achieve the most favorable balance between productivity and physiological stability, whereas buffaloes beyond the third parity showed clear signs of functional decline, characterized by reduced milk quality and diminished udder resilience.

3.4.1. Effect of AFC on milk production and composition traits

In the present study, daily milk yield increased with advancing AFC, corroborating previous findings in primiparous buffaloes (Rautela et al., 2024; Calanni Macchio et al., 2025). This trend likely reflects the greater physiological maturity and more advanced mammary gland development achieved at calving by animals with greater AFC (Macias and Hinck, 2012; Challana et al., 2014). However, the influence of AFC on milk yield diminished in later parities, as also reported by Zicarelli et al. (2007) in a cohort of 953 Italian Mediterranean buffaloes. This attenuation may be explained by the predominant effect of AFC during first lactation, when insufficient BW or suboptimal body conditions at calving can exacerbate negative energy balance and reduce milk yield potential. In agreement with previous studies in both buffaloes and dairy cows (Catillo et al., 2002; Bilal et al., 2014), fat content was largely unaffected by AFC across parities. This stability is consistent with the tight physiological regulation of milk fat synthesis, which depends more on energy balance than on developmental stage or parity order (Bauman and Griinari, 2003; Jenkins et al., 2008). Nonetheless, in primiparous buffaloes, a slight numerical increase in fat content was associated with greater AFC, as also observed by Calanni Macchio et al. (2025). This effect may be linked to improved body reserves and greater metabolic readiness at the onset of lactation in animals calving later. In contrast, protein content showed a slight but consistent decline (~0.02–0.03%) in buffaloes with AFC above 37 mo, across all parities except for primiparous. While seemingly modest, such reduction can negatively affect cheese yield by impairing caseification efficiency (Fasale et al., 2017; Sales et al., 2020). This is particularly relevant in the Italian context, where buffalo milk is primarily destined for Mozzarella production (Catillo et al., 2002; Rosati and Van Vleck, 2002). Furthermore, SCS significantly decreased as AFC increased, except in primiparous which had higher SCS when calving between 37-38 mo. Previous studies have reported that greater AFC may be associated with increased SCS and greater mastitis susceptibility in later lactations, potentially compromising lifetime udder function and productivity (Costa et al., 2019). Our findings indicate also that anticipating the AFC may have long-term negative effects on udder health in subsequent lactations, suggesting that excessively early calving could predispose buffaloes to higher SCS and mastitis risk later in life. Conversely, when AFC is excessively delayed, primiparous animals may experience suboptimal udder health and increase susceptibility to disorders. Overall, these results highlight the need to identify an optimal AFC range that balances early-lactation performance with long-term udder health and herd productivity.

3.4.2. Effect of AFC on reproductive traits, cumulative milk yields

Although a lower AFC is often targeted to reduce rearing costs, and delayed calving may result from poor management or biological constraints, both extremes are associated with reduced longevity and an increased risk of premature culling, largely due to suboptimal milk yield and impaired reproductive performance (Galeazzi et al., 2010; Fodor et al., 2019). In dairy cattle, where physiological maturity is reached earlier than in buffaloes (around 24–25 mo), excessively early calving has been associated with a higher incidence of fertility disorders and involuntary culling (Wathes et al., 2008). Conversely, achieving an optimal AFC can improve lifetime productivity and lower replacement rates, both of which are essential for sustainable buffalo farming (Aspilcueta-Borquis et al., 2022; Gomez-Carpio et al., 2023, 2025). In this study, buffaloes with an AFC between 32 and 37 mo exhibited the most favorable reproductive performance, with significantly shorter CI and DO, corroborating previous findings in primiparous animals (Calanni Macchio et al., 2025). The greater total yields of milk, fat, and protein recorded in this AFC class may be partly attributable to the shorter DO, as a reduced interval between calving and conception increases the proportion of time spent in lactation within a given time frame. Thus, while greater AFC within this optimal range appears to support both improved reproductive efficiency and greater lactation yields, part of this production advantage may be mediated through its effect on DO, rather than being solely an independent outcome of AFC. This highlights the complex interdependence between reproductive management and productive performance.

