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GENETIC ANALYSIS OF FERTILITY AND SEASONALITY TRAITS IN BUBALUS BUBALIS

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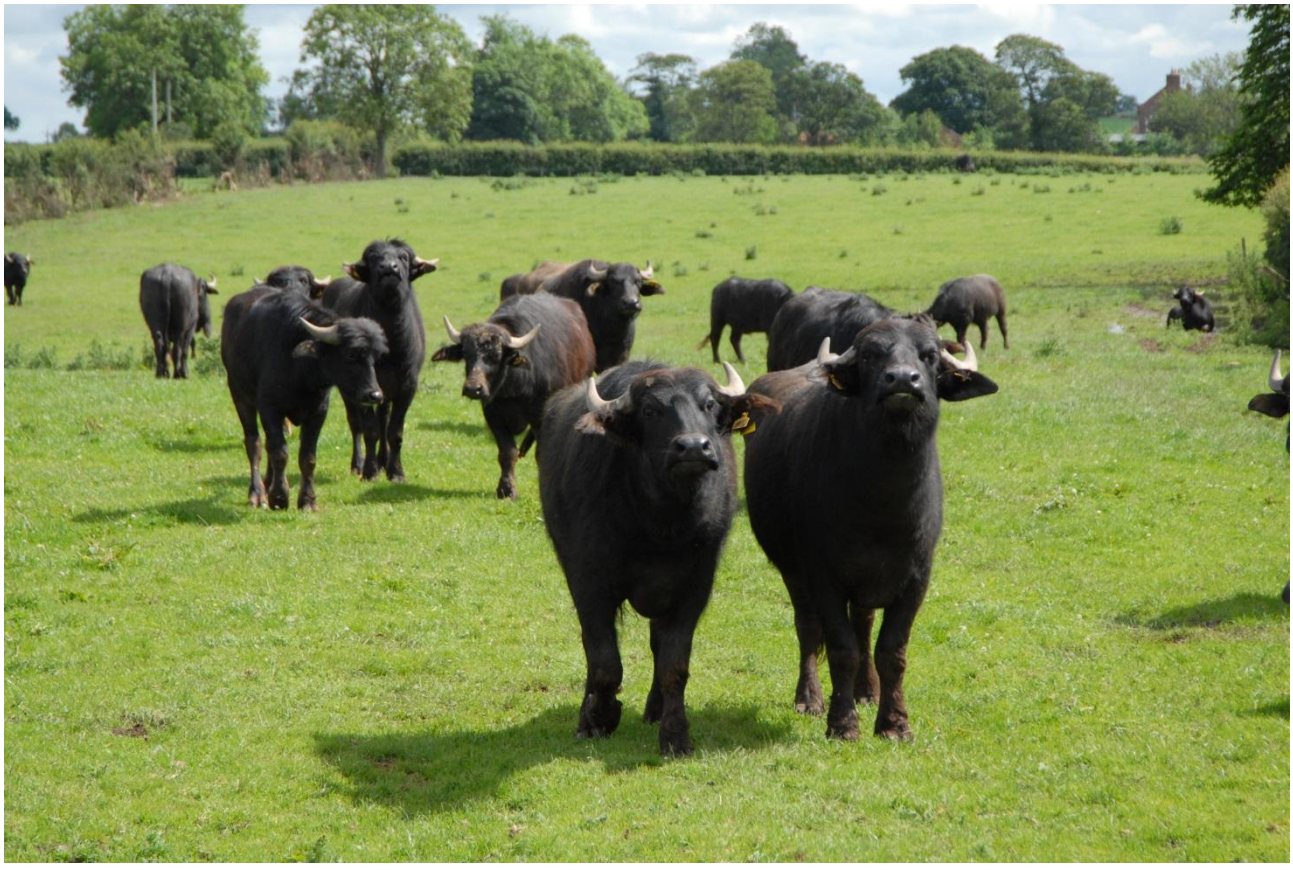

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GENETIC ANALYSIS OF FERTILITY AND SEASONALITY TRAITS IN *BUBALUS BUBALIS*

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1. INTRODUCTION

Water buffalo is an important livestock resource, which occupies a critical niche in many ecologically disadvantaged agricultural systems, providing milk, meat, and work power. In Italy, the most important production related to buffalo breeding is milk, traditionally processed into mozzarella cheese. This peculiar product gained a well-defined profile in the market, under the name of "*Mozzarella di Bufala Campana*", enhanced and protected by a Denomination of Protected Origin (DPO) trademark, recognized in an increasing number of countries. The major problem of buffaloes is the poor reproductive efficiency, mainly caused by late age at maturity, long calving intervals, and silent heat. On Italian farms where natural mating is practiced and bulls are always present in the herd, the calving interval is approximately 400 days and the culling rate is lower than 15% (Zicarelli, 2010). Moreover, artificial insemination is not often performed because of the weakness of oestrus symptoms and the variability of oestrus length, which makes oestrus detection very difficult. Fertility is a problem also in cattle species, where a negative genetic correlation with milk yield has been observed (Van Arendonk et a., 1989; VanRaden et al., 2004; Wang et al 2009). It is now well established that the large improvement in milk yield obtained over the last 40 years, was accompanied by a strong decline in fertility in this species (Washburn et al., 2002; Hare et al., 2006; Norman et al., 2009).

Another fundamental factor affecting buffalo breeding is seasonality of calving. Particularly in Italy this is a big issue, since it implies that the greater milk production does not coincide with the increased market demand for mozzarella cheese, the main income of buffalo breeding. Under Mediterranean latitudes, reproductive efficiency of buffaloes is usually negatively affected by increasing day-length. Buffaloes become sexually active in late summer to early autumn (Zicarelli 1997). The main environmental factor affecting seasonality is photoperiod, which regulates changes in the daily melatonin secretion by the pineal gland. The pattern of melatonin secretion provides photoperiodic information to cells within the brain that possess the relevant receptors and control reproductive function (Migaud et al., 2005).

During the last 10,000 years our domestic species have been genetically adapted for various purposes and to different environmental conditions. In the past years the developments in understanding animal genetics have opened the possibility to evolve the genetic evaluation of livestock species. Improvement in animal traits through genetic selection is advantageous, because genetic gain is cumulative over generations. The genetic improvement of livestock breeds has been traditionally based on phenotypic selection. The development of molecular biology tools during the past decades created new means for studying livestock genetics and animal breeding, allowing a more accurate selection of individuals also without phenotypic information. The availability of molecular markers largely distributed throughout the genome, makes them key players in animal genetics, also as an useful tool for animal identification and genetic distance estimation. Extensive genetic maps designed in the last few decades in a variety of animal species such as cattle, sheep, swine, were used for marker assisted selection, quantitative trait loci segregating analysis and for detection of major genes (Rohrer et al., 1994; Kinghorn, 1997; Vignal et al., 2002). Single nucleotide polymorphisms (SNPs), single base variation in a DNA sequence, are now the most widely used class of genetic marker, as they are easy to evaluate and interpret and are widely distributed within genomes. A total of 4.4 million human SNP were genotyped during phase II of the HapMap project (Frazer et al., 2007). In recent years, the discovery and validation of millions of Single Nucleotide Polymorphisms also in the major livestock genomes has been made possible by

the release of their complete sequences. These are now available for cattle (Elsik et al., 2009), sheep (Archibald et al., 2010) and horse (Wade et al., 2009), pig (Groenen et al., 2012) and chicken (Hillier et al., 2004). The release of complete genome sequences also for goat and buffalo has been recently announced, but are not yet available to the scientific community. In domestic animals Genome-wide association study (GWAS) has become feasible thanks to the development of large collections of SNPs and the development of more cost-effective methods for large-scale SNP analysis and also in buffalo species, a genomic SNP chip tool is now developing.

Even with these new genetic tools, the selection for fertility trait is hampered by low heritability. In cattle, where the BovineSNP50 chip tool is now widely used for association studies, it has been observed that the low heritability and polygenic nature of fertility traits limit the improvements in reliabilities achieved by incorporation of genomic information compared to other traits (Cochran et al., 2013). Consequently, it has been suggested that incorporation of candidate gene SNPs into genomic tests for reproduction is required to select causative SNPs or SNPs physically more close to causative SNPs in cattle, as it was already demonstrated in detection of genomic associations with disease (Amos et al., 2011).

Aim of this work is to conduct a candidate-gene association study in genes related to fertility and seasonality traits in Mediterranean Italian buffalo. Candidate genes analyzed are: signal transducer and activator of transcription 5A (*STAT5A*), serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 14 (*SERPINA14*) and tumor necrosis factor alpha (*TNFA*) for fertility, and melatonin receptor 1A (*MTNR1A*) for seasonality.

1.2. WATER BUFFALO

The water buffalo (*Bubalus bubalis*) is one of the most important dairy animals, concentrated largely in tropical and sub-tropical countries. Water Buffalo milk production, although only produced in a few countries, is increasing and it constitutes 13% of the world milk production, principally in Asia (Ferreira et al., 2013).

1.2.1. Origins

The phylogeny of water buffalo is still a matter of debate (Perera, 2011). Buffaloes form two groups: the Asian (genus *Bubalus*) and the African (genus *Syncerus*). Since the African buffalo can be tamed and has bred in captivity, but it has never been domesticated, the wild water buffalo is thought to be the founder of all domestic buffaloes in the world today (Cockrill, 1993). MacGregor (1941) classified water buffaloes in two groups: the Swamp buffaloes of South-east Asia and the River buffaloes of the Indian subcontinent. These two types can be distinguished using karyotyping, as they differ in the number of chromosomes (Iannuzzi, 1994), first described by Fischer & Ulbrich in 1968 (figures 1.2.1.1. and 1.2.1.2.), as well as morphological and ethological criteria (Cockrill, 1981). Swamp buffaloes are the most similar to the wild Asian progenitor *Bubalus arnee* (Clutton-Brock 2001); compared to Indian breeds, they usually have in fact more massive horns, heavily striated and grow outward from the head laterally and upwards to form a semi-circle as in the wild *arni*. Overall colour is dark slate grey; a red tinge in the long hairs of the coat is common (Cockrill, 1984). The River buffaloes are usually black, have curled or sickle-shaped horns and are primarily dairy animals (Cockrill, 1984). The main breeds of dairy buffalo belong to the river type and include the Murrah, Surti, Jafarabadi and Nili-Ravi. The swamp type has no specialized breeds but selective breeding in some countries has resulted in populations with characteristic features (Perera, 2011). The Mediterranean buffalo, which some consider to be a third type, is derived from the river type.

From the practical aspects of buffalo breeding, the disparity in the number of chromosomes in swamp (2n=48) and river (2n= 50) buffalo has relevance (Huang et al., 2003). The F1 hybrids have 49 chromosomes, while the F2 hybrids have 48, 49 or 50 chromosomes. The backcrosses have two different karyotype categories each, with 2n = 48 and 2n = 49 in the three quarters swamp types and 2n = 49 and 2n = 50 in the three quarters river types (Harisah et al., 1989). The distribution of chromosome categories among the F2 hybrids and backcrosses suggests that only genetically balance gametes of the F1 hybrids are capable of producing viable F2 and backcross generations, and that crossbreds with 2n=49 had lower fertility than crossbreds with 2n=50 (Huang et al., 2003).

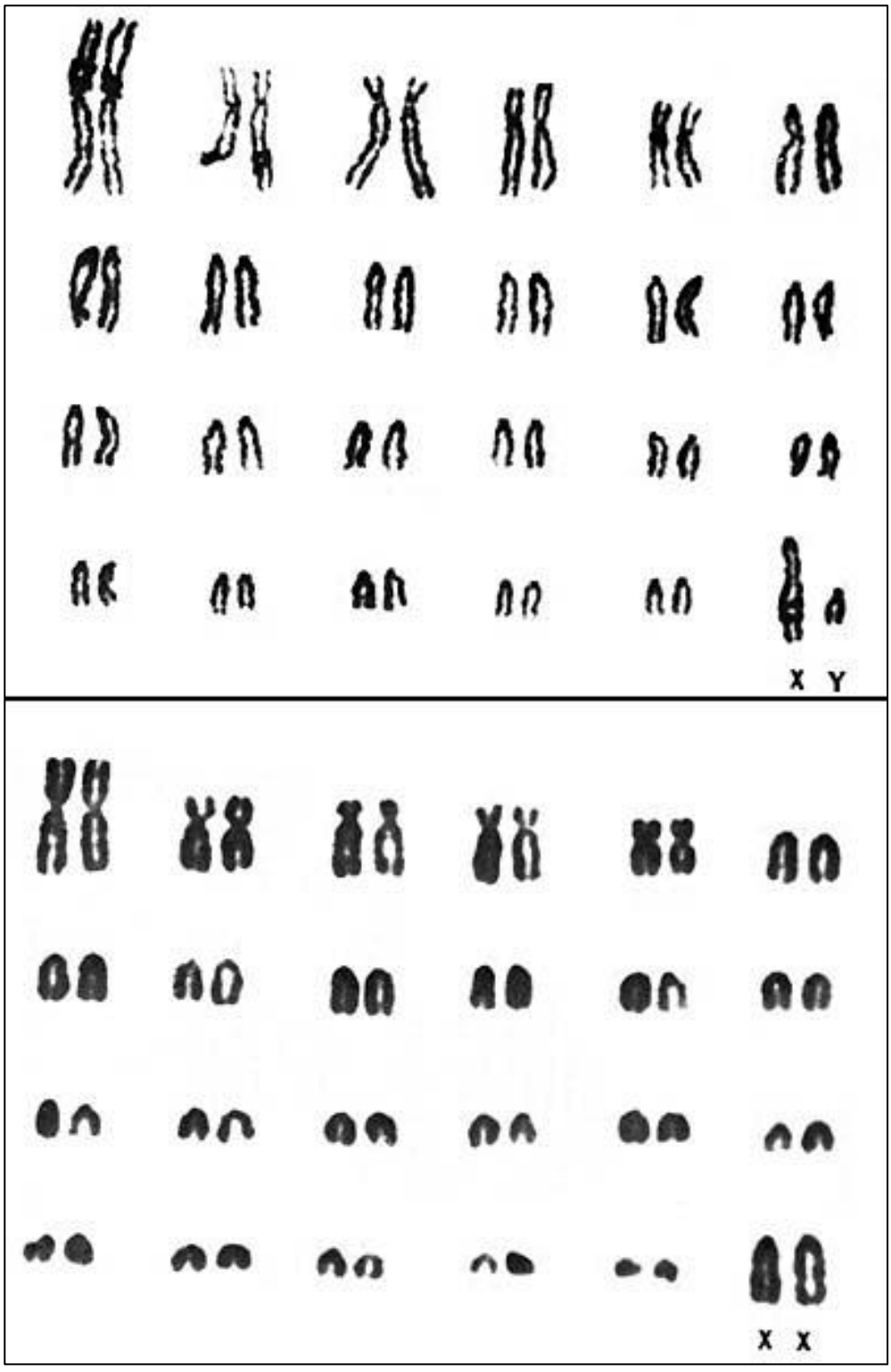


Figure 1.2.1.1.: Swamp buffalo karyotype (Fischer & Ulbrich, 1968).

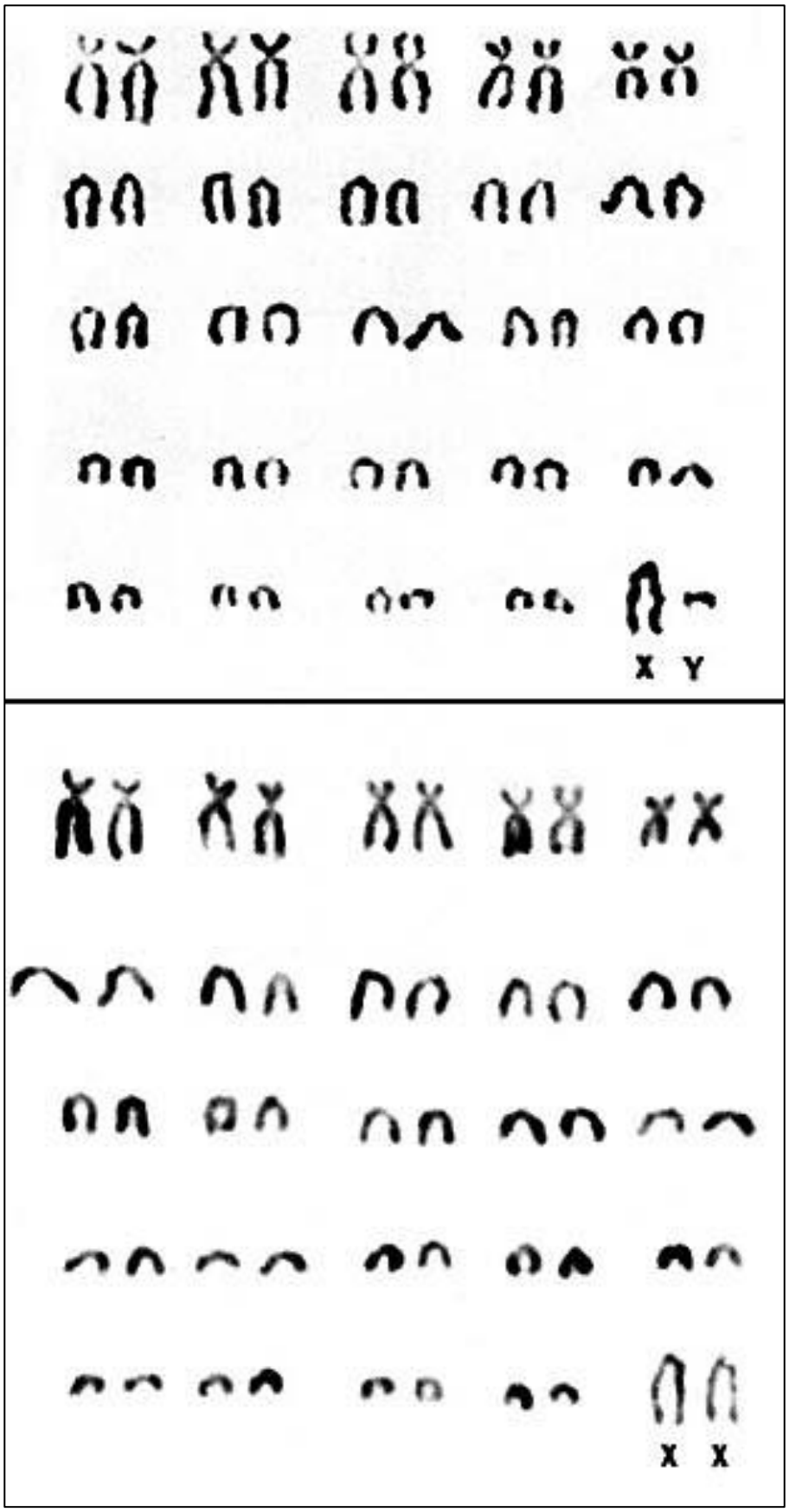


Figure 1.2.1.2. River buffalo karyotype (Fischer & Ulbrich, 1968).

1.2.2. Domestication

The domestication of buffaloes most likely took place in the civilization of the Indus, the Yangtze, and the Euphrates and Tigris in the third millennium BC (Nachtsheim and Stengel, 1977; Cockrill, 1981). A seal dating back to the Indus Valley Civilization of Mohenjo-daro, states that at the time (2500 BC), the buffalo was already domesticated in the region corresponding to modern Pakistan; another seal coming from the same period was discovered in the Cemetery of Ur in Mesopotamia (Clutton-Brock 2001). Some evidence indicates that wild prehistoric buffaloes lived in the Middle East. It has been suggested (Bökönyi et al., 1973) that the discovery of a Sassanid silver plate, dating back to a period between the sixth and seventh century AD, decorated in relief with depictions of a hunting scene, could prove that at that time the buffalo still existed in Iran in the wild (Fig 1.2.2.1.).

Figure 1.2.2.1. Silver plate of Sassanid manufacture, from Iran, which depicts the hunt wild buffalo and other animals, VI-VII century AD, Russia (picture from Clutton-Brock 2001).

Based on studies of mitochondrial DNA (mtDNA) of swamp and river buffalo, together with analysis of data published from South-East Asian and Australian water buffalo, Kierstein and colleagues concluded that both swamp and river buffaloes descend from one domestication event, probably in the Indian subcontinent (Kierstein et al., 2004). They also found evidence for introgression of wild *Bubalus arnee* mtDNA into domestic swamp buffalo. However, another research proved that river and swamp buffalo are distinguished into two distinct clades, indicating that the two types were domesticated independently (Kumar et al., 2007). This was supported by studies in China (Lei et al., 2007) that showed two mtDNA lineages with divergence estimated at

18,000 years ago, indicating independent domestication events for the swamp buffalo from China and the river buffalo from the Indian subcontinent. Finally, in a recent work (Yue et al., 2013) authors report that there are three possible scenarios regarding the original domestication center of buffalo: (1) swamp buffalo was domesticated in multiple centers, including Southeastern Asian and Southwestern China; (2) swamp buffalo was first domesticated in Southeastern Asia, and then introduced to Southwestern China before spreading to the adjacent regions; and (3) swamp buffalo was first domesticated in Southwestern China, but archaeologists have not discovered the early buffalo remains. Authors conclude that more DNA tests on buffalo from Southeastern Asia, particularly Myanmar, Laos, Vietnam, and Thailand, need to be done in order to compare with the data from China.

In Italy, buffaloes were introduced from central Europe in the sixth century or by the Bey of Tunis in the seventh century at the time of the Arab conquest (Salerno, (1974). Importation of water buffaloes to Africa, Australia, and South America took place only recently (Kierstein et al., 2004).

1.2.3. Role of the buffalo in livestock production

In recent decades, there has been an increase in the international interest in water buffalo species, made evident by the popularization of buffalo farming in Mediterranean area to Latin America and in Central/Northern Europe as well (Barile, 2005). The world buffalo population is continuously increasing and was estimated at over 195 million head in 2011. More than 97% of the population is in Asia, above all India, where buffaloes play an important role in rural livestock production. In fact, the good feed conversion efficiency of buffaloes and the relatively low maintenance requirements make them ideal in low-input, low-cost production systems (Paul et al., 2002).

Buffaloes are important production animals also in developed countries (Zicarelli, 1994). In Europe, buffalo population was estimated at about 390,000 heads in 2011, the most part of which is concentrated in Italy, with more than 365,000 heads (FAO, 2013).

The average milk yield per animal, where checked, is 940 kg (Table 1.2.3.1.).

Country	Milk buffaloes (Head)	Buffalo milk Yield (kg/animal)	Milk production (tonnes)
Albania	20	500	10
Bangladesh	90000	400	36000
Bhutan	209	400	84
Brunei Darussalam	450	200	90
Bulgaria	5444	1629	8868
China	5706400	543	3100000
Egypt	1680000	1579	2653240
Georgia	9500	579	5500
Greece	200	800	160
India	37131000	1679	62350000
Iran (Islamic Republic of)	150000	933	140000
Iraq	29500	922	27206
Italy	**244599**	**787**	**192540**
Malaysia	9700	1150	11155
Myanmar	560000	541	302974
Nepal	1291660	859	1109330
Pakistan	11864000	1935	22955000
Sri Lanka	86220	537	46330
Syrian Arab Republic	3398	1766	6000
Turkey	40218	1004	40372
Vietnam	32000	1000	32000

Table 1.2.3.1. World production of buffalo milk in 2011 (www.fao.org).

1.2.4. Mediterranean Italian Buffalo

In recent decades, buffalo farming has expanded greatly in Mediterranean areas. In Italy, buffalo was estimated at over 365 thousand head in 2012 (FAO, 2013) and its importance and competitiveness is confirmed by the positive trend observed in the national buffalo population in the last ten years (Figure 1.2.4.1.).

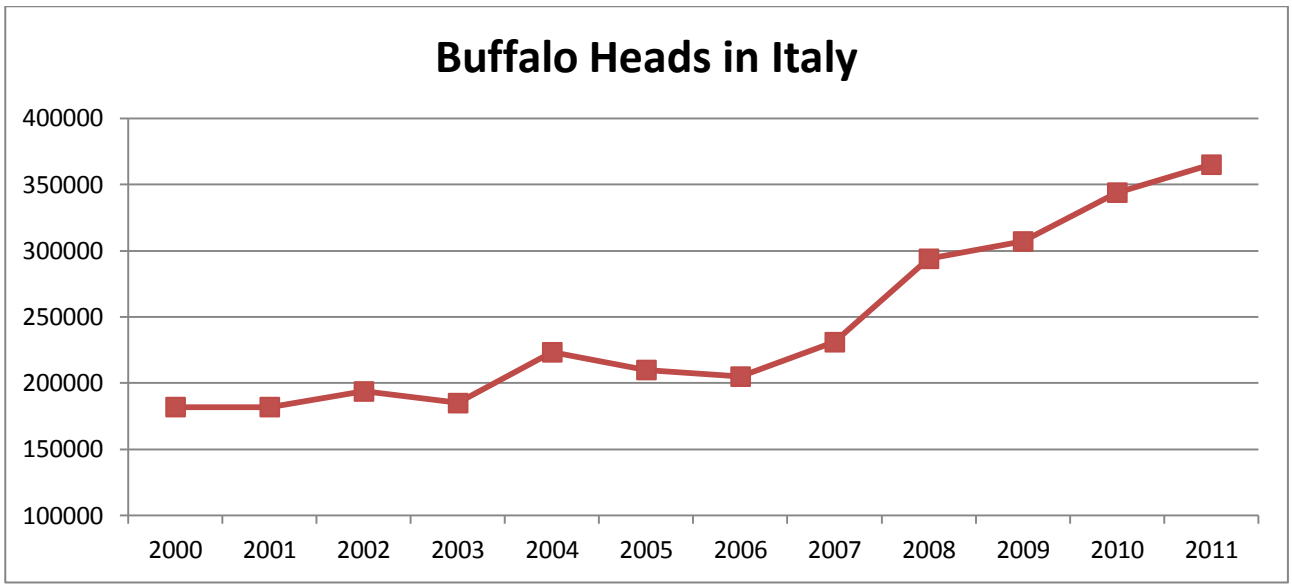


Figure 1.2.4.1. Buffaloes population trend in Italy from 2000 to 2011 (www.fao.org).

In the Italian ANASB (Italian Buffalo Breeders' Association) database (www.anasb.it), 295 breeding farms resulted in 2012, with on average about 190 heads per farm and a total population of about 56,000 buffaloes, which means that there is a big difference in the estimated population relative to that checked. The largest concentration of heads checked can be found in southern and Center of Italy, where 95 percent of the Italian buffalo population is reared.

The increasing in Buffalo farming in Italy is due to the growing market demand for buffalo milk that is utilized exclusively for the production of "mozzarella cheese". Another economic benefit deriving from buffalo milk production is that buffalo milk is not restricted by the specific European Union (EU) directive called "milk quotas", introduced to regulate cow milk production. In fact, this regulation induced some farmers, in areas where Friesian cattle are traditionally reared, to consider the option of breeding milking buffaloes for the production of "mozzarella" cheese. This led to an expansion of buffalo breeding also in the north of Italy, away from the customary area in southern Italy (Borghese, 2005).

The buffalo farming in Italy has been reaching significant productive standards thanks to an intense work of selection and research carried out during the past years. These efforts have led to an officially recognized breed known as Mediterranean Italian Buffalo. Many improvements have been also seen in management. In fact, in Italy, the type of farming has changed over time and the scheme with extensive use of meadows and pastures of the past has given way to an intensive farming, modeled on the same modern systems used for dairy cows. Concerning nutritional aspects, the "unifeed" technique has become very widespread for buffalo, both during lactation and when dried off. Buffaloes are divided into at least two groups: the dried off and the lactating ones. The lactating ones are usually divided in two groups based on lactation stage (milk yield). Dry matter intakes for the lactating buffalo depend on live weight, milk yield, lactation stage (the highest consumption of dry matter is between the 50th and the 150th day from calving), forage concentrate ratio and quality (Bartocci and Terramoccia 2004).

For Mediterranean Italian Buffalo, the recording activity is administrated by the Italian Breeders Association (A.I.A.) through productive controls performed monthly by Recording Inspectors. The collecting activity is organized all over the Italian territory by the Provincial Breeders' Associations

(A.P.A.'s). Data are then transmitted to A.I.A.'s central office to be processed. The productive controls referred to the conventional lactations (270 days or less) longer than 150 days, and regard: milk yield (kg), fat and protein content (kg and %) and somatic cell count. The beginning of the official lactation starts at calving, the first control cannot be carried before the five days from calving and not beyond 75 days. Every milk control must be made on all the milking ordinarily practiced by the breeder in the 24 hours, also annotating: time of the control, amount of milk (kg), and milk weight, measured with a balance or determined with milk flow-meters (Borghese, 2013).

Productive controls are recorded at the end of lactation, and are divided into four categories, based on the parity order:

1-animals at first calving

2-animals at second calving

3-animals at third calving

4-animals from the fourth calving onwards

Data recorded for each category are: the number of controlled buffaloes, the number of closed lactations, the average milk production and the average fat and protein percentage, the average age at calving, the average length of lactation. For the dairy productivity controls, only the conventional lactations longer than 150 days are considered.

All data collected are shared with ANASB, to be processed for the elaboration of productive and morphological breeding values. These are the result of genetic evaluation carried out by the method Blup-Animal Model, which will be explained in more details in paragraph 1.5. The major selective goal for production traits is to increase the mozzarella cheese yield, expressed in the selection index PKM, that is the most important product for buffalo species in Italy, as well as both milk yield and quality. The production traits analyzed for this purpose are:

• Mozzarella cheese yield (PKM)

• Milk yield (kg)

• Fat content (kg and %)

• Protein content (kg and %)

The breeding values are based on open and closed lactations whose length ranges from 140 to 570 days. All productions referred to a standard lactation of 270 days and those reporting value of less than 1,300 kg are not considered.

The milk average production recorded in 2012 was over 2,200 kg, with 8.3% fat and 4.7% protein content (ANASB, 2013; see Table 1.2.4.2.).

Year	Average milk yield (kg)	Fat (%)	Proteins (%)	heads checked (n)	Farms checked (n)	Average heads/farm (n)
2002	2168	8.28	4.73	35755	292	122.4
2003	2175	8.1	4.65	36966	287	128.8
2004	2184	8.06	4.68	39439	294	134.1
2005	2169	8.07	4.69	39925	282	141.5
2006	2178	8.09	4.67	40425	286	141.3
2007	2211	8.18	4.66	44430	290	153.2
2008	2221	8.24	4.66	46799	290	161.3
2009	2182	8.39	4.61	48535	288	168.5
2010	2180	8.47	4.59	50240	292	172,1
2011	2223	8.5	4.66	54548	302	180,6
2012	2218	8.3	4.7	56075	295	190.1

Table 1.2.4.2. Parameters regarding buffalo population and milk production in the last decade in Italy (ANASB 2013).

The production of mozzarella cheese guarantees the profitability of buffalo species in Italy. Buffalo mozzarella is a €300million ($430million) a year industry, which produces around 33,000 tons of mozzarella cheese every year, with 16% sold abroad, mostly in the European Union. France and Germany are the main importers, but sales to Japan and Russia are expanding. With the inclusion in the European Union register of Denomination of Protected Origin in 1996, as "*Mozzarella di Bufala Campana*", the organoleptic and merchandise characteristics of this typical cheese were officially recognized. The *Mozzarella di Bufala Campana* cheese is produced exclusively with buffalo fresh milk from the area of origin and performed with a specific and regulated technological process. The production district of this cheese is defined in its law specifications and encompasses seven provinces across two regions of southern Italy (Campania and Lazio) (Bonizzi et al., 2007).

The composition of buffalo milk is different from that of other animal species, such as cow and sheep. It is richer in protein, fats and calcium. These chemical characteristics allow to obtain cheese yields equal to about twice those usually obtained with cow's milk. In table 1.3.4.3., average production performances of the two most important dairy cow breeds reared in Italy, Italian Holstein and Italian Brown, together with two native northern Italian and less productive breeds, Grey Alpine and Rendena, are compared to those registered for Mediterranean Italian Buffalo.

Breed	Year	N. lactations recorded	Milk yield (kg)	Fat (%)	Protein (%)
Italian Holstein	2012	1,130,270	9,320	3.72	3.38
Italian Brown	2012	56,014	7,089	3.98	3.55
Grey Alpine	2013	6,214	5,015	5.015	3.72
Rendena	2012	2,717	5,206	3.42	3.31
Mediterranean Italian Buffalo	2012	56,075	2,218	8.30	4.70

Table 1.2.4.3. Average production performances recorded for Italian Holstein (www.anafi.it), Italian Brown (www.anarb.it), Grey Alpine (www.grigioalpina.it), Rendena (www.anare.it) and Mediterranean Italian Buffalo (www.anasb.it).

1.3. BUFFALO LOW FERTILITY

Problem of silent heat coupled with late maturity, poor expression of oestrus, irregular oestrous cycle, seasonality in breeding, anestrous, low conception rate, long postpartum interval are some of the major constraints in buffalo productivity and improvement through artificial breeding (Madan et al., 1990). In table 1.3.1. are summarized the principal parameters of buffalo reproduction.

Parameter	Mean	Range
Age at puberty (months)	30	16–46
Weight at puberty (kg)	275	200–350
Length of oestrous cycle (days)	21	17–26
Length of oestrus (h)	10	5–27
Time of ovulation		
After onset of oestrus (h)	34	24–48
After end of oestrus (h)	14	6–21
Length of gestation (days)		
River type	310	300–320
Swamp type	330	320–340
Birth weight of calves (kg)	26	22–36
Involution of uterus (days)	30	25–35

Table 1.3.1. Summary of reproductive characteristics of buffalo (Perera, 2008).

Reproduction is a complex biological process which involves a series of physiological events properly regulated by the endocrine system. In the sexual reproduction of all organisms except bacteria, haploid, uninucleate gametes are produced that join in fertilization to form a diploid, uninucleate zygote, which suddenly develops in a new individual. The gametes differ essentially from somatic cells in having undergone meiosis, a process in which the number of chromosomes is reduced to one-half of the diploid ($2n$) number found in somatic cells. The resulting sex cells thus receive only half the number of chromosomes present in the somatic cell. Furthermore, the sex cells are generally capable of developing into a new individual only after two have united in a process called fertilization. The gametes have two forms: the female sex cell (ovum, or egg), derived from

an oocyte (immature egg), and the male sex cell (spermatozoon or sperm), derived from a spermatocyte.

Different aspects of reproduction in buffaloes, compared to cows, along with the physiological mechanisms involved are discussed in the following paragraphs.

1.3.1. Gamete formation

Reproductive development and physiology are evolutionarily conserved processes across eutherian mammalian species gonads, the site of future gamete production (Matzuk and Lamb, 2002). The indifferent gonad forms during fetal development in the female: primordial germ cells (PGCs) enter the gonad primordium and the tissue eventually differentiates along an ovarian pathway; this differentiation dictates the formation of the secondary sex organs (Xu et al., 1996). The haploid germ cells are produced in the adult gonad, but the diploid germ-cell line is established during early embryogenesis. When the gonads become morphologically differentiated into ovary, the germ cells of the female are termed oogonia and divide mitotically until meiosis initiated. After beginning the meiotic process, germ cells can no longer divide mitotically, and they are therefore incapable of increasing their number. This is crucial for female reproduction since all germ cells are transformed to oocytes during early stages of development. Most of the millions of primordial oocytes formed in the fetal gonads eventually undergo atresia before birth and during postnatal development (Foote, 1975). Thus, the female starts the fertile life with a finite number of germ cells and, unlike the male, the female gonad contains only a limited number of potential gametes in the adult (Austin and Short, 1982). This concept, which has been considered a dogma for many years, has recently been challenged by authors suggesting that neo-oogenesis takes place during adult life in the mouse ovary from germline stem cells in the surface epithelium of the ovary (Johnson et al., 2004). However, even if several studies have supported this theory (Virant-Klun and Skutella, 2010), it remains to be confirmed, as other works failed to find evidence that any cells contribute to the formation of new oocytes in the adult (Notarianni, 2011).

Based on the concept of a limited, fixed supply of oocytes in the female, buffaloes are severely disadvantaged compared to cows. In fact, the ovaries of post-pubertal buffalo heifers have a reservoir of only 10,000 to 20,000 primordial follicles compared with over 100,000 in cattle (Perera, 2011).

1.3.2. Development and function of the reproductive system

The reproductive process in mammals is governed by the central nervous system. Information emanating from a variety of external cues (e.g. visual, auditory, tactile, olfactory) is fed into the central nervous system and converges on the hypothalamus. The hypothalamus is the portion of the anterior end of the diencephalon that lies below the hypothalamic sulcus and in front of the interpeduncular nuclei. The pituitary gland, or hypophysis, consists of two major subdivisions, the anterior and the posterior lobe. The posterior lobe is made up of neural tissue and is connected to the rest of the brain via the infundibular stem, or pituitary stalk (Austin and Short 1984). Thus, there is a direct neural link between the posterior pituitary and the brain. The anterior lobe of the pituitary (or adenohypophysis) is further subdivided into the pars distalis, pars intermedia and pars tuberalis. The pars tuberalis surrounds the infundibular stem like a cuff and extends upwards to lie beneath the portion of the median eminence. The anterior pituitary communicates with the brain by a vascular connection, the hypothalamo-hypophyseal portal system (Austin and Short 1984).

The anterior pituitary secretes six hormones, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Pituitary FSH is essential for development and maintenance of ovarian follicles in single and multiple ovulating species (Kaneko et al., 1991). Inhibin (INHBE), produced by granulosa cells of ovarian follicles, is a glycoprotein hormone which suppresses production and/or secretion of FSH through negative feedback at pituitary level (Burger et al., 2008). In figure 1.3.2.1. the protein interactions involved in this processes are represented. These interactions comprise also growth differentiation factor-9 (GDF9), expressed in oocytes, which plays an important role in the development of primary follicles in the ovary (Juengel et al., 2004). It has a critical role in granulosa cell and theca cell growth, as well as in differentiation and maturation of the oocyte (Su et al., 2004).

