Received: 5 August 2021 | Accepted: 2 September 2021

DOI: 10.1111/rda.14013

ORIGINAL ARTICLE

Reproduction in Domestic Animals WILEY

Effects of age and season on conception rate of Mediterranean Italian Dairy Buffalo (*Bubalus bubalis*) following oestrus synchronization and fixed-time artificial insemination

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Funding information
Executive Agency for Higher Education, Research, Development and Innovation Funding. PN-III-P1-1.2-PCCDI-2017-0361, Grant/Award Number: Contract no: 7PCCDI/2018; Institutional Development Fund of the National Council for Financing in Higher Education 2021, Grant/Award Number: Contract no: CNFIS-FDI-2021-0309

Abstract

The objective of this project was to report the effects of season and age on conception rate amongst Italian Mediterranean Buffalo subjected to an Ovsynch/Resynch (O/R) reproductive management protocol. The study utilized nulliparous (heifers), primiparous buffalo cows (PBC) and multiparous buffalo cows (MBC). The primiparous and multiparous groups were subjected to the synchronization protocol throughout the entire year, but heifers were synchronized and inseminated only during the spring/summer seasons. The conception rate obtained following the OvSynch oestrus synchronization protocol, applied during spring, was 68.4% for heifers, 83.3% for PBC and 67.7% for MBC. The overall total conception rates following the complete O/R protocol were 84.27%, 94.4% and 79%. Conception rates achieved during summer were heifers 52%, PBC 47.2% and MBC 49%, whilst overall conception rates following the full O/R protocol were 72%, 69.8% and 58.2% respectively. In the autumn seasons, PBC conceived 58.9% and MBC 52.1% following initial Ovsynch, which improved to total overall conception rates of 87.5% and 78.7% following the full O/R protocol. Similarly, in the winter season, PBC experienced a conception rate of 47.5% following Ovsynch and 72.5% after a follow-up Resynch. MBC experienced 60.0% and 74.4% conception following Ovsynch and full O/R, respectively, during winter. Total conception rates during all seasons were quite acceptable following the O/R protocol. There was a significant decrease from spring to summer in conception rate for all parity groups, but heifers were not a severely affected as older buffalo cows. This finding agrees with that of other investigators indicating that heifer fertility is not as negatively impacted by long photoperiod and higher ambient temperature as that of older animals. The O/R protocol as utilized in this study is an effective means of reproductive management for dairy buffalo cows and is effective for improving fertility during out-of-season breeding.

KEYWORDS
conception rate, dairy Italian Mediterranean Buffalo, oestrus synchronization, OvSynch, ReSynch

1 | INTRODUCTION

The domestic water buffalo (*Bubalus bubalis*) is an important dairy species worldwide (Crudeli et al., 2019; Zicarelli, 1997). This is certainly true in the Mediterranean region. Whilst buffalo populations have decreased in many Mediterranean countries during the last 40 years (Borghese, 2010), there has been a resurgence of buffalo population in Italy in part due to the popularity of *Mozzarella di Bufala Campana*, a buffalo cheese with EU Protected Designation of Origin. The Italian Mediterranean Buffalo is a breed that has been specifically selected for dairy production. Demand for buffalo dairy products peaks in spring and summer, in part due to tourist demand for specialty cheeses manufactured with buffalo milk. As a result, milk prices tend to be the highest during this time of year. Buffalo, however, exhibit a moderately strong seasonality in their reproductive pattern, which is counter cyclical to product demand peaks (Zicarelli 1994, 1997; Baruselli et al., 2003). This has led to considerable interest in technologies for oestrous cycle management of dairy buffalo for the purpose of increasing fertility during those portions of the year when it is low.