3.4.3. Effect of AFC on economic return

The economic analysis showed that all AFC classes generated positive net profits, confirming the overall economic efficiency of buffalo farming under current production and market conditions. However, a clear pattern emerged: intermediate AFC values, particularly between 34 and 42 mo, were consistently associated with the highest net returns, especially in second- and third-parity animals. These findings support the view that a moderate delay in first calving enhances physiological maturity and productive performance, thereby improving the cost-effectiveness of rearing investments. Recent large-scale evidence on more than 50,000 Italian Mediterranean buffaloes further confirmed that calving at 34-37 mo provides the most advantageous combination of shorter CI, lower SCS, and sustained daily milk yields, ultimately boosting farm profitability (Calanni Macchio et al., 2025). By contrast, both early (<30 mo) and late (>38–42 mo) AFC were associated with reduced productivity, prolonged CI, and higher culling risk, which undermine long-term economic sustainability (Rautela et al., 2024). Although an earlier AFC may appear economically favorable by reducing rearing time and heifer management costs, these savings are often outweighed by lower milk yield, impaired reproductive efficiency and greater metabolic stress in the first lactation when calving occurs before full physiological maturity (Sung et al., 2016).

From a cost-structure perspective, the total production cost of buffalo milk averaged €1.38/L, including direct, indirect, overhead, and imputed components, in line with incremental costing methodologies adopted by CREA (Council for Agricultural Research and Economics) and supported in dairy economics literature (Ghelfi, 2000). Direct costs represented the largest share (52.2%), followed by overheads (37.0%) and imputed costs for family labor and land use (10.9%), highlighting the central role of operational expenditures in determining profitability. This distribution emphasizes that net returns depend not only on biological performance (milk yield, udder health, reproductive efficiency) but also on managerial choices related to feeding strategies, reproduction, and resource allocation. In agreement, previous studies have shown that optimizing both animal performance and feed resource management can substantially increase profitability in buffalo systems (Öztürk et al., 2022). Overall, the evidence indicates that aligning AFC with the physiological development of animals, optimally between 35 and 38 mo, represents a key strategy to enhance both farm profitability and long-term economic resilience in buffalo dairy systems.

However, some limitations should be acknowledged when interpreting these findings. Although the models accounted for major fixed effects and herd variability, residual confounding by management strategies, nutritional plans, and body condition at calving cannot be fully excluded. Differences in farm management intensity, housing systems, and labor organization may also have influenced both biological performance and cost structure. The influence of herd-specific environmental and management factors on dairy cows performance has long been recognized in animal breeding research, highlighting the challenge of disentangling genetic and environmental effects in field data (Weigel et al., 2017). Although the editing procedure helped to control potential confounding factors, selection bias might exist, as heifers reaching first calving at different ages could have followed distinct growth trajectories or been subject to varying culling pressures, factors not entirely captured by the available data. Despite these limitations, the high number of animals outcomes analyzed supports the robustness of the observed relationships between AFC, productivity, and economic outcomes.

3.5. Conclusions

Age at first calving emerges as a strategic variable in the management of buffalo dairy systems, with long-term implications not only for individual animal performance but also for the economic sustainability of the entire herd. The present findings, based on observational evidence, highlight that the goal should not be to minimize AFC at all costs, but rather to identify and target an optimal window that maximizes both biological and economic efficiency. However, given the observational nature of the data, the translation of these results into management practices should be approached with caution and validated under site-specific conditions. In practical terms, herd managers are encouraged to adopt proactive strategies that ensure heifers are physiologically mature at the time of first calving. This includes careful monitoring of growth and body condition, nutritional plans that promote balanced development without compromising mammary gland integrity, and reproductive management based on readiness rather than chronological age alone. Aligning AFC decisions with the animal's actual preparedness and the farm's production goals can help reduce early-life reproductive failures, improve lifetime milk yields, and lower involuntary culling rates. Furthermore, incorporating AFC as a routine decision-making parameter in buffalo herd management and genetic selection programs may significantly enhance farm resilience and profitability. In this context, the results of this study provide a valuable framework for defining AFC targets that balance biological performance with economic sustainability, while underscoring the need for further experimental validation across different production environments.

3.6. NOTES

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Nonstandard abbreviations used: AFC = Age at first calving, CI = calving interval, DO. = days open.