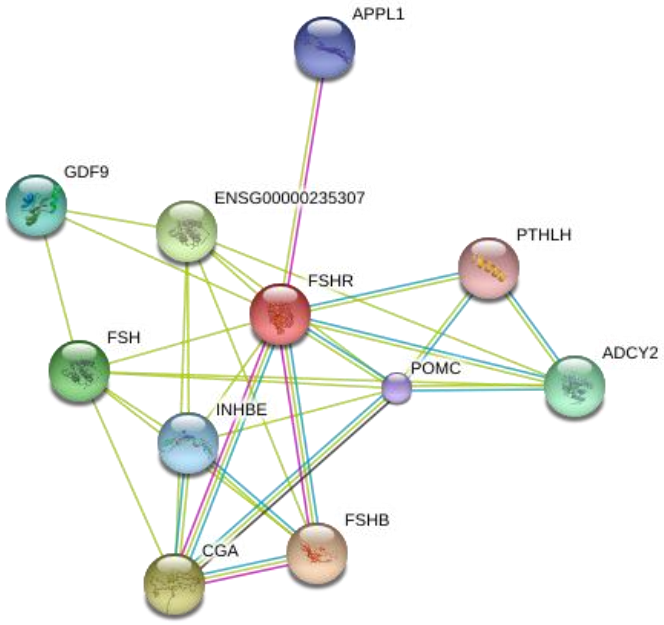


Figure 1.3.2.1. Representation of known and predicted protein interactions of man FSH hormone from STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) v9.1 (Franceschini et al., 2013).

Leptin is another important hormone involved in reproductive processes and it serves as a metabolic signal that acts on the hypothalamic-pituitary-ovarian axis to enhance Gonadotropin releasing hormone (GnRH), and LH secretion and ovarian function (Terzano et al 2012). GnRH is a decapeptide neurohormone that plays a key role in the reproductive axis, ultimately modulating the release of gonadal steroid hormones. It has been reported in several farm animals, that leptin stimulates steroidogenesis and modulated follicular development (Agarwal et al., 1999; Brannian et al., 1999). Leptin effects on gonadotropin-releasing hormone and luteinizing hormone secretion are mediated by neuropeptide Y (NPY) and kisspeptin, thus, leptin appears to be an important link between metabolic status, the neuroendocrine axis and subsequent fertility in farm animals (Barb and Kraeling, 2004). In figure 1.3.2.2. a schematic representation indicating how kisspeptin neurons regulate GnRH neurons is reported.

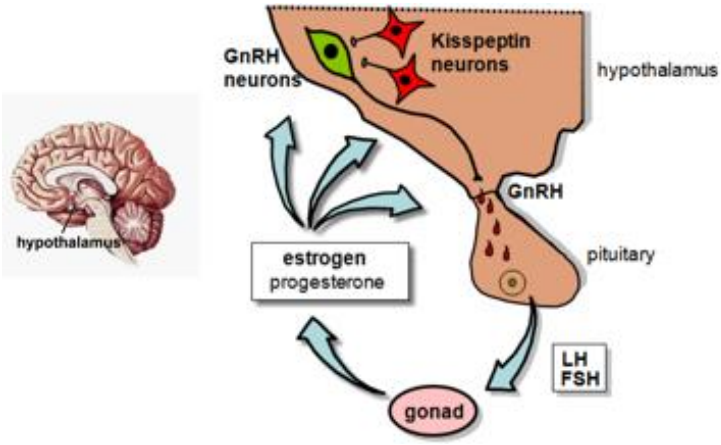


Figure 1.3.2.2. Diagram of kisspeptin neurons regulation of GnRH neurons (Yeo, 2007).

The LH is important in studies of ovarian activity since its pre-ovulatory peak is responsible for the follicular wall rupture and ovulation (Terzano et al, 2012). Both FSH and LH play a fundamental role also in the onset of puberty in mammals.

1.3.3. Puberty

Puberty is the culmination of a complex series of maturational events that lead to the completion of sexual and somatic development and the acquisition of reproductive competence (Tena-Sempere, 2013). In female, puberty is characterized by the manifestation of oestrus and ovulation. This is a developmental process with genetic drivers conserved among species (Matzuk and Lamb, 2002).

Puberty is a product of increased activity of the hypothalamic-pituitary-gonadal axis leading to production of gonadal steroids and other growth-associated hormones (McCarthy, 2013). GnRH from the hypothalamus regulates the pituitary gonadotrope production of follicle stimulating hormone and luteinizing hormone (Matzuk and Lamb, 2002), which in turn reaches the gonad and promotes steroidogenesis (figure 1.3.3.1.).

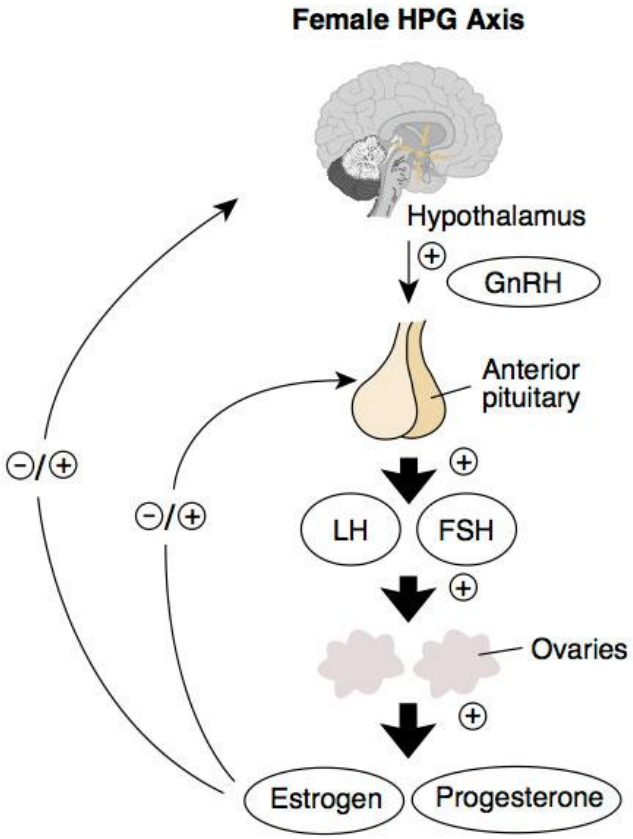


Figure 1.3.3.1. Schematic representation of the Female Hypotalamus-Pituitary- Gonad Endocrine Axis, (Hiller-Sturmhofel and Bartke, 1998). The hypothalamus secretes GnRH that acts on the pituitary gland stimulating its releasing of gonadotropins (i.e., LH and FSH).

During pubertal development, GnRH secretion transforms from low-level irregular pattern to a pattern of regular, pulsatile secretion , which is critical in initiating this process (Plant et al., 2006). The increased GnRH secretion at puberty is determined by a cascade of events. During the prepubertal period an inhibitory neuronal system suppresses GnRH release and during the subsequent maturation of the hypothalamus this prepubertal inhibition is removed, allowing the adult pattern of pulsatile GnRH secretion (Terasawa and Fernandez, 2001). However, interruption of inhibition proves insufficient for induction of puberty; there is also the need for an accelerator, which must include fine-tuned temporal control of GnRH neurons in what is referred to as the GnRH pulse generator, so that luteinizing hormone is released from the pituitary at the appropriate frequency and amount (Windsor-Engnell et al., 2007). In this regard, during the past decade evidence has accumulated suggesting GnRH secretory activity is modulated by a specific glial-neuronal gene family which synthesizes adhesion/signaling proteins involved in the functional and structural integrity of bi-directional glial-neuronal communications (Srivastava et al., 2011).

One hypothesis about the possible mechanism of GnRH neuron activation that has received considerable recent attention is one in which neurons synthesizing the neuropeptide kisspeptin stimulate GnRH neurons to initiate puberty(McCarthy, 2013). Kisspeptin is a small RF-amide peptide from a phylogenetically diverse family of peptides that share a common C-terminal arginine

and an amidated phenylalanine and have critical roles in the control of reproduction, food intake and energy expenditure (Ebling and Luckman, 2008). Kisspeptins are the peptide products of *KISS1* gene, which operate via the G-protein-coupled receptor *GPR54* (Navarro, 2013).The critical role of kisspeptin in pubertal maturation was first demonstrated in 2003 when studies in both humans and mice (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003) reported that mutations or deletion of *GPR54* prevented normal pubertal maturation resulting in infertility. Subsequently, *KISS1* knockout mice, i.e. designed to produce a null mutation in the *KISS1* gene, were reported to exhibit a similar phenotype to the *GPR54* knockout mice, indicating that kisspeptin signaling through *GPR54* is essential for normal pubertal maturation to occur, and loss of *KISS1* cannot be overcome by compensatory mechanisms. (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). In figure 1.3.3.2., the *KISS1* network in man is represented, indicating interactions between *KISS1* and GnRH.

KISS1 neurons are localized to the arcuate nucleus and the anteroventral periventricular nucleus, two brain regions that play a key role in the control of GnRH neuronal activity, hence kisspeptin was established as being fundamental for GnRH secretion, but also for luteinizing hormone release and, ultimately, puberty (Kauffman, 2010). In fact, since kisspeptin cells provide direct synaptic input to GnRH cells (Kinoshita et al., 2005) and kisspeptin is a potent stimulator of GnRH secretion (Messager et al., 2005), these cells are ideally placed to transmit estrogen feedback information to the brain cells that drive the reproductive process (Clarke et al., 2009).

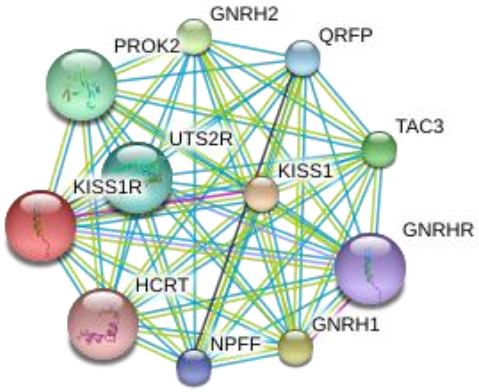


Figure 1.3.3.2. *KISS1* network in man, from STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) v9.1 (Franceschini et al., 2013).

The timing of puberty onset is an important phenotype for the livestock industry because late puberty has negative effects on reproduction rates and profitability. In buffalo, compared with cattle, puberty is delayed. Buffalo heifers usually attain puberty when they reach about 55-60%, of their adult body weight, around 250-400 kg for the river type (Perera, 2011). The age at which buffalo heifers attain puberty can be highly variable, ranging from 18 to 46 months, and it is significantly influenced by nutrition, management, social environment, climate, season of birth, growth rate and diseases (Barile, 2005). The pre- weaning and weaning systems are important in promoting growth and achieving puberty, therefore heifer management needs start from birth to ensure a correct weight increase. In fact, the animals that showed a higher daily gain before the

trials reached puberty in a shorter time (Borghese, 2005). The delay in puberty and the consequent delay in conception, is one of the causes of low reproductive efficiency of bubaline species thus lengthening non-productive life. In recent years, there has been a general improvement of this parameter. In a study performed on 86 farms (30,735 primiparous buffaloes, which calved between 1975 and 2005), it was observed that the mean age at first calving decreased by 1 month every 5 years (Zicarelli et al., 2007). However it must be said that, since the age at puberty is hard to establish because of difficulties in estrous detection, in this species most estimations are extrapolated from the age at first calving (Barile, 2005).

1.3.4. Oestrous cycle

From the time of puberty, the female begins to express the oestrous cycle which, in buffaloes, can vary from 16 to 28 days (Baruselli et al., 1997). The periods of oestrous cycle are oestrus, metoestrus, dioestrus and pro-oestrus.

1.3.4.1. Oestrus

During oestrus period, the female is receptive to the male and will stand for mating. Near the start of oestrus, there is dramatic surges in FSH, which promotes follicular growth and estrogen production by granulosa cells in ovarian follicles, and LH, together with estradiol. The interval between the onset of oestrus and the LH surge can vary from 1 to 12 h in buffaloes and ovulation occurs between 26 and 33 hours after the LH surge (Seren and Parmeggiani, 1997), with differences according to the reproductive method adopted. For example, studies on Italian buffalo show that they have the interval from peak LH concentration to ovulation being about 25 hours in animals that conceived to artificial insemination (AI) and 46 hours in those that did not (Moioli et al., 1998).

Behavioural symptoms of oestrous are induced by the action of estrogens on the central nervous system. These are hormones produced by the ovary and transported by carrier proteins, the most important of which is estradiol 17β (E2). Peripheral plasma E2 profile in buffalo is not very different from that reported in cattle, with peak concentrations observed before and during the preovulatory surge of gonadotropins, after which the levels come down to base values in the next few days, with minor fluctuations throughout the oestrous cycle (Terzano et al., 2012). A marked difference between buffalo and cattle is that external signs of oestrus are less obvious in the former, with less oestrus-associated mounting behavior (Roy and Prakash, 2009). The main behavioral signs are restlessness, bellowing and frequent voiding of small quantities of urine, but these are not consistently exhibited by all animals. Externally detectable physical changes include swelling of the vulva, resulting in removal of the horizontal wrinkles that are present on its external surface and this, together with vestibular reddening, can be detected by regular examination of individual animals under confined systems (Perera, 2008). Mucus, secreted from the cervix during oestrus, is less copious than in cattle and does not usually hang as strands from the vulva but tends to accumulate on the floor of the vagina and be discharged either when the animal is lying down or with the urine. These factors have contributed to the observation that silent ovulation (also termed as silent oestrus) is more common in buffalo than in cattle (Perera, 2008).

1.3.4.2. Metoestrus

After ovulation, dramatic changes occur in the follicle, which result in the formation of a transient ovarian organ, the *corpus luteum* (CL). The mammalian CL is composed of a heterogeneous mixture of cell types. There are at least two types of steroidogenic cells, large and small luteal cells, which originate from the granulosa and thecal cells of the follicle ruptured at ovulation, respectively

(Skarzynski et al., 2008). The primary product of the CL is progesterone (P4), required for establishment and maintenance of pregnancy. P4 concentration in blood increases during the developing luteal stage. The CL continues to secrete a high level of P4 until late luteal stage, and then its ability rapidly decreases in the regressing luteal stage (Skarzynski et al., 2008). In domestic animals LH released in a pulsatile fashion from the anterior pituitary plays a major role in the regulation, synthesis and secretion of P4 in the CL (Niswender et al., 2007). In buffalo, P4 levels continue to increase in animals that conceive, but drop 3 days before the next oestrus in those that fail to conceive (Batra et al., 1979). Variation in progesterone concentration during oestrus cycle in buffaloes can also be observed according to season (Srivastava et al., 1999) and nutritional status (Ronchi et al., 2001).

1.3.4.3. Dioestrus

Dioestrus id characterized as the period in the cycle when the *corpus luteum* is fully functional and reaches the maximum size. Progesterone attains the highest values in this stage, preventing secretion of GnRH by the hypothalamus. The duration of this phase is directly related to the time that the corpus luteum remains functional. High progesterone levels prompt the uterus to prepare a suitable environment for development of the embryo, and eventual attachment of the conceptus to the endometrium (implantation).

1.3.4.4. Pro-oestrus

If fertilization does not occur, the pro-oestrus period begins, during which the regression of CL, or luteolysis, takes place. This is essential for normal cyclicity as it allows the development of a new ovulatory follicle (Skarzynski et al., 2008). In mammals, luteolysis consists of two phases, functional luteolysis and structural luteolysis (McCracken et al. 1999). A rapid functional regression of CL is characterized by a decrease of P4 production, followed by a phase of structural regression (McCracken et al. 1999). The onset of the decline in P4 concentrations is variable, depending upon the time of regression of CL (Terzano et al., 2012). The CL regressing implies that the hypothalamus is no longer inhibited and oestrogen levels are rising, due to the formation of follicles, promoting GnRH secretion. It has been proposed that the regression of the CL in buffaloes is a more extended process than in cattle, based on a more gradual decline in circulating concentrations of progesterone in the former (Avallone et al 1987). In support of this suggestion, some authors demonstrated that the RNA/DNA ratio of CL tissue in buffaloes did not change during the developing, developed and regressing phases. Moreover, on a tissue weight basis, total DNA and RNA did not differ between developing and regressing CL of buffalo (Ghosh and Mondal, 2006). On the contrary, in cattle the RNA/DNA ratio was reported to decline during the regressing phase of the CL (Hafs and Armstrong, 1968; Mares et al., 1962).

The following figure 1.3.4.4.1. represents a mammalian ovary with the sequential development of the follicle and the formation of *corpus luteum*.

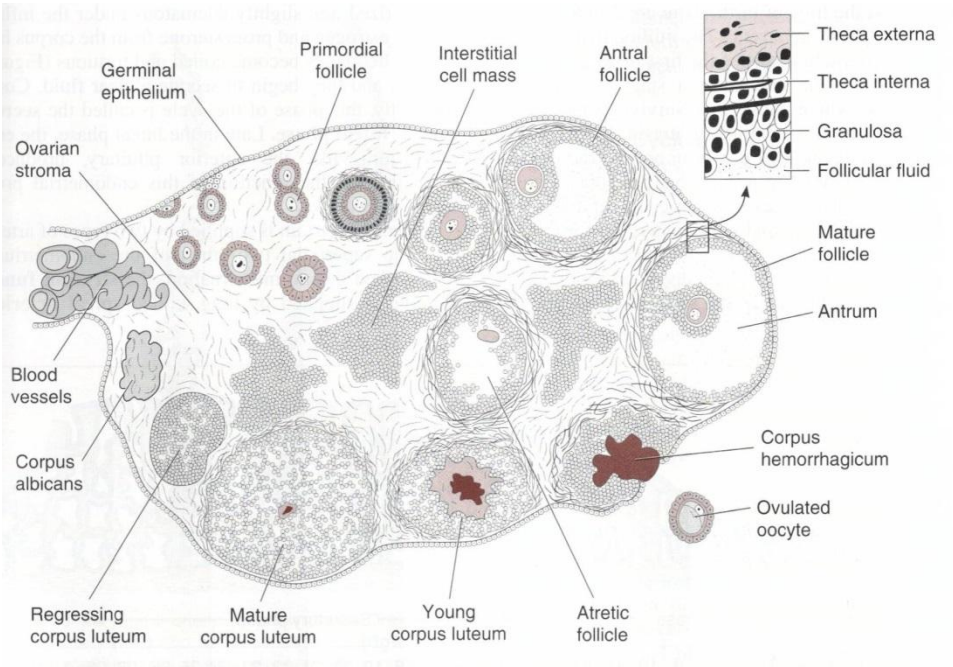


Figure 1.3.4.4.1. Diagram of a mammalian ovary, showing the sequential development of a follicle, formation of a *corpus luteum*, and, in the center, follicular atresia. A section of the wall of a mature follicle is enlarged at the upper right. (Gorbman and Bern, 1962).

1.3.5. Fertilization, pregnancy and embryo development

Fertilization comprises a series of steps beginning with penetration of the egg coats followed by the incorporation of the spermatozoon into the cytoplasm of the egg. When the mammalian oocyte is fertilized, it is still arrested at metaphase II until the sperm's entrance activates a release of calcium from storage sites into the ooplasm in a wave-like pattern (Swann and Yu, 2008). The repeated oscillations of cytosolic Ca^{2+} give rise to a set of event known as oocyte activation (Khatib, 2012). Next there occurs a transformation of the sperm nucleus and of the remaining haploid set of egg chromosomes so as to form, respectively, the male and female pronuclei (Austin, 1982). Fertilization results with a diploid cell, the zygote, containing the genetic code for a new individual.

With fertilization starts gestation, which ranges from 300 to 330 days with a mean of approximately 310 days for river type buffaloes (Perera et al., 1987).

To establish a successful pregnancy, a number of well-orchestrated events must take place in a precise order. An 'in time' resumption of ovarian activity and cyclicity should result in the completion, selection and growth of a healthy follicle, enclosing a competent oocyte, and ultimately in ovulation, fertilization and uterine attachment of a viable embryo (Van Soom et al., 2006). Early embryo development is a process of series of repeated cell divisions known as cleavage. Each cell in an early mammalian embryo is called a blastomere, and there are no morphologic differences among individual blastomeres (Chen et al 2010). However, from a molecular point of view, many complex processes take place in each individual blastomere in order to attain cell division and begin cell differentiation (Khatib, 2012). During the first cell cycles, the preimplantation embryo is controlled by maternal genomic information that is accumulated during oogenesis (Telford et al 1990). Around the 8-cell stage in the bovine embryo, the embryo starts transcribing its own RNA (Memili and First, 2000).When the embryo passes from the oviduct into uterus, an amorphous mass of cells forms a structure called morula. During the next few days, fluid collecting in the

intercellular spaces will push to the center forming the blastocyst, a structure with a fluid-filled cavity (the blastocele) surrounded by a layer of cells (Bearden and Fuquay, 1984).

In ruminants, the establishment and maintenance of pregnancy is strongly dependent on progesterone production. This is related to the correct functionality of *corpus luteum* at the early stages of embryo development, and supported by additional progesterone production by the fetoplacental unit at later stages of pregnancy (Jainudeen and Hafez, 1980). In buffaloes, it has been reported that progesterone concentration linked to maintenance or loss of pregnancy, follows a seasonal pattern, with a differential production according to the period of the year (Di Francesco et al., 2012). However, the reasons for higher embryonic mortality in buffaloes during specific periods of the year are not fully understood (Campanile et al., 2013).

1.3.6. Embryonic mortality

The high incidence of embryonic mortality is one major reproductive problem recognized in subfertile animals. Embryonic development in buffaloes is advanced by 12–24 h compared with embryonic development in cattle, as it has been demonstrated by both in vivo (Gasparrini, 2002) and in vitro studies (Neglia et al., 2003). The windows for embryonic mortality in buffaloes occur between day 15 and day 24 (early embryonic mortality) and days 25–45 (late embryonic mortality), with fetal mortality typically occurring from day 46 to day 90 (Vecchio et al., 2010). This is a big issue in buffalo as in cattle, where more than 40% of the conceptuses are lost within two weeks post insemination, suggesting that oocyte and early embryo quality may be compromised (Wathes et al., 2008). Furthermore, in cattle late embryonic losses (after Day 28 post insemination) can account for 20% of pregnancy losses and 5% of cows lose their fetus during later pregnancy (Wathes et al., 2008).

Many factors are involved in embryonic mortality and the failure to maintain pregnancy. Concerning early embryonic mortality, it is clear that the oocyte's microenvironment during oocyte maturation has a profound effect on oocyte quality and subsequent embryo developmental potential (Leroy et al., 2011). During follicular growth, maternal genes are transcribed and the resulting mRNA and protein molecules are synthesised and accumulated in the oocyte (van den Hurk and Zhao, 2005). These processes are crucial to guarantee the survival of the early embryo before embryonic genome activation. Once genome activation has occurred, the embryo starts using its own newly formed DNA to make transcription factors (Leroy et al., 2011). This means that, even when a perfect fertilization has taken place, adverse follicular conditions during oocyte growth and maturation can impact on the viability of the embryo later on (Leroy et al., 2011). Embryonic mortality at this stage is unobserved by the farmer. However, adverse conditions within the follicle during oocyte growth and maturation may originate from disturbed maternal metabolism, which can lead to an incompetent oocyte and thus to impaired fertility (Leroy et al., 2011). Also late embryonic mortality is likely mediated through inadequate oocyte competence and a compromised maternal environment. Oocyte competence increases with follicular maturity and is dependent upon acquisition of a complete complement of mRNA transcripts and establishment of the appropriate epigenetic marking of the oocyte genome before the preovulatory gonadotropin surge (Pohler et al., 2012).

1.3.7. Postpartum anoestrus

As in cattle, postpartum uterine involution in buffalo is usually completed in 25-35 days after calving (Perera et al., 1987). At this moment, there are a low number of ovarian follicles and

follicular waves and few cycles occur. If conception does not take place, therefore, an anoestrus of variable length begins (Zicarelli, 1994). The period of postpartum anoestrus is a major cause of infertility resulting in economic loss to buffalo breeders in many countries (El-Wishy, 2007), is highly variable and is usually longer than in cattle under comparative management conditions. Under optimal conditions, buffalo can resume ovarian activity after calving by 30-90 days. The presence of the bull in the herd appears to have a biostimulating effect on postpartum ovarian activity of buffalo cows, reducing cyclic irregularities and advancing the time of ovulation (Moioli et al., 1998).

Abnormal estrous cycles postpartum lead to prolonged calving to first insemination intervals and, consequently, to prolonged calving intervals, that are important parameters to evaluate the productive and reproductive efficiency in a farm and/or in a population (Singh and Lal, 1992). Actually, long calving intervals are a major problem in buffalo breeding. On Italian farms where natural mating is practiced and bulls are always present in the herd, the calving interval is approximately 400 days and the culling rate is lower than 15% (Zicarelli, 2010).

1.3.8. Application of reproductive biotechnologies in buffalo breeding

From the above, it is clear that inherent reproductive problems, namely weak/silent oestrous signs, seasonal anoestrus, a long post-partum anoestrus period, delayed age of puberty and low conception rates limit the productivity of buffalo. Assisted reproductive technologies have been introduced to overcome the reproductive inefficiencies, thus contributing to increased genetic gain (Nandi et al., 2002). These technologies, allowing planning selective directions in a shorter time, allow the distribution of elite genes, the reduction in generation interval and provide continued genetic gain and increased production. In fact, in spite of the expansion of buffalo farming, the improvement, for example, in milk or meat production was poor, and was mainly due to a progress in management techniques rather than to genetic selection (Barile, 2005).

Manipulation of animal reproduction is probably as old as domestication itself. Since man started to keep animals in captivity, he exerted a profound influence on the natural behavior and reproduction of domesticated species. The purpose of this manipulation was always an attempt to optimize production traits, whether it is milk, meat, wool, labor or any other advantage a particular species could possibly offer. Until only several decades ago, the productivity of our livestock species improved by application of the principle ‘breed the best to the best’. Thus, crossbreeding the most productive animals or those with the best conformation characteristics resulted in a gradual improvement of certain traits, depending on their inheritability and expression (Bols et al., 2010).

Today, assisted reproduction and biotechnology allow breeders to design and direct the reproductive course, disseminate desired traits and accelerate genetic improvement (Basrur and King, 2005). Therefore, reproduction biotechnology is without doubt one of the most emblematic products for research to genetic improvement in the field of animal science and it has left a decisive mark on the evolution of farming over these last sixty years (Thibier, 2005).

1.3.8.1. Artificial Insemination (AI)

The first generation of reproduction biotechnology applied is artificial insemination (AI). The use of frozen-thawed semen for AI, along with sire testing and selection has markedly affected genetic quality of livestock, especially in dairy cattle (Bazer and Spencer, 2005). Through the choice of the best males this technique allows to improve the genetic make-up of the entire population. AI gives the opportunity to quickly obtain a numerous progeny for a given sire, thus allowing a quick

evaluation of its genetic value, carried out through the productive performances of its daughters. The use of frozen semen for AI has also made it possible both for genes to migrate from one population to another through the marketing of male germoplasm and for female of seasonal breeders to be bred during the non-breeding season, and to preserve and use the germoplasm of a meritorious male beyond his reproductive lifespan (Foote, 1999).

There are in Italy two Bull Buffalo Centers for semen production: the COFA (Cooperativa Fecondazione Artificiale) in Cremona Province, Lombardia Region, in North of Italy and the Chiacchierini Bull Centre in Perugia Province, Umbria Region, in Central Italy, which currently produces semen from 16 tested bulls (Borghese, 2013). However, the application of AI in the buffalo species has always found strong limitations due to intrinsic physiological features such as reduced signs of oestrus behavior, variable duration of oestrus and time interval between LH surge and ovulation (Baruselli et al., 1997; Campanile et al., 2008). The failure in estrus detection causes a decrease in the reproductive performance and a consequent increase in the breeding period and in the calving interval, with serious economic losses for the breeder (Baruselli et al., 2007). Therefore, the use of management schemes not requiring the identification of estrus, contribute to the increase of AI in buffalo herds, mainly because it is easy to perform. The objectives of these schemes are to synchronize the luteal phase, the follicular growth and the ovulation allowing the AI in all animals of the farm, even those that are not showing estrus or cyclicity. The use of these protocols, which have been improved with the spread of artificial insemination technique in buffalo herds, enables genetic improvement, increasing milk and meat yield (Baruselli et al., 2007).

1.3.8.2. Synchronization of ovulations and oestrus induction

Systematic reproductive manipulation of the estrous cycle using exogenous compounds such as progesterone and gonadotropins to mimic physiological levels of naturally occurring hormones are routinely used in dairy herds world-wide. These hormonal interventions normally improve fertility performance and decrease proportion of animals culled at the end of lactation due to reproductive failure (van Werven et al., 2013). Various protocols to synchronize estrus and ovulation have been evaluated in buffaloes in an attempt to overcome the difficulty of applying AI in spontaneously ovulating animals (Zicarelli et al., 2007). Hormonal treatments have been tested to induce and synchronize oestrus in buffalo heifers, with economic impact as a greater proportion heifers can be bred early (Barile et al., 2001). At the period of seasonal anoestrus buffaloes present absence of estrous behavior and a lack of ovulation and progesterone secretion by the ovary. Thus, at this period ovarian follicular turnover occurs. To induce oestrus in buffalo, prostaglandins have been used to control both luteal and follicular functions, providing the possibilities for synchronization of follicular growth and ovulation (Kharche and Srivastava, 2001). Synchronisation with different compounds (PGF2α-prostaglandin2α, GnRH-gonadotrophin releasing hormone, CIDR-controlled internal drug relesing device, PRID-progesterone releasing intravaginal device, CRESTAR-progestagen ear implant) have been used to synchronize the follicular wave and/or luteal regression (Borghese, 2013). The use of gonadotropin-releasing hormone (GnRH) followed seven days later by prostaglandin F2α (PGF2α) can synchronise oestrus and improves the conception rate (De Rensis et al., 2005; Karen and Darwish, 2010). These hormonal treatments to synchronize oestrus in buffalo are widely used also to induce out-of season reproduction. Nevertheless, it must be considered that the demand for free-hormone products is increasing and this leads to a search for alternative methods.

Between the other important reproductive technologies are: induction of multiple ovulation followed by embryo transfer (MOET), which allows the decreasing of generation interval for the obtaining of a higher number of progenies during the reproductive female life; ovum pick-up (OPU) and in vitro embryo production (IVEP), technologies that allow to obtain from each female a greater number of transferable embryos (Gasparrini, 2002), permitting the repeated production of embryos from live donors of particular value and is a serious alternative to multiple ovulation (Galli et al., 2000); use of sexed semen or embryos to produce female progeny.

1.4. SEASONALITY OF REPRODUCTION

In buffalo, the successful application of reproductive technologies can be hampered, among other conditions already mentioned, by the seasonality of reproduction.

Seasonality is a survival strategy adopted by many wild mammals to ensure that the offspring are born at the most favorable season of the year. This biological programming of births, or synchronization of reproductive response to appropriate environmental conditions, clearly leads to distinct advantages for the offspring, being born at the time of most suitable weather and maximal food availability during the early stages of life (Wood et al., 2006). Although domestication processes generally reduced seasonality of reproduction compared to what is observed in their wild counterparts, a majority of animal-derived products remain accessible only seasonally and this characteristic is still present in some genetic types of bovine extensively bred, such as Podolica, Sarda, Maremmana, Bos Indicus and the Highland bovine (Zicarelli, 1997).

In buffalo, the differential seasonal output in terms of cyclicity, pregnancy rates, and calving is clearly evident when animals are subjected to controlled breeding through the adoption of reproductive technologies, as well as when they are left to naturally occurring mating with the exclusion of any human intervention (Di Palo et al., 2009). The buffalo cows seasonal change in displaying oestrus, conception rate and calving rate is clearly manifested. Buffalo is a short day breeder, as it is sexually active in response to decreasing day length that is, under Mediterranean latitudes, in late summer to early autumn (Zicarelli, 1997). On the other hand, during the spring and summer, the cow shows stages of partial anoestrous or even deep anoestrous. Calving occurs mainly between July and December and the calving interval is longer for deliveries occurring between February and June, indicating a decrease in the conception rate during the spring-summer seasons. This is a big issue in Italy, where the market demand for buffalo dairy products is concentrated in the spring-summer period. To produce milk in synchrony with the market requirements, a special procedure was defined and developed, the out of breeding mating season (OBMS). It consists in the interruption of sexual promiscuity in the herd between September and December during the first year of application, and from September to March in the following steps of the technique (Zicarelli, 1997). Currently, the OBMS technique is practiced in more than 60% of the farms in Campania region, where the largest concentration of heads can be found. It must be considered, however, that in Italy the OBMS technique leads to a decline in fertility. In fact, when the OBMS technique was not applied, calving intervals of 400 to 445 days were recorded, while more recently, a mean inter-calving interval of 487±133 days was reported (Zicarelli et al. 2007).

When the AI is applied, the females are generally inseminated in February-March after oestrus induction, to obtain calving before spring. However, buffaloes that undergo oestrus synchronization and artificial insemination during a period of increasing day length have a relatively low conception rate, about 50%. Therefore, a month after artificial insemination the empty females are naturally mated to increase conceptions of a further 30% with a total mean conception rate of 80% (Borghese 2013). Buffaloes calving during the unfavorable season express long calving intervals, because they do not resume their ovarian activity until the following proper season, decreasing their reproductive efficiency (Borghese, 2005). In particular, it has been reported that buffaloes that deliver between January and March delay their conception until August to September, after three months of decreasing day length. Similarly, buffaloes that deliver in the period April to September show the shortest inter-calving period because after 58 days decreasing day length begins (Zicarelli, 2010). However, also buffaloes that delivered in the period October-December show high calving

intervals, due to the out of breeding season mating technique, that does not allow the conception between October and February, the period of maximum sexual activity. The shortest calving intervals are generally observed in the last two quarters, when the resumption of ovarian activity coincides with the decreasing day length (Zicarelli 2007).

Among the environmental factors influencing reproductive seasonality, photoperiod is of critical importance. The annual photoperiod cycle provides a critical environmental signal, which entrains seasonal physiology (Dupre et al., 2008). Nocturnal secretion of the pineal hormone melatonin reflects these seasonal changes in photoperiod and thereby provides the brain with an internal hormonal representation of external photoperiod changes. Together with reproduction, seasonal cycles in melatonin modulates multiple physiological systems including food intake, adiposity, body temperature regulation, and many neuroendocrine pathways (Bartness et al., 1993). In buffaloes living in environments where there is no significant annual variation in photoperiod (equatorial zones) nutrition has a major influence on reproduction (Vale et al., 2002). On the other hand, they become increasingly influenced by photoperiod with distance from the equator, even if nutrition remains important (Zicarelli et al., 1997; Campanile et al., 2010). In the following paragraphs, the principal mechanisms behind mammalian seasonality are presented.

1.4.1. The basis of circannual timing

At the basis of reproduction seasonality are long-term timing mechanisms that allow organisms to anticipate environmental events months or years in advance and to optimize survival and reproductive success (Lincoln et al., 2003). Two types of mechanisms are used by mammals for the long-term timekeeping. The first, already mentioned, is photoperiodism, which registers the change in the annual cycle in day-length and translates this into the timed control of physiology and behavior (Tamarkin et al., 1985). The second timing mechanism is circannual rhythm generation, which occurs in mammalian groups from all latitudes. These species express annual cycles in the wild, and continue to express circannual cyclicity when maintained indoors under constant conditions, often for many years or throughout life (Woodfill et al., 1994).

1.4.2. Photoperiodism

Photoperiodism generates timing through *photoinduction* which is a genetically programmed response to a change from short to long days, or *vice versa* (Lincoln et al., 2003). The response to photoperiod is mediated by endogenous rhythms, controlled by one neuronal pathway. The photoperiod is converted into neuroendocrine signals via a dedicated photo-neuroendocrine pathway, which involves the master biological clock located in the suprachiasmatic nuclei (SCN) and other hypothalamic nuclei, which in turn synchronize various biological activities with the time of the day and year (Kalsbeek et al., 2006). The mammalian pineal gland converts external signals (principally light) to an endocrine message: melatonin, produced exclusively at night, with duration depending on the length of the night. Therefore, photoperiodic variations in circulating levels of melatonin throughout the year informs the animal of the day length, providing the body with a strong and reproducible representation of the seasons (Simonneaux and Ribelayga, 2003).

Melatonin is secreted into the peripheral blood and cerebral spinal fluid where the highest concentrations occur (Malpaux et al., 2001). Experimental studies in different species, among which sheep, clearly demonstrate that pinealectomy, or any surgical procedure that disrupts the daily melatonin signal, blocks seasonal photoperiodic responsiveness (Karsch et al., 1989; Lincoln, 2006). Variable long-term cycles in gonadal activity and other characteristics persist, however,

reflecting the intrinsic control (Lincoln, 2006). The action of melatonin is apparently unique, as the same signal can both stimulate and inhibit reproductive activity, depending on the species. The reproduction of short-day breeders, that are sexually active in fall, such as buffalo, is stimulated by melatonin via exerting a stimulating effect on Gonadotropin-releasing hormone secretion by the hypothalamus. On the contrary, in long-day breeders, such as horse, increased melatonin exposure has the opposite effect, inhibiting GnRH release by the hypothalamus.