Buffalo fertility is highest during autumn and winter seasons of the year (Zicarelli, 1994), and therefore, primary calving season in Italy is during a period from September to December with a peak in October (Zicarelli, 1997). Low fertility associated with the spring and summer seasons has been attributed to a variety of causes including reductions in nutritional status, higher ambient temperature and increasing and/or long photoperiod. Even in Italy, where most dairy buffalo cows are maintained on total mixed rations (TMR) that are essentially constant throughout the entire year, seasonality is quite prominent and is likely due to increasing photoperiod coupled with higher ambient temperature (Zicarelli, 1994, 1997; Baruselli et al., 2003, Tailor et al., 1990; Berber et al., 2002). Campanile et al. (2005) have also demonstrated that embryo mortality is higher during the warm seasons of the year and attribute that effect to lower progesterone production in early pregnancy. Furthermore (Das & Khan, 2010; Mohan et al., 2010; Prakash, 2002; Prakash et al., 2005), buffalo tend to exhibit a higher incidence of silent oestrus during warm seasons (April, 70%) which continually declines into December (10.5%).

Even during seasons of high fertility, to maintain an efficient and effective reproductive management programme, buffalo cows must consistently conceive within 90 days post-calving (Zicarelli, 1997). This requires significant time investment for oestrus detection and record keeping if an artificial insemination (AI) programme is to be utilized—the fixed-time artificial insemination (FTAI) firstly used by Pursley et al. (1997) has a limited use worldwide in buffalo (Barile, 2019). Utilization of ovulation synchronization protocols such as Ovsynch or Ovsynch followed by a Resynch protocol can improve the efficiency of reproduction management in dairy buffalo.

The Ovsynch protocol has been effectively applied to buffalo cows both during periods of high natural fertility and those of low or absent reproductive activity (Baruselli & Carvalho, 2005). However, many buffalo cows show a prolonged period of sexual inactivity during seasons that are unfavourable for mating (spring/summer) and do not respond properly to OvSynch treatment alone. Protocols that use progestins, oestradiol, prostaglandin, gonadotropin-releasing hormone (GnRH) and equine chorionic gonadotropin improve the rates of conception in buffalo cows. The conception rate following FTAI, even during seasons that are favourable for reproduction, is further influenced by many management factors including body condition score and parity of the animals (Baruselli et al., 2003).

The objective of this project was to report the effects of season and age on conception rate amongst Italian Mediterranean Buffalo subjected to an Ovsynch/Resynch (O/R) reproductive management protocol.

2 | MATERIAL AND METHODS

2.1 | Experimental design

This study was conducted at the *'Bradano River'* dairy buffalo farm, in SE Italy, in the Irsina locality. The farm has the following coordinates: N 40°44'36.9639'', E 16°10'50.484, at 212 m altitude above sea level. The subject animals were Italian Mediterranean buffalo cows, in free stalls housing system. The research was conducted during October 2015-December 2017.

Lactating buffalo cows received TMR with 10 kg triticale silage, 6 kg corn silage, 2 kg alfalfa hay, 1 kg triticale hay, 0.5 kg straw, 1.8 kg soy meal, 3.5 kg cornmeal, 4 kg of mixed feedstuffs (wheat bran 38%, barley 38%, beet pulp 12% and cotton seeds 12%) and 0.4 kg of vitamin-mineral supplements. In order to counteract the negative effect of thermal stress, feeding was done twice a day in a feeding zone with cooling system by water sprinklers and cooling fans in high volume' shelters.

The conception rate was calculated consecutive to the implementation of an Ovsynch / Resynch oestrus synchronization protocol. The mean insemination age for heifers was 20.8 ± 2.46 months and 3 ± 0.4 years for primiparous buffalo cows (PBC). The PBC had an open days (OD) interval of 112.7 ± 52.1 days. The mean age of the multiparous buffalo cows (MBC) taken into study was 6.4 ± 2.7 years, and the OD interval was 123.77 ± 152.1 days.

The oestrus synchronization protocol used 20 μg per animal GnRH analogue (Buserelin 4 μg/ml, Receptal MSD Animal Health Italia) intramuscularly administered and 500 μg per animal Prostaglandin $F_{2\alpha}$analogue (Cloprostenol 250 μg/ml; Estrumate, Shering-Plough Animal health, S.P.A., Milan, Italia).