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4. General Discussions

General Discussions

The present thesis demonstrated that AFC exerts an influence on milk yield and composition, fertility traits, and economic sustainability throughout the productive life of Italian Mediterranean buffaloes. By integrating long-term phenotypic data spanning ten years with an economic assessment, this work identified an optimal AFC range that reconciles biological efficiency with reproductive performance and farm profitability. AFC represents a critical management variable in dairy buffaloes because the first calving marks the onset of lactation at a stage when animals may have not yet completed somatic growth, making the first lactation a period of pronounced physiological and metabolic challenge. Unlike multiparous buffaloes, which have already achieved physiological maturity and can allocate energy predominantly to milk synthesis and reproductive recovery (Ettema and Santos, 2004; Atashi et al., 2021), primiparous animals may experience a condition of marked metabolic vulnerability, particularly when body energy reserves at calving are suboptimal, either insufficient or excessive, increasing the risk of severe negative energy balance (Campanile et al., 2010). Within this framework, a key outcome of the present thesis was the identification of an optimal AFC window between 34 and 38 months. Animals calving either earlier or later than this range exhibited lower milk production, higher SCS, and reduced estimated net income compared with buffaloes calving within the optimal AFC interval. Calving before 30–32 months, often pursued to reduce rearing costs, proved counterproductive in terms of long-term profitability, resulting in a reduction in net income ranging from €10 to €27 per lactation compared with the optimal AFC group. Moreover, buffaloes calving the first time at a younger age exhibited greater SCS, suggesting increased susceptibility to mammary inflammation, which may be linked to immune suppression associated with negative energy balance. Conversely, delayed AFC (>38 months) was associated with higher milk yield during the first lactation, reflecting more advanced mammary and somatic development (Macias and Hinck, 2012). However, this advantage was offset by poorer reproductive performance, as evidenced by longer CI and DO, alongside reduced economic returns. In addition, these animals showed higher SCS, potentially due to prolonged exposure to environmental pathogens during extended rearing periods (Eastham et al., 2018). A further contributing mechanism may involve health selection, whereby heifers that calve later may have experienced early-life growth or health constraints that delayed puberty and conception, ultimately reducing their resilience to udder inflammation later in life (Atashi et al., 2021).

These findings indicate that extending the rearing period without targeted management fails to optimize profitability. Therefore, the management objective should shift from achieving a specific chronological age to ensuring that heifers enter the 35–38 month AFC window in a metabolically prepared state. This requires nutritional strategies that promote skeletal growth during the *pre*-weaning and *pre*-pubertal phases, enabling heifers to reach approximately 340–360 kg of body weight at conception (Campanile et al., 2010). At the same time, excessive fat deposition must be avoided, as high BCS can impair mammary development and compromise future fertility (Roche et al., 2009). BCS represents a key integrative indicator of energy balance, influencing reproductive efficiency, oocyte competence, and *post-partum* recovery. In buffaloes, including the Italian Mediterranean breed, an adequate BCS at calving, generally within a moderate-to-high range on a five-point scale, is associated with improved resumption of ovarian cyclicity, higher conception rates, and reduced *post-partum* disorders (Campanile et al., 2010; Saqib et al., 2022). Nutritional management during heifer rearing and the *pre-partum* period is therefore pivotal. Campanile et al. (2010) demonstrated that buffalo heifers receiving high-energy diets achieved significantly higher BCS compared with animals fed restricted-energy diets (4.20 vs. 3.40), alongside superior oocyte developmental competence. Similarly, poor BCS has been associated with increased anestrus (Baruselli et al., 2001) and reduced pregnancy rates (Kumar et al., 2020). Comparable evidence is well established in dairy cattle, where primiparous cows with inadequate BCS or body weight at calving often exhibit heightened inflammatory responses, delayed uterine involution, and impaired resumption of ovarian activity (Mammi et al., 2021). Conversely, maintaining moderate BCS has been linked to greater longevity and stay-ability beyond the third lactation (Buonaiuto et al., 2023) in cows, while excessive BCS predisposes animals to metabolic disorders and calving difficulties due to adiposity-related insulin resistance and hepatic lipid overload.