Light regulates the melatonin rhythm by two different mechanisms: first, periodic light stimuli every 24 h act to entrain the circadian clockwork of the SCN, and to regulate clock genes and electrophysiological activity of SCN neurons (Sumova et al. 1995, Nuesslein-Hildesheim et al. 2000), which control the timing of many aspects of daily rhythmicity, as well as the nocturnal-associated release of melatonin (Lincoln et al., 2003); secondly, light inhibits melatonin secretion regardless of circadian time, via a retinal–hypothalamic–sympathetic innervation to the pinealocytes and the control of the rate-limiting enzyme N-acetyltransferase (NAT), the key enzyme in the melatonin biosynthetic pathway (Klein et al. 1997). These two mechanisms determine that melatonin is released only at night, providing an endocrine index of night length and thus day length (Lincoln et al., 2005).

Concerning circadian rhythms, the molecular machinery that regulates these processes comprises of a set of genes, known as "clock" genes, the products of which interact to generate and maintain the rhythms. Clock genes are strongly involved in the molecular mechanism that decodes the duration of the melatonin signal to produce a summer or winter phenotype (Lincoln, 2006). In figure 1.4.2.1. is reported the pathway map representing today knowledge on the molecular interaction and reaction networks for circadian rhythm in man. The first negative feedback loop is a rhythmic transcription of period genes (*PER1*, *PER2*, and *PER3*) and chryptochrome genes (*CRY1* and *CRY2*). *PER* and *CRY* proteins form a heterodimer, which acts on the *CLOCK/BMAL1* heterodimer to repress its own transcription. *PER* and *CRY* proteins are phosphorylated by *casein kinase I epsilon* (CKIepsilon), which leads to degradation and restarting of the cycle. The second loop is a positive feedback loop driven by the *CLOCK/BMAL1* heterodimer, which initiates transcription of target genes containing E-box cis-regulatory enhancer sequences (Kanehisa et al., 2012).

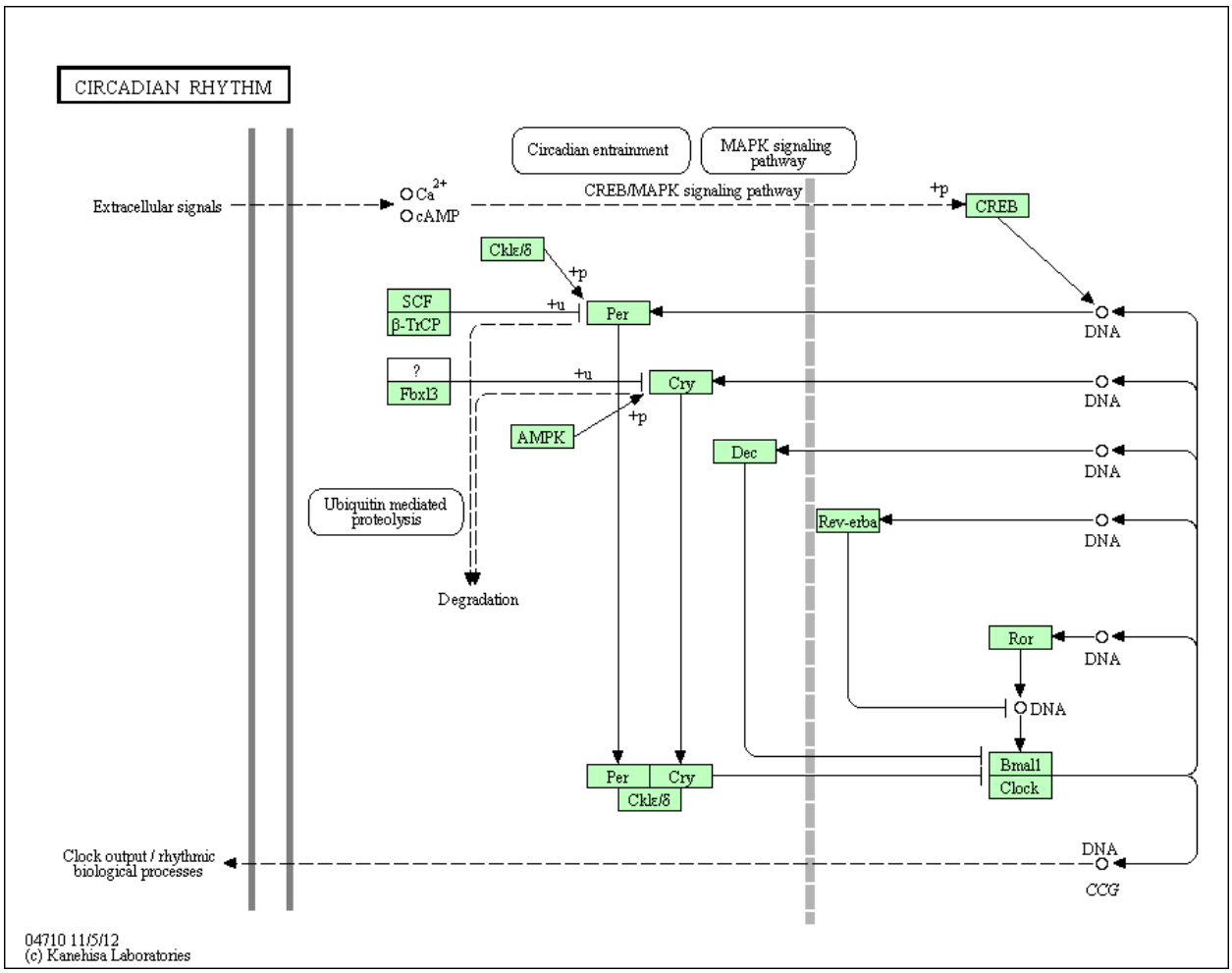


Figure 1.4.2.1. Pathway map of circadian rhythm in *Homo sapiens*, from KEGG database (Kanehisa and Goto, 2000), update October 2013.

Light stimuli are received by the SCN directly through the complex retino-hypothalamic pathway of phototransduction (see figure 1.4.2.2.). This is a biochemical process by which the photoreceptor cells generate electrical signals in response to the caption of photons made by the photoreceptive pigments, the rhodopsins.

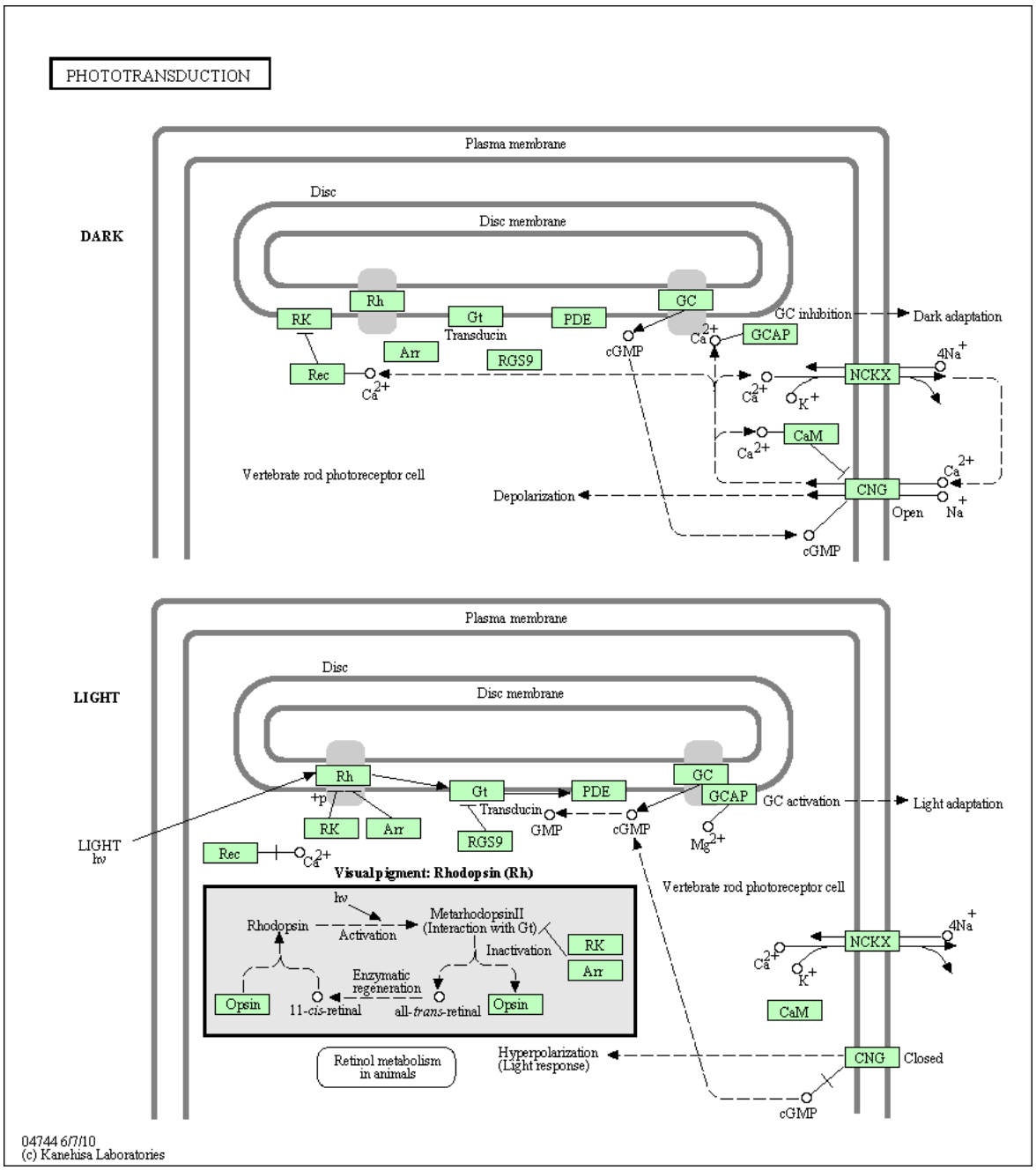


Figure 1.4.2.2. Pathway map of Phototransduction in Bos taurus, from KEGG database (Kanehisa and Goto, 2000), update October 2013. The photon isomerizes 11-cis-retinal to all-trans-retinal which induces a structural change that activates the opsin. This triggers hydrolysis of cGMP by activating a transducinphosphodiesterase 6 (PDE6) cascade, which results in closure of the cGMP-gated cation channels (CNG) in the plasma membrane and membrane hyperpolarization. The hyperpolarization of the membrane potential of the photoreceptor cell modulates the release of neurotransmitters to downstream cells. Recovery from light involves the deactivation of the light- activated intermediates: photolyzed rhodopsin is phosphorylated by rhodopsin kinase (RK) and subsequently capped off by arrestin; GTP-binding transducin alpha subunit deactivates through a process that is stimulated by RGS9 (Kanehisa et al., 2012).

Through this complex process, light active SCN neurons, synchronizing a circadian pacemaker in the SCN that controls the activity of the pineal gland (figure 1.4.2.3.). This "central pacemaker" in the SCN receives signals from the environment and coordinates the oscillating activity of peripheral clocks that are located in almost all tissues (Schibler and Sassone-Corsi, 2002).

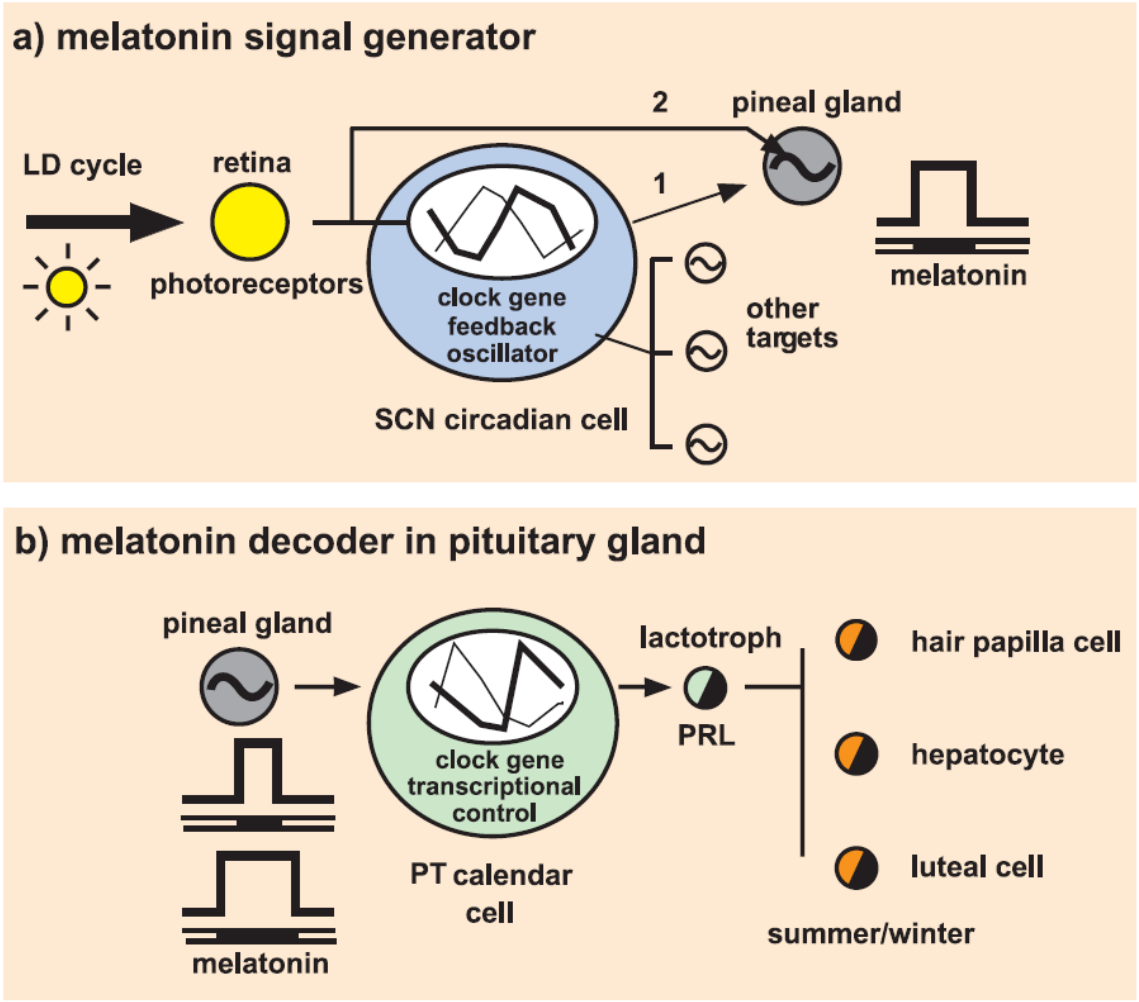


Figure 1.4.2.3. (Lincoln et al., 2003) Representation of the two mechanisms by which light regulates the melatonin rhythm: (a) the generation of a 24-h nocturnal melatonin signal by the pineal gland that reflects the night-length and thus day-length, and (b) the decoding of this signal in specialized target tissues, including the pars tuberalis (PT) of the pituitary gland that is thought to regulate seasonal cycles in prolactin secretion from lactotrophs. Light of the daily light/darkness cycle is detected by photoreceptors in the retina, and influences the melatonin signal through entrainment of the SCN circadian cell (pacemaker pathway - 1) and by inhibition of melatonin secretion (inhibitory pathway - 2). The wave form of circadian clock gene expression, and patterns of electrophysiological activity in the SCN pacemaker, are known to vary with photoperiod; however long-term time keeping depends principally on the melatonin target cells – here called calendar cells. These are thought to use a clock gene-based mechanism to decode melatonin duration to produce a long or short day physiology. For the PT/lactotroph axis, LD activate an increase in prolactin release and drive associated seasonal changes in the hair papilla cell (pelage moult cycle), hepatocyte (liver function), luteal cell (uterine activity/implantation) and other tissues.

One important feature of the circadian clocks is that they are self-sustained: circadian oscillations intrinsic to each cell can occur autonomously, without any environmental signals. However, because the period of oscillation is not exactly 24 h, the endogenous clock needs to be synchronized by external cues, a process called *entrainment* (Masri et al., 2012). External cues, above all light, reset the system daily and thereby prevent the endogenous clock from free-running out of phase (Quintero et al., 2003). Unlike temperature, availability of food, rainfall or other environmental cues, photoperiod provides information about the season and remains constant from year to year.

1.4.3. Circannual rhythm generation

Circannual rhythm generation is the second timing mechanism used by mammals for long-term timekeeping. Under static conditions, complete endogenous long-term cycles of physiology and behavior, alternating between summer and winter phenotypes, may persist for years or even throughout the entire life cycle. In longer-lived species (e.g. ground squirrels, mustelids, sheep, and deer), exposure to a fixed light period can also cause refractoriness and reversion to a winter-like physiology, and when the photoperiod is held constant for a sufficiently long period, these animals express alternating transitions in seasonal physiology every 10–12 months as an endogenous circannual rhythm (Freeman and Zucker, 2001).

In some species (e.g. hibernating ground squirrels, tropical fruits bats), the intrinsic circannual cycle predominates irrespective of photoperiod, while in others (e.g. Sika deer, Suffolk sheep) both circannual timing and photoperiodism are combined to regulate seasonality (Lincoln et al., 2003).

Analogous to multi-oscillator circadian organization, circannual rhythm generation occurs in multiple tissues with hypothalamic and pituitary sites serving as central pacemakers (Hazlerigg and Lincoln, 2011). In photoperiodic species, the innate nature of the timing mechanisms is revealed by exposure to constant day length and temperature extended over long periods, analogous to the use of constant light or dark regimens in the study of circadian biology (Hazlerigg and Lincoln, 2011). Melatonin target tissues read the changes in the nightly melatonin signal to time seasonal physiology to the annual photoperiodic cycle. The interpreting of the changes in melatonin signal duration that govern seasonal physiology depends on specialized melatonin target cells in the brain, pituitary gland and, it is believed, in peripheral tissues. These cells are required to express high affinity with melatonin receptors to register the systemic signal, and to distinguish between short (6–10 h) and long (12–16 h) daily exposure to melatonin (Lincoln et al., 2003). Long duration melatonin signal stimulate a winter physiology and short duration signals promote a summer physiology. However, the cellular sites of melatonin action for the seasonal control of reproductive activity are still unknown (Simonneaux et al., 2013). A high density of melatonin receptors has been identified in the pars tuberalis of the adenohypophysis in a number of mammalian species (Masson-Pevet and Gauer, 1994), and recent studies point to this structure as a crucial site for the effect of melatonin on seasonal functions, particularly reproduction (Nakao et al., 2008; Dardente et al., 2010).

An important role in the seasonal regulation of reproduction in mammals has been recently demonstrated for kisspeptins and RFamide-related peptide-3 (RFRP3) neurons (Simonneaux et al., 2013). Kisspeptins have been already mentioned in the previous paragraphs for their role in reproductive processes, including the onset of puberty. In fact, decisive evidence has mounted in recent years that KISS1 neurons, expressing kisspeptins, are part of such a hypothalamic network involved in the metabolic regulation of GnRH neurons (Castellano et al., 2010). On the other hand, the *RFRP* gene in mammals is expressed in neurons situated in the mediobasal hypothalamus and it encodes a precursor that produces two peptides, RFRP-1 and RFRP-3 (Kriegsfeld et al., 2006; Revel et al., 2008; Rizwan et al., 2009). RFRP neurons project to multiple brain regions including the preoptic area, the arcuate nucleus, the lateral septum, the anterior hypothalamus, and the bed nucleus of the stria terminalis (Ukena and Tsutsui, 2001; Mason et al., 2010). In a recent work performed on Syrian hamster species, it has been reported that *RFRP-3* expression in the dorsomedial hypothalamus is strongly inhibited by melatonin in a short-day photoperiod and that both kisspeptins and RFRP-3 neurons regulate GnRH neuron activity. In addition, central RFRP-3

infusion was associated with a significant increase in arcuate kisspeptins expression (Simonneaux et al., 2013). Hence, the role of kisspeptins and RFRP3 neurons is thought to be essential in regulation of reproductive seasonality in mammals.

Interesting hypotheses have been presented to explain the long time domains that characterize circannual rhythms, recognizing that histogenic processes running at low rates over months to years are the core of this mechanism (Hazlerigg and Lincoln, 2011). Authors proposed that circannual rhythm generation depends on tissue-autonomous, reiterated cycles of cell division, functional differentiation, and cell death (Hazlerigg and Lincoln, 2011). According to authors, while circadian timing is clock gene and cell based, circannual timing is tissue based and the local environments surrounding adult stem cells (so-called stem cell niches) play a fundamental role. They stated also that entrainment of circannual rhythms by photoperiod or other inputs operates through the CNS via hypothalamic or pituitary stem cell niches (Hazlerigg and Lincoln, 2011), focusing on two potential cell stem niches as being of crucial importance: the subventricular zone adjacent to the walls of the third ventricle in the basal hypothalamus and the cleft region in the anterior pituitary. Based on this hypothesis, the new challenge would be to identify genes that govern the timing of cyclic hystogenesis in the adult, confirming their role in circannual timing by tissue-specific ablation (Hazlerigg and Lincoln, 2011).

1.5. SINGLE NUCLEOTIDE POLYMORPHISM (SNP) MARKERS IN ANIMAL GENOME ANALYSIS

From all the foregoing that fertility and reproductive seasonality, regardless of the species, breed or sex of the individual, are very complex processes. In addition, an antagonistic relationship between fertility and milk production parameters has been well-established in numerous studies in cattle (Roxstrom et al., 2001; Royal et al., 2002; Berry et al., 2003). This unfavorable genetic correlation has led to a decline in reproduction in dairy animals, at least in part due to an insufficient consideration of this trait when selecting for a higher milk production. Undesirable trends are expected for the reproductive traits if they are not included in the breeding goal and many countries have lately implemented genetic evaluation for fertility traits in cattle species. More traits are gradually being evaluated and more sophisticated evaluation methods are being implemented (Berglund, 2008).

Today's breeding is international, intensive and uses modern reproductive and molecular genetic techniques. For Mediterranean Italian Buffalo, the selection indexes were published for the first time in 1997 by the Italian ANASB association, which collects phenotypes and determines the selection goals. For farmers participation in the controls is voluntary and milk records registered are: milk yield (kg) fat and protein content (kg and %), mozzarella cheese yield (kg), somatic cells and morphological traits. No reproductive parameters are currently included in selection schemes.

Mediterranean Italian Buffalo genetic evaluation is based on the Best Linear Unbiased Prediction (BLUP) of bulls, which relies on modeling of phenotypic and pedigree information to estimate the genetic value of animals. The BLUP method enables the distinction of the genetic and non-genetic components of the phenotypic values (Henderson, 1975). Since in buffalo, as in all dairy animals, many traits included in the selection index are sex-limited (i.e. milk yield and quality traits) and can only be collected on females, phenotypes are recorded on the daughters of bulls under evaluation. This selection scheme, called Progeny Test (PT), is designed to increase genetic progress by optimizing the accuracy of selection and the generation interval.

In the genetic evaluations of buffaloes, it is necessary to consider that BLUP methods assume that all known genetic relationships among individuals included in the analysis are correct (Parlato and Van Vleck, 2012). However, in the Italian buffalo population, rates of sire and dam misidentification were found to be 24% and 20% (ANASB, Caserta, Italy; E. Parlato, 2010). This is a big challenge, since pedigree errors are expected to bias estimation of genetic parameters (Senneke et al., 2004), breeding values of individuals (Banos et al., 2001), correct ranking of tested bulls in progeny testing, and expected genetic progress (Israel and Weller, 2000; Weller et al., 2006).

As already stated, natural mating is the system applied by most Italian buffalo enterprises. Breeding is, generally, carried out by group mating (2 bulls with 25 cows) and calving takes place on open ranges. Under these conditions, paternity is hard to establish. Thus, to avoid pedigree errors due to sire misidentification, only sires identified by DNA testing are included in the relationship matrix for the genetic evaluation. Sires in the reported pedigree are classified as unknown (Parlato and Van Vleck, 2012). These limitations in registering the genealogies, together with the difficulties in applying the AI necessary for the planned progeny test, strongly hamper the genetic improvement in buffalo species.

Recently, use of DNA markers has provided a more accurate method of identifying individuals and verifying parentage. For the buffalo population, the DNA markers of choice in parental testing are microsatellites, i.e. repeating sequences of 2-6 base pairs of DNA (Heyen et al., 1997). The probability of exclusion depends on the marker type, number of alleles, and allelic frequencies in the population to be used for paternity testing. In a recent work on the effect of parentage misidentification on estimates of genetic parameters, it was demonstrated that including only bulls identified by DNA testing in the pedigree for the genetic evaluation will lead to a much greater genetic progress. Authors concluded that implementation of reproductive biotechnologies, particularly AI programs, and DNA testing of sires are the keys to increasing genetic progress in the Italian buffalo population (Parlato and Van Vleck, 2012).

With recent advances in genetics it is now possible, and highly recommended, to use molecular markers as auxiliary tools for animal breeding. Molecular biological tools are the methods of choice also to provide an insight into the limitations currently associated with reproductive technologies in buffalo species (Singh et al., 2009). The next challenge is to integrate the knowledge gained from molecular genomic investigations into optimizing buffalo production systems through the selection of desirable animals and their use in breeding programs.

1.5.1. The generation of single nucleotide polymorphisms

Molecular genetics techniques are widely used in today's breeding, with an extent depending on the species. In fact, the genetic polymorphism at the DNA sequence level has provided a large number of markers and revealed potential utility of application in animal breeding (Teneva, 2009), opening the possibility to evolve the genetic evaluation of all species of livestock.

The most widely used class of molecular markers in today's genetics are single nucleotide polymorphisms (SNPs), as they are easy to evaluate and interpret and they are widely distributed within genomes. In fact, SNPs are the most common form of polymorphism among individuals, approximately one every 200 base pairs in livestock (Williams, 2005). Both during the DNA duplication that occurs when cells divide and as the result of external factors (e.g., exposure to radiation or certain chemicals), changes in the nucleotide sequence (i.e., mutations) can occur. A SNP marker is just a single base variation in a DNA sequence, with a usual alternative of two possible nucleotides at a given position. For such a base position with sequence alternatives in genomic DNA to be considered as an SNP, it is assumed that the least frequent allele should have a frequency of 1% or greater (Vignal et al., 2002). SNPs with a minor allele frequency (MAF) <5% are usually considered as rare, and the others are called "common". SNPs may be within (intragenic) or outside genes (intergenic). Within a gene, a SNP may be located within a coding sequence (exonic) or within a non-coding sequence (intronic). In turn, exonic SNPs can be split into synonymous (no amino acid change in the protein) and non-synonymous (amino acid change in the protein). Non-synonymous SNPs are potentially functional.

Furthermore, many mutations occur in non-coding DNA regions and therefore do not result in protein variants that are associated with an altered phenotype or increased disease risk. Under two conditions, however, even mutations in non-coding regions might result in an altered phenotype. First, mutations that occur in regulatory regions, such as promoters or intron splice sites, could alter gene activity and, consequently, the phenotype determined by that gene. Second, non-coding mutations that occur in an intron or outside a gene could be associated with an altered phenotype if they are positioned close to a functional mutation, according to the phenomenon known as linkage

disequilibrium (LD). This is defined as the correlation of alleles at two loci, and depends on the distance between the loci. The greater the distance, the higher is the probability of a recombination event to occur between the two loci. On the other hand, the alleles of two marker loci that are very close together will have a very low probability of recombination and will mostly segregate together in the population. Hence, non-coding mutations that are very close to a functional mutation (typically within 200,000 nucleotides) are almost always inherited together with the functional mutation itself (Kwon and Goate, 2000).

1.5.2. The human genome example

As often in molecular genetics, work progress in the human genome is the most advanced. The availability of genetic markers has led to extraordinary progress in human genetics in the past 25 years, including the elucidation of the molecular genetic basis of many Mendelian disorders or traits. In 2005, the International HapMap Project genotyped one million SNPs (Consortium, 2005) and, in a second phase, a total of 4.4 million human SNP were genotyped (Frazer et al., 2007). At the time being more than 20 million genetic markers are deposited in public databases (see the NCBI Single Nucleotide Polymorphism database). These variants constitute the major source of inter-individual genetic and phenotypic variation (Beckmann et al., 2007).

The literature is continuously expanding with experimental uses of genetic variation. To date, SNPs are the variant type of choice for association studies in common diseases and complex traits. In fact most human variation that is influenced by genes can be traced to SNPs, especially in such medically and commercially important traits, and even when a SNP is not directly responsible, the overall number of SNPs means they can also be used to locate genes that influence such traits (Li and Sadler, 1991). Nucleotide diversity is also a sensitive indicator of biological and historical factors that have affected the human genome (Chakravarti, 1999). Gene diversity depends on the mutation rate of genes, the size and demographic history of the population in which these mutations occur, the time over which such diversity accumulates and biological factors such as selection. Human population history studies in the past relied largely on single genetic loci, such as mitochondrial DNA. The year 2010 saw the publication of the first three ancient hominid nuclear genome sequences (Green et al., 2010; Reich et al., 2010), the first results from the 1000 Genomes Project (Consortium, 2010), and several other human genome and exome sequences (Li et al., 2010; Schuster et al., 2010). These new developments could help to better understand human population history (Stoneking and Krause, 2011).

1.5.3. Use of SNP markers in animal science

Following the human model, we assist in the last years to a high improvement in genetic studies for animal species. SNPs are particularly interesting for this purpose occurring, for example, at a frequency of about one SNP per 500 base pairs (bp) in cattle (Heaton et al. 2001), which is twice the frequency observed in man, attesting at about one SNP per kb.

In recent years, large collections of SNP have been identified in the main livestock species, like chicken (2.9 million), dog (3.2 million), mouse (15.5 million), horse (1.15 million) and cow (9.2 million) (Archive EnsEMBL release 68 - July 2012). With the advent of next-generation sequencing, more high density SNP arrays were made commercially available, for example Illumina BovineHD BeadChip with more than 777,000 SNPs (Matukumalli et al., 2011), which is 15-fold denser as compared to the previous BovineSNP50 array (Van Tassell et al., 2008). Today the use of SNP markers plays a fundamental part in different fields of animal breeding and genetics, such as

food traceability and authentication, conservation and the most commonly used Marker Assisted Selection, or MAS and genomic selection.

In following paragraphs, some of the most common applications of molecular genetics in animal science are presented.

1.5.3.1. Traceability

Genetic traceability is the identification/authentication of animals and their products through the analysis of DNA, which is unique for each individual and highly variable among individuals (except for monozygotic twins and clones) (Alford and Caskey, 1994; Cunningham and Meghen, 2001). It is inalterable during animal life, sex and age independent, quite stable in processed food (at least subjected to standard treatments) and present in almost all of the cells of the organism. Once DNA is extracted from a biological sample (blood, muscle, hair, sperm or even a processed food such as cheese or canned meat), it is analysed by molecular markers to assess variations between individual (Nicoloso et al., 2013). Individual identification and paternity testing are based on the principle of molecular fingerprinting (Cegelski et al., 2003; Koskinen, 2003; Lirón et al., 2004). Animal assignment exploits the difference in allele frequencies among breeds. Although less informative (Krawczak, 1999), SNPs are rapidly replacing microsatellites due to a more robust genotyping and data interpretation (Weller et al., 2006), and a strong potential for automation (Lindblad-Toh et al., 2000).

SNPs in the coding regions of candidate genes might be breed specific markers exploitable in a diagnostic assay to identify and protect typical products tied to a specific breed. EU - resolution 2009/c 286 E/10 invokes new management approaches that reconcile animal food production with the conservation and sustainable use of biodiversity, promotes the delivery of ecosystem services and benefits the agricultural sector and society as a whole. This resolution calls on member states to sponsor traditional products, to improve the traceability of animals and products, to sustain Protected Geographical Indication and Protected Designation of Origin brands and to introduce a mandatory EU labelling regulation system (Nicoloso et al., 2013). In this context, breed DNA traceability and authentication methods might represent a valid marketing strategy and operative tool to support and protect high quality products from local breeds linked to traditional farming and production methods (Schwägele, 2005; Smith et al., 2005).

1.5.3.2. Conservation and genetic diversity

DNA-based polymorphisms are now the markers of choice also for molecular-based surveys of genetic diversity. Wide agreement exists on the need to conserve the genetic diversity of animal genetic resources (AnGR). Genetic diversity is necessary for genetic change within a biological population. Genetic diversity of AnGR allows for the sustained ability of a breed or population to respond to selection to increase productivity and for adaptation to changing environmental conditions, including not only those conditions associated with climate, but also to changes in markets, management and husbandry practices, and disease challenges (Boettcher et al., 2010). In turn, conservation of diversity of AnGR helps ensure long-term food security. In addition, conservation of specific AnGR may be necessary to preserve particular cultural and historical values, to sustain the request value of livestock, and to fulfill the rights of an existing genetic resource to continue to exist (Hanotte and Jianlin, 2005). Conservation is one of the four Strategic Priority Areas of the recently adopted Global Plan of Action for Animal Genetic Resources

(Hoffmann et al., 2011), underlining the need for governments to address this topic in national plans for management of AnGR.

1.5.3.3. Marker and Assisted Selection

One of the most commonly used applications of markers in animal breeding programs is Marker Assisted Selection, or MAS. This is the process of the selection for a particular trait using genetic markers, DNA segments associated with and hence segregating in a predictable pattern as the trait. The basic idea of MAS is to exploit linkage disequilibrium existing in the joint distribution of markers and quantitative trait loci (QTL) genotypes (Gianola et al., 2003), which can be used to improve predictions of genetic merit of candidates for selection in a breeding program (Fernando and Grossman, 1989). MAS can accelerate the rate of genetic progress by increasing accuracy of selection and by reducing the generation interval. The advantages of MAS are that it is greatest for traits with low heritability. It facilitates increased rate of genetic gain by allowing measurement in young stock thereby reducing generation interval, which is very important above all for traits that can be identified and selected only after growing the organism to maturity. MAS could also enhance future prospects for breeding for such traits as tolerance or resistance to environmental stresses, including diseases (Soetan and Salako, 2010). Several strategies to incorporate markers information on breeding programs have been proposed, as selection index, two and single step analyses and non-parametric methods (VanRaden, 2008; Legarra et al., 2009). SNP markers are also employed to determine the genetic relatedness in animals (Heaton et al., 2002). Through the DNA fingerprinting method, it is possible to accurately trace offspring to parents or genetic source (Soetan and Salako, 2010).

1.5.4. Association Studies

Genetic association studies are performed to determine whether a genetic variant is associated with a trait: if association is present, a particular allele, genotype or haplotype of a polymorphism or polymorphisms will be seen more often than expected by chance in an individual carrying the trait. Thus, association studies are based on the examination of one or more genetic variants in different individuals to see if any variant is associated with a trait. Microsatellite markers selected from genome-wide linkage have been used to localize QTL with effects on several economically important traits in cattle (Boichard et al. 2003; Casas et al. 2003; Ashwell et al. 2004; Hu et al. 2007). However, genotyping microsatellite markers is labour-intensive and allele calls are laboratory-specific. In addition, these anonymous markers provide no information on the genes underlying QTL. SNPs are more abundant than microsatellite loci and, despite being biallelic and so having lower information, SNPs within genes may also be the causative mutations responsible for variations in the phenotypes (Williams et al., 2009).

There are two approaches for dissections of complex and quantitative traits using single nucleotide polymorphisms, i.e., genome-wide association study and candidate gene association study.

1.5.4.1. Genome-Wide Association Studies

Genome-wide association study (GWAS) has become feasible in humans as well as in domestic animals thanks to the development of large collections of SNPs and the development of more cost-effective methods for large-scale SNP analysis. The number of SNPs required for a GWAS depends on the pattern of linkage disequilibrium in the population. In humans, significant LD usually extends over only short distances (tens of kb) and therefore a large number of SNPs is required for GWAS, on the order of 500,000 SNPs. On the contrary, the population structure of domestic

animals makes them particularly useful for GWAS. Populations of domestic animals resemble to some extent recombinant inbred lines although most domestic animals are not highly inbred. But breeds have been formed from large populations by dividing them into many smaller often closed populations. This has led to a reduced genetic diversity within breeds and large haplotype blocks. Therefore, a more modest number of SNPs is required for GWAS in domestic animals (Andersson, 2009).

The number of studies based on this technique in animal sciences is increasing. In cattle, to name a few as example, in a recent study authors performed a GWAS for rectal temperature during heat stress in lactating Holstein cows, identifying SNPs that serve as QTL for that trait, which could prove useful in genetic selection and for identification of genes involved in physiological responses to heat stress (Dikmen et al., 2013). In another recent GWAS, authors genotyped more than 700 animals with phenotypes on several carcass quality traits, providing useful information to further assist the identification of chromosome regions and subsequently genes affecting carcass quality traits in beef cattle (Lu et al., 2013). Pig is another species well studied and for which several genome wide association studies have been performed. For example, a whole-genome analysis performed in one porcine crossbreeding population, revealed molecular networks and potential candidate genes for the expression of lean meat water-holding capacity (Heidt et al., 2013).

These studies are only few examples of the increasing number of researches based on genome-wide analysis. This is because genomic selection has two important properties: (1) it strongly increases the potential genetic gain, by up to 80% because of a reduced generation interval (Schaeffer, 2006); and (2) it disconnects the phenotype recording in a reference population from the selection of the candidates evaluated from their genomic information. However, this does not mean that phenotyping is now less important than in the past. On the contrary, the difficulties in obtaining accurate phenotypes and biological samples, particularly for tissues other than blood, which are difficult to obtain from live animals, are important limitations. The real added value of these approaches relies on researchers ability to transform these raw data without interest on their own at the farmer level into informative diagnosis through indicator traits (Boichard and Brochard, 2012).