Animals were synchronized using the Ovsynch protocol followed by a Resynch protocol (Figure 1). This programme is based on the administration of 20 μg of GnRH analogue to all buffalo cows on day 0, followed by the administration of 500 μg prostaglandin $F_{2\alpha}$analogue (PGF) on day 7 and the administration of a second dose of GnRH analogue on day 9. Artificial insemination was performed 16–20 hr following the final administration of the GnRH analogue. The Resynch protocol is performed by administration of the GnRH analogue, without prior pregnancy determination, within 28 days following

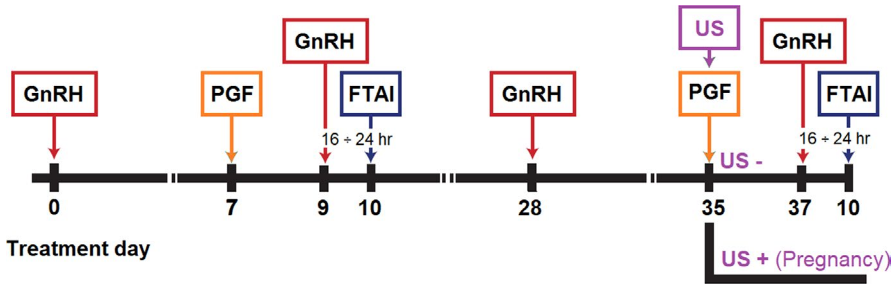

FIGURE 1 Diagram of Ovsynch/Resynch protocol with FTAI at 16–24 hr following administration of second GnRH dose. Abbreviations: FTAI, fixed-time artificial insemination; GnRH, gonadotropin-releasing hormone; PGF, prostaglandin F2α analogue; US, ultrasound

AI. The administration of the GnRH analogue does not have a negative influence on pregnancy if already present (Buttery et al., 2007). Gestation diagnostic via ultrasound (US) was conducted on day 35 following AI (day 7 after the initiation of the second OvSynch protocol) using an Esaote Tringa portable US instrument. The buffalo cows that were not confirmed as being pregnant received an injection of prostaglandin$F_{2\alpha}$. After 37 days following the initial AI (day 9 from the beginning of the Resynch protocol), the buffalo cows were administered a second dose of GnRH analogue. Artificial insemination was performed within 16–20 hr following the second GnRH injection.

The GnRH analogue (Buserelin) and PG $F_{2\alpha}$analogue (Cloprostenol) doses were chosen according to literature data. Thus, the dose of GnRH used to synchronize oestrus in buffalo cows ranged between 8 and 100 μg per animal, whilst the doses of Cloprostenol varied between 375 and 524 μg per animal (De Araujo Berber et al., 2002; Baruselli et al., 2003; Berber et al., 2002; Gudev et al., 2007; Neglia et al., 2003).

Conception rate was calculated for each season. A total of 619 AIs were performed during October 2015–December 2017. The buffalo cows were grouped into gynaecological categories (parities), and the seasons were calculated according to the astronomical calendar. Artificial insemination was carried out using thawed semen provided by COFA (Centro Operativo di Fecondatione Artificiale) Cremona Italia, which provided semen from two buffalo bulls (Otello and Black Star with Bacterial Load—maximum 5,000 CFUs/ml; hypo-osmotic swelling test—≥40%; acrosome integrity in fresh semen—≥ 70%; per cent intact acrosome—≥65%; sperm concentration—20 million spermatozoa per straw).

The study was performed in compliance with EU Directive 63/2010 EU and 43/2014 RO laws regarding the protection of animals used for scientific purposes—the activities as considered *'recognized acts of animal husbandry'* in accordance with Statement in the frame of authorization no 535/2016.