From a nutritional standpoint, balancing dietary energy and protein is fundamental to sustain growth and metabolic health. In Italian Mediterranean buffaloes, diets providing approximately 13–15% crude protein have been shown to support optimal growth, reproductive performance, and *pre-partum* BCS (Sabia et al., 2015; Fantuz et al., 2024). Italian buffalo production systems, typically based on total mixed rations, generally allow adequate energy–protein balance for growing and pregnant heifers. This contrasts with feeding systems adopted in tropical buffalo populations, such as Murrah or Nili-Ravi, where reliance on low-quality fibrous residues often limits nutrient intake, delays attainment of breeding weight, and results in suboptimal BCS at calving (Sarwar et al., 2002). For this reason, these breeds typically reach puberty later than Mediterranean buffaloes, resulting in an AFC that is often higher than that observed in Italian production systems (Rautela et al., 2024). These differences highlight the strong interaction between environmental conditions, management practices, and nutritional strategies in shaping reproductive outcomes. Overall, controlled feeding approaches that promote gradual growth, adequate protein supply, and avoidance of excessive fat deposition appear to enhance reproductive longevity and lifetime productivity (Barile, 2005; Sarwar et al., 2009), in line with the concept of metabolic programming.

4.1. Reproductive Efficiency and Seasonality

In Italian buffalo herds, reproductive physiology is inherently asynchronous with market demand. Fertility naturally peaks during the short photoperiod of autumn and winter in Italian Mediterranean buffaloes (D'Occhio et al., 2020), whereas milk demand driven by mozzarella production is highest in spring and summer (Otava et al., 2021). As previously introduced, to solve this mismatch, farmers commonly adopt out-of-breeding season mating strategies to shift calving toward late winter and spring (Baruselli et al., 2003; D'Occhio et al., 2020), when milk is required. Although effective in aligning milk supply with market needs, these strategies often result in extended CI and increased AFC. Importantly, AFC is not determined by a single event but reflects the cumulative outcome of management decisions applied throughout the *pre*-pubertal, breeding, and *pre-partum* phases. In primiparous buffaloes, synchronizing all favorable conditions, adequate growth rate, timely onset of puberty, appropriate timing of first insemination, and optimal BCS at calving, is particularly challenging. Any imbalance along this trajectory may compromise reproductive efficiency during the first and subsequent lactations. Within this context, the interaction between AFC and seasonality further complicates reproductive performance at the individual-animal level. Our analysis showed that young primiparous buffaloes (<30 months) experienced prolonged reproductive intervals in subsequent lactations. This outcome reflects the combined effect of management-driven voluntary waiting periods and biological constraints. While delayed rebreeding is often intentionally applied to concentrate calving from late winter onward, young primiparous animals are also characterized by limited metabolic reserves and incomplete somatic maturity. As a consequence, these animals exhibit a reduced capacity to counteract the inhibitory effects of increasing day length on ovarian activity. Compared with mature buffaloes, primiparous animals facing the simultaneous demands of residual growth and lactation are more susceptible to photoperiod-driven reproductive suppression (Campanile et al., 2009; Salzano et al., 2019), leading to a disproportionate prolongation of reproductive intervals and increased issues related to the seasonal breeding rhythm required for market-oriented milk production (Dash et al., 2016). Therefore, longitudinal management is essential to prevent reproductive delays not only in the first lactation, but also across subsequent productive lactations, thereby enhancing the long-term efficiency and sustainability of primiparous buffaloes.

4.2. Economic Sustainability and Cost Structure

Economic sustainability in buffalo dairy systems cannot be interpreted as the simple outcome of shortening the rearing phase or maximizing biological output in early lactation. Rather, profitability emerges from the interaction between biological efficiency, market structure, and farm-specific constraints. Although reducing AFC theoretically lowers non-productive rearing costs, the present analysis demonstrates that net economic margins are highly sensitive to milk pricing schemes and feeding strategies, which mediate how biological performance is converted into income. In the Italian buffalo sector, milk payment systems are predominantly based on either flat-rate pricing or dual-pricing models with seasonal premiums according to the dairy. Flat-rate schemes reduce the economic pressure to manipulate reproductive seasonality, allowing management decisions to prioritize biological robustness and reproductive recovery. In contrast, dual-pricing systems reward milk delivered during spring and summer, thereby incentivizing producers to concentrate calving and peak lactation in biologically suboptimal periods characterized by long photoperiods and elevated thermal load. Achieving this temporal alignment requires intensified reproductive interventions, increased veterinary input, and stricter nutritional management, all of which raise variable costs and amplify biological risk. Under these conditions, the economic advantage of higher milk prices may be partially or fully offset by reduced conception rates, increased DO, and higher involuntary culling. The narrow gap between production costs and farm-gate prices further constrains the economic resilience of buffalo farms. With an average milk production cost of €1.38/L and market prices typically ranging from €1.50 to €1.80/L depending on quality and seasonality, profitability remains highly vulnerable to inefficiencies. Farms that fail to synchronize production with favorable market windows may be forced to sell milk at spot prices close to or below break-even, a vulnerability exacerbated by recent increases in feed and energy costs. In this context, economic sustainability depends less on maximizing short-term output and more on maintaining biological stability and production continuity over multiple lactations.