1.5.4.2. Candidate Gene Association Studies (CGAS)

Candidate gene approach is based on the *a priori* hypothesis of the involvement of a gene in pathways playing a role in the determination of a phenotype of interest. This approach is based on: generating hypotheses about, and identifying candidate genes that might have a role in, the aetiology of the trait; identifying variants in or near those genes that might either cause a change in the protein or its expression, or be in linkage disequilibrium with functional changes; genotyping the variants in a population; and on using statistical methods to determine whether there is a correlation between those variants and the phenotype (Tabor et al., 2002).

Therefore, candidate genes are generally the genes with known biological function directly or indirectly regulating the developmental processes of the investigated traits, which could be confirmed by evaluating the effects of the causative gene variants in an association analysis (Zhu and Zhao, 2007). The first studies focused the attention on single polymorphisms, in single genes, thought having a major role determining functional changing in the transduced protein, or in close regions regulating the gene expression. In the candidate gene approach, differently from other genetic designs, is easier to find a linked intermediate phenotype which can confirm the hypothesis,

since the selection of gene is based on *a priori* hypothesis of involvement of its function in a certain phenotype (Gianfagna et al., 2012).

There are two main strategies in selecting candidate genes for an association study.

One is position-dependent strategy, in which the identification of candidate gene is mainly based on the physical linkage information in a QTL-identified chromosomal segment (Zhu and Zhao, 2007). This approach aims at the proximity of known QTLs, and candidate genes are identified from tens to hundreds of gene members harbored in the targeted chromosomal region. Some successful applications of position-dependent strategy have already been reported in different fields, including the classical examples of *DGAT1* in cattle (Grisart et al., 2002), *GDF8* in sheep (Johnson et al., 2005) and *IGF2* in swine (Van Laere et al., 2003).

The other strategy is the functional candidate gene approach, where a putative candidate gene is the one that could be statistically detected from the genes controlling large components of inheritable gene expression variation. One method used to find out a gene's function is based on the use of knockout mouse models, which are typically designed to produce a null mutation in the target gene and thus they are useful to reveal non-redundant and essential functions of genes (Matzuk and Lamb, 2002). Although functional information from gene knockout and transgenic animal and cellular models can provide distinct clues about candidate genes responsible for phenotypes of interest, there is little practical information available because of the difficulty of producing gene knockout and transgenic animals in livestock (Zhu and Zhao, 2007). In general, important biological features of traits are directly reflected by transcript pattern, and quantitative traits were usually the consequence of the structure of genetic regulatory networks and the parameters that control the dynamics of those networks (Frank, 2003).

Many researchers used the candidate gene approach in different fields. For instances in goat species, recent works based on the candidate gene approach found polymorphisms associated with birth weight and weaning weight (Supakorn and Pralomkarn, 2013), dairy (Crepaldi et al., 2013) and growth (Zhang et al., 2013) traits.

1.5.4.3. Comparison between the two techniques

Until recently, genetic association testing of quantitative or binary traits was synonymous with candidate gene analysis, as the number of markers required for genome-wide coverage made studies of one or a few candidate genes the only practical option (Singer, 2009). With the onset of microarray genotyping, the whole-genome association analysis became feasible, and also in buffalo species a genomic SNP chip tool is now developing. However, candidate gene associations still remain a powerful method, especially for the association analysis of particular traits, such as fertility.

The relatively sparse number of polymorphisms tested in whole-genome association analysis provides high power to identify significant associations. However, because the candidate polymorphisms have been specifically selected by the researcher, the credibility and interpretability of those significant associations are frequently greater than is the case for associations identified in a genome-wide study (Singer, 2009). In fact, the most important problem in *a posteriori* designs is that the plausible mechanism linking genotype and phenotype is often not known. In most of GWAS, SNPs found to be associated to a particular trait are located in regions distant from genes (Gianfagna et al., 2012). In fact, genome-wide scanning usually proceeds without any presuppositions regarding the relevance of specific functional features of the analyzed traits. In

general, genome-wide scanning only locates the glancing chromosomal regions of quantitative trait loci (QTLs) at cM-level with the aid of DNA markers under family-based or population-based experimental designs, which usually embed a large number of candidate genes. In fact, up to now, genome-wide association studies have used panels of SNP markers selected to give a uniform distribution across the genome. In comparison, the alternative candidate gene approach has been proven to be extremely powerful for studying the genetic architecture of complex traits, which is a far more effective and economical method for direct gene discovery (Zhu and Zhao, 2007). Indeed, to identify the causative mutations within genes or regulatory sequences, SNPs within genes provide a higher power for association analysis, even when the SNPs themselves do not produce functional variations, compared with using a SNP set with uniform genome-wide distribution (Jorgenson and Witte, 2006). This conclusion is based on the observation that, in general, linkage disequilibrium in regions with a high density of coding sequences is shorter than in gene-sparse regions of the genome. As a consequence, SNPs within genes are more likely to be in LD with the causative variations than SNPs in flanking non-coding regions (Williams et al., 2009).

A number of important discoveries have recently been made from GWAS, with important advance in our understanding of the genetic basis of many traits of interest. Nevertheless, the associated loci that have been identified usually have small individual effects on phenotype, and even collectively tend to explain only a small fraction of the heritable component (Kruglyak, 2008). Moreover, for some traits studied no significant loci have been identified with this method (Gibson and Goldstein, 2007). This failure to detect loci that explain the bulk of the heritable components of the phenotypes studied could be imputable to several factors. First, because the detected loci have small effects, the power to detect them is low, and more such loci remain to be discovered as sample sizes increase. Second, association studies can only detect the effects that are due to relatively common alleles. Rare alleles remain to be discovered — both at the loci that are identified by GWAS because they also have common alleles with phenotypic effects, and at other loci that do not have such common alleles (Kruglyak, 2008). The former can be found by focused resequencing studies of the loci identified by GWAS; finding the latter might require resequencing of other genes in the relevant pathways, of the exons of all genes (Porreca et al., 2007) or of the entire genome. Third, we might be missing the effects of structural variation, of other less well studied types of genome alterations, and of interactions among variants and between genetic and environmental factors (Kruglyak, 2008).

So, in general, candidate gene studies tend to have rather high statistical power but are incapable of discovering new genes or gene combinations, while GWA studies can identify genes regardless of whether their function was known before (Cooke et al., 2008), but have low power owing to the number of independent tests performed (Wu et al., 2010). Indeed, the problem of false positives, already an issue in early studies deploying a few hundred microsatellite markers, is becoming acute as we move into the era when SNPs are replacing microsatellites and more than a million markers may be used (Amos et al., 2011). A bias on population genetics parameters caused by the use of SNP subsets discovered in different breeds has been also assessed. In fact, existing SNP panels were developed in breeds unrelated or poorly related to, for example, those used in extensive agriculture. As a result, they may not be fully informative to detect selective sweeps along the genome in many breeds (Negrini et al, 2010). The limited SNP density, or marker resolution, of the BovineSNP50 assay significantly impacted the rate of false discovery of selective sweeps. This bias led to the identification of recent selective sweeps associated with breed formation and common to

only a small number of breeds rather than ancient events associated with domestication which could potentially be common to all European taurines (Ramey et al., 2013).

On the other hand, the two approaches of genome-wide and candidate gene analysis could be seen as two complementary tools in the study of complex traits. In fact, GWAS provides useful information to further assist the identification of chromosome regions and subsequently genes affecting traits. Candidate genes identified this way, can then be studied to find polymorphisms that are most likely to functionally affect the trait. This strategy, proved to be useful in the analysis of different traits and species. For example, in a recent work authors performed a GWAS to analyze fatty acid composition in more than 500 pigs, identifying a total of 46 loci surpassing the suggestive significance level with specific effects on fatty acid composition and finding several promising candidate genes in the adjacent regions of the lead SNPs at the genome-wide significant loci, such as *SCD* and *ELOVL7* (Yang et al., 2013). To cite another example, a genome-wide association approach utilizing the Illumina BovineSNP50 BeadChip was presented to seek genomic regions that potentially harbor genes underlying variation in carcass quality of beef cattle, providing useful information to further assist the identification of chromosome regions and subsequently genes affecting these traits (Lu et al., 2013).

1.5.5. The genetic information in buffalo species

Despite the increasing interest in buffalo, the genetic information is still underdeveloped in this species as compared to other, such as cattle. The backwardness in the information of bubaline genome is a big challenge, since genome resources in water buffalo would provide knowledge and technologies that could help optimize production potentials and reproduction efficiency in the species.

For the whole genome mapping, the first method used in buffalo species is the radiation hybrid (RH) and in situ hybridization. Iannuzzi and Di Meo (2009) reported 309 mapped loci on all chromosome arms mostly assigned by FISH (Fluorescent In Situ Hybridization). As water buffalo and domestic cattle, both members of the Bovidae family, are closely related, the vast amount of genomic resources for cattle research has served as shortcuts for the water buffalo community to initiate genome science in the species (Michelizzi et al., 2010). RH maps were constructed for river buffalo and cattle Y chromosomes (Stafuzza et al., 2009) and the first generation whole genome RH map for river buffalo when compared to Btau_4.0 genome sequence assembly showed the marker order with in linkage groups was consistent with cow assembly (Michelizzi et al., 2010). These studies encouraged researchers interested in buffalo genomics to undertake buffalo genome mapping initiatives using cow genome resources. The first version of assembly of a single female Murrah buffalo was constructed with Illumina paired end and mate pair short read sequencing using the cattle genome (Btau 4.0 assembly) as a reference (Tantia et al., 2011). This buffalo assembly represented ~91%-95% coverage in comparison to the cattle assembly and the analysis also reveiled about 300 structural variants in the buffalo genome (Tantia et al., 2011). In Italy, an *International Buffalo genome Consortium* was founded, within an international collaborative project, with the aim to sequence and analyze the buffalo genome, starting with de novo sequencing, assembly and annotation of a Mediterranean Buffalo female (Williams J, 2013).

For now, the complete annotated genome sequence for buffalo is not yet available in public genetic resources. Likewise, is not yet available a genome-wide chip tool for a GWAS for this species, even if it is developing. In a recent work authors made an investigation of transferability of

BovineSNP50 BeadChip from cattle to water buffalo for genome wide association study (Wu et al., 2013). A total of 40,766 (75.5%) bovine SNPs were found in the water buffalo genome in this work, but 49,936 (92.5%) were with only one allele, and finally 935 were identified to be polymorphic and useful for association analysis in water buffalo. Therefore, even if the genome sequences of water buffalo and cattle shared a high level of homology, the polymorphic status of the bovine SNPs varied between these two species. Authors concluded that more works in larger sample size are needed in future to verify these candidate SNPs for water buffalo.

For these reasons, studies on important economic traits in buffalo are still prevalently based on candidate gene approach analysis. For example, a recent candidate gene association study was performed to verify the existence of polymorphisms in the ghrelin gene and their associations with milk, fat and protein yield and percentage (Gil et al., 2013). Three out of SNPs found by authors resulted differently associated with fat yield and percentage and protein percentage, and could be used as molecular markers to assist selection. In another recent study, buffalo β-casein gene and its promoter were characterized and several nucleotide substitutions were found, of which three located in exon VII of β-casein and seven in its 5' untranslated region (UTR) (P V et al., 2013).

For their aforementioned close relationship, a useful starting point in the candidate gene association studies on buffalo species is represented by what has already been found in cattle. For example, in a study performed on four buffalo breeds, authors investigated several polymorphisms in *DGAT1*, *GH*, *GHR*, *PRL* and *PRLR* genes, which have been proved to be strongly associated with milk composition traits in dairy cattle (Yao et al., 1996; Winter et al., 2002; Yardibi et al., 2009), Ghasemi et al., 2009). In this work an indirect evidence that water buffalo have fixed alleles with genotypes reported in cattle, which is thought to be responsible for high milk fat, high protein content and low milk yield, was reported. Moreover, three new intra-specific SNPs were found (Shi et al., 2011).

1.5.6. Genetic evaluation of fertility

Concerning fertility, despite the explosion of molecular, genomic and computer techniques, our understanding still is far from complete.

First of all, the term “fertility” itself is not so precise because it does not clearly explain the issue of what must be measured: fertilization or conception to form a zygote, formation of a blastula, attachment of an embryo to the uterine lining, an embryo-induced increase in some molecule detectable in peripheral maternal blood, a conceptus detectable via transrectal palpation or ultrasound, or a live-born calf. Producers would define success as a live calf (Amann and DeJarnette, 2012). Indeed, many reproduction traits are difficult to handle in parameter estimation and genetic evaluation. The low heritabilities, usually less than 5%, are mainly due to a large influence of management and environmental effects. Moreover, fertility is the result of a process involving both male and female and it is not so easy to distinguish their contribute to the success or otherwise of reproductive process. In cattle it has been demonstrated that a portion of the embryo death before Day ~ 8 is caused by the fertilizing spermatozoon (Walters et al., 2006), but most subsequent embryonic mortality is due to the female, environmental factors, or defects in the embryo (Santos et al., 2004). Moreover, calving performance traits are influenced by an effect of the young (Hansen et al., 2004) which may be difficult to correctly estimate.

Therefore, a challenge in achieving a good and expected selection response in reproduction traits is the data collection and the quality of data. Traditionally, most fertility traits are based on calving

and insemination data and each trait has its strengths and weaknesses. For female fertility, there are measures reflecting the ability to resume oestrous cycles after calving and the ability to conceive, or measures combining these abilities like calving to last insemination, often also called days open (Berglund, 2008). Information about calving are recorded by ANASB for Mediterranean Italian buffaloes but, as already stated, natural mating is the system applied by most Italian buffalo enterprises and calving takes place on open ranges. Furthermore, as buffalo is characterized by seasonal anoestrus, is common practice at breeders, to avoid missing the pregnancy, to assign two or even three bulls at the same time to one group of breedable buffaloes (Catillo et al., 2001). This practice, while increasing fertility rate, does not allow paternity assessment. This is a big issue in buffalo genetic evaluation, since phenotypic recordings of traits for individuals and their pedigree are fundamental prerequisites.

The study of reproductive biology in mammals appears very difficult also for the *in vitro* analyses. Two major obstacles have historically hindered this branch of research. First, germ cells in mammals develop in a microenvironment of supporting stromal cells of somatic origin that interacts with the former through autocrine/ paracrine mechanisms as well as direct cell-to-cell interactions (Matzuk and Lamb, 2002). Devoid of this support, isolated germ cells in culture fail to survive and maintain their characteristics, making it extremely difficult to study molecular interactions and pathways that are unique to these cells. The second, but related, difficulty has been the inability to develop an appropriate cellular context in vitro that would allow undifferentiated germ cells to differentiate into specialized gametes that preserve their ability to fertilize and produce healthy offspring (Roy and Matzuk, 2006). Our understanding of the molecular mechanisms of mammalian reproduction therefore has been largely dependent on loss-of-function mutagenesis in mice, based on the use of knockout mouse models (Roy and Matzuk, 2006). However, there is little practical information available because of the difficulty of producing gene knockout and transgenic animals in livestock (Zhu and Zhao, 2007).

In figure 1.5.6.1. is represented a scheme of the key proteins of female fertility pathway in mammals, defined through knockout mouse models (Matzuk and Lamb, 2002).

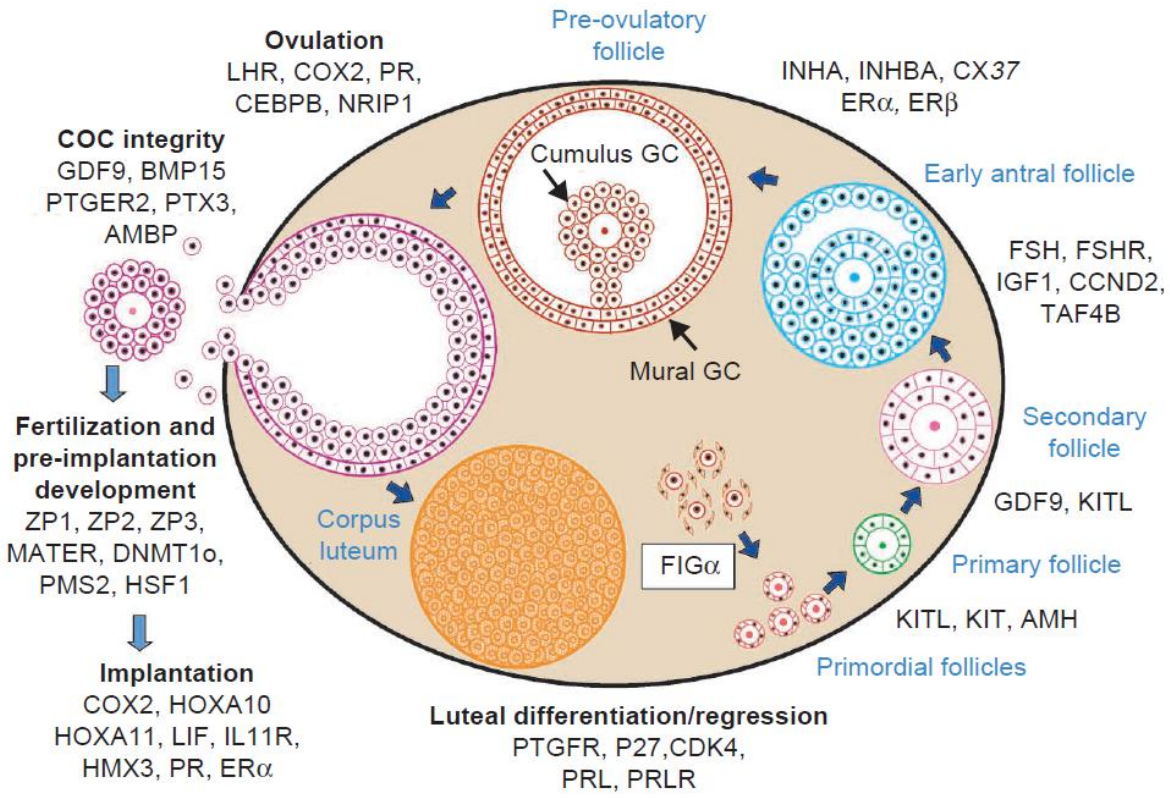


Figure 1.5.6.1. Female fertility proteins. Knockout mouse models have defined key proteins that function at various stages of follicle formation, folliculogenesis, ovulation, and post-ovulatory events. FIGα is required for primordial follicle formation, and several proteins are needed for oocyte and granulosa cell (GC) growth and differentiation, ovulation, and the integrity of the cumulus oocyte complex (COC) (Matzuk and Lamb, 2002).

In the genetic study of reproductive parameters, there are other complications that must be considered.

The analysis of gene expression profiles is a useful method to increase our understanding of the mechanisms behind reproductive functions and their phenotypic expression. However, reproduction traits are regulated by a multitude of genes and environmental factors in a complex relationship (Berglund, 2008).

In figures 1.5.6.2, 1.5.6.3, 1.5.6.4., and 1.5.6.5. are reported pathway maps representing today knowledge on the molecular interaction and reaction networks for endocrine systems related to female fertility.

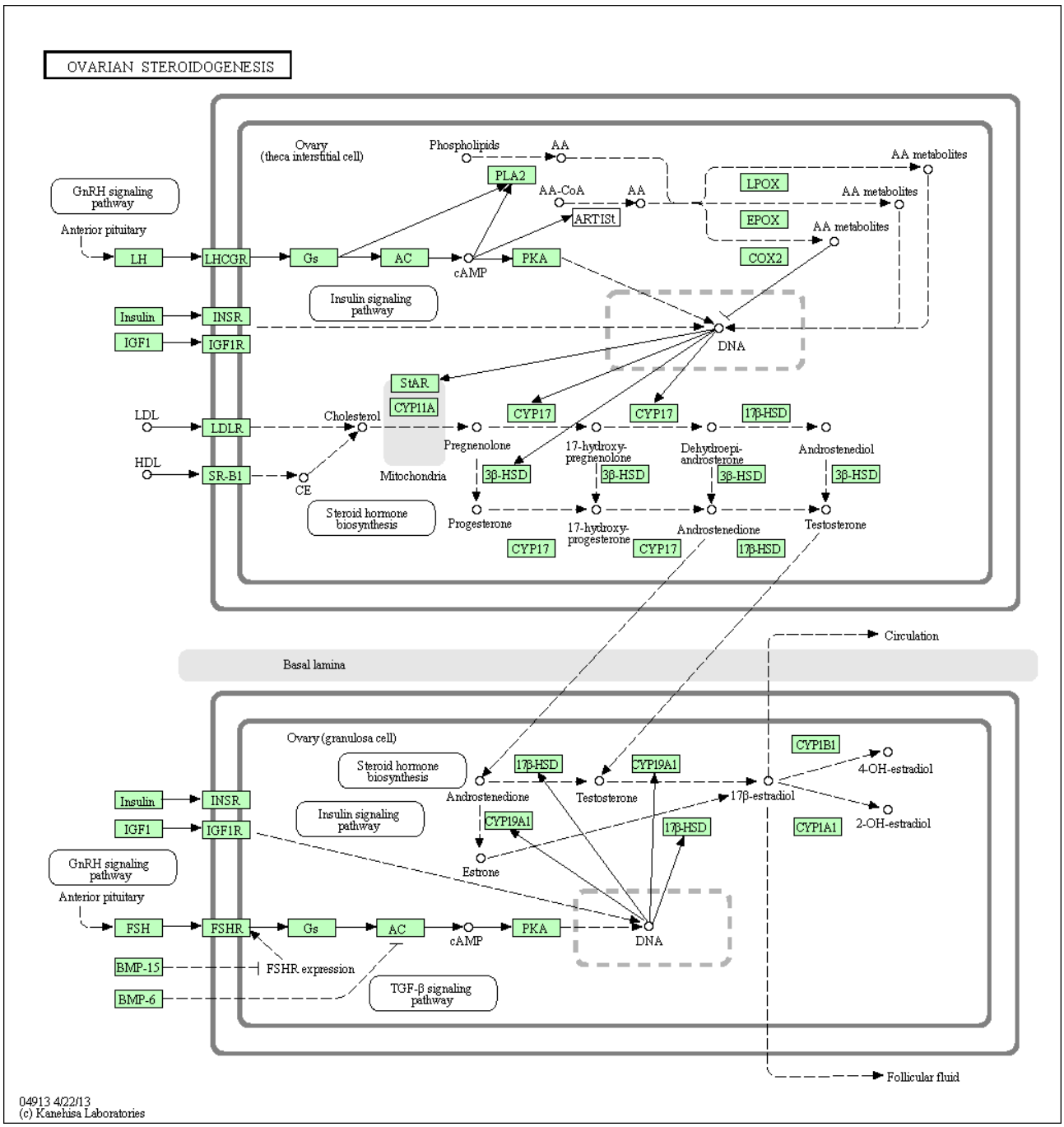


Figure 1.5.6.2. Pathway map of ovarian steroidogenesis in *Bos taurus*, from KEGG database (Kanehisa and Goto, 2000), update October 2013. Theca cells respond to LH signaling by increasing the expression of enzymes necessary for the conversion of cholesterol to androgens, such as androstenedione (A) and testosterone (T). Granulosa cells respond to FSH signaling by increasing the expression of enzymes necessary for the conversion of theca-derived androgens into estrogens (E2 and estrone).

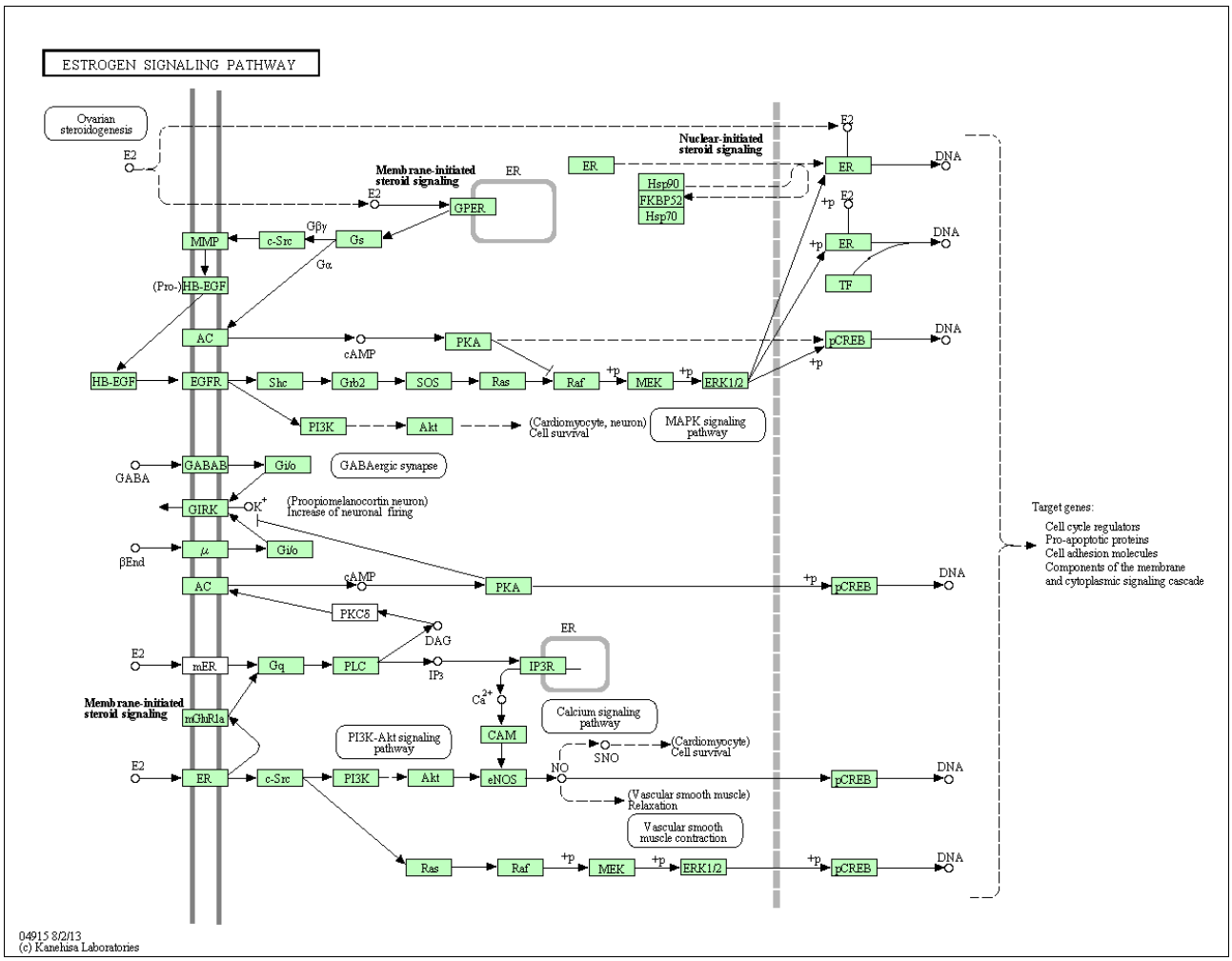


Figure 1.5.6.3. Pathway map of ovarian estrogen signaling in *Bos taurus*, from KEGG database (Kanehisa and Goto, 2000), update October 2013. Estrogen mediates its cellular actions through two signaling pathways classified as "nuclear-initiated steroid signaling" and "membrane-initiated steroid signaling". In the "nuclear" pathway, estrogen binds either ERalpha or ERbeta, which in turn translocates to the nucleus, binds DNA at ERE elements and activates the expression of ERE-dependent genes. In "membrane" pathway, Estrogen can exert its actions through a subpopulation of ER at the plasma membrane (mER) or novel G-protein coupled E2 receptors (*GPER*). Upon activation of these receptors various signaling pathways (i.e. Ca2+, cAMP, protein kinase cascades) are rapidly activated and ultimately influence downstream transcription factors (Kanehisa et al., 2012).

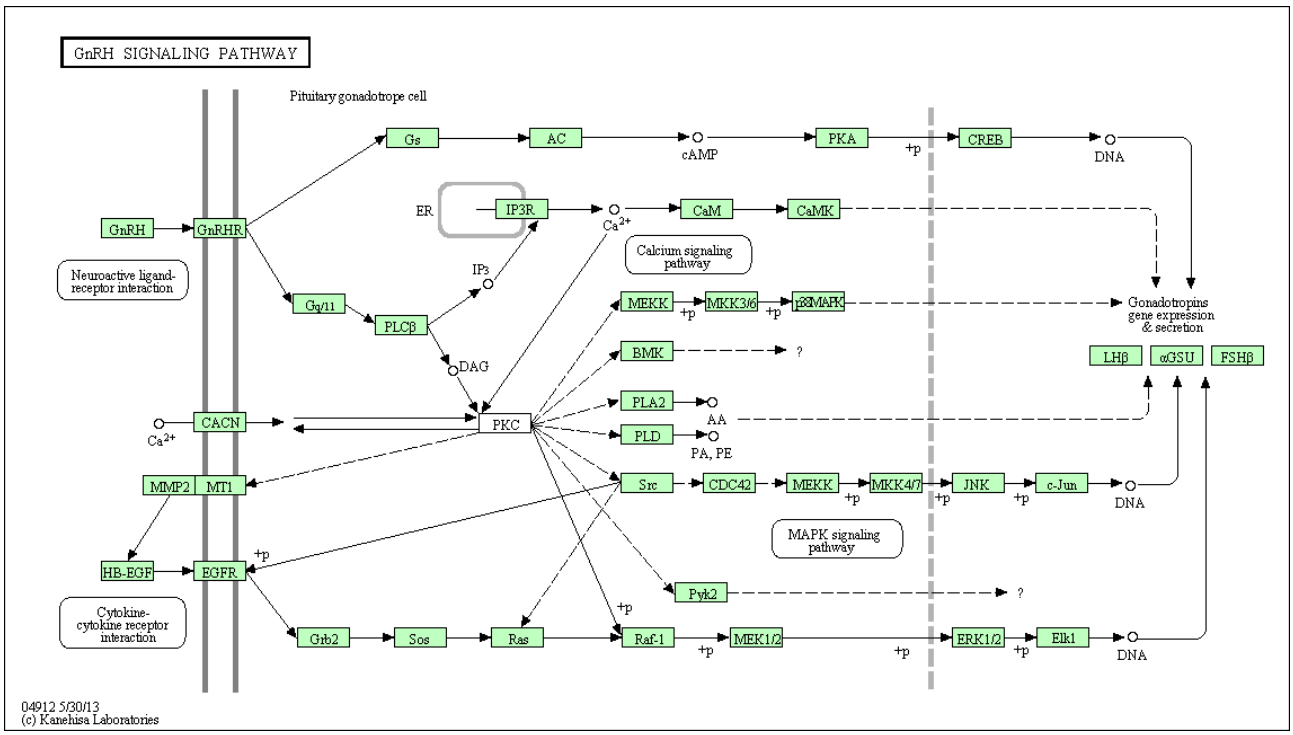


Figure 1.5.6.4. Pathway map of GnRH signaling in *Bos taurus*, from KEGG database (Kanehisa and Goto, 2000), update October 2013. GnRH secretion from the hypothalamus acts upon its receptor in the anterior pituitary to regulate the production and release of the gonadotropins, LH and FSH. The *GnRHR* is coupled to Gq/11 proteins to activate phospholipase C which transmits its signal to diacylglycerol (*DAG*) and inositol 1,4,5-trisphosphate (*IP3*). *DAG* activates the intracellular protein kinase C (PKC) pathway and IP3 stimulates release of intracellular calcium. In addition to the classical Gq/11, coupling of Gs is occasionally observed in a cell-specific fashion. Signaling downstream of protein kinase C (PKC) leads to transactivation of the epidermal growth factor (*EGF*) receptor and activation of mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated kinase (*ERK*), Jun N-terminal kinase (*JNK*) and p38 *MAPK*. Active MAPKs translocate to the nucleus, resulting in activation of transcription factors and rapid induction of early genes.

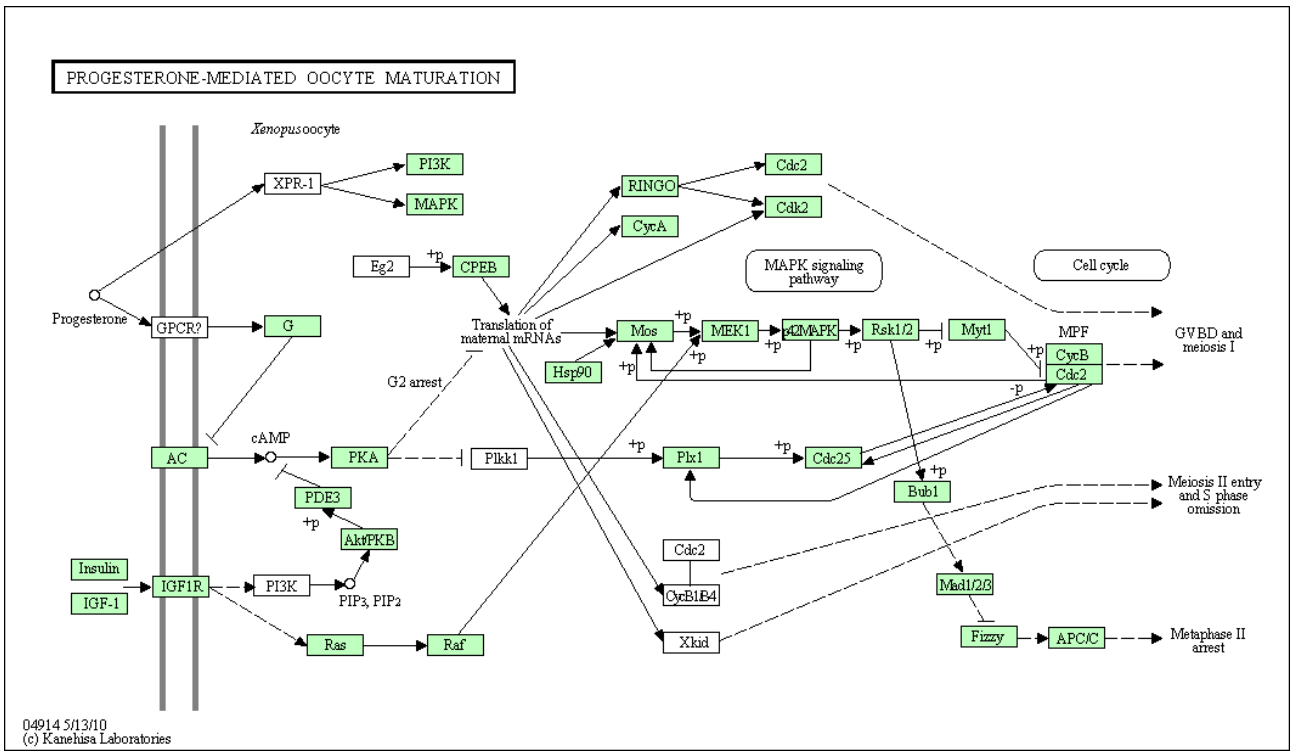


Figure 1.5.6.5. Pathway map of GnRH progesterone-mediated oocyte maturation in *Bos taurus*, from KEGG database (Kanehisa and Goto, 2000), update October 2013. Xenopus oocytes are naturally arrested at G2 of meiosis I. Exposure to either insulin/IGF-1 or the steroid hormone progesterone breaks this arrest and induces resumption of the two meiotic division cycles and maturation of the oocyte into a mature, fertilizable egg. This process is termed oocyte maturation. The transition is accompanied by an increase in maturation promoting factor (*MPF* or Cdc2/cyclin B) which precedes germinal vesicle breakdown. Most reports point towards the Mos-*MEK1-ERK2* pathway [where *ERK* is an extracellular signal-related protein kinase, *MEK* is a *MAPK*/*ERK* kinase and Mos is a p42(*MAPK*) activator] and the polo-like kinase/*CDC25* pathway as responsible for the activation of *MPF* in meiosis, most likely triggered by a decrease in *cAMP*.

1.5.7. Epigenetic aspects in reproductive cyclicity

A new interesting aspect concerning a possible role of epigenetics in various aspects of fertility has recently been revealed. The term epigenetics refers to changes in the phenotype not due to DNA sequence variations but caused by chromatin modifications that regulate gene activity (Saitou et al. 2012). Chemical modification of DNA or chromatin-associated proteins, particularly histones, has a major influence on chromatin structure and gene expression. DNA methylation is a primary epigenetic mechanism by which gene expression is controlled and, in mammals, it is based on the covalent attachment of a methyl group to the C5 position of the cytosine ring of CpG pairs (Illingworth and Bird, 2009). This modified residue is distributed throughout the majority of the genome including gene bodies, endogenous repeats and transposable elements and functions to repress transcription (Eckhardt et al., 2006). Methylcytosine spontaneously deaminates to thymine resulting in the under representation of CpG (21% of that expected in the human genome) (Lander et al., 2001). However, non-methylated DNA sequences called CpG islands can be found along the entire genome. These have an elevated G + C content and little CpG suppression (Gardiner-Garden and Frommer, 1987). CpG islands have been shown to colocalize with the promoters of all constitutively expressed genes and approximately 40% of those displaying a tissue restricted expression profile in human genome (Zhu et al., 2008). Hence, it is clear that increased methylation of CpG residues in the promoter of genes is associated with reduced gene expression.

In several works, an important role of epigenetics in female fertility has been assessed, particularly for the onset of puberty. As already stated, the timing of puberty is controlled by many genes but the elements coordinating this process have not been identified (Herbison, 2007). It was proposed that the highest level of intra-network control is provided by transcriptional regulators that, by directing expression of key subordinate genes, impose genetic coordination to the neuronal and glial subsets involved in initiating the pubertal process (Ojeda et al., 2003). However, the explanation of how inherited, permanent changes in DNA sequence can regulate gene expression dynamically while also defining an encompassing level of coordination and transcriptional plasticity on the gene networks involved was discovered only recently, hypothesizing that a biological regulatory system behind these mechanisms may be, in fact, epigenetics (Lomniczi et al., 2013).