2.2 | Statistical evaluation methods

Two experiments were organized to complete the study. The first experiment was based on the evaluation of the conception rate of three parities (heifers, PBC and MBC) throughout spring–summer seasons. The second experiment was based on the analysis of the conception rate in PBC and MBC during the autumn–winter seasons. To provide a measure of variance for statistical analysis, the buffalo cows from each parity were randomized into three groups.

Data analysis was performed using factorial design two-way ANOVA with fixed factors of parity (three levels in Experiment 1 and two levels in experiment2) and season (two levels for each experiment) (Table 1). Means were separated using Tukey's test. Any mean pairs with calculated q values exceeding the critical q for $p = .05$ or $p = .01$ were considered significant at their respective levels.

3 | RESULTS

Table 2 presents the separate conception rates following the initial Ovsynch protocol, and the subsequent Resynch protocol administered to those buffalo cows who did not conceive after the initial Ovsynch. The total of the Ovsynch +Resynch conception rates were used for statistical analysis for both Experiments 1 and 2 (Tables 3 and 4).

TABLE 1 Total number of animals in each parity X season category

Crosstabulation by Parity and Season		Season				
		Experiment 1		Experiment 2		
		Spring	Summer	Autumn	Winter	Total
Parity	Heifers	57	50	0	0	107
	Primiparous	18	53	58	40	167
	Multiparous	62	98	94	90	344
Total		137	201	150	130	618

Note: Each category was divided into three subgroups, so that a variance estimate could be calculated for conception rate within category.

TABLE 2 Conception rate percentages by parity and season for Experiments 1 and 2

Parity	Season							
	Spring		Summer		Autumn		Winter	
	Ovsynch	Resynch	Ovsynch	Resynch	Ovsynch	Resynch	Ovsynch	Resynch
Heifers	68.4	15.8	52.0	20.0	-	-	-	-
Primiparous	83.3	11.1	47.2	22.6	58.9	28.6	47.5	25.0
Multiparous	67.7	11.3	49.0	9.2	52.1	26.6	60.0	14.4

Note: Conception rates were calculated as number of pregnant buffalo cows at 35 days as determined by ultrasound diagnostic, divided by the number of buffalo cows inseminated at the previous fixed-time artificial insemination, for both the Ovsynch and Resynch protocols.

TABLE 3 Experiment 1 mean total conception rates for each parity by season and parity following Ovsynch/Resynch protocol

	Conception Rate %		
Parity[a]	Spring	Summer	Overall[b]
Nulliparous	84.2	72.0[c]	78.1
Primiparous	94.4	69.8[c]	82.1
Multiparous	79.0	58.2	68.6
Overall[b]	85.9	66.7	-

[a]All combinations of parity and season differ with $p < .01$ except as noted below.

[b]Overall means for parity and season differ; $p < .01$.

[c]These means differ with $p < .05$.

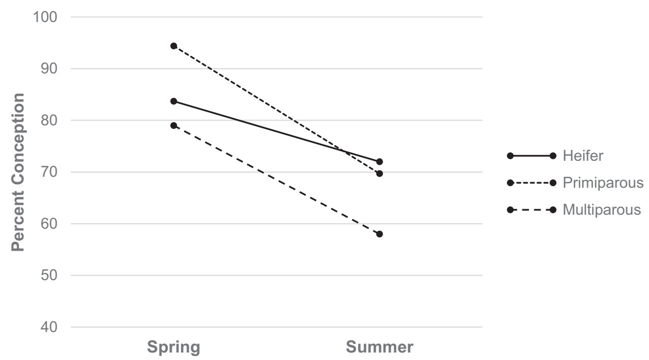

FIGURE 2 Effect of parity and season on conception rates of buffalo cows across the spring and summer seasons following an Ovsynch/Resynch protocol (Experiment 1)

TABLE 4 Experiment 2 mean total conception rates for each season and parity following an Ovsynch/Resynch protocol

	Conception rate		
Parity	Autumn	Winter	Overall[a]
Primiparous	87.5[b]	72.5[c]	80.0
Multiparous	78.7[b]	74.4[c]	76.6
Overall[d]	83.1	73. 5	-

[a]Overall means for Parity differ; $p < .05$.