Despite the use of a standardized milk price (€1.50/L) in the economic model due to the absence of long-term historical price series, the observed patterns were robust. The highest net returns consistently emerged in the intermediate AFC group (34–38 months), with average net profits of approximately €339 per lactation in mid-parity animals. This AFC window appears to represent an economic equilibrium in which rearing costs are sufficiently contained while biological maturity at first calving supports efficient lactation, faster reproductive recovery, and extended productive lifespan. Animals calving earlier incurred higher downstream risks related to metabolic stress and reproductive failure, whereas delayed AFC increased fixed rearing costs without proportional gains in lifetime production. Taken together, these findings indicate that economic optimization in Italian Mediterranean buffalo systems is achieved not by selecting for extreme precocity or maximal first-lactation yield, but by targeting an AFC that aligns physiological maturity with market realities and management capacity. The economically ideal buffalo is therefore one that enters production at an optimal developmental stage and sustains consistent, high-quality milk production across multiple parities, minimizing biological disruption and stabilizing farm income over time (Gomez-Carpio et al., 2023).).

4.3. Future Perspectives: Towards Precision Rearing and Genomics

The practical implications of these findings call for a shift toward precision rearing strategies. Managing replacement stock as a homogeneous group is increasingly inadequate, particularly for primiparous buffaloes exposed to concurrent metabolic and social stressors. Precision livestock farming (PLF) enables individual-level monitoring through continuous, real-time sensor data, supporting timely identification of metabolic imbalance, and reproductive dysfunctions (Matera et al., 2025). In dairy buffaloes, automated tracking of feeding behavior, activity patterns can detect *pre-partum* changes, facilitating targeted interventions and mitigating *peri-partum* disturbances that delay uterine recovery, estrus expression, and subsequent conception (Giaretta et al., 2021). When coupled with AI-based decision support, PLF can further strengthen estrus detection, pregnancy monitoring, and transition management, thereby improving the probability of reaching the target AFC without compromising growth, welfare, or subsequent milk performance (Das et al., 2023). In production systems undergoing progressive intensification, these tools also address the structural limitation of manual individual monitoring, supporting proactive reproductive management, reducing involuntary culling, and improving lifetime productivity through tighter control of AFC trajectories.

Beyond management, genetic and genomic evidence indicates that AFC is a viable selection target in dairy buffaloes. In Murrah, AFC shows low-to-moderate heritability (≈0.13–0.19), consistent with a polygenic architecture and meaningful additive variance despite strong environmental sensitivity (Dos Santos et al., 2024). This supports its integration into multi-trait breeding objectives alongside production traits (e.g., milk and protein yield), where AFC and CI are core indicators of reproductive efficiency. Genomic evaluations have directly assessed single-step genomic BLUP (ssGBLUP) and weighted variants for AFC in Murrah, reporting higher prediction accuracy for genotyped animals compared with pedigree BLUP (e.g., ~0.30 with PBLUP vs ~0.39 with weighted ssGBLUP) and reduced bias/dispersion, implying faster genetic gain for sexual precocity (Dos Santos et al., 2024; Melo et al., 2024). Reaction-norm genomic models further indicate genotype-by-environment interaction for AFC, with heritability increasing in favorable environments (up to ~0.39) and genomic regions associated with robustness across management gradients. Genome-wide scans in Brazilian Murrah and Murrah-derived dairy populations have highlighted candidate regions and pathways linked to AFC and first calving interval, including loci harboring ROCK2, PMVK, ADCY2, MAP2K6, BMP10 and GFPT1 across multiple buffalo autosomes (De Araujo Neto et al., 2020). Although buffalo reference populations and genomic resources remain smaller than in dairy cattle, current evidence supports the operational inclusion of reproductive traits in genomic selection schemes for specialized dairy buffaloes (Shao et al., 2021; Calanni Macchio et al., 2025).