The epigenetics influences on the onset of puberty was demonstrated in a recent work though pharmacological inhibition of the enzymes responsible for methylating DNA. Authors identified silencers of the Polycomb group (PcG) as principal contributors to this mechanism and showed that PcG proteins repress *KISS1*, a puberty-activating gene already mentioned. Therefore epigenetic silencing has been proven to be a mechanism underlying the neuroendocrine control of female puberty (Lomniczi et al., 2013). In another recent work it was demonstrated that daily treatment of prepubertal female mice with 5-azacytidine, a DNA methyltransferase inhibitor, substantially delayed puberty onset, suggesting that some gene or genes must be repressed by methylation for puberty to occur (McCarthy 2013).

Epigenetics seems also to be involved in oocyte maturation processes. During cytoplasmic maturation, there is accumulation of RNA, proteins, nutrients and substrates that are fundamental for completing oocyte maturation and subsequent embryo development (Watson, 2007). Final mRNA and protein production along with epigenetic modifications seem to be essential for acquisition of oocyte competence, even if the details have not been well characterized. Sirard and colleagues proved that, while many oocytes attain meiotic and cytoplasmic competence, the molecular milieu of an oocyte may determine the potential for embryonic and fetal development culminating in birth of viable offspring. Although molecular changes within the cytoplasm are difficult to investigate, authors suggested that these final changes in the days preceding ovulation may be the “capacitators” that result in a normal pregnancy (Sirard et al., 2006).

An important role of epigenetics has also been demonstrated in the cyclicity of many mammalian processes. In fact, epigenetic regulation of certain genes has been shown to be cyclic, exhibiting a periodicity that results in a rapid, tight and dynamic control of gene expression (Metivier et al., 2008). It is now clear that epigenetic information is essential for circadian rhythms (Nakahata et al., 2008). As already mentioned above, circadian rhythms impact on a wide range of physiological mechanisms and this impact extends to fertility, such that disruptions to timing systems can impact upon reproductive capacity (Kennaway et al., 2012). For example, photic condition were suggested to be one of the modulators of growth hormone secretion, involved in the regulation of male and female infertility, in ruminants. A significant effect of day length on the secretion of GH in sheep and goats has long been observed (Barenton et al., 1988; Gazal et al., 2002). Epigenetic control has also been implicated in the modulation of biological timekeeping, regulated by the circadian clock machinery on a systemic level (Reppert and Weaver, 2001), and cellular metabolism and epigenetic state seem to be closely linked (Masri and Sassone-Corsi, 2010).

1.6. CANDIDATE GENES FOR FERTILITY TRAITS

Today, in the genetic study of fertility, for which genetic selection is hampered by low heritability, the candidate gene approach is still the most used method also in species where the whole-genome tools are now widely adopted. For example in cattle, where the BovineSNP50 chip tool is now the most extensively used approach for association studies, it has been observed that the low heritability and polygenic nature of reproduction traits limit the improvements in reliabilities obtained through incorporation of genomic information compared to other traits (Cochran et al 2013). Therefore, the candidate gene approach in an association study for fertility traits could be the best method to select causative SNPs or SNPs physically more close to causative SNPs, as it was already demonstrated in detection of genomic associations with disease (Amos et al., 2011).

Few studies have been performed in buffalo species to dissect the genetics of fertility parameters. Here are some examples of the characterization of genes which can be considered candidate for female fertility.

In a recent study, Kandasamy and colleagues studied the expression profile of bubaline ghrelin, a novel motilin-related endogenous ligand for growth hormone secretagouge receptor, implicated in various biological functions, including regulation of female reproduction. The results obtained indicate the persistent expression of ghrelin in the uterine endometrium throughout the estrous cycle and in early pregnancy, which might be helpful in determining its role in buffalo reproduction (Kandasamy et al., 2013). In another recent work, *GDF9* gene, which plays a vital role in determination of oocyte competence, was isolated and characterized in buffalo, using orthologous primers based on the bovine *GDF9* sequence (Roy et al., 2013). *GDF9* gene, together with bone morphogenetic protein15 (*BMP15*), were analyzed to evaluate the association of their mRNA expression in cumulus-oocyte complexes of buffalo ovary during in vitro maturation. Authors concluded that these two genes are differentially expressed during the period of oocyte maturation process and that *BMP15* expression pattern is associated specifically with the period of cumulus cell expansion (Kathirvel et al., 2013). In databases for buffalo species are mRNA and complete coding sequences for both these two genes, while in bovine they are well characterized: *GDF9* is located on chromosome 7, it has 1 transcript with a coding sequence 1790 bp long and the encoded protein is made up of 453 aa. 43 SNPs along all genomic sequence are known. *BMP15* is located in X chromosome and it is 1185 bp long, there is one transcript made up of just two exons and the encoded protein has 394 aminoacids. 10 SNPs are known of which two are missense variations (ensembl release 73 - September 2013).

Another important candidate gene for female fertility is *CYP19* (cytochrome P450, family 19) which produces aromatase, the key enzyme in estrogen biosynthesis, catalyzing the conversion of androgens into estrogens by irreversible aromatization step. The *CYP19* gene is regulated by different tissue specific promoters (Fürbass et al., 1997). Aromatase expression shows a gradual increase from small to large follicles, indicating that the increased aromatase expression may be essential for the follicular development and maturation in buffalo ovary (Lenz et al., 2004). The bubaline *CYP19* cDNA was characterized in granulosa cells of large follicles (Kumar et al., 2009) and the expression pattern during ovarian follicular growth, development and maturation has been also characterized in this species (Babitha et al., 2013). In databases are bubaline mRNA sequence, promoter region, 5'UTR and a partial coding sequence for this gene. In bovine species this gene has a 5178 bp cds and it has 10 exons, with 3 known SNPs reported, of which one in the 5'UTR and two in the 3'UTR, that is unusually extended.

FSH (follicle-stimulating hormone) is a key regulator of the reproductive process in mammals, and its receptor (FSHR) is located at the plasma membrane of target cells (Sprengel et al., 1990). *FSHR* gene has been studied in many livestock species including cattle (Houde et al., 1994), sheep (Yarney et al., 1993), horse (Robert et al., 1994), donkey (Richard et al., 1997), poultry (Wakabayashi et al., 1997), monkey (Gromoll et al., 1993), mouse (Tena-Sempere et al., 1999), rat (Sprengel et al., 1990) and domestic guineapig (Suzuki et al., 2003). A study has been conducted to clone and characterize the *FSHR* gene of buffalo (Minj et al., 2008) and sequence analysis indicated that the buffalo *FSHR* cDNA sequence comprised of an open reading frame of 2085 bp encoding a 695 amino acid protein. Its nucleotide sequence showed more than 80% similarity to the homologous genes of mammalian species. At amino acid level buffalo *FSHR* exhibited a high percentage (84–96.7%) of identity with the corresponding mammalian homologs. In bovine *FSHR* gene have 19 known SNPs in databases, of which two synonymous in exons 1 and 2, while two are non-synonymous and are located in exons 4 and 9, two SNPs are located in splicing sites, at the end of exons 2 and 6, one is located in the 3'UTR and the others are located in introns.

FGF2 (fibroblast growth factor 2) is present in the uterine lumen during early pregnancy and plays an active role in regulating the establishment and maintenance of pregnancy in ruminants (Michael et al., 2006). It is involved in oocyte maturation and it has been associated with embryonic mortality in cattle (Khatib et al., 2008a). Buffalo mRNA e complete coding sequence are in databases for this gene. In bovine species the coding sequence of this gene is 6594 bp long, the encoded protein is composed by 155 amino acids and there are 3 exons with an extended 3'UTR. Moreover, as many as 228 SNPs are known in cattle (ensembl release 73 - September 2013).

PRL-PRLR (prolactin- prolactin receptor): prolactin plays a crucial role in mammary gland differentiation by favoring mammary growth, initiating milk synthesis. *PRL* and its homologs accomplish their biological effects through the *PRL* receptor (*PRLR*). *PRLR* deficiency results in implantation failure. Uterine *PRLR* is supposed to be essential for the support of late gestation (Reese et al 2000). This gene has been studied in bubaline species and mRNA and complete coding sequence are in databases, moreover 2 SNPs in exon 3, one of which non-synonymous (Shi et al., 2011) are known. In bovine species this gene is made up of 5 exons and has a 902 bp long coding sequence. There are 4 known SNPs, of which two synonymous in exons 1 and 4 and two in introns. Finally this gene has a homology equal to 80% with pig, 76% with rabbit, 74 % with horse and 72% with human. In man for *PRL* gene are in databases 16 known SNPs, two of which are silent SNPs and one, located in exon 2 provokes a stop codon gain (ensembl release 73 - September 2013).

The genetic variability in leptin gene, candidate for milk quality and female fertility traits, was analyzed in buffalo species but no significant effects were found by authors (Zetouni et al., 2013). Another work has been performed on buffalo bulls with the aim to identify polymorphisms in the osteopontin gene and their associations with certain semen production traits of water buffaloes in the Brazilian Amazon (Rolim Filho et al., 2013). Also for this gene mRNA e complete coding sequence are in databases for buffalo.

In bovine species, an important finding has been recently made by VanRaden and colleagues, who reported the discovery of five haplotypes with deleterious effects on fertility in three breeds of dairy cattle, including one recessive in Brown Swiss cattle, three in Holsteins, and one in Jerseys (VanRaden et al., 2011). Based on this previous discovery, a whole-genome resequencing has been performed in a following study, identifying a nonsense mutation located in *CWC15* gene, which

resulted the causative mutation associated with early embryonic loss in Jersey cattle (Sonstegard et al., 2013).

2. AIM OF THE WORK

Fertility and seasonality traits are of critical importance in buffalo species, as discussed in the introduction. Up to now, even if a complete genomic sequence was announced, as well as the development of a high density SNP chip tool, little genetic information are available for this species.

For this reason, aim of this work was to perform a polymorphism detection and an association study in candidate genes involved in fertility and seasonality of reproduction in Mediterranean Italian Buffalo.

3. MATERIALS AND METHODS

3.1. SNP discovery analysis

3.1.1. Animals

SNP discovery analysis was conducted on two sets made up of 12 animals each. For fertility estimation 6 animals with calving interval >200 days and 6 with calving interval <100 days were selected. For seasonality trait analysis 6 animals with calving occurred between March and May and 6 with calving occurred between October and December were chosen.

3.1.2. DNA isolation and PCR analyses

DNA extraction from frozen blood samples has been performed using a commercially available kit (Promega ReliaPrep^{TM} Blood gDNA Miniprep System), according to the manufacturer's instructions. Primers used for PCR amplifications with annealing temperatures are reported in table….. and amplicons were investigated for SNP discovery. A typical PCR reaction mix (20µl) comprised: 1µl of gDNA, 5X PCR Buffer (Promega), 5mM MgCl2, 0.4µl of each primer, dNTPs each at …. mM, 0.2µl of Taq DNA Polymerase (Promega). PCR products were purified and sequenced. The sequence alignment was then performed using the BioEdit software (Hall, 1999).

3.2. SNP genotyping

Entire blood of a total of 491 female buffaloes was collected and DNA extraction was performed as already described. DNA samples were then genotyped for the identified SNPs (http://lgcgenomics.com).

3.2.1. Animals and parameters analyzed

Samples were collected from four farms located in south of Italy, province of Caserta. In the analyzed farms the OBMS technique is performed, which interrupts sexual promiscuity in the herd during the autumn season.

Phenotypic data obtained for all samples are the following:

- date of calving
- milk yield (kg)
- protein (kg and %)
- fat (kg and %)
- lactation number
- age at the control
- days with the bull
- days in milk at the control
- date of sampling
- farm

3.3. Statistical analysis

Population genetics parameters were calculated using PowerMarker v3.25 (http://statgen.ncsu.edu/powermarker; Liu and Muse, 2005). In particular, minor allele frequency, expected and observed heterozygosity, and Hardy-Weinberg equilibrium exact P-values were calculated.

Associations between the analyzed traits and each single SNP at the 5 candidate genes were tested using the following mixed linear model:

$$[1] \qquad Yijk = \mu + \text{FLOCK}i + \text{bAGE} + \text{NLACT}j + \text{SEA}k + \text{SNP}l + e,$$

where *Yijk* = calving interval (days), seasonality (2 levels), milk yield (kg), protein or fat yield (kg) and protein and fat content (%); μ = overall mean; FLOCK*i* = random effect of the flock (4 levels); bAGE= covariable represented by the age at calving (days); NLACT*j* = fixed effect of the number of lactation (from 1 to 7); SEA*k* = fixed effect of the calving season; SNP*l* = fixed effect of the SNP genotype (3 levels); and *e* = random residual.

All the factors included in the model (flock, age at calving, lactation number and seasonality of calving) were prior described through a one-way analysis of variance.

The parentage information was limited and no information were available for the sires of the buffaloes analyzed. However, the sires were not shared between flocks, so the random effect of flock was considered also to account for the effect of the bull.

For the SNP association analysis, being that each SNP was tested separately, the level of significance was corrected gene wide using the Bonferroni adjustment.

Average gene substitution effect (α) was calculated using a model with the same structure of model 1 but with the gene effect treated as a covariable (Banos et al., 2008; Pauciullo et al., 2012). The SNP allelic effect was described as 0, 1, or 2, in each case corresponding to as many copies of the substitution SNP base. The coding of the 3 genotypes was based on the number of copies of the first allele in alphabetical order.

In a separate series of analyses, interactions between alleles in a SNP locus were also fitted to assess possible dominance effects . In this case, the model 1 was repeated with the SNP effect treated as a covariable and described as 0, if the SNP is homozygous, and 1 if the SNP is heterozygous (Dagnachew et al., 2011).

Variance associated with the SNP genotype (σ^2_{SNP}) was estimated by running a model with the same structure of model 1 but with the SNP treated as random. Thus, a variance component associated with the SNP locus was estimated (Crepaldi et al., 2013). Contributions of the SNP locus (r^2_{SNP}) to the total phenotypic variance of the trait considered were calculated as

$$r^2_{SNP} = \frac{\sigma^2_{SNP}}{\sigma^2_{total}}$$

4. RESULTS AND DISCUSSION

4.1. Sample description

4.1.1. Dataset editing

For the 491 buffaloes genotyped in present study, as already mentioned in materials and methods, phenotypic data made available by the Italian Buffalo Breeders' Association (ANASB) are the periodic milking recording data, referring to: farm, date of calving, lactation number, year, age of animal, days in milk at recording (referring to a standard lactation of 270 days where it resulted longer) and mean values of all the controls recorded throughout the lactation for milk yield (kg), protein (kg and %) and fat (kg and %).

For the purpose of this research, the data provided have required a massive editing work.

The dates of calving were used in present work to have information related to seasonality and fertility parameters. For all animals, the month of every registered calving was pointed out to analyse the reproductive seasonality. Moreover calving interval, that is one of the most used and reliable field datum for the analysis of fertility traits, was calculated through the distance in days between two consecutive dates of calving for every animal having more than one calving date recorded.

The analysis of the registered dates of calving, required a considerable revision phase to delete all the recorded data which were not considered reliable. For this purpose, the characteristics of the biology of reproduction in water buffalo were considered. Gestation ranges from 300 to 330 days with a mean of approximately 310 days for river type buffaloes (Perera et al., 1987; Campanile et al., 2005). Considering that, under optimal conditions, buffalo can resume ovarian activity after calving by 30-90 days (Moioli et al., 1998), only calving intervals falling in a range between 330 and 1000 days were considered reliable. After this correction, 9 animals, for a total of 43 observations, were removed from the dataset.

Another important parameter, the number of lactation, required a careful control. In fact, ANASB usually records as first lactation the first controlled, even if this corresponds to a different order of physiological lactation. A lactation number equal to 1 refers to the first lactation recorded for the animal, but it often does not correspond to its first calving. For example, in the dataset were animals with age equal to 12 or 9 years at first lactation. This is not reliable and means that, most likely, the previous deliveries of these animals have not been recorded. This may be the result of inaccuracy of registration or even of the birth of male calves that have not been declared. Since the lactation number is a fundamental parameter to be considered in the analyses performed in the present research, a correction was made to consider only the reliable lactation number data, based on the age of animals and the dates of calving. In particular, all animals having more than 4 years at first lactation, for a total of 17 animals and 64 observations, were removed from the dataset. Finally, the age at calving (days) was obtained for every animal from the distance in days between the date of birth and the date of calving.

4.1.2. Descriptive statistics

The descriptive statistics for all the traits considered are reported in tables 4.1.2.1. and 4.1.2.2..

Trait	Age at calving (years)					Calving interval				
Lactation number	**N. obs.**	**Mean**	**SD**	**Max**	**Min**	**N. obs.**	**Mean**	**SD**	**Max**	**Min**
all	1441	5.02	2.45	18.6	1.9	947	446.92	109.58	982	331
1	436	2.83	0.61	4.4	1.9					
2	341	4.11	0.78	6.2	2.9	340	474.62	121.47	982	332
3	239	5.37	1.08	9.8	3.9	236	439.04	113.45	947	332
4	140	6.52	1.32	11.0	4.9	140	414.09	75.94	770	331
5	85	7.69	1.52	12.0	5.9	85	424.24	85.51	883	334
6	52	8.77	1.14	13.4	7.0	52	422.69	71.14	697	336
7	29	10.02	1.20	14.6	8.8	29	444.72	134.13	879	352
8	12	11.03	1.61	15.6	9.5	11	402.82	63.11	543	337
9	8	12.24	1.85	16.5	10.5	8	420.13	73.12	572	347
10-12	4	10.55	4.94	14.2	11.7	3	492.33	66.86	567	438

Table 4.1.2.1. Descriptive statistics for age at calving and calving interval on the samples analyzed.

Trait	Protein yield (kg)					Fat yield (kg)					Milk yield (kg)				
Lactation number	**N. obs.**	**Mean**	**SD**	**Max**	**Min**	**N. obs.**	**Mean**	**SD**	**Max**	**Min**	**N. obs.**	**Mean**	**SD**	**Max**	**Min**
all	1060	114.51	28.863	228	30	1060	211.716	54.037	413	59	1063	2462.31	607.75	4980	559
1	366	108.52	25.224	198	47	366	199.825	45.975	376	81	368	2339.21	535.31	4079	1015
2	260	116.16	26.810	215	42	260	215.969	51.451	376.4	69	260	2497.08	564.93	4620	842
3	166	117.73	30.961	207	30	166	218.042	58.148	381	59	166	2518.11	628.91	4242	559
4	99	121.12	32.950	208	39	99	225.596	62.73	413	75	99	2612.45	699.43	4396	898
5	58	124.14	36.526	228	49	58	227.5	65.266	399	94	58	2681.69	781.66	4980	1049
6	37	118.14	29.185	170	61	37	218.568	62.062	382	105	37	2559.35	638.59	3759	1337
7	16	135.88	27.122	189	88	16	237.313	43.657	320	154	16	2893.88	563.02	4159	2036
8	6	128	38.838	174	75	6	237.167	71.648	336	140	6	2679.17	809.37	3662	1559
9	4	109	51.459	163	45	4	198.5	110.711	336	78	4	2334.50	1124.42	3597	975
10-12	3	127	28.214	153	97	3	220	52.716	265	162	3	2607.67	583.02	3176	2011
	Protein %					**Fat %**									
all	1064	4.623	0.189	5.3	3.7	1065	8.591	0.820	11.8	6.5					
1	366	4.627	0.192	5.3	3.7	367	8.564	0.856	11.8	6.6					
2	262	4.621	0.178	5.2	3.9	262	8.631	0.808	11.2	6.6					
3	166	4.634	0.205	5.3	4	166	8.629	0.804	11.4	6.9					
4	100	4.603	0.204	5.1	4.1	100	8.598	0.808	11.2	7					
5	58	4.593	0.168	4.9	4.1	58	8.522	0.807	10.9	6.5					
6	37	4.589	0.173	4.9	4.2	37	8.481	0.805	10.2	6.8					
7	16	4.650	0.179	4.9	4.3	16	8.175	0.505	8.9	7.4					
8	6	4.767	0.151	5	4.6	6	8.850	0.414	9.5	8.2					
9	4	4.650	0.129	4.8	4.5	4	8.275	0.727	9.3	7.6					
10-12	3	4.833	0.058	4.9	4.8	3	8.367	0.404	8.8	8					

Table 4.1.2.2. Descriptive statistics for milk, protein and fat yield (kg) and for protein and fat (%) recorded on the samples analyzed.

The calving intervals observed in our sample showed, as expected, a highly asymmetric distribution, with a skewness value equal to 2.04. This is an indication of a highly right-tailed distribution, as can be observed in the following figure (4.1.2.1.). Median value for this parameter is equal to 412 days with upper and lower quartiles equal to, respectively, 481 and 375 days. Due to the lack of homoscedasticity in respect to the number of lactation shown by this parameter at Levene's test, subsets for every lactation number were created and considered separately for the subsequent SNP association analyses.

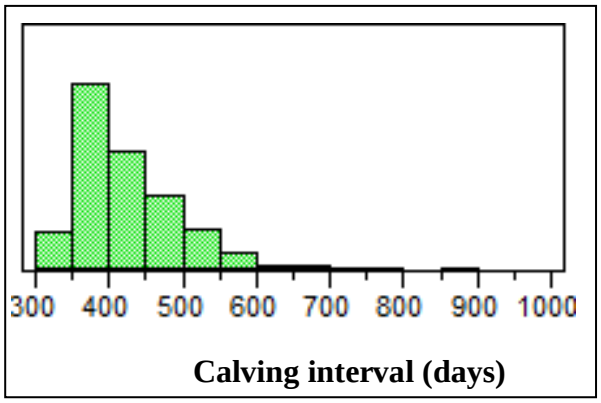


Figure 4.1.2.1. Calving interval distribution in the entire dataset.

The highest mean value for calving interval, equal to 982 days, was reached between the first and the second parturition, while the lowest one, 331 days, was observed at fourth lactation. The average calving intervals recorded in the different subsets analyzed are quite high compared to the mean value reported in literature for Italian farms, that is 400 days (Zicarelli, 2010). However, the latter is related to farms where natural mating is practiced and bulls are always present in the herd. But, in the farms analyzed in present work, the Out of Breeding Mating Season (OBMS) technique is performed, which interrupts sexual promiscuity in the herd during the autumn season. Since, in this case, the resumption of ovarian activity coincides with the decreasing day-length at Italian latitude, this practice can cause longer intercalving periods (Zicarelli et al., 2007).

The OBMS technique operated in the analyzed farms appears evident analyzing the calving distribution. This showed an opposite trend compared to the natural breeding conditions (figure 4.1.2.2.), as 71% of the observations fall in the out of breeding season period, with high deliveries concentration between June and July.

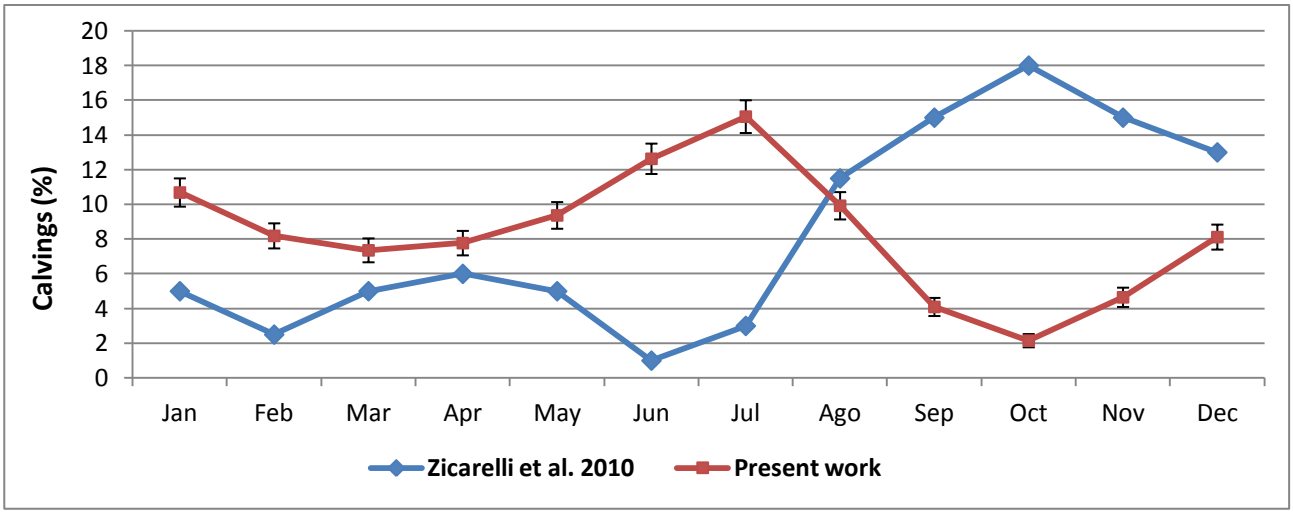


Figure 4.1.2.2. Monthly calving distribution in Mediterranean Italian buffalo under natural conditions (Zicarelli, 2010) compared to animals analyzed in present work.

After this analysis, the dates of calving were divided into "seasonal" (from August to December) and "out of season" (from January to July). The calving distribution at different number of lactation in these two period is represented in figure 4.1.2.3. Notably, at first lactation almost 80% of calvings occurred in the out of breeding season. The low seasonality showed by females at first calving is probably due to a more successful application of the OBMS technique operated in the analyzed farms, as heifers are less sensitive to photoperiod (Zicarelli, 1997).

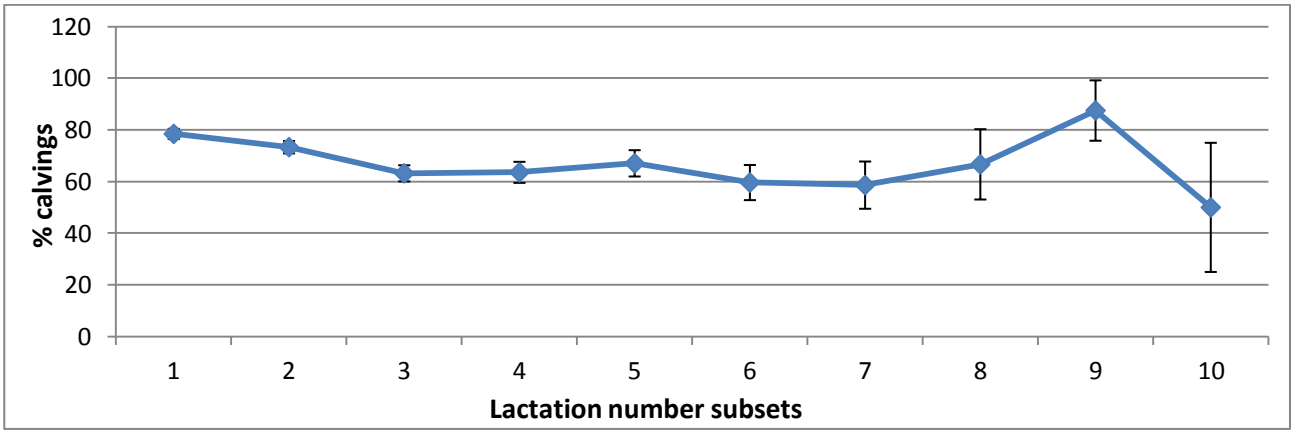


Figure 4.1.2.3. Average percentage of calvings observed in the out of breeding season in the different subsets of lactation number.

Median value for age at calving amounted to 4.4 years, with upper and lower quartiles pair to, respectively, 6.1 and 3.1 years, but in most of calvings recorded animals were three years old (figure 4.1.2.3.). The mean value ± SD for age at first calving was pair to 2.81±0.4 years, that is lower compared to the official statistics reported for Mediterranean Italian Buffalo by the Italian Breeders' Association (AIA), where the average age reported for buffalo cows at first calving is 3 years, 4 months and 29 days (www.aia.it).

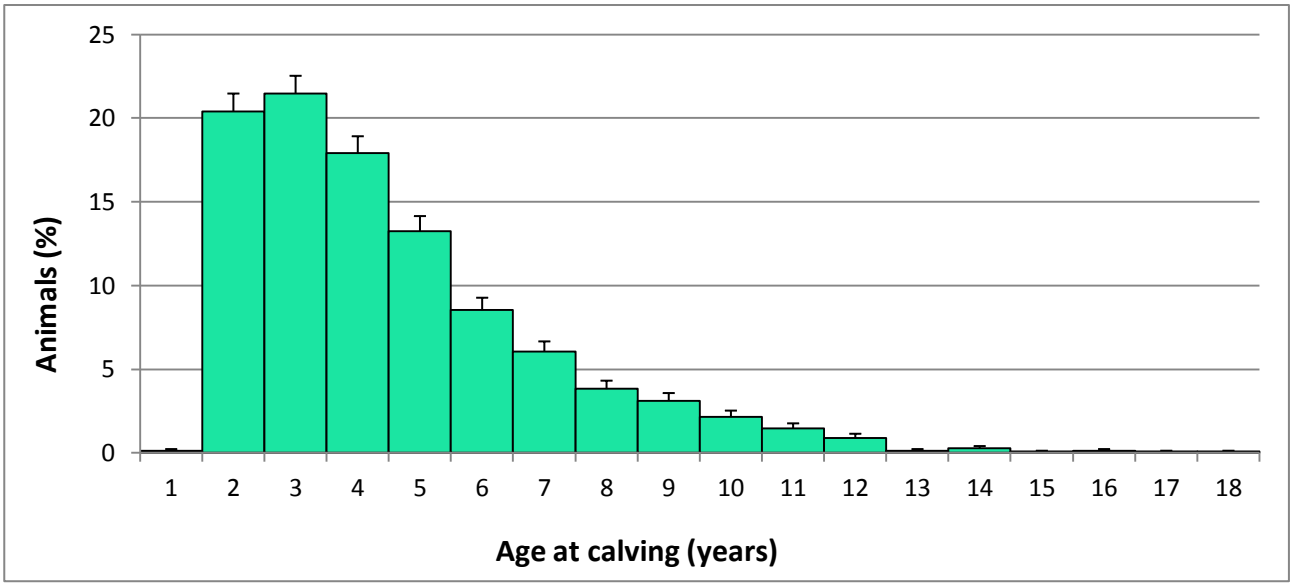


Figure 4.1.2.4. Average percentage of animals recorded at different classes of age at calving.

Concerning productive parameters, milk yield showed a normal distribution in the complete dataset, with a mean value ± SD equal to 2462.31±607.75 kg. The maximum value recorded is pair to 4980 kg, while 559 kg is the minimum one. These performances are considerably high compared to those reported for Mediterranean Italian Buffalo, as the mean value ± SD declared for milk yield for year 2012 is equal to 2218±602 kg (www.anasb.it). This divergence could be due to a particularly high management level of the farms where the animals analyzed in the present research were sampled.
Protein yield (kg) showed a normal distribution but, with respect to the classes of lactation number, homoscedasticity at Levene's test was not respected, because the variance at first lactation resulted out of range compared to the other classes. For this reason, the subset including data related to first lactation was analyzed separately from the others in the subsequent SNP association analyses. The same applies to fat yield (kg) parameter, which exhibited a similar trend.

Protein (%) and fat (kg and %) did not show normal distributions, as verified with the Shapiro-Wilk test. For this reason, transformations were calculated to be used for the subsequent analyses. For fat yield and fat percentage parameters, a root square and a logarithmic transformation, respectively, were calculated to meet the Shapiro-Wilk test for normality. Nevertheless, for protein % no transformations could overcome the lack of normality.

As can be observed in table 4.1.2.2., protein yield distribution showed mean value ± SD equal to 114.51 ± 28.9 kg. High variation is observed at this parameter, with a maximum value equal to 228 kg, reached at the fifth lactation, and a minimum of 30 kg, at the third one.

High variation was shown also by fat yields, with mean ± SD of 211.72 ± 54.04 kg in the entire dataset, maximum, observed at fourth lactation, equal to 413 kg and minimum, at third lactation, equal to 59 kg.

Protein and fat percentage parameters respected the homoscedasticity condition based on the classes of lactation number. Average value ± SD for protein content amounts to 4.62 ± 0.19% in the entire dataset, while maximum and minimum values are equal to 5.3 and 3.7 respectively, the former observed at first and third lactation subsets, the latter at the first one. Fat content showed mean

value ± SD equal to 8.59 ± 0.82 % in the entire dataset, maximum of 11.8 %, recorded at first lactation, and minimum 6.5 %, observed at fifth lactation. Mean values observed in the analyzed population for these parameters are comparable with data reported for Mediterranean Italian Buffalo for year 2012, where mean values for protein and fat percentage amount to, respectively, 4.7 % and 8.3 % (www.anasb.it).

4.1.3. ANOVA for production, fertility and seasonality parameters

A one –way analysis of variance (ANOVA) was performed to describe the factors included in the model used for the subsequent SNP association analyses. These are: seasonality of calving, flock, classes of lactation number and age at calving (days).

4.1.3.1. Seasonality of calving

In the entire dataset, seasonality of calving affected calving intervals ($P<0.0054$), with higher values (mean ± SD equal to 461.71 ± 111.15 days) for the seasonal buffaloes compared to animals calving out of the breeding season (mean ± SD equal to 440.06 ± 113.92 days). Seasonality of calving seems to exert strong influence also on the production parameters, as previously reported (Pauciullo et al., 2012). In fact, animals that calved in the out of breeding season period exhibited significantly higher ($P<0.0312$) milk yields (mean ± SD equal to 2489.22 ± 579.33 kg) compared to the seasonal ones (mean ± SD equal to 2401.59 ± 666.56 kg). No statistically significant differences emerged for protein and fat yields (kg), in relation to the seasonality of calving. An opposite trend ($P<0.0016$) compared to milk yield, as expected, was shown by fat percentage in response to the season of calving, with lower values (SE) for buffaloes that calved in the out of season period, 8.5 (1.003) %, compared to the seasonal ones, 8.7 (1.005) %. The production across different calving seasons did not show statistically significant differences for protein percentage.

4.1.3.2. Flock

Flock exerts a strong effect on all traits ($P<0.0001$). It is important to note that this parameter includes also the sire effect, for this reason it was included as random factor in the ANOVA model used to estimate the SNP effects. In fact, the use of AI is very limited in buffalo species and in most cases bulls are not shared by different farms. Above all, there is a very low availability of parentage information, which is a big issue for the genetic improvement of this species.

Regarding seasonality, as can be seen in the figure reported below (4.1.3.2.1.), a strong effect was detected for Z flock, which showed much better performances for this trait. In fact, 82.5% of animals calved in the out of breeding season in this farm. The trend observed between the other three flocks analyzed did not show statistically significant differences for seasonality of calving. Moreover, the Z flock showed better performances also for fertility trait (figure 4.1.3.2.2.a), with an average calving interval (SE) equal to 409 (6.01) days.

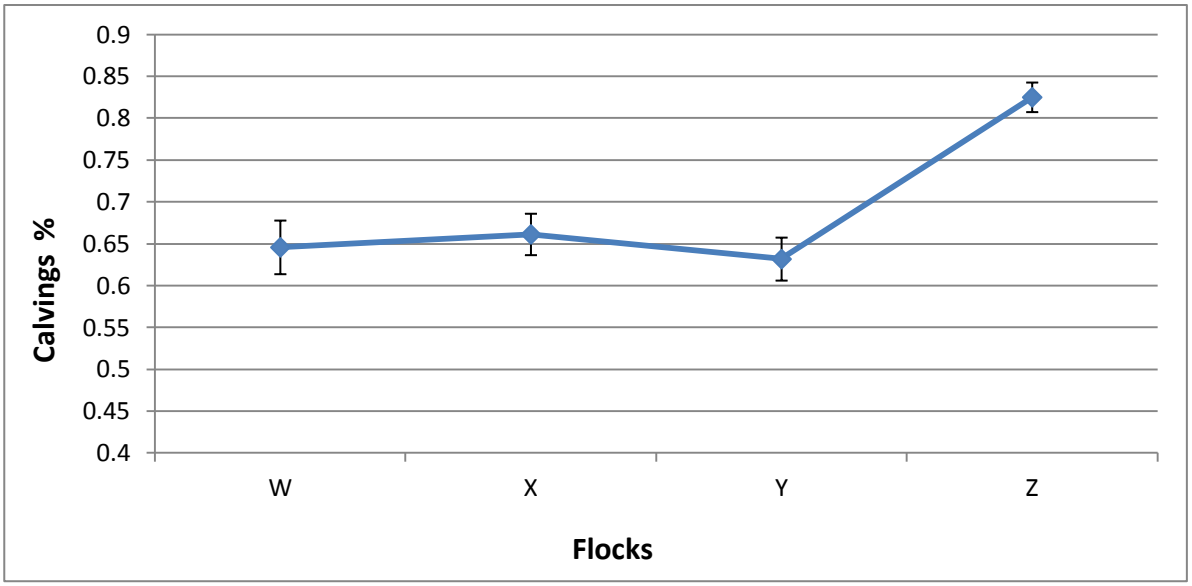


Figure 4.1.3.2.1. Percentage of calvings occurred in the out of breeding season at different flocks (P<0.0001).

From the point of view of production, the Y flock evidenced the highest milk yields, with mean value (SE) pair to 2700 (31.8) kg (figure 4.1.3.2.2. b). This trend was also observed for fat and protein yields (figure 4.1.3.2.3. a, b) with mean values equal to, respectively, 232.5 (0.01) kg and 128.9 (1.48) kg. On the contrary, X flock exhibits fat and protein yields significantly low compared to the other farms analyzed, with mean values equal to, respectively, 177.38 (0.01)kg and 94.24 (1.47)kg. The Y flock exhibited the highest values also for protein percentage, with mean (SE) equal to 4.5 (0.01) %, while for fat percentage parameter, the best performances were recorded for X and Y flocks, with mean values (SE) equal to, respectively, 8.75 (0.05) % and 8.71 (0.05) % (figure 4.1.3.2.2. c and d).