[b]Cells not sharing a common superscript differ; $p < .01$.

[c]No significant difference; $p > .05$.

[d]Overall means for Season differ; $p < .01$.

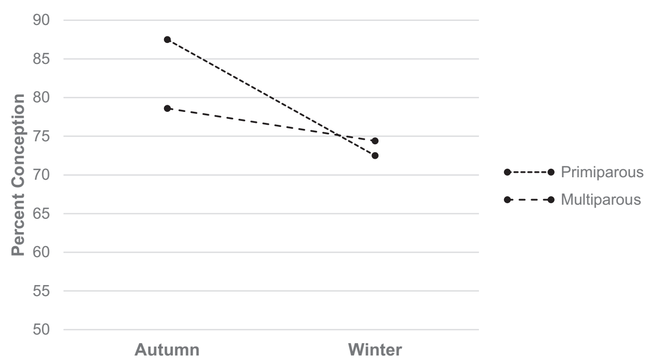

FIGURE 3 Effect of parity and season on conception rates of buffalo cow synchronized with an Ovsynch/Resynch protocol across the autumn and winter seasons (Experiment 2)

3.1 | Experiment 1

Table 3 and Figure 2 present results from Experiment 1. There was significant interaction ($p < .01$) for parity by season in Experiment 1.

All parity groups achieved conception rates that were significantly different from one another at either the $p < .01$ or $p < .05$ level (Table 3). PBC exhibited the highest overall conception rate (82.1), with heifers intermediate (78.1) and MBC the lowest (68.6). Season also produced a significant effect with conception across all parities higher in the spring (85.9%) than summer (66.7%) ($p < .01$).

There was, however, significant interaction in the magnitude of this effect (Figure 2). The reductions in conception rate from spring to summer for the PBC and MBC were relatively close in magnitude, PBC with reduction of 24.6% and MBC with a reduction of 20.8%. Heifers, on the other hand, clearly showed a substantially smaller reduction from spring to summer of 12.2%. Water buffalo heifers synchronized with an O/R protocol appear to be less affected by seasonal change than higher parity buffalo cows under the conditions of this experiment.

3.2 | Experiment 2

Table 4 and Figure 3 present results from Experiment 2. Again, there was significant interaction ($p < .01$) for parity by season in this experiment.

The overall conception rate between the two parity groups differed ($p < .05$) with PBC higher at 80.0% than multiparous buffalo cow at 76.6%. The effect of season was also significant ($p < .01$) with higher conception rates achieved in the autumn season compared to winter (83.1 and 73.5 respectively).

When looking at individual category effects, during the autumn season, PBC had higher ($p < .01$) conception rates (87.5%) compared with MBC (78.7%). However, during the winter season, there was no difference in the conception rate between the two parity groups. Figure 3 illustrates the interaction of the effects of parity and season. PBC experience a substantially greater reduction in conception rate from autumn to winter (reduction of 15.0%) than did MBC (reduction of 4.3%). The conception rates achieved by MBC synchronized with an O/R protocol are less affected by season between autumn and winter than those of PBC under the conditions of this experiment.

For PBC and MBC, the non-parametric method Kruskal–Wallis rank test showed differences ($p < .001$) in survival curves across synchronization protocols for the median-day interval between calving and artificial insemination (IA) with days in milk at conception (DIM at conception), which was 89 days for cows assigned to the Ovsynch programme, whereas it was 102 days for the Resynch cows (Figure 4). In case of nulliparous, the Kruskal–Wallis rank test showed differences ($p < .001$) in survival curves across synchronization protocols for the median—age of the first conception (AFC), which was 622 days for heifers assigned to the Ovsynch programme and 651 days for the Resynch heifers (Figure 5).