In the Mediterranean Italian buffalo, selection has historically prioritized milk yield and milk components, consistent with the predominance of production traits in buffalo breeding goals (ANASB, 2025). Recent population-wide analyses provided quantitative support for expanding breeding objectives to reproductive efficiency traits. Using ANASB field data, AFC in Italian Mediterranean buffaloes averaged 1,024 days (≈36 months; range 705–1,425 days) with moderate dispersion (CV 13%), whereas CI traits were more variable (CI in primiparous: 473 days; CI in multiparous: 452 days), indicating stronger sensitivity to environmental and managerial factors. Genetic parameter estimates confirmed low heritability for fertility traits (h^2 ≈0.09 for AFC; 0.08 for CI in primiparous; 0.03 for CI in multiparous), and models including a herd (farm) random effect attributed a substantial proportion of the phenotypic variation to herd-level structure, consistent with the influence of reproductive policies such as synchronization and voluntary waiting periods. The lower h^2 in Italian buffalo population may reflect the important role of reproductive management which is a characteristic of the Italian buffalo system. This context is relevant for interpreting genetic trends: the genetic trend for AFC did not show a clear directional pattern, which is coherent with the historical absence of explicit selection on this trait. Importantly, results also indicated that the relationship between fertility and milk production in Italian Mediterranean buffaloes is not exclusively environmental. When milk yield is considered as a correlated trait in a multi-trait framework, genetic correlations between fertility traits and production remain different from zero, supporting a genetic component to the association. Genetic correlations among fertility traits were estimated at ~0.10 between AFC and CI in primiparous buffaloes and ~0.41 between AFC and CI in multiparous buffaloes, while both CI measurements were strongly correlated (~0.92). Together, these estimates suggest that selection targeting AFC is expected to induce correlated responses in CI, and that balanced selection indices are required to avoid unintended shifts in reproductive performance when selection pressure remains concentrated on production traits. In line with this rationale, probably a standardized reproductive index, including an aggregated reproduction index that combines AFC and CI components with equal weights, may accelerate balanced improvement in reproductive efficiency within the Italian Mediterranean buffalo breeding scheme. Overall, integrating precision management with breeding strategies that explicitly account for AFC provides a coherent pathway toward early-maturing and robust buffalo phenotypes. In practice, PLF can reduce the environmental and management noise that currently dominates fertility variation by enabling earlier detection and correction of individual risk trajectories, while genetic/genomic selection can consolidate long-term gains through targeted, index-based improvement of reproductive efficiency without sacrificing the productive traits underpinning the economic value of dairy buffalo systems.

4.4. REFERENCES

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5. Conclusions

Conclusions

This research establishes the AFC as a pivotal management variable in Italian Mediterranean buffalo production, linking it directly to reproductive efficiency, milk performance, and farm profitability. Across large-scale commercial herds, the results consistently identify an optimal AFC range of 34 to 38 months, representing the best compromise between biological maturity and economic return. Within this window, buffaloes achieve higher milk yields, shorter reproductive intervals, and improved udder health, while minimizing the costs and reproductive inefficiencies associated with both premature and delayed calving. Importantly, the findings underscore that the objective in buffalo herd management should not be to minimize AFC indiscriminately, but rather to optimize it, ensuring that first calving occurs when animals are physiologically and metabolically prepared. Achieving this balance depends on integrated management strategies combining nutritional planning, body condition monitoring, and seasonally adapted breeding programs. Improving preweaning and *pre-partum* feeding regimens and refining reproductive technologies to account for seasonal fertility patterns, can support earlier but still biologically appropriate calving. From a broader perspective, AFC should be considered not only a reproductive milestone but also a strategic parameter for herd-level decision-making and genetic selection. Incorporating AFC and related fertility traits into breeding indices, alongside traditional production traits, offers a path toward faster genetic progress, improved longevity, and enhanced resilience of buffalo dairy systems. While the conclusions are based on observational data, their practical implications are clear: aligning first calving with the animal's physiological readiness rather than chronological age can reduce reproductive failures, improve lifetime productivity, and strengthen farm profitability. Future research should focus on experimental validation of these findings under diverse production environments and on the development of precision management tools to guide AFC-related decisions in real time.