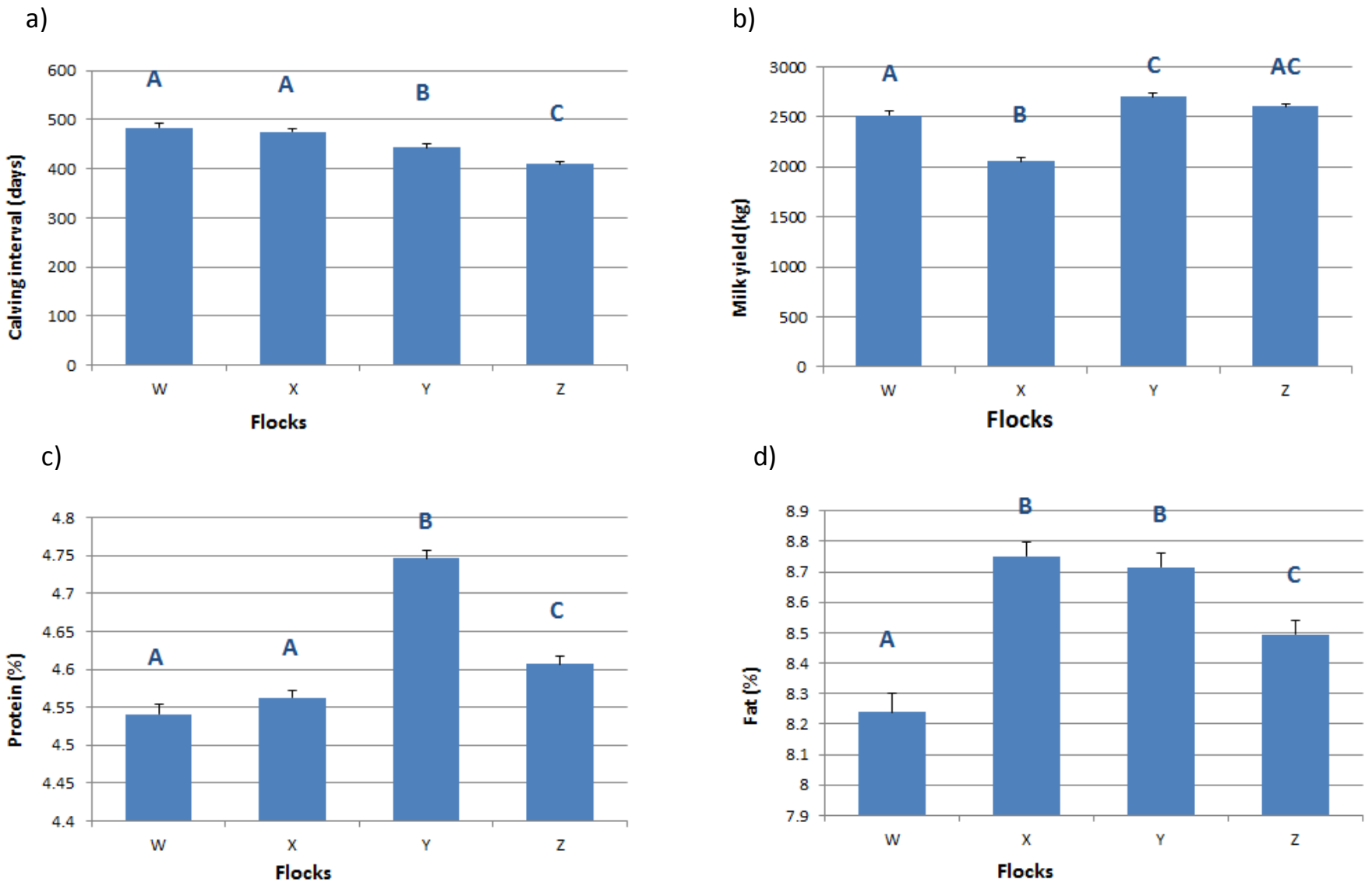
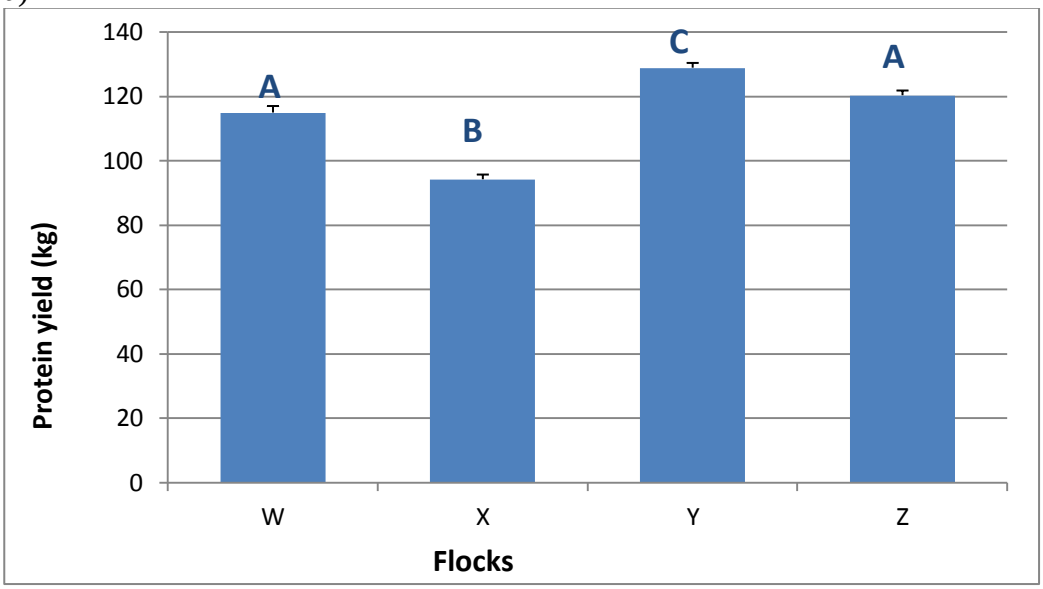


Figure 4.1.3.2.2. Mean values observed for calving interval (a), milk yield (b), protein% (c) and fat% (d) in the four analyzed flocks (P<0.0001).

a)



b)

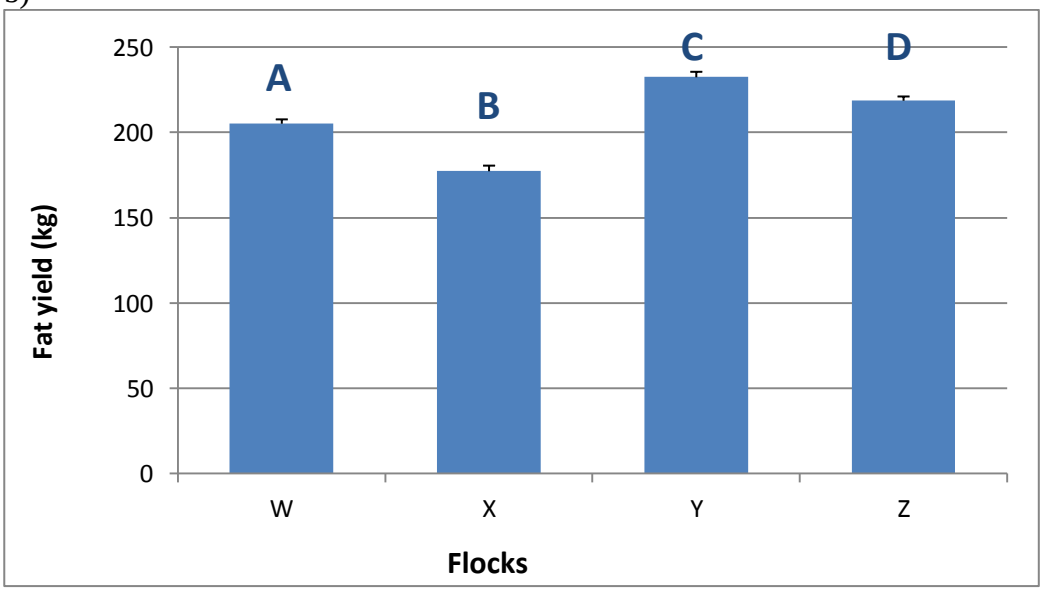


Figure 4.1.3.2.3. Mean values observed for fat (a) and protein (b) yield (kg) in the four analyzed flocks (P<0.0001).

4.1.3.3. Lactation number

Another fundamental parameter to be considered in the study of the analyzed traits is the number of lactation.

In the analyzed sample, the average number of lactation ± SD was equal to 2.63 ± 1.76. This is low compared to the official statistics for Mediterranean Italian Buffalo in Italy in 2012, where an average number of lactation equal to 3.29 is reported (www.aia.it). For buffaloes analyzed in present work, 86% of data are related to the first 4 lactations, while fewer observations are related to superior lactation classes. In particular, 6% of the observations fall in the 5th lactation, 4% in the 6th and 2% in the 7th. Consequently, for the ANOVA analysis, data related to lactations superior to 7 were included in the latter, due to a lack of numerosity.

This parameter significantly (P<0.0001) affects all traits, except for fat and protein percentage.

Calving intervals resulted significantly higher between first and second lactation (figure 4.1.3.3.1.), with a mean value (SE) of 474.34 (5.8) days. This was expected, as postpartum anoestrus in buffaloes, which is one of the main factors responsible for long calving interval, is usually reported with higher frequency in primiparous cows (Presicce et al., 2005). The lowest value for this parameter was recorded between the third and fourth parturition, with mean value (SE) equal to 414.11 (9.03). However, no statistically significant differences could be detected between lactations superior to the second one.

Concerning the seasonality of reproduction, the tendency in calving out of the typical breeding season observed in the analyzed farms appears more evident at first lactation (Figure 4.1.3.3.2.). as already underlined, the low seasonality showed by females at first calving can be due to a more successful application of the OBMS technique operated in the analyzed farms, as heifers are less sensitive to photoperiod (Zicarelli, 1997).

In the analyzed sample, milk yield tends to increase with the class of lactation number, reaching maximum value at 7th lactation, with a mean (SE) equal to 2742.7 (111.3) kg. The lowest mean values for this parameter, as expected, were observed at first lactation, with 2341 (31.3) kg of milk (figure 4.1.3.3.3.). No statistically significant differences were observed for protein and fat content (kg and %).

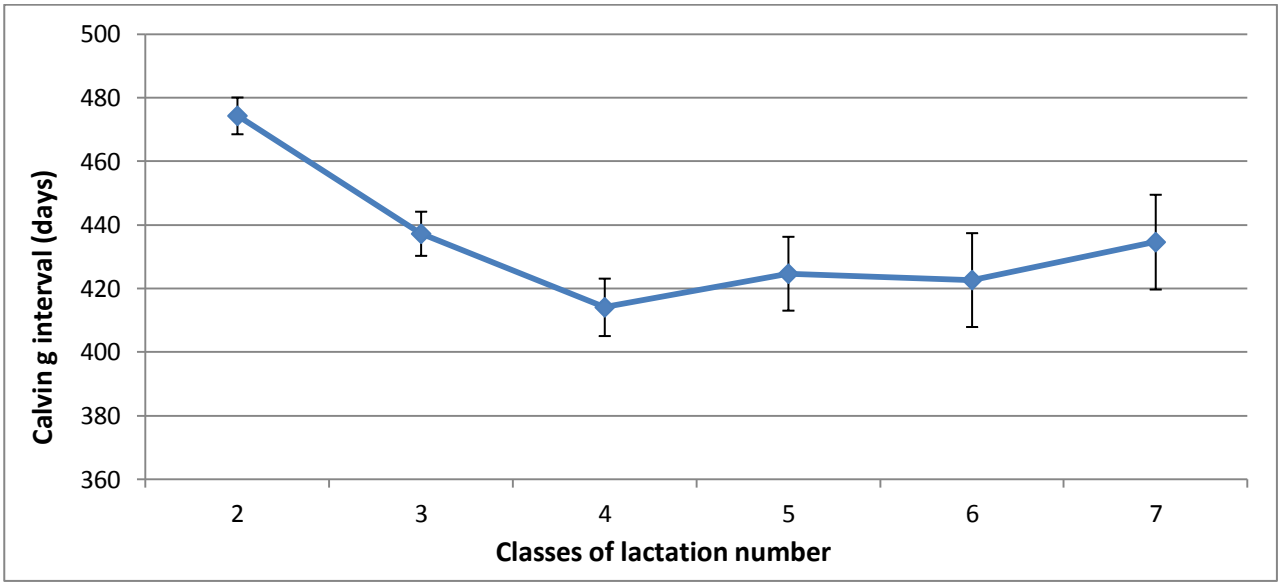


Figure 4.1.3.3.1. Mean values observed for calving interval at different classes of lactation number (P<0.0001).

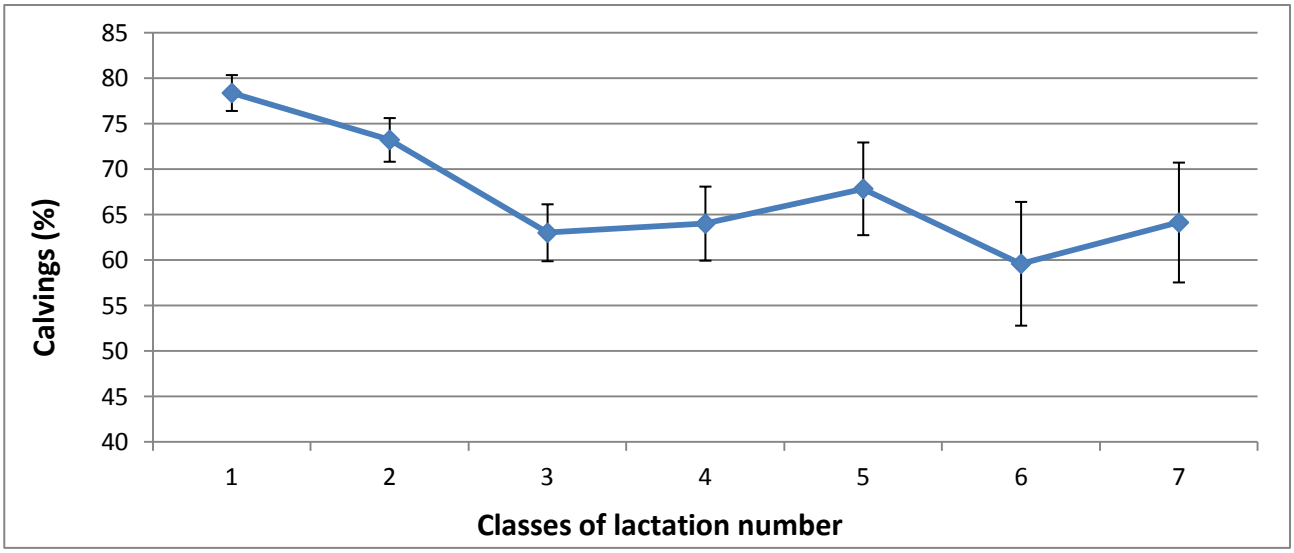


Figure 4.1.3.3.2. Average percentage of calvings observed in the out of breeding season at different classes of lactation number (P<0.0001).

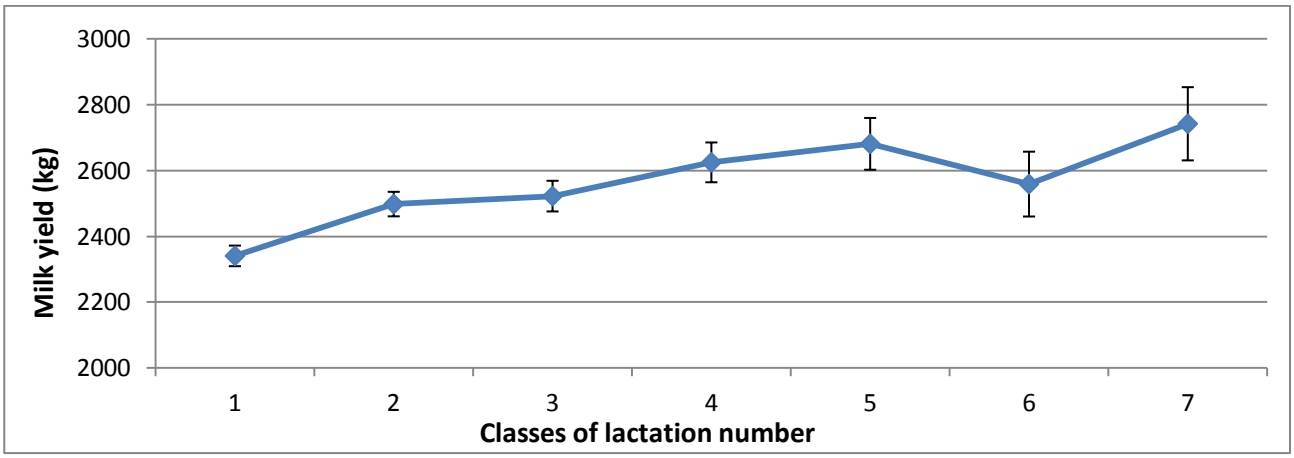


Figure 4.1.3.3.3. Mean values observed for milk yield at different classes of lactation number (P<0.0001).

4.1.3.4. Age at calving

Age at calving was also considered in the association analyses. Only 2 animals were 1 year old at calving, that is doubtful from a biological point of view, consequently they were removed from the dataset. Moreover, data related to ages of calving superior to 10 years were included in the latter class, because of lack of numerosity (number of observations per class less than 20).

Concerning calving interval, just one animal had data related to an age lower than 3 years, so it was removed from this analysis, due to a lack of numerosity for this class. The statistically significant (P<0.0012) difference observed for this parameter in the analyzed sample is due to the high mean value registered at 4 years of age, equal to 464.4 (SE=6.9) days, compared to those recorded at 3 and 6 years of age, equal to, respectively, 420.3 (SE=8.5) and 424.3 (SE=10.1) days (figure 4.1.3.4.1.).

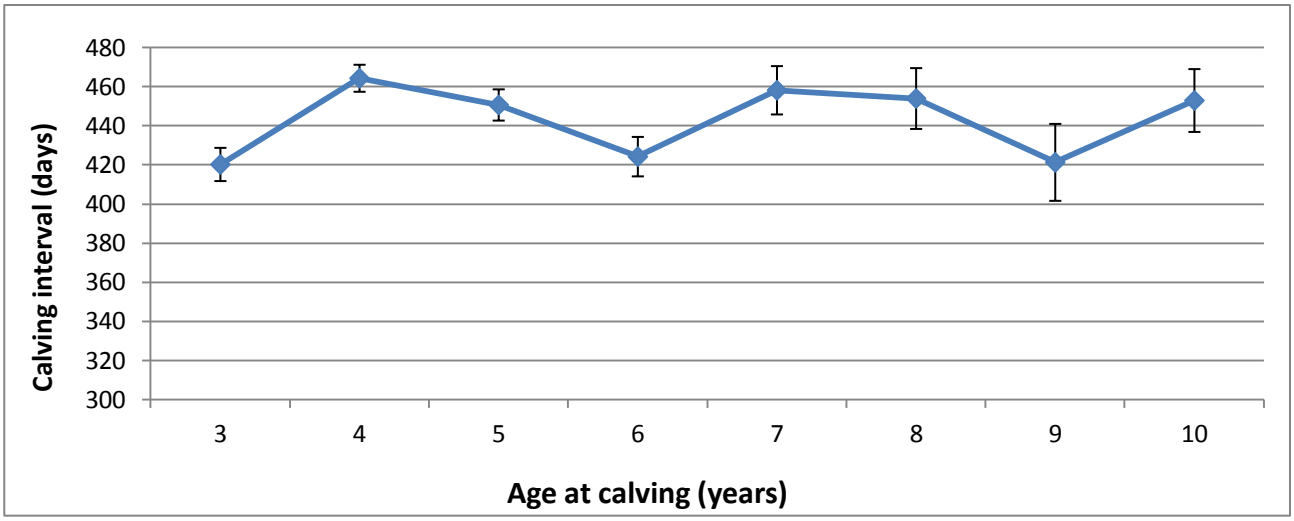


Figure 4.1.3.4.1. Mean values observed for calving interval at different classes of age at calving (P<0.0012).

The seasonality of deliveries for age at calving, reflects what was shown by the classes of lactation number, with lower seasonality, in terms of percentage of calvings observed in the out of breeding season, for animals that have calved younger, above all in the first three years of age (figure 4.1.3.4.2.), which correspond to lactations number 1 and 2.

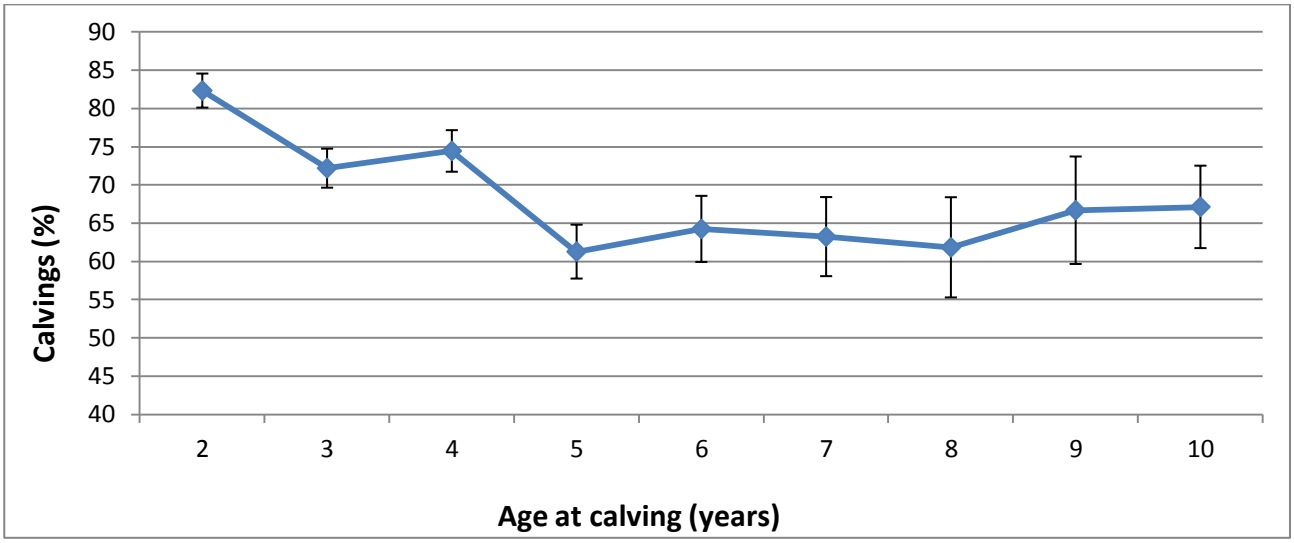
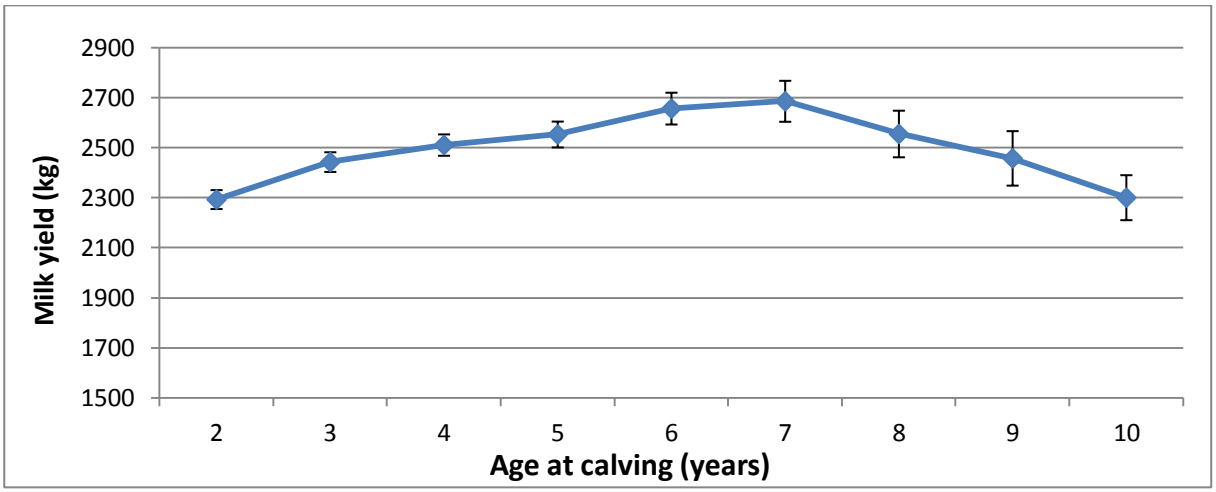


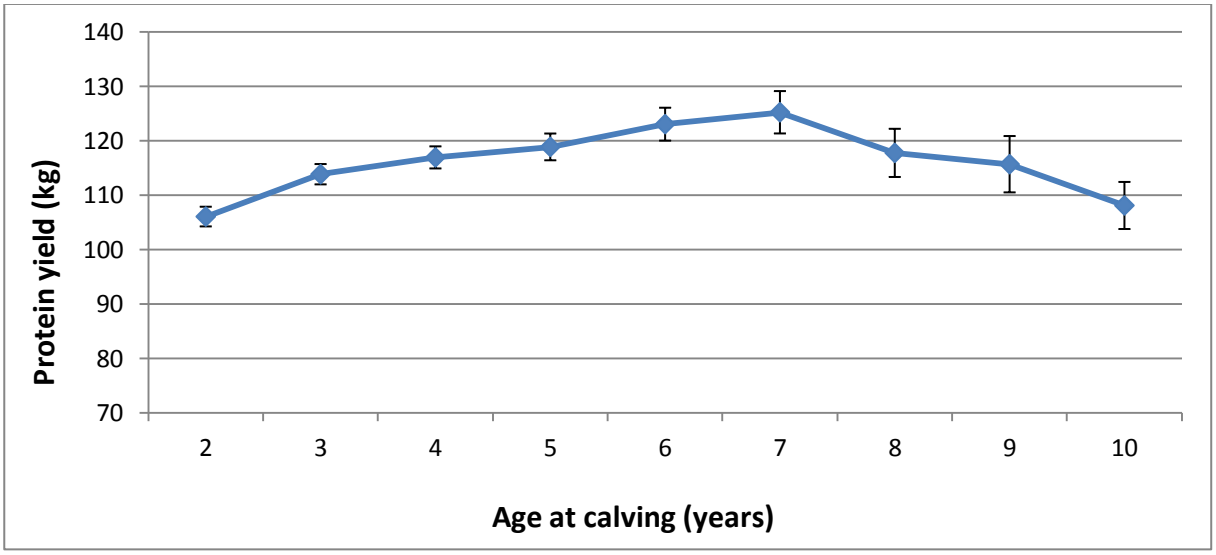
Figure 4.1.3.4.2. Average percentage of calvings observed in the out of breeding season at different ages at calving (P<0.0001).

Production parameters were also affected by the age at calving. No differences could be detected for protein and fat percentage, while milk, protein and fat yields (kg) tend to increase with age at parturition, reaching the maximum value at 7 years, with a mean (S.E.) equal to 2686.51 (81.95) kg, 125.24 (4.82) kg and 225.95 (0.1) kg, respectively (figure 4.1.3.4.3.).

a)



b)



c)

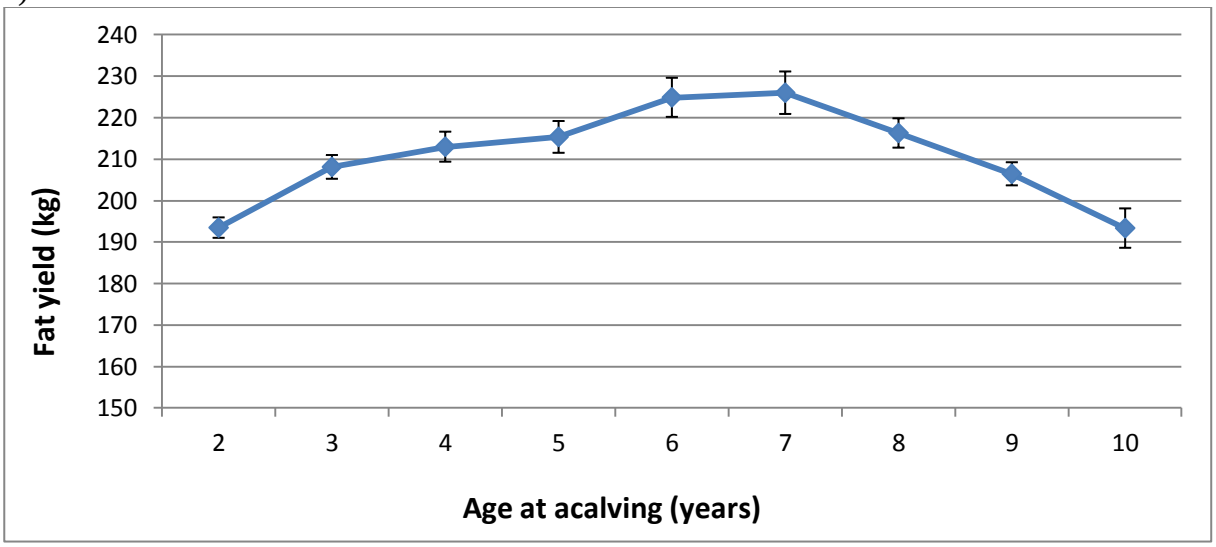


Figure 4.1.3.4.3. Mean values for milk (a), protein (b) and fat (c) yields observed at different ages at calving (P<0.0001).

4.2. Candidate genes analysis for SNP discovery

As is clear from the above, the characters examined in present research are very complex. For this reason, it was not easy to identify a single criterion for the selection of candidate genes that could provide a complete description of the complexity of the problem.

After an extensive study of literature and databases, also considering information available for other species, such as cattle, the candidate genes chosen for SNP discovery analysis were: *STAT5A*, *SERPINA14* and *TNFA* genes, involved in fertility, and *MTNR1A* gene, previously associated with seasonality of reproduction. These are discussed in detail in the following paragraphs.

4.2.1. *SERPINA14*

The *SERPINA14* (serpin peptidase inhibitor, clade A, alpha-1 antiproteinase, antitrypsin, member 14) is member of a large serpin super family of serine protease inhibitors (Ing and Roberts, 1989), that are secreted from the uterine endometrium mainly under the influence of progesterone (Leslie and Hansen, 1991) in many ungulate animal species including bovine (Mathialagan and Hansen, 1996), ovine (Ing et al., 1989), caprine (Tekin et al., 2005) and porcine (Malathy et al., 1990). The SERPINA14 protein performs diverse biological functions which include direct nutrition to the conceptus, growth control, inhibition of proteolytic activities and suppression of the local maternal immune system for sustaining pregnancy (Roberts and Bazer, 1988). This gene has been studied in bubaline species (Kandasamy et al., 2010) where authors found a differential spatio-temporal expression of *SERPINA14* gene in the uterine endometrium of buffalo which suggests its plausible important roles in reproduction.

In bovine species this gene is located on chromosome 21 and it has a 1464 bp long coding sequence, it is composed by 5 exons and there are 124 known SNPs (see figure 4.2.1.1.) in literature, of which: 22 are in the *upstream* region; one in the 5'UTR; 14 are located in exons, of which 6 are synonymous; 7 are missense variants and 1 is placed within the region of the splice site; 32 SNPs are in introns; one is in the 3'UTR and finally 55 are located in the downstream gene region. The homology between bovine and other species is quite low, equal to 60% with swine and 45% with horse.

Concerning buffalo species, in databases is the complete mRNA and coding sequences for this gene (National Center for Biotechnology Information, Accession: HM590822.1).

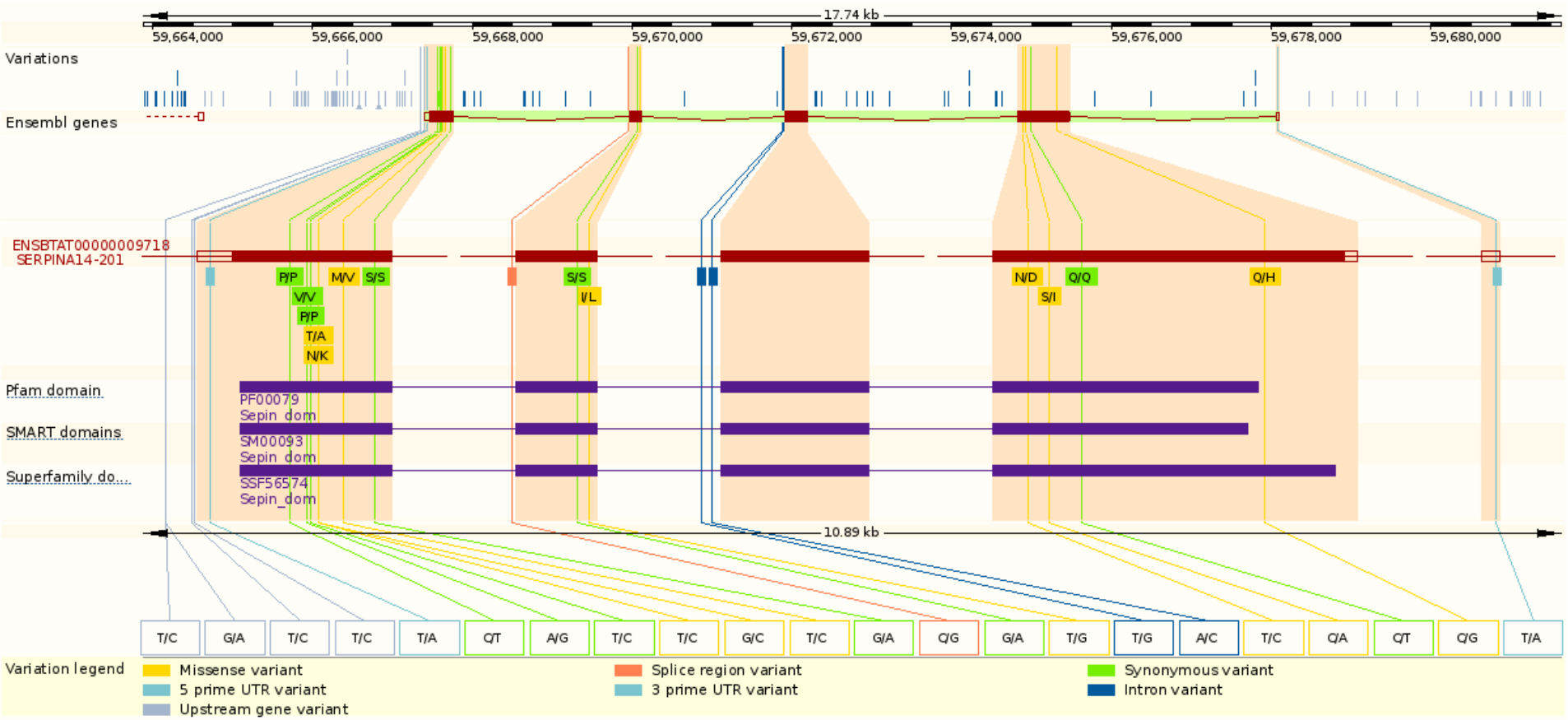


Figure 4.2.1.1. *Bos taurus SERPINA14* gene structure and known polymorphisms (Ensembl release 73 - September 2013). 29 intronic variations are not represented in the image.

4.2.2. *STAT5A*

The signal transducer and activator (STAT) proteins are known to play a vital role in cytokine signaling pathways (figure 4.2.2.1.). The STAT proteins are transcription factors that are specifically activated to regulate gene transcription when cells encounter cytokines and growth factors. Hence, they act as signal transducers in the cytoplasm and transcription activators in the nucleus (Kisseleva et al., 2002). In mammals, the STAT proteins comprise a family of 7 structurally and functionally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Darnell, 1997). The 7 mammalian STAT proteins range in size from 750 to 850 AA. The chromosomal distribution of these STAT, as well as the identification of STAT proteins in more primitive eukaryotes, suggest that this family arose from a single primordial gene (Chen et al., 1998). In addition, STAT share a number of structurally and functionally conserved domains.

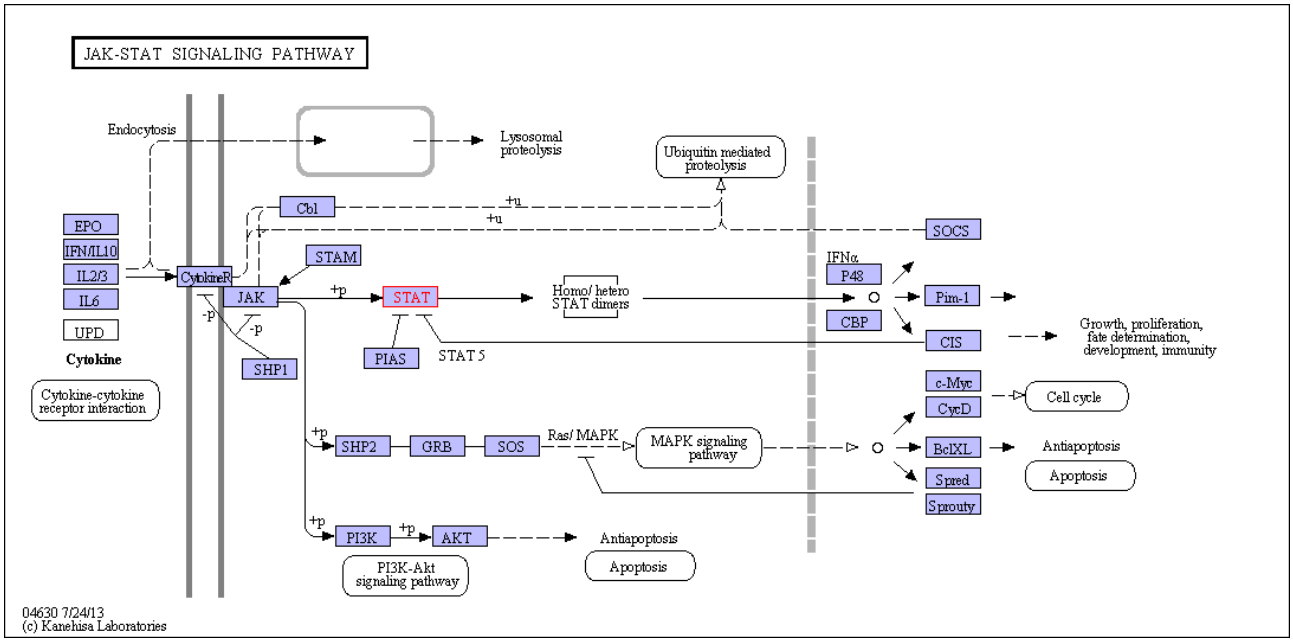


Figure 4.2.2.1. Jak-STAT signaling pathway in man from KEGG database (Kanehisa and Goto, 2000), update October 2013.