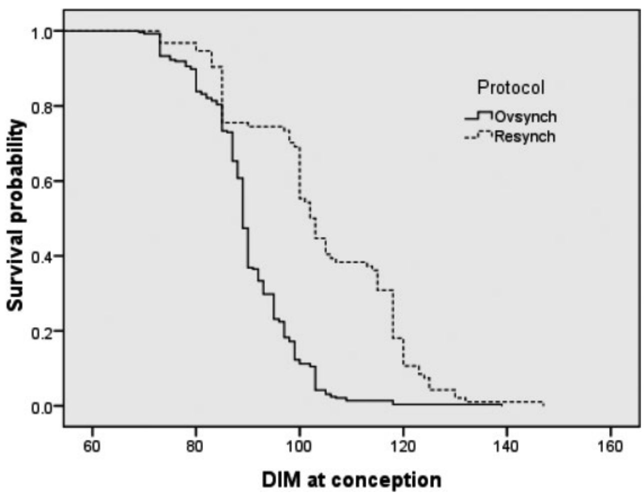

FIGURE 4 Kaplan–Meier survival curves for effect of treatments (Ovsynch and Resynch) of primiparous buffalo cows and multiparous buffalo cows from experimental groups on interval calving to conception (DIM at conception, established after ultrasound control at 35 of pregnancy). Days in milk (DIM) to conception differed between experimental groups (log-rank p =.000, Wilcoxon p =.000)

4 | DISCUSSION

The primary findings of this study are comparisons of conception rates achieved by Italian Mediterranean dairy buffalo to O/R ovulation synchronization as influenced by season of the year and parity of the female animals. The study found differences in conception rate between spring and summer seasons, and between autumn and winter seasons following an O/R administration. It also demonstrated differences between parity groups, in particular interactions between parity and season in conception rates achieved.

Regarding seasonal effects In Experiment 1, overall conception rate during the spring/summer seasons were 68.6% for MBC, 78.1% for heifers and 82.1% for PBC. Furthermore, the highest conception rates were observed during the spring season, with heifers at 84.2%, PBC at 94.4% and MBC at 79.0%. Clearly, the O/R protocol used in our study was very effective during what is generally considered a low fertility season for water buffalo. Whilst our study does not have a direct comparison between spring/summer and autumn/winter, an anecdotal comparison of Experiment 1 with Experiment 2 shows very similar responses between these two portions of the year amongst buffalo cows in this study. Crudeli et al. (2019) reported an overall pregnancy rate of 73.2% across four modifications of protocols utilizing O/R in Argentinian meat-type Mediterranean buffalo. One of those modifications (TRT1) was the same protocol used in our study. After full O/R protocol administration, our study's conception rates ranged from 58.0% (MBC in summer season) to 94.4% (PBC in spring season). Our results roughly mirror those of Crudeli et al. (2019).

Seasonal effects were also observed in Experiment 2 with overall conception rates during the autumn/winter seasons at 80.0% for PBC and 76.6% for MBC. Whilst these percentages were statistically different, from a practical perspective, they are essentially comparable. The conception rates achieved for Experiment 2 were comparable or higher than those reported by other researchers for trials conducted during the breeding season. Crudeli et al. (2019) reported a 73.2% pregnancy rate using a similar O/R protocol with Mediterranean buffalo in Argentina.

This study demonstrated differences in response between parity groups and interactions of parity by season in conception rate. Experiment 1 exhibited a significant interaction effect of parity by season with heifers showing a substantially lessened conception rate decrease spring to summer than was the case for PBC and MBC. This was not unexpected as buffalo heifers have been shown to be less sensitive to photoperiod than older animals (Avallone et al., 1994; Pursley et al., 1997; Zicarelli, 1994). In Experiment 2, we observed a 9.6% drop for overall conception rate from autumn to winter across parities, which also exhibited a parity by season interaction. PBC exhibited a higher rate in autumn (87.5%) than MBC (78.7%). However, during winter, the conception rate achieved by the two parity groups was not different (PBC 72.5% and MBC 74.4%). The reduction in conception rate from autumn to winter is not unexpected for species that are short-day breeders since day length begins to increase during the winter season. A similar phenomenon is observed with

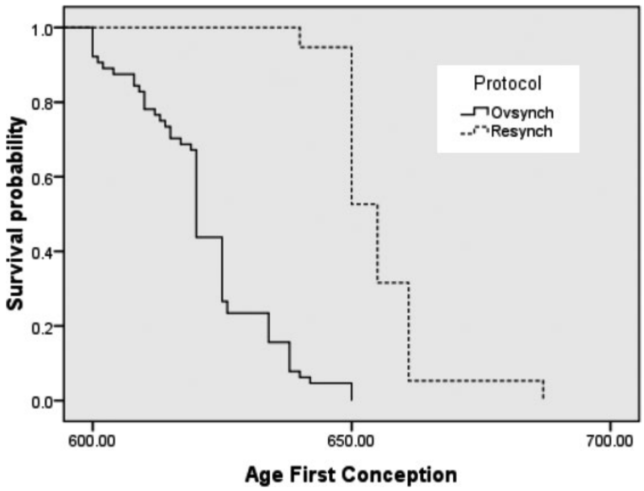

FIGURE 5 Kaplan–Meier survival curves for effect of treatments (Ovsynch and Resynch) of nulliparous from experiment 1 on age at conception (age of the first conception, established by ultrasound control at 35 day after FTAI). Age at first conception differed between experimental groups (log-rank p =.000, Wilcoxon p =.000)

sheep for example. Reasons for the greater decrease in conception from autumn to winter amongst PBC relative to MBC are less obvious. Curiously, de Araujo Berber et al. (2002) reported a significantly higher conception rate for MBC versus. PBC (60% versus. 30% respectively) following a single Ovsynch protocol performed during the breeding season. The reasons for this discrepancy relative to our results are not obvious. Further investigation is required to determine whether there is truly a consistent influence of parity, and if so, what the physiological or endocrinological aetiology might be.

Whilst this study primarily explored conception rate following an O/R protocol, it also makes possible anecdotal conception rate comparisons following administration of Ovsynch alone as these percentages were intermediate data achieved during the progress of the trial. Ovsynch protocol induced a good ovulation (70%) in low-breeding season of buffalo (Sharma et al., 2017). Conception following a single Ovsynch protocol administered to buffalo cows reported elsewhere include 33.3% (Paul & Prakash, 2005) and 35.7% without progestin supplementation (De Rensis et al., 2005). Others have reported pregnancy rate of 40% for heifers and 42.8% for MBC (Presicce et al., 2005) during the spring/summer seasons of the year. Our study generated conception rates ranging from 47.1% (PBC in summer season) to 83.3% (PBC in spring season) following initial Ovsynch. The reasons that our study achieved generally higher conception rates are unclear. We hypothesize that overall excellent nutritional management coupled with environmental management (fans, shade and well-maintained free-stall housing) and excellent overall reproduction management at the Bradano River Farm contributed to the success of the initial Ovsynch procedure.