The *STAT5A* gene has been studied in bovine species for its influence on both reproduction and milk production traits. (Khatib et al 2009). In particular, an association between allele G of SNP12195 and a decrease in both protein and fat percentages was found, and this G allele was also associated with low embryonic survival and fertilization rate. More recently, *STAT5A* gene was associated with oestrous expression in cattle (Homer et al., 2013). Again, the expression of this gene during early bovine embryogenesis was analyzed, demonstrating that the embryonic *STAT5A* gene is primarily activated by maternal gene products (Flisikowski et al., 2013).

In bovine species this gene has a 2585 bp long cds, it is made up of 20 exons and there are 144 known SNPs, of which: 46 are located in within the upstream gene region; 4 are located in exons and do not result in an aminoacid change; one is a splice acceptor variant, i.e. it changes the 2 base region at the 3' end of the intron; one SNP is located on the splicing site at the end of exon 8, while all the other 68 SNPs are located in introns (figure 4.2.2.2.). *STAT5A* gene in bovine species shows high homology with other livestock species, equal to 97% with horse, 96% with swine and 95% with rabbit. In Figure 14 gene structure and known polymorphisms are reported.

In buffalo, nucleotide sequences encompassing exons from 6 to 11 and a partial coding sequence are reported in databases (National Center for Biotechnology Information).

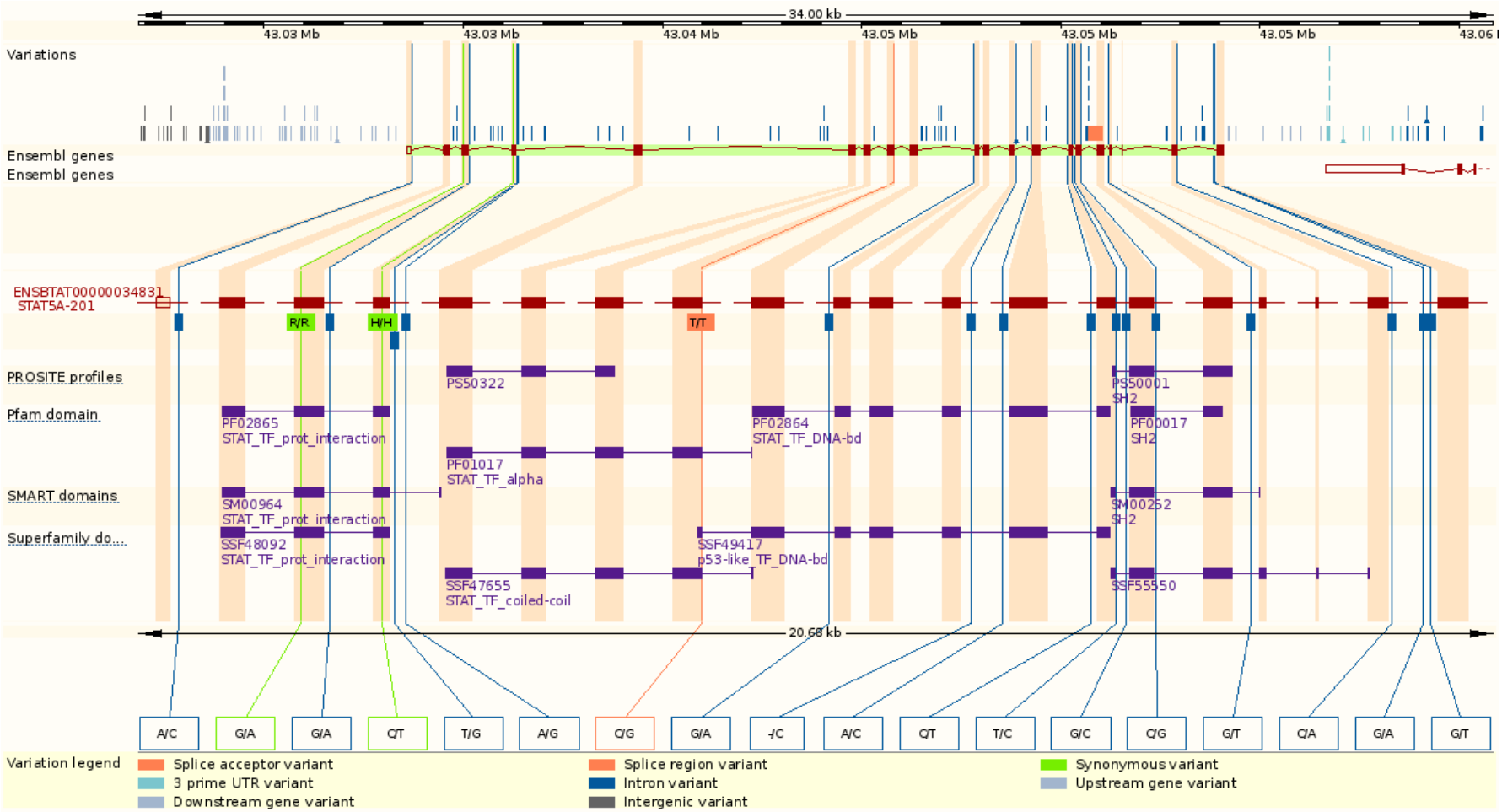


Figure 4.2.2.2. *Bos taurus STAT5A* gene structure and known polymorphisms (Ensembl release 73 - September 2013). 53 intronic variations are not represented in the image.

4.2.3. *TNFα*

The *TNFα* (tumor necrosis factor-α) gene has been associated with male fertility in man, in particular with low sperm count, altered sperm motility (Tronchon et al., 2008; Zalata et al., 2013)), significantly decreased normal sperm morphology, acrosin activity, and seminal α-glucosidase (Zalata et al., 2013). Polymorphisms of *TNFα* gene both in exon and promoter regions have been also associated with female fertility, in particular with the early first ovulation within 3 weeks after parturition, in the high-producing dairy cow (Shirasuna et al., 2011).

For buffalo species, no nucleotide sequences are reported in databases (National Center for Biotechnology Information). On the contrary, the bovine *TNFα* is well characterized and it is located on chromosome 23, the transcript is composed by 4 exons for a total of 1,689 bps and a translation of 234 residues length. 112 known SNPs are reported in databases, of which: 51 in the upstream gene region; 4 in coding sequence and synonymous; 8 in introns; 3in the 3'UTR and 46 in the downstream gene region (figure 4.2.3.1.).

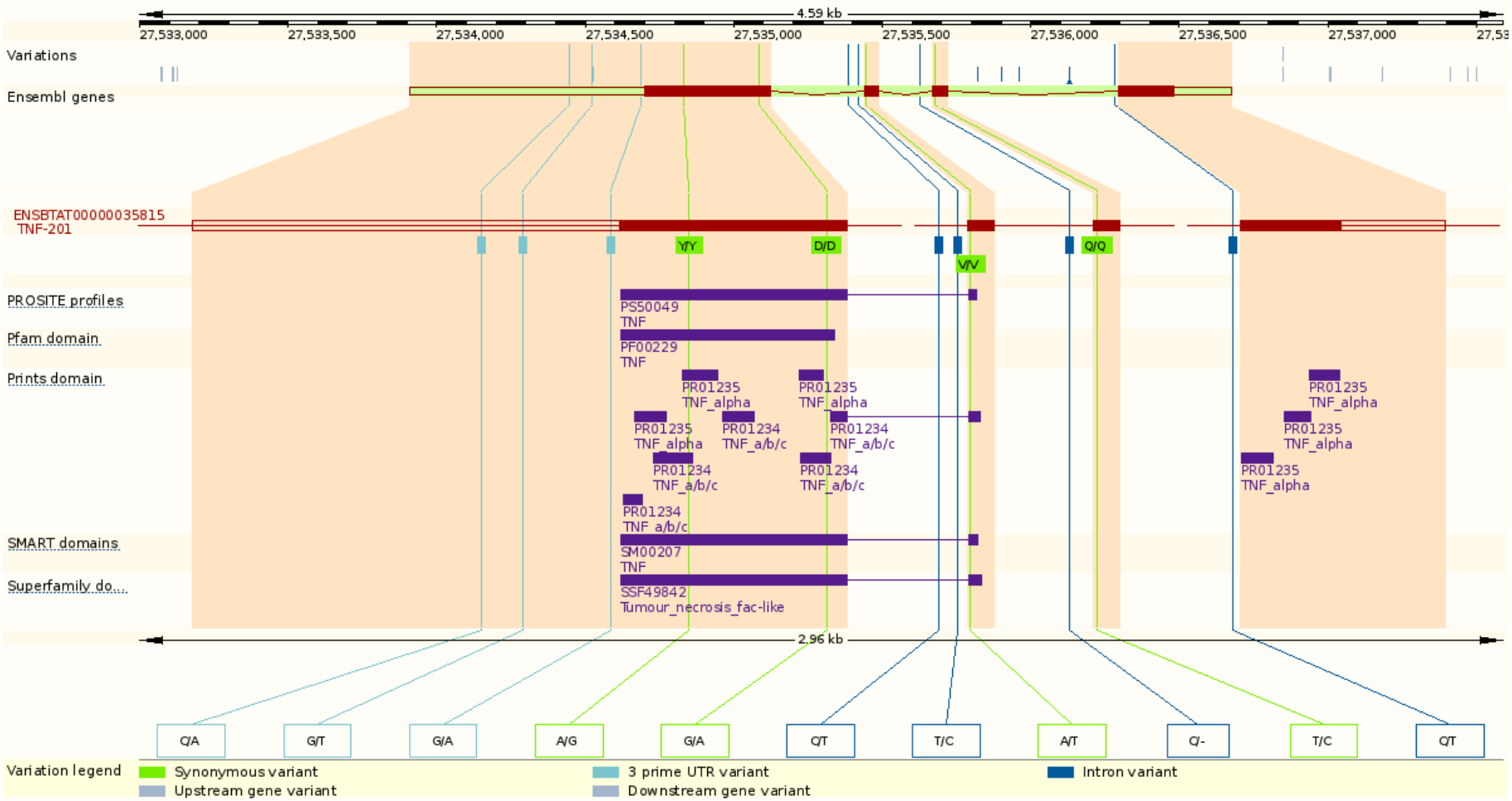


Figure 4.2.3.1. *Bos taurus TNFα* gene structure and known polymorphisms (Ensembl release 73 - September 2013). 4 intronic variations are not represented in the image.

4.2.4. *MTNR1A*

As it was explained in the previous paragraphs, there is evidence that melatonin, through its impact on the timing of puberty and adult breeding activity, plays a key role in the control of reproductive events in a wide range of species. Melatonin secretion is mediated by photoperiod, the main environmental factor affecting the regulation of reproductive seasonality (Kennaway and Hugel, 1992). The melatonin effect is carried out at hypothalamic level, by regulating of GnRH secretion (Malpaux et al., 1998). Melatonin receptors are classified in MTNR1A and MTNR1B subtypes but only the first seems to be involved in the regulation of seasonal reproductive activity (Dubocovich et al., 2003). In several sheep, goat and cattle breeds, polymorphic sites in *MTNR1A* (melatonin receptor 1A) gene exon 2 were found (Messer et al., 1997). One G to an A substitution in position 612 in sheep and a G to an A substitution in position 52 in goat of the sequence of *MTNR1A* gene was found to lead to a less seasonal reproductive activity (Carcangiu et al., 2009a; Carcangiu et al., 2009b). In a more recent work (Carcangiu et al., 2011) it has been demonstrated that buffaloes carrying C/C genotype showed the reproductive activity principally during decreasing day-length whereas those with T/T genotype showed mating period largely during increasing day-length. Animals carrying T/T genotype could be allocated to reproduction during long photoperiod instead the C/C subjects during natural mating season. In a more recent study performed on *MTNR1A* gene in Mediterranean Italian buffalo, no associations between genotype, first mating and subsequent calving date were found, but the duration from first to second calving was longer in buffaloes with the C/C genotype compared with those with the T/T and C/T genotypes ($P<0.01$). Moreover, the period of calving for buffaloes with the C/C genotype was mainly from July to September, whereas that for buffaloes with the T/T genotype was largely from March to May and the association between the T/T genotype and reproductive activity during days with a long photoperiod indicates that this polymorphism may be considered a genetic marker to identify buffaloes that are able to reproduce out of the breeding season (Luridiana et al., 2012).

In databases a partial coding sequence, including the exon 2, is reported for buffalo species. The bovine *MTNR1A* has a 771 bps long coding sequence and it is made up of 2 exons. 183 SNPs are reported in this species, of which: 40 in the upstream gene region; 8 in exons, of which 3 missense and 5 synonymous; 128 in introns and 7 in the downstream gene region (figure 4.2.4.1.).

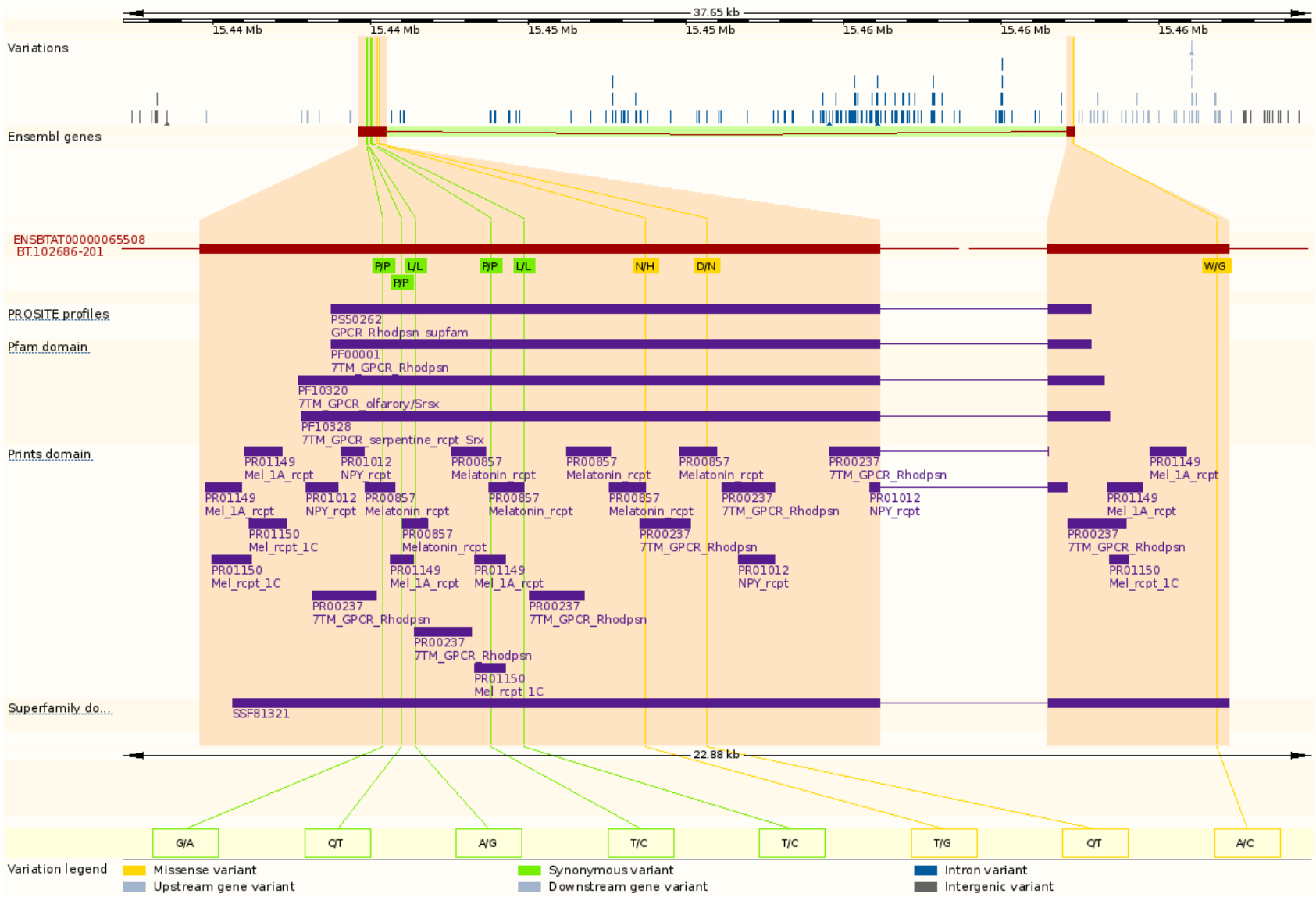


Figure 4.2.4.1. *Bos taurus MTNR1A* gene structure and known polymorphisms (Ensembl release 73 - September 2013). 128 intronic variations are not represented in the image.

4.3. SNP detection and population parameters

The polymerase chain reaction (PCR) analyses and the alignment of fragments allowed the identification of 12 single nucleotide polymorphisms in the analyzed genes. Of these, 11 were identified for the first time in present work, while a C>T substitution in *MTNR1A* gene was already found in literature and associated to seasonality in buffalo species (Carcangiu et al., 2011). The SNPs identified are described in table 4.3.1.

GENE NAME	SNP NAME	LOCATION	SNP TYPE	AA CHANGE	REFERENCES	REFSEQ-SNP IDENTIFICATION[1]
MTNR1A	**c.318C>T**	**exon 2**	**C/T**	**no**	**Carcangiu et al. (2011)**	**GU817415**
SERPINA14	c.70C>T	exon 2	C/T	Leu>Phe	Present Work	HM462262.1
	c.717G>A	exon 3	A/G	Asn>Asp	Present Work	HM462262.1
TNFA	**c.323C>A**	**exon 4**	**A/C**	**no**	**Present Work**	**NM_173966.2**
	c.371G>A	**exon 4**	**A/G**	**no**	**Present Work**	**NM_173966.2**
STAT5A	**c.128+179G**	**intron 2-3**	**G/C**	**-**	**Present Work**	**NM_001012673.1**
	c.924C>T	**exon 8**	**C/T**	**no**	**Present Work**	**NM_001012673.1**
	c.989+144G	intron 8-9	G/T	-	Present Work	NM_001012673.1
	c.989+344C	**intron 8-9**	**C/T**	**-**	**Present Work**	**NM_001012673.1**
	c.1342+99A	**intron 10-11**	**A/G**	**-**	**Present Work**	**NM_001012673.1**
	c.2057T>A	exon 16	T/A	no	Present Work	NM_001012673.1
	c.2331C>T	exon 19	CT	no	Present Work	NM_001012673.1

Table 4.3.1. Single Nucleotide Polymorphisms detected in present work.
[1]Sequenced reference (SeqRef) identification in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov).

A first genotyping analysis on a sample of 36 buffaloes was performed in outsourcing for all the SNPs found. For two polymorphisms located at *STAT5A* gene, c.989+144G and c.2331C>T, the setup of SNP genotyping failed at the laboratory where the analysis was performed. Seven SNPs, highlighted in bold in table 4.3.1., resulted polymorphic and were subjected to the subsequent analyses, while other 3 SNPs among those identified by PCR sequencing resulted monomorphic in the sample of 36 buffaloes. Of these, the two SNPs within the *SERPINA14* gene were detected by comparing the sequences of animals analyzed in present work with the one found in database for *Bubalus bubalis* (GenBank: HM462262.1). The other polymorphism which resulted monomorphic in the 36 buffaloes genotyped, the STAT5Ac.2057T>A, was identified through a well-defined double pick in the chromatogram of one of the six animals analyzed in the SNP discovery. This probably means that it is a very rare polymorphism, which was not considered for the subsequent analyses in present work.

Of the seven polymorphisms resulted polymorphic, apart from that already present in literature, two are located in exon 4 of *TNFA* gene and four in *STAT5A* gene, of which one in exon 8 and three in introns. All the coding SNPs are synonymous, i.e. they do not change the aminoacid sequence of the encoded protein. Even if synonymous codons are nucleotide triplets that are translated into the same amino acid, they deserve to be analyzed also because of the phenomenon known as codon bias. This

means an unequal frequency usage of the codons from the same synonymous group, and is a characteristic to all organisms from bacteria to multicellular eukaryotes (Nabiyouni et al., 2013). Also, Chamary and co-authors reviewed evidence that variable sites in synonymous codons are important in mRNA stability and proper splicing (Chamary et al., 2006). Synonymous SNP, as well as non-coding SNP, could also be associated with an altered phenotype if they are in linkage disequilibrium with a functional mutation, being almost always inherited together with the functional mutation itself. Moreover, SNP located in introns may have effects on important mechanisms such as transcription, translation and splicing.

The seven polymorphic *loci* were genotyped on the sample of 491 female Mediterranean Italian Buffaloes characterized by the phenotypes described above. Population parameters for these SNPs are reported in table 4.3.2. The markers MTNR1Ac.318C>T and STAT5Ac.128+179G deviated from the Hardy-Weinberg equilibrium, with significantly different values for observed and expected heterozygosity. Noticeably, even if these two polymorphisms had comparable value for the minor allele frequency (MAF) (equal to 0.292 and 0.319, respectively), for the SNP at *STAT5A* gene the homozygous class for the minor allele C resulted highly under-represented, with a frequency for this genotype equal to 0.073, that is very low. This can be due to a selection against this allele, which remains in the population mostly in heterozygous form.

A similar hypothesis can be done for the homozygous class of minor allele A of the marker at position c.323C>A of *TNFA* gene, which showed a very low frequency, equal to 0.016, in the analyzed population. In fact, just 8 animals of the 491 analyzed were AA. This was expected, as already in the first sample of 36 buffaloes genotyped this SNP reported a very low frequency for the A allele, equal to 0.097, and no animals were homozygous for this allele.

The other SNP genotyped at *TNFA* gene, located at position c.371G>A, showed a MAF lower than 1%, so it was removed from the subsequent association analyses. The A allele at this locus was rare also in the initial sample of 36 buffaloes, with just one animal carrying this allele in heterozygous form. This animal was the same analyzed for SNP discovery, and it allowed the detection of this rare polymorphism. Even if just one buffalo among the 36 genotyped carried the A allele, this SNP was analyzed also in the larger sample of 491 animals, in order to maximize information on *TNFA* gene, as only another one SNP was found at this interesting *locus*.

For all the other markers, the MAF resulted around 30%, little higher (36%) for the SNP c.924C>T at *STAT5A* gene.

SNP		Genotype and frequency			Allele and MAF	No. of obs.	Availability	Het exp	Het obs	HW exact *P*-value
MTNR1A	c.318C>T	CC	CT	TT	T					
		0.520	0.378	0.103	0.292	487	0.992	0.413	0.378	0.051*
TNFA	c.323C>A	AA	AC	CC	A					
		0.016	0.189	0.794	0.111	491	1.000	0.197	0.189	0.346
	c.371G>A	AA	AG	GG	A					
		-	0.002	0.998	0.001	487	0.992	0.002	0.002	1.000
STAT5A	c.128+179G	CC	CG	GG	C					
		0.073	0.493	0.435	0.319	467	0.951	0.435	0.493	0.004*
	c.924C>T	CC	CT	TT	T					
		0.422	0.439	0.139	0.358	483	0.984	0.460	0.439	0.274
	c.989+344C	CC	CT	TT	T					
		0.474	0.410	0.115	0.321	485	0.988	0.436	0.410	0.167
	c.1342+99A	AA	AG	GG	G					
		0.550	0.368	0.083	0.267	484	0.986	0.391	0.368	0.210

Table 4.3.2. Population genetics parameters: genotype frequencies, minor allele frequencies (MAF), observed and expected heterozygosity (Het obs and Het exp, respectively), and Hardy-Weinberg (HW) equilibrium exact *P*-values at the investigate SNPs.

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4.4. Associations with SNPs

4.4.1. *TNFA* gene

The SNP located at the 3rd exon of *TNFA* gene showed a statistically significant association (P<0.0404) with the parameter of calving interval. This trait was analyzed separately for every class of lactation number, and the association observed with the TNFAc.323CA polymorphism could be assessed only in the fourth lactation subset. However, just one animal, with very low value for calving interval (equal to 331 days) resulted homozygous for the A allele in this subset, so it was removed from the dataset. After repeating the analysis with this correction, all the factors in the model resulted significantly associated with calving interval (table 4.4.1.1.). In particular, a statistically significant (P<0.0318) effect was highlighted between buffaloes homozygous for the C allele, showing significantly longer calving intervals compared to the CA animals, with least square means (SE) equal to, respectively, 425.72 (7.14) days and 390.31 (15.33) days (figure 4.4.1.1.). This confirms the improving effect of the A allele observed for the homozygous AA animal, which reported a very low level for calving interval. In fact, the A allele at this locus showed a large (P<0.012) negative substitution effect, equal to -38.11 (SE=15.03) days (table 4.4.3.2.). Also dominance effect resulted statistically significant (P<0.038), with a negative value of -34.32 (SE=16.37) days. Since just one animal carried the AA genotype in the subset analyzed and both allelic substitution and dominance effects are calculated based on the allelic frequencies, is to assess whether, in a sample with other frequencies, these effects are confirmed. The SNP contribution to the variance observed is equal to 0.029 and is the highest between the polymorphisms analyzed (table 4.4.3.2.).

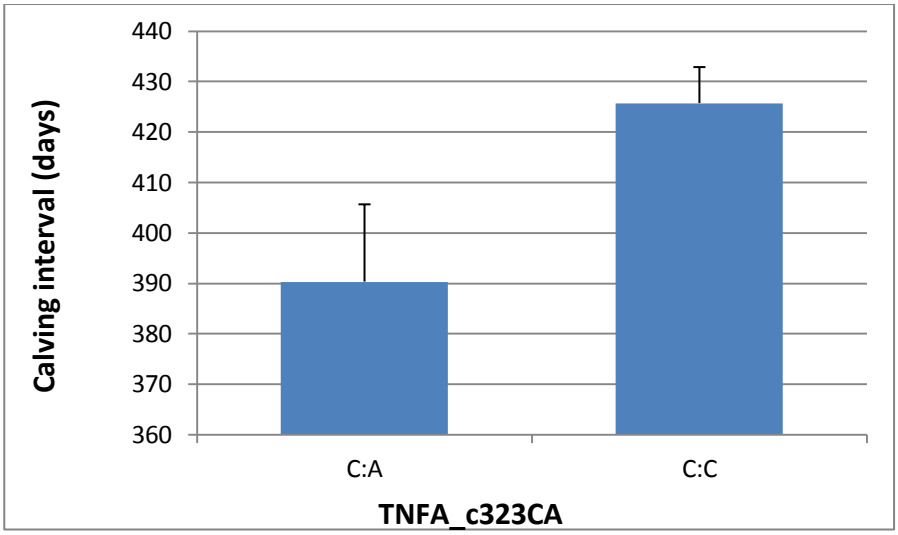


Figure 4.4.1.1. Least square means for calving interval at the TNFAc.323CA *locus* in the 4th lactation subset (P<0.0318).

Trait	Dataset	Effects	Sum of Squares	Degree of freedom	Mean Square	Prob>F
Calving interval	Lactation 4	Flock	43954.6	3	14651.5	0.0329
		Seasonality of calving	19987.9	1	19987.9	0.045
		Age at calving (days)	48363.5	1	48363.5	0.002
		TNFA_c323	22969.2	1	22969.2	0.0318
		Error	639157.1	131	4879.1	
		C Total	794531.98	137		<.0001

Table 4.4.1.1. Model results for the TNFAc.323C>A polymorphism association analysis with calving interval parameter in the 4th lactation subset.

The TNFAc.323CA polymorphism did not show any other statistically significant associations, even considering the subset including data related to only the 4th lactation. Nevertheless, the frequency of A allele seems to decrease from the first lactation on, as can be observed in table 4.4.1.2. In the first lactation, 8 AA animals were recorded while in the fourth one just one animal carried this genotype and it was not present from the 6th lactation on. Since in our sample the A allele resulted significantly associated with a reduction of the calving interval, its decrease in the population is to be considered a negative effect. Moreover, no statistically significant associations were detected for this polymorphism with the other parameters studied. Therefore, further analyses on other traits, which could have been selected reducing the A allele frequency in the buffalo population, deserve to be conducted.

Lactation number	Animals	A allele frequency	SD
1	96	0.1103	0.0110
2	80	0.1176	0.0128
3	50	0.1050	0.0145
4	26	0.0935	0.0173
5	14	0.0833	0.0220
6	6	0.0577	0.0222
7	4	0.0690	0.0320

Table 4.4.1.2. Frequency of A allele for SNP TNFAc.323CA in the different lactation number subsets analyzed.

Contrary to buffalo, for which there is no information on the sequence in the database, the bovine *TNFA* is well characterized and it is located on chromosome 23. The transcript is composed by 4 exons for a total of 1,689 bps and a translation of 234 residues length. 112 known SNPs are reported in databases, of which: 51 in the upstream gene region; 4 in coding sequence and synonymous; 8 in introns; 3in the 3'UTR and 46 in the downstream gene region (figure 4.2.3.1.).

The *TNFA* gene has been previously associated with male fertility in man, in particular with low count, motility and morphology of sperm (Tronchon et al., 2008; Zalata et al., 2013). Calving interval measures the cow's ability to be successfully inseminated again quickly post calving. Since the outcome of an insemination depends on both male and female fertility, previous studies performed in cattle species have compared bull fertility estimation based on the reproductive performance of field data, such as calving interval, on cows (Clay and McDaniel, 2001; Averill et al., 2004). Surely, the polymorphism found in buffalo species in the present research deserves to be

studied also on male buffaloes, in order to investigate associations with male fertility parameters. However, polymorphisms of *TNFA* gene both in exon and promoter regions have been directly associated with female fertility in cattle, in particular with the early first ovulation within 3 weeks after parturition, which can result in the elongation of calving interval period, in the high-producing dairy cow (Shirasuna et al., 2011). In this recent work, which was not published when the PCR performed in present research were set up, Shirasuna and co-authors found an association with a polymorphism located at the 4th exon of *TNFA* gene, in addition to a non-coding mutation. Given the relevant effect of the SNP located in the 3rd exon, highlighted in the present work for buffalo, it would be interesting to analyze also the remaining part of the *TNFA* gene, particularly the 4th exon region, in this species.

None of the other polymorphisms, identified in candidate genes for fertility traits, showed statistically significant associations with calving interval. It must be said, however, that this parameter, obtained from the dates of calving, was the only enough reliable phenotype which could be related to fertility. However, as discussed above, fertility is a complex issue, involving many mechanisms and very difficult to be measured with field data. This is true above all in buffalo species, where artificial insemination is rarely practiced and very little parentage information is available. More accurate fertility phenotypes should be obtained to find associations with the genes analyzed in present study.

4.4.2. *MTNR1A* gene

The *MTNR1A* gene was a candidate gene for seasonality of reproduction. Concerning this parameter, the SNP association analysis in present work was performed between "seasonal" (from August to December) and "out of season" (from January to July) dates of calving.

The C>T substitution in *MTNR1A* gene found in literature and associated to seasonality in buffalo species (Carcangiu et al., 2011), was confirmed in our sample. The association analysis performed in the present research, did not allow establishing a relationship between this polymorphism and seasonal breeding. Carcangiu and co-authors reported, for the buffaloes with C/C genotype, the highest number of mating in the semester between August and January, with a peak in October and November, which is the typical breeding period for buffalo species. On the other hand, animals with T/T genotype mated mostly in the semester between February and July and calving occurred largely from March to May, specifically in the out of breeding season period. In another study, the same polymorphism in *MTNR1A* gene was analyzed in a smaller population of Mediterranean Italian Buffaloes, confirming the association reported by Carcangiu and colleagues with seasonality of reproduction (Luridiana et al., 2012). In fact, Luridiana and co-authors found that reproductive activity in buffaloes with the T/T genotype was unaffected by fluctuations in the photoperiod. In contrast, buffaloes with the C/C genotype exhibited a more seasonal reproductive pattern, with sexual activity mainly during days with a short photoperiod.

Some differences between animals studied in present work and those analyzed by the other authors should be underlined. In both the cited papers one herd, of different size, of Mediterranean Italian Buffaloes reared in the South of Sardinia was analyzed, while the animals studied in present work belong to 4 different farms, located in South of Italy. Moreover, all animals studied by Carcangiu (2011) and Luridiana (2012) and co-authors were under natural photoperiod conditions, with bulls

kept always within the herd, while in our sample all the buffaloes were subjected to the OBMS technique, which interrupts sexual promiscuity in the herd during the autumn season.

In the population analyzed in present research, the C allele resulted more frequent (allele frequency equal to 0.72) than T (0.28). Also this data is in contrast with the findings of previous authors, who reported a more balanced allele frequency for this SNP. In particular, Carcangiu and colleagues in 2011 found a frequency for the C allele equal to 0.44, and 0.56 for T allele.

The trend of genotypic frequencies at this *locus* showed even more remarkable differences from the results reported by other authors. In fact, in present work just 50 buffaloes showed the T/T genotype (10.2%), while 253 were C/C (51.5%) and 184 C/T (37.5%). Carcangiu and colleagues reported instead genotype frequencies equal to 34% for T/T, 26% for C/C and 40% for C/T. These results are similar to those reported for the same SNP in the other study performed on 60 buffaloes by Luridiana et al. in 2012, where allele frequencies for C were 0.42 and 0.58 for T allele, and genotype frequencies for C/C, C/T and T/T were equal to, respectively, 28%, 38% and 34%. The number of homozygous animals for the C allele observed by other authors is significantly lower than that found in the population analyzed in present work. Likewise, in our population the number of TT buffaloes is significantly lower compared to those analyzed by the other authors. Our sample included data related to all lactations, while in that studied by Carcangiu and colleagues, primiparous and old cows were removed from the dataset. However, no statistically significant differences were detected for the MTNR1Ac.318C>T polymorphism in respect to the number of lactation in our population. As already underlined, in the farms analyzed in present research the OBMS technique was performed. In fact, 71% of calvings recorded for buffaloes analyzed in present work fall in the out of breeding season period, while 29% are seasonal, i.e. between August and December. In present work, seasonality of reproduction was studied in animals subjected to OBMS practice because, on one hand, this represents the usual management condition for the Mediterranean Italian Buffalo farms and, on the other hand, it seemed interesting to investigate animals which, even under OBMS conditions, were not able to show sexual activity in the out of breeding season period.

Given the differences emerged in comparison to other researches, underlined and discussed above, and since the animals studied in present work belong to 4 different farms, the previous analysis of the flock variable in respect to the seasonality of calving parameter was investigated. As was presented in the previous paragraph (4.1.3.2.), a strong effect on reproductive seasonality parameter was assessed for Z farm, where 82.5% of calvings occurred in the out of breeding season. Consequently, the association analysis of this trait with the MTNR1Ac.318C>T polymorphism was repeated removing all data related to this flock. Actually, in this case an association with this SNP could be detected ($P<0.0415$), with a tendency in calving in the out of breeding season period showed by the animals with genotype TT. The Z flock presented two significant differences in respect to the other three analyzed farms: a significantly higher number of TT animals (14.60 %) and buffaloes with a significantly lower age at calving, with a mean (SE) equal to 1700 (39.47) days in the Z flock and a mean of 1864 (27.69) days in the other three farms.

A statistically significant ($P<0.0012$) allelic substitution effect was observed, with a negative value equal to -0.286 (SE=0.0113) for the C allele. No dominance effects could be instead detected.

The results obtained with this posterior analysis, seem to be in accordance with those reported from the other authors who associated the reproductive seasonality trait with this polymorphism at *MTNR1A* gene. However, this association could be seen in our population only after the removing of data related to one of the flocks analyzed. Hence, this effect should be validated on a sample collected from a higher number of farms.

In the present research, the MTNR1Ac.318C>T polymorphism resulted associated also with dairy parameters. In particular, a statistically significant effect emerged on milk (P>0.0043), fat (P<0.0101) and protein (P<0.0101) yield (kg) parameters. In all these cases, TT animals showed lower performances. In particular, animals homozygous for T allele reported a significantly reduced milk yield production, with least square mean (SE) equal to 2340.53 (68.41) kg, compared to CC and CT, showing least square mean values (SE) of 2515.15 (56.50) kg and 2512.89 (57.89) kg, respectively (figure 4.4.2.1.a). The C allele at this *locus* had a large (P<0.0123) substitution effect, equal to (SE) 63.03 (25.15) kg, while the genotype dominance effect resulted not statistically significant. Little contribution, equal to 0.0085, is given by this SNP to the total variance observed (table 4.4.3.2.). All the effects in the model resulted statistically significant for this parameter, except for the age at calving (table 4.4.2.1.).