Advanced technologies are useful for reproductive management of buffalo dairy herds during the entire year, but they are particularly desired for increasing the percentage of calving during seasons when buffalo milk demand is high, which means buffalo cows must conceive during a time of year when fertility is lower. Buffalo experience their lowest fertility during spring and summer seasons. Buffalo are highly responsive to photoperiod and are short photoperiod breeders. Therefore, during spring, they are relatively infertile due to increasing photoperiod (Tailor et al., 1990). During summer season, their fertility is even lower than in spring, apparently due to long (but no longer increasing) photoperiod coupled with high temperature (Prakash et al., 2005; Zicarelli, 1997). Various endocrinological aetiologies for this lowering of fertility have been proposed. These include changes in melatonin secretion rate and diurnal periodicity (Phogat et al., 2016) with animals exhibiting greater seasonality having higher melatonin levels two hours post-sunset than was the case for animals showing lesser seasonality. Others have proposed that increasing day length and increasing environmental temperatures can cause hyperprolactinemia, limit the secretion of luteinizing hormone and suppress gonadotropin secretion—all phenomena which lead to alterations in ovarian steroidogenesis. Stress caused by high temperatures and high relative humidity affects folliculogenesis, the quality of follicular fluid and the quality of the oocyte (Palta et al., 1997; Presicce et al., 2005). Furthermore, hypothyroidism associated with high environmental temperature has been proposed as a primary causative factor leading to hyperprolactinemia (Campanile et al., 2005). In addition, lower fertility during warm seasons has been partially attributed to increased early embryonic mortality resulting from lowered levels of progesterone secretion by corpora lutea during thermal stress (Campanile et al., 2005). Other researchers have observed decreased response to Ovsynch during spring/summer seasons (Prakash et al., 2005). Our findings illustrate that an O/R protocol is an effective tool for reproductive management of dairy water buffalo during all seasons of the year, that when coupled with excellent environmental management, can generate very acceptable conception rates.

We find it interesting that PBC experienced the highest overall conception rates both during the breeding season and the off-season, that is both Experiments 2 and 1 respectively, contrary to El-Tarabany (2018) results. Weigel (2021) has summarized the relationships between milk production genetic potential and reproduction efficiency in US dairy cattle. He points out that conception rate to the first service is higher for first parity buffalo cows than for older buffalo cows. The buffalo in this study exhibit that same tendency. However, with *Bos taurus* dairy cattle, heifers exhibit the highest fertility of all age groups (Wathes et al., 2014) in a general sense. However, Pursley et al. (1997) observed lower conception rates than expected relative to older cattle, in heifers subjected to Ovsynch. Similarly, in our Experiment 1, buffalo heifers did not experience conception rates as high as PBC.

5 | CONCLUSIONS

These results indicate that for Italian Mediterranean buffalo subjected to an O/R protocol, seasonal differences in response occur, with lower conception rates in summer compared to spring, and lower rates in winter relative to autumn. Animal age also affects conception rate. Nulliparous buffalo cows do not appear to experience as great a reduction between spring and summer as higher parity animals. When comparing winter to autumn, MBC appear to experience a smaller reduction in conception rate than PBC animals although this needs to be confirmed through further study.

The use of an O/R protocol is an effective method for improving fertility in Italian Mediterranean water buffalo. Conception rates during the peak fertility season with O/R were comparable to those achieved with other methods, with the added advantage of FTAI. During the non-fertile season, conception rates using O/R were substantially improved relative to those achieved with other approaches.

ACKNOWLEDGEMENTS

This paper is financed for open access by the CNFIS-FDI-2021-0309 project assigned to the Banat's University of Agricultural Sciences and Veterinary Medicine from Timisoara through the Institutional Development Fund of the National Council for Financing in Higher Education 2021. The authors thank the owner and staff of the farm *"Bradano River"*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Gabriel Otava involved in conceptualization, data curation, and investigated the original draft. **Stefano Squicciarini** investigated the original draft. **Simona Marc** reviewed and edited the methodology. **Tijana Suici** involved in writing the draft. **Gary William Onan** involved in methodology, statistics, writing the review and editing. **Ioan Hutu** reviewed and edited the formal analysis. **Iuliu Torda** investigation and data curation **Calin Mircu** involved in resources, Writing- editing.

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How to cite this article: Otava, G., Squicciarini, S., Marc, S., Suici, T., William Onan, G., Hutu, I., Torda, I., & Mircu, C. (2021). Effects of age and season on conception rate of Mediterranean Italian Dairy Buffalo (*Bubalus bubalis*) following oestrus synchronization and fixed-time artificial insemination. *Reproduction in Domestic Animals*, 00, 1–8. https://doi.org/10.1111/rda.14013