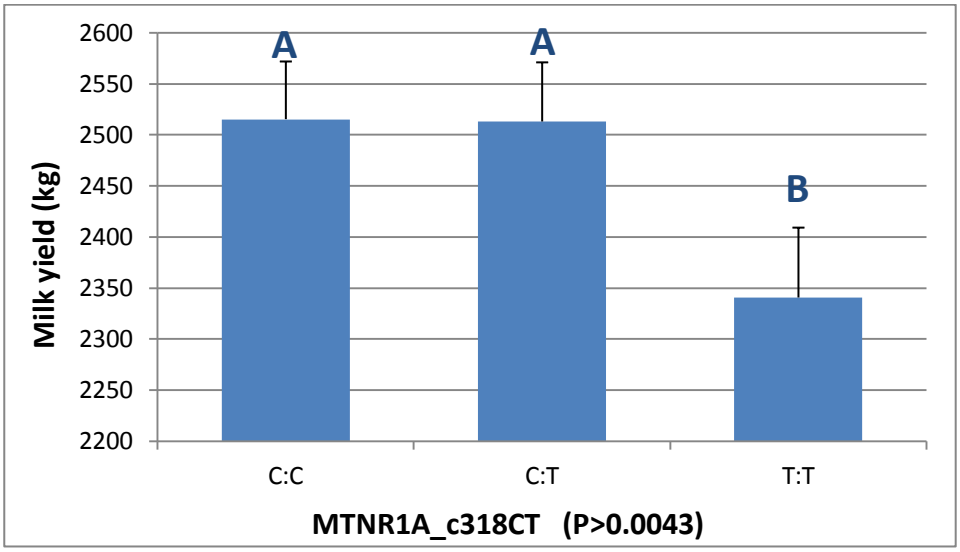
Milk yield parameter showed a normal distribution in the analyzed population and also homoscedasticity was satisfied, as reported above. Therefore, just one dataset, including data related to all lactations, was considered. On the contrary, fat and protein yields were analyzed separately for the first lactation subset, which showed an out of range variance for the classes of lactation number. Concerning fat yield, no statistically significant differences were observed in the primiparous dataset. On the other hand, while both the age at calving and the number of lactation did not affect this parameter (table 4.4.2.1.), an effect (P<0.018) was shown by the MTNR1Ac.318C>T SNP in the subset including lactations from 2 to 7 (figure 4.4.2.2.a). In particular, heterozygous animals showed significantly higher values for this parameter, with least square mean $\pm$ SD equal to 215.02 $\pm$ 3.7 kg, compared to the homozygous for the C allele (211.65 $\pm$ 4.28 kg) and for the T allele (196.93 $\pm$ 2.26 kg), the latter being noticeably lower. The substitution effect for the C allele resulted not statistically significant at this *locus*, as well as the genotype dominance effect. However, it is noticeable that CT animals showed performances comparable with the CC ones, and were not at intermediate level between the two homozygous classes for this parameter. The SNP variance contribution at this *locus* was low and equal to 0.0099 (table 4.4.3.2.).

Also the association of this polymorphism with protein yield was observed in the subset including lactations from 2 to 7, while no statistically significant associations could be detected in the primiparous subset. As for the fat yield parameter, both the age at calving and the number of lactation resulted not significant in the model, while a strong effect was assessed for the SNP at *MTNR1A* gene (table 4.4.2.1.). Buffaloes carrying the CC genotype at this *locus* showed significantly higher performances, with least square mean values (SE), equal to 118.64 (2.62) kg, while CT and TT animals, showed least square means (SE) equal to, respectively, 116.84 (2.75) kg and 108.89 (3.45) kg (figure 4.4.2.2.b). Allelic substitution effect for the C allele at this *locus* resulted statistically significant (P<0.0062), with α (SE) equal to 4.07 (1.48) kg, while no dominance effects could be detected (table 4.4.3.2.).

A statistically significant effect of MTNR1Ac.318C>T polymorphism was also assessed for protein percentage, analyzed in the entire dataset, as homoscedasticity was satisfied for this parameter. The

trend observed for the three classes of genotype in respect to protein % was the same of the other associations previously discussed. In fact, buffaloes carrying the CC genotype at the SNP in *MTNR1A* gene showed better performances (P<0.0375), with least square mean values (SE), pair to 4.61 (0.018) %, while CT and TT animals showed comparable values, with least square means (SE) equal to, respectively, 4.58 (0.02) % and 4.59 (0.02) % (figure 4.4.2.1.b). No allelic substitution effects could be observed for this parameter, while a significant (P<0.019) genotype dominance effect was detected, equal to -0.027 (SE=0.011) %. Interestingly, in this case an intermediate level for protein % is shown not by the heterozygous animals, but by the homozygous for the T allele. Finally, SNP variances contribution resulted low for both protein yield and percentage and equal to, respectively, 0.01 and 0.0054 (table 4.4.3.2.).

a)



b)

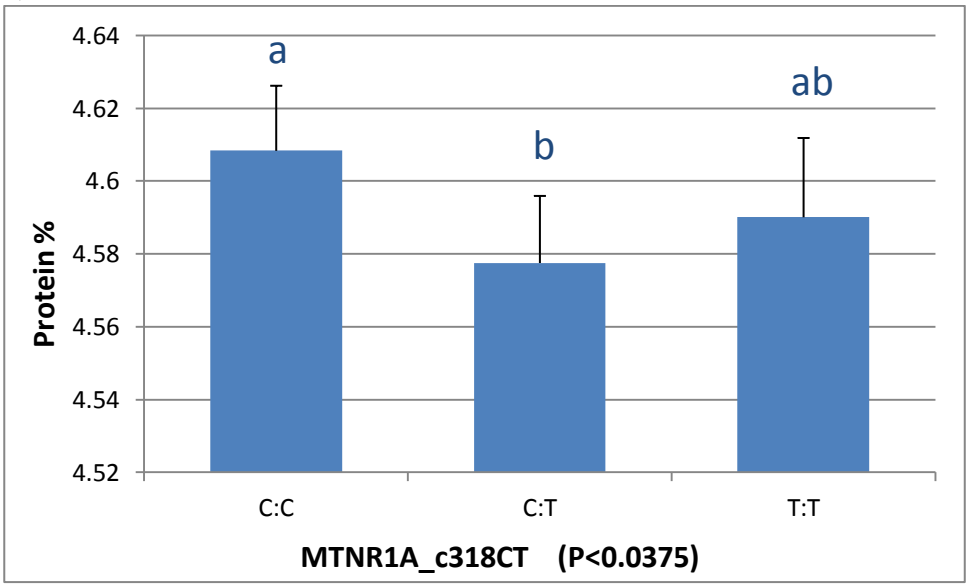
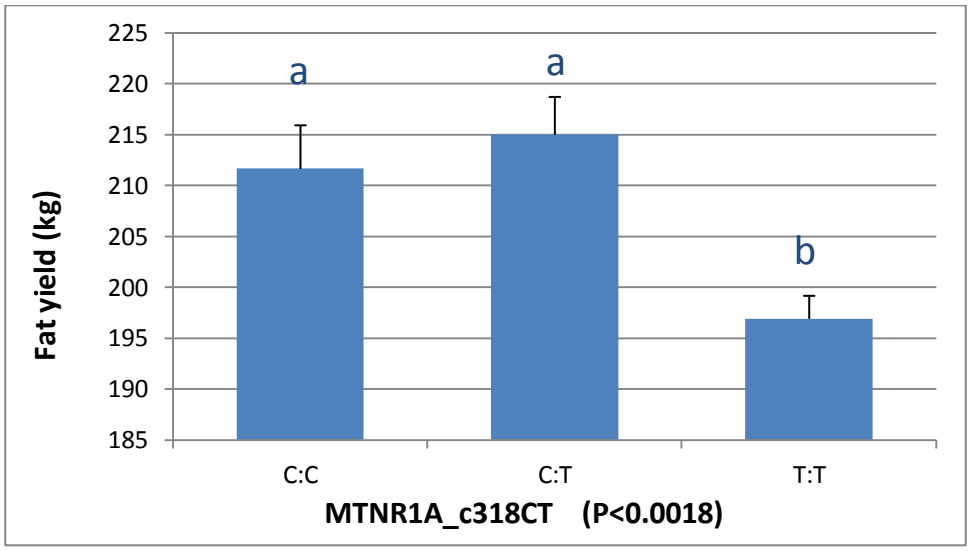


Figure 4.4.2.1. Least square means at the MTNR1Ac318C>T *locus* for milk yield (a) and protein percentage (b) in the entire dataset.

a)



b)

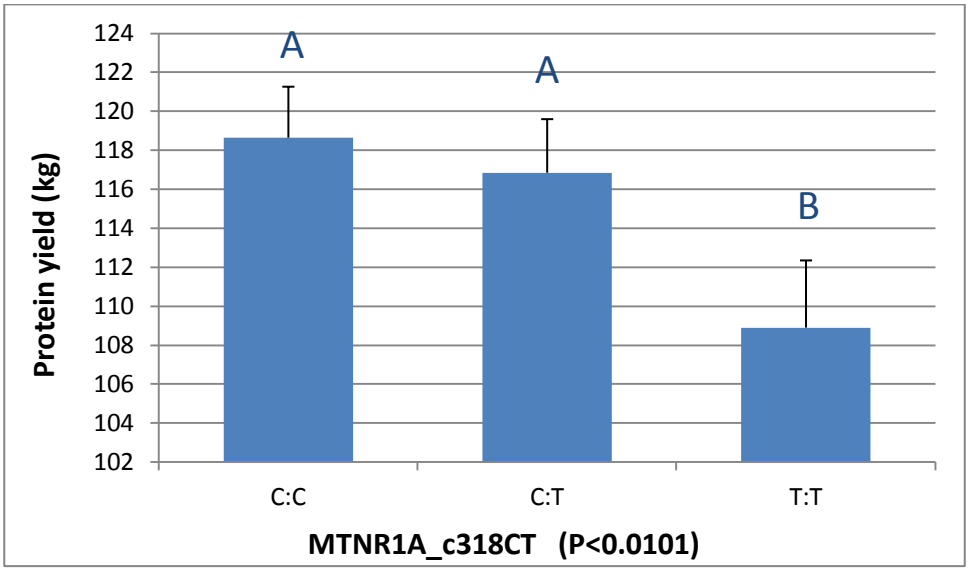


Figure 4.4.2.2. Least square means at the MTNR1Ac318C>T *locus* for fat (a) and protein (b) yield in the lactations from 2 to 7.

Trait	Dataset	Effects	Sum of Squares	Degree of freedom	Mean Square	Prob>F
Milk yield	all lactations	Flock	67600000	3	2.25E+07	<.0001
		Seasonality of calving	2531469	1	2531469	0.0032
		Number of lactation	4742027	6	790338	0.0124
		Age at calving (days)	128363	1	128363	0.5058
		MTNR1A_c318	128363	2	1586687	0.0043
		Error	288298434	995	289747	
		C Total	374339183	1008		<.0001
Fat yield (kg)	lactations 2-7	Flock	530.905	3	176.968	<.0001
		Seasonality of calving	10.5453	1	10.5453	0.062
		Number of lactation	12.3946	5	2.47893	0.5343
		Age at calving (days)	0.13021	1	0.13021	0.8355
		MTNR1A_c318	24.3821	2	12.1911	0.018
		Error	1906.1562	632	3.0161	
		C Total	2472.705	644		<.0001
Protein yield (kg)	lactations 2-7	Flock	161957	3	53985.6	<.0001
		Seasonality of calving	3399.11	1	3399.11	0.0237
		Number of lactation	2985.19	5	597.038	0.4781
		Age at calving (days)	60.9539	1	60.9539	0.7614
		MTNR1A_c318	6119.92	2	3059.96	0.0101
		Error	417545.56	632	660.7	
		C Total	589378.44	644		<.0001

Table 4.4.2.1. Model results for the MTNR1Ac318C>T polymorphism association analysis with milk, fat and protein yield parameters.

The SNP at *MTNR1A* gene, although already found in literature, has never been previously analyzed for dairy traits in buffalo species. The strong negative effect associated to the TT genotype in the productive parameters analyzed, seems to be a possible explanation of the low frequency observed for this genotype in the sample analyzed in present work. In fact, even if other authors reported a positive effect for seasonality of reproduction for TT genotype at MTNR1Ac.318C>T *locus* (Carcangiu et al., 2011; Luridiana et al., 2012), the effect on productive parameter, assessed for the first time in present work, is opposite. Moreover, the relationship found by previous authors with seasonality of reproduction was only in part confirmed in present work, and it deserves further analyses on a sample collected in a larger number of flocks.

The reducing of the TT genotype frequency could be the result of a selection of more productive animals in the analyzed farms. In fact, a high management level of the flocks studied can be deduced from the high dairy performances reported, compared to the official statistics published for Mediterranean Italian Buffalo. Another element is given by the high efficiency of the application of OBMS technique in the farms studied in present work, as a high percentage of the recorded calvings occurred in the out of breeding season period.

The MTNR1Ac.318C>T *locus* proved to be very interesting for buffalo species. There is an apparent antagonistic relationship between the seasonality of reproduction, based on works made by other authors and only partially confirmed in current work, in respect to productive parameters

analyzed in present research. A study of productive performances on the herds analyzed by the other authors, would be very useful to better understand this important topic.

4.4.3. *STAT5A* gene

An association with seasonality of calving was assessed in the analyzed sample for two *loci* at *STAT5A* gene: c.989+344C (P<0.0046) and c.1342+99A (P<0.0144). In particular, concerning the former SNP, 81.1 % of animals homozygous for the minor allele T calved in the out of breeding season period. On the other hand, no statistically significant differences could be observed in respect to the season of calving for buffaloes carrying the other two genotypes (table 4.4.3.1.). Consequently, the T allele in homozygous form seems to be associated to a less seasonal reproductive pattern. In fact, the allelic substitution effect resulted statistically significant for this *locus* (P<0.0149), with α (SE) equal to -0.24 (0.098) calvings, even if no statistically significant dominance effect was detected (table 4.4.3.2.).

Another effect (P<0.0136) for this polymorphism was assessed in present work with protein percentage. Even if the association resulted not statistically significant based on the Bonferroni-adjusted significance level, a trend is observed for this SNP. In particular, buffaloes homozygous for the T allele showed higher performances, with least square mean (SE) equal to 4.628 (0.024) %, compared to the heterozygous, 4.577 (0.018) %. The homozygous for the other allele, showed intermediate performances, equal to 4.599 (0.019) % (figure 4.4.3.1.). The substitution effect for the C allele at the c.989+344C *locus* did not show a statistically significant effect, while a negative dominance effect of genotype was observed (P<0.012), amounting to -0.028 (SE=0.011) %. Also in this case, as for the SNP at *MTNR1A* gene, an intermediate level for protein % is shown not by the heterozygous animals, but by the homozygous for the T allele. These trends suggest, on the basis of classical Mendelian genetics, the presence of an overdominance effect. This is a form of dominance in which the expression of the heterozygote is outside the range defined by the expressions of the homozygous genotypes and most closely resembles the expression of the homozygous dominant genotype (Bourdon R.M. 1997).

The variance contribution of this SNP resulted equal to 0.0072 (table 4.4.3.2.).

It must be noticed that, even if the TT genotype seems to be associated to better performances for both seasonality and protein percentage traits, its frequency in the analyzed population is highly under-represented, equal to only 11.5%. Also the frequency of the T allele is low (32%). Moreover, this low frequency remained with the increasing of the number of lactation. In fact, the SNP was not affected by this parameter. However, based on the overdominance effect explained above, a selection against the TC genotype, which showed significantly negative performances for the analyzed traits, could have occurred, leading to a reduction of the T allele in the analyzed population. Further association analyses of this polymorphism on more traits could help in understanding the interesting mechanisms emerged in present work.

Regarding the other SNP, located at position c.1342+99A on *STAT5A* gene, associated with seasonality of reproduction, 74.44% of AA animals calved in the out of breeding season period, and this trend is observed also for the heterozygous buffaloes, with 67.96% of calvings occurred in this period. The homozygous for the minor allele G, showed more balanced trend, with percentage of calving in the out of breeding season equal to 58.6% and 41.4% in the breeding season. Hence, the GG genotype seems to be associated to a reduced sensitiveness to photoperiod. The allelic

substitution effect resulted statistically significant (P<0.0083), with α (SE) equal to 0.302 (0.114) calvings. On the other hand, no statistically significant dominance effect was detected (table 4.4.3.2.). The G allele was the minor allele in the analyzed population, with a frequency equal to about 27 %. Moreover, the GG genotype is very under-represented, with a frequency equal to 0.8%. No other statistically significant effects were detected for this polymorphism in the analyzed sample. Hence, considering the positive effect on seasonality trait observed in present work for this genotype, its frequency in the population should be enhanced. It must be said, however, that with Bonferroni correction the effect at this SNP resulted not statistically significant.

Also for the STAT5A_c989+344C and STAT5A_c1342+99A polymoprhisms, as was done for the SNP on *MTNR1A* gene, the association analysis for seasonality parameter was repeated removing all data related to the Z flock, which reported significantly high percentage of calving in the out of breeding season period. The associations already found resulted confirmed (P<0.0101 for STAT5A_c989+344C and P<0.0013 for STAT5A_c1342+99A) and, differently from the previous analysis, the significance at the c.1342+99A *locus* overcame the Bonferroni correction.

SNP	**STAT5A_c989+344C**		
Genotype	C:C	T:C	T:T
out of season (%)	44.93	41.85	13.22
seasonal (%)	47.8	44.6	7.6
SNP	**STAT5A_c1342+99A**		
Genotype	A:A	G:A	G:G
out of season (%)	59.52	34.72	5.75
seasonal (%)	50.00	40.05	9.95

Table 4.4.3.1. Percentage of animals with different genotypes at the two SNPs STAT5A_c.989+344C and STAT5A_c.1342+99A, calving in the two periods considered.

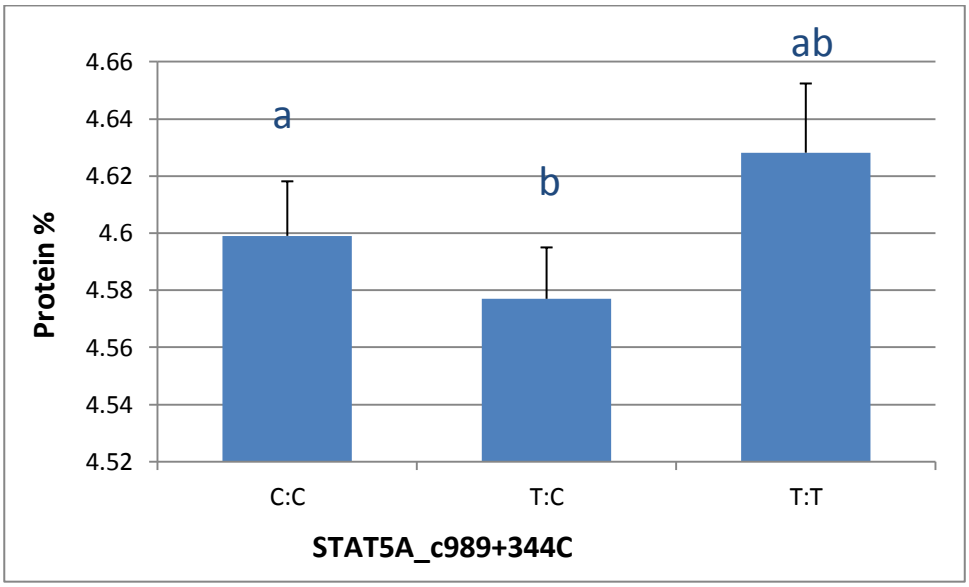


Figure 4.4.3.1. Least square means at the STAT5A_c.989+344C *locus* for protein percentage in the entire dataset (P<0.0136).

Another polymorphism detected on the *STAT5A* gene, the C to G substitution at position c.128, showed a trend (P<0.0567) in respect to the protein yield (kg) in the subset including data related to

classes of lactation number from 2 to 7. In particular, buffaloes carrying the CC genotype registered significantly higher performances, with least square mean (SE) equal to 123.87 (5.75) kg, compared to the GG ones, least square mean (SE) of 118.12 (2.62) kg. Interestingly, the lowest average protein yields, with least square mean (SE) pair to 114.04 (2.71), were recorded for the heterozygous animals (figure 4.4.3.2.). Based on these results, also in this case an overdominance effect may be hypothesized.

A high dominance effect was observed at this *locus* (P<0.032), equal to (SE) -4.531 (2.104) kg. However, no statistically significant effect was detected for allelic substitution. Noticeably, the CC genotype class at this *locus* resulted highly under-represented in the analyzed population, with a frequency equal to 0.073. A hypothesis behind this could be based on the negative performances associated to the heterozygous animals, which may have led to a selection against the TC genotype, even if the effect was not statistically significant.

Finally, the SNP variance contribution, equal to 0.0099, resulted comparable with the other emerged in the present work on polymorphisms STAT5A_c989+344C, STAT5A_c1342+99A and MTNR1Ac.318C>T (table 4.4.3.2.).

The SNPs found at *STAT5A* gene are not in Linkage Disequilibrium.

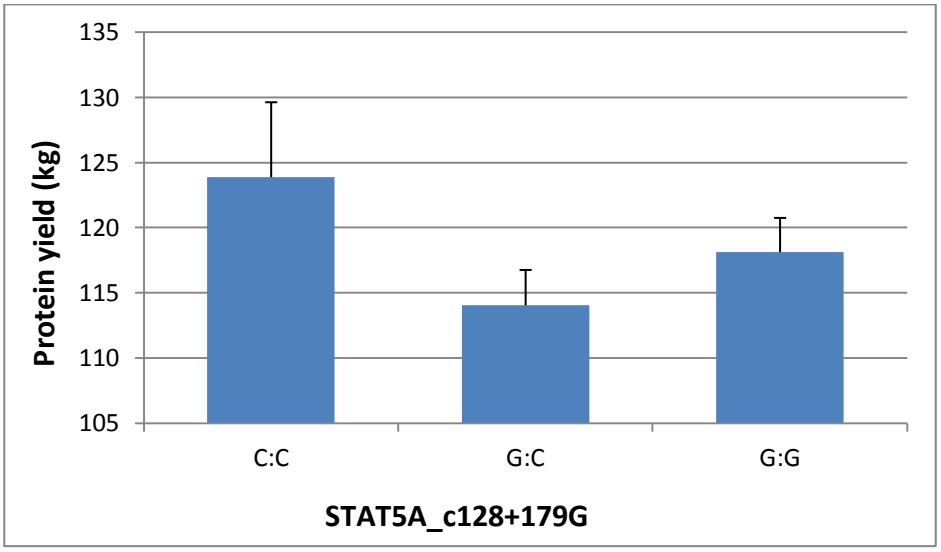


Figure 4.4.3.2. Least square means at the STAT5A_c.128+1798G *locus* for protein yield in the lactations from 2 to 7 subset (P<0.0546).

The trends observed in present work for milk protein (kg and %) and fat (kg) content, in respect to polymorphisms found in *STAT5A* sequence, highlighted an influence of this gene on milk production traits in buffalo species. The *STAT5A* gene has been studied in cattle for its influence on both reproduction and dairy traits. (Khatib et al 2009). In particular, a SNP located in this gene has been associated with protein and fat percentages in bovine species (Khatib et al., 2008b).

On the other hand, in the present research no effects were detected for the fertility parameter of calving interval in buffalo. Instead, in cattle this gene was previously associated with oestrous expression (Homer et al., 2013) and embryonic survival (Khatib et al., 2008b).

As already explained, among the phenotypes available for the buffaloes analyzed in present work, the only one which could be reliable for the study of fertility was calving interval, obtained from the

dates of calving. However, this is only an aspect of the problem and it is difficult to find correct parameters to capture the complexity of fertility, which can be also sufficiently accurate. This is a big issue above all in buffalo species, where artificial insemination is rarely practiced and very little parentage information is available. More accurate phenotypes should be obtained to find more associations with the genes analyzed in present study and to better understand the molecular basis of reproduction processes.

				Effect[1]				Variance contribution[2]
Variable	**dataset**	**SNP**	**allele**	**α**	**P-value**	**d**	**P-value**	**r^2_{SNP}**
Calving interval (days)	lactation 4	TNFAc.323C>A	A	-38.11 ± 15.03	0.012	-34.32 ± 16.37	0.038	0.029
Seasonality of calving	All lactations	STAT5Ac.989+344C	C	-0.24 + 0.098	0.015	-0.14 + 0.129	0.275	
Seasonality of calving	All lactations	STAT5Ac.1342+99A	A	0.302 + 0.114	0.008	-0.047 + 0.135	0.730	
Milk yield (kg)	all lactations	MTNR1Ac.318C>T	C	63.03 ± 25.15	0.012	33.75 ± 36.17	0.351	0.0085
Protein yield (kg)	lactations 2-7	MTNR1Ac.318C>T	C	4.069 ± 1.482	0.006	0.341 ± 2.183	0.876	0.01
	lactations 2-7	STAT5Ac.128+179G	C	-1.582 ± 1.841	0.390	-4.531 ± 2.104	0.032	0.007
Protein content (%)	all lactations	MTNR1Ac.318C>T	C	0.015 ± 0.008	0.057	-0.027 ± 0.011	0.019	0.0054
	all lactations	STAT5Ac.989+344C	C	-0.0026 ± 0.009	0.766	-0.028 ± 0.011	0.012	0.0072
Fat yield (kg)	lactations 2-7	MTNR1Ac.318C>T	C	0.16 ± 0.1	0.110	0.228 ± 0.147	0.121	0.0099

Table 4.4.3.2. Allele substitution effect (mean + SE) and contribution of each significant SNP to the total phenotypic variance of calving interval, milk yield (kg), protein content (kg and %) and fat yield (kg). for the statistically significant associations described in present work.

[1]α=substitution effect, expressed in the same units of corresponding variables; d=dominance effect, expressed in the same units of corresponding variables.
[2]r^2_{SNP}= contribution to the variance of SNP genotype to the total genotypic variance.

5. CONCLUSIONS

Fertility and seasonality traits are of critical importance in buffalo species and, until now, even if a complete genomic sequence was announced, as well as the development of a high density SNP chip tool, little genetic information is available for this species.

In the present research, a polymorphism detection and an association study in candidate genes involved in fertility and seasonality of reproduction in Mediterranean Italian Buffalo were performed. The candidate genes analyzed were: signal transducer and activator of transcription 5A (*STAT5A*), serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 14 (*SERPINA14*) and tumor necrosis factor alpha (*TNFA*) for fertility, and melatonin receptor 1A (*MTNR1A*) for seasonality. The single nucleotide polymorphism (SNP) detection analysis allowed the identification of a total of 12 SNPs. Of these, 11 were identified for the first time in present work, while a C>T substitution in *MTNR1A* gene was already found in literature and associated to seasonality in buffalo species. After a first genotyping on a sample of 36 buffaloes, seven SNPs resulted polymorphic, including the one already found in literature and six identified in this work for the first time. Of these, two are located in exon 4 of *TNFA* gene and four in *STAT5A* gene, of which one in exon 8 and three in introns. All the coding SNPs are synonymous, i.e. they do not change the aminoacid sequence of the encoded protein. These SNPs were genotyped and used for the association analysis. In fact, even non-coding and synonymous mutations can be associated with an altered phenotype, affecting mechanisms such as transcription, translation and splicing or being in linkage with a functional mutation.

The genotyping analysis of these seven polymorphisms was performed on a total of 491 female Mediterranean Italian Buffaloes, characterized by the periodic milking recording data and the dates of calving, provided by the Italian Buffalo Breeders' Association (ANASB).

The data obtained have required a massive editing work. For the study of fertility and seasonality traits, the dates of calving were considered the most suitable phenotype. However, these required a considerable revision phase to delete all the recorded data which were not considered reliable. For this purpose, the characteristics of the biology of reproduction in water buffalo were considered. Another important parameter, which required a considerable revision phase, was the number of lactation. In fact, in the ANASB registrations the lactation identified with a number equal to 1 refers to the first lactation recorded for one animal, but it often does not correspond to its first calving. This may be the result of inaccuracy of registration or even of the birth of male calves that have not been declared. Since, for the purpose of this research, the lactation number is a fundamental parameter to be considered, a correction was made to evaluate only the reliable lactation number data, based on the age of animals and the dates of calving.

After these corrections, 26 animals, for a total of 107 observations, were removed from the dataset.

Another fundamental element considered in the analyses performed in present work, was the lack of parentage information. Natural mating is the system applied by most Italian buffalo enterprises. Breeding is generally carried out by group mating of two or even three bulls at the same time to one group of breedable buffaloes and calving takes place on open ranges. Under these conditions, paternity is hard to establish. For this reason, since the sires were not shared between flocks, a random effect of flock was considered in the SNP association analyses also to account for the effect of the bull.

In the analyzed population calving interval distribution showed quite high value compared to that reported in literature for Italian farms. This trend reflects the higher intercalving periods usually ascribed to the Out of Breeding Mating Season technique, which interrupts sexual promiscuity in the herd during the autumn season, performed in the analyzed population. This is shown by the calving distribution trend, which is opposite compared to the natural breeding conditions, with high deliveries concentration in the out of breeding season period, especially at first lactation. Compared to the official statistics reported for Mediterranean Italian Buffalo by the Italian Breeders' Association (AIA), the mean value recorded for age at first calving in our sample is lower, while milk yields show considerable high performances, suggesting that the analyzed farms reach high management level. However, milk protein and fat content observed in the analyzed population are comparable with published data. In our sample, animals that calved in the out of breeding season showed shorter calving intervals and higher milk yield compared to the seasonal ones, but a significant influence on all the analyzed traits was also detected for the flock variable. This was expected considering that this parameter includes the sire effect.

For the analyzed buffaloes, most of data are related to the first 4 lactations and the average number of lactations observed is low compared to the official statistics for Mediterranean Italian Buffalo. The number of lactation significantly affects all traits, except fat and protein percentage. Milk yield tends to increase with the class of lactation number, reaching maximum value at 7^{th} lactation, and the minimum one, as expected, is observed at first lactation. A statistically significant effect is also shown by the age at calving, which affected all the analyzed parameters, except for protein and fat percentage.

Phenotypic parameters were used for an association study of the SNPs found in the analyzed genes. An interesting result emerged for the SNP located at the 3^{rd} exon of *TNFA* gene. This is a candidate gene for fertility traits and a statistically significant association with calving interval was assessed in the fourth lactation subset. Noticeably, just one animal, with very low value for calving interval, resulted homozygous for the A allele but, even removing this subject from the dataset, a statistically significant effect was highlighted, confirming the improving effect of the A allele observed for the homozygous AA animal. In fact, the A allele at this *locus* showed large negative substitution effect, leading to a reduction of the calving interval, and also the genotype dominance effect resulted statistically significant. These effects deserve to be evaluated on a larger sample with more balanced genotype frequencies at this SNP. However, it is relevant that the frequency of A allele decreases from the first lactation on. Since in present work this allele resulted significantly associated with a reduction of the calving interval, its decrease in the population is to be considered a negative effect. Moreover, as no statistically significant effects were detected for this polymorphism on the other parameters analyzed, further analyses on other traits, which could have been selected reducing the A allele frequency in the buffalo population, deserve to be conducted.

None of the other polymorphisms identified in candidate genes for fertility traits showed statistically significant associations with calving interval. However, fertility is a complex issue, involving many mechanisms and very difficult to be measured with field data. This is true above all in buffalo species, where artificial insemination is rarely practiced and very little parentage information is available. With the availability of more accurate fertility phenotypes, the association study with polymorphisms analyzed in present work could underline further relevant effects.

Concerning seasonality parameter, the SNP association analysis was performed between "seasonal" (from August to December) and "out of season" (from January to July) dates of calving. In the present work, the C>T substitution in *MTNR1A* gene found in literature and associated to seasonality in buffalo species was confirmed, but a relationship between this polymorphism and seasonal breeding was not completely established. Other authors reported a statistically significant difference in mating behavior for animals homozygous for the T allele, showing sexual activity in the out of breeding season period, compared to the CC ones, which resulted more seasonal. An opposite trend emerged for the TT genotype frequency, which resulted significantly lower in our population compared to that reported by other authors. Some differences between the sample analyzed by the other authors and that studied in present work can be underlined. In fact, animals studied in the other works belong to one herd, of different size, of Mediterranean Italian Buffaloes reared in the South of Sardinia, while our sample belong to 4 different farms, located in South of Italy. Moreover, the association analysis of the SNP on *MTNR1A* gene was performed by other authors on animals reared under natural photoperiod conditions, with bulls kept always within the herd, while in our sample all the buffaloes were subjected to the OBMS technique, which interrupts sexual promiscuity in the herd during the autumn season. In fact, most of calvings recorded in our sample fall in the out of breeding season period. In present work, the seasonality of reproduction was studied in animals subjected to OBMS practice because, on one hand, this represents the usual management condition for the Mediterranean Italian Buffalo farms and, on the other hand, it seemed interesting to investigate animals which, even under OBMS conditions, were not able to show sexual activity in the out of breeding season period.

Given the differences emerged in comparison to other researches, and since a strong effect on reproductive seasonality parameter was assessed for Z farm, the association analysis of this trait with the MTNR1Ac.318C>T polymorphism was repeated removing all data related to this flock. Actually, in this case an association with this SNP could be detected, with a tendency in calving in the out of breeding season period showed by the animals with genotype TT. The Z flock presented two significant differences in respect to the other three analyzed farms: a significantly higher number of TT animals and buffaloes with a significantly lower age at calving. A strong allelic substitution effect was observed, while no dominance effects could be detected. The results obtained with this posterior analysis, seem to be in accordance with those reported from the other authors who associated the reproductive seasonality trait with this polymorphism at *MTNR1A* gene. However, this association could be seen in our population only after the removing of data related to one of the flocks analyzed. Hence, this effect should be validated on a sample collected from a higher number of farms.

The SNP at *MTNR1A* gene, although already found in literature, has never been previously analyzed for dairy traits in buffalo species. In the present research, a statistically significant effect emerged on milk, fat and protein yield (kg) and with protein percentage parameters, with large substitution effects for milk and protein yield. The effects for fat and protein yield were observed in the subset excluding data related to the first lactation. In all cases, TT animals showed lower performances compared to the CC ones.

The strong negative effect associated to the TT genotype for productive parameters analyzed, seems to be a possible explanation of the low frequency observed for this genotype in the sample analyzed in present work. In fact, even if other authors reported a positive influence for seasonality of

reproduction of TT genotype at MTNR1Ac.318C>T *locus*, the effect on productive parameter, assessed for the first time in present work, is opposite. Moreover, the relationship found by previous authors with seasonality of reproduction was only in part confirmed in present work, and it deserves further analyses on a sample collected in a larger number of flocks. Hence, the reducing of the TT genotype frequency could be the result of a selection of more productive animals in the analyzed farms. In fact, a high management level of the flocks analyzed can be deduced from the high dairy performances reported, compared to the official statistics published for Mediterranean Italian Buffalo. Another element is given by the high efficiency of the application of OBMS technique in the farms analyzed in present work, as a high percentage of the recorded calvings occurred in the out of breeding season period.

The MTNR1Ac.318C>T *locus* proved to be very interesting for buffalo species. There is an apparent antagonistic relationship between the seasonality of reproduction, based on works made by other authors and only partially confirmed in current work, in respect to productive performances. A study of the dairy traits recorded on the herds analyzed by the other authors, would be very useful to better understand this important topic.

Concerning the *STAT5A* gene, an association with seasonality of calving was assessed in the analyzed sample for two *loci*: c.989+344C and c.1342+99A. In particular, in relation to the former SNP, the T allele in homozygous form seems to be associated to a less seasonal reproductive pattern. In fact, the allelic substitution effect resulted statistically significant for this *locus*, even if no statistically significant dominance effect was detected. For this polymorphism, a trend is also observed in respect to the protein percentage parameter, even if the effect was not statistically significant with the Bonferroni correction. In particular, buffaloes homozygous for the T allele showed higher performances. Also in this case, as for the SNP at *MTNR1A* gene, an intermediate level for protein % is shown not by the heterozygous animals, but by the homozygous for the T allele. These trends suggest, on the basis of classical Mendelian genetics, the presence of an overdominance effect.

It must be noticed that, even if the TT genotype seems to be associated to better performances for both seasonality and protein percentage traits, its frequency in the analyzed population is highly under-represented and also the frequency of the T allele is low. Based on the overdominance effect, a selection against the TC genotype, which showed significantly negative performances for the analyzed traits, could have occurred, reducing the T allele frequency in the population. Further association analyses of this polymorphism on more traits could help in understanding the interesting mechanisms emerged in present work.

Regarding the other SNP on *STAT5A* gene, located at position c.1342+99A and associated with seasonality of reproduction, the GG animals showed a reduced sensitiveness to photoperiod. The allelic substitution effect resulted statistically significant, while no statistically significant dominance effect was detected. The G allele was the minor allele in the analyzed population and the homozygous genotype GG is very under-represented. No other statistically significant effects were detected for this polymorphism in the analyzed sample. Hence, considering the positive effect on seasonality trait observed in present work for this genotype, its frequency in the population should be enhanced. It must be said, however, that with Bonferroni correction the effect at this SNP resulted not statistically significant.

Also for the STAT5A_c989+344C and STAT5A_c1342+99A polymorphisms, as was done for the SNP on *MTNR1A* gene, the association analysis with the seasonality parameter was repeated removing all data related to the Z flock, which reported significantly high percentage of calving in the out of breeding season period. The associations already found resulted confirmed and, differently from the previous analysis, the effect of the c.1342+99A *locus* overcame the Bonferroni correction.

Another polymorphism detected on the *STAT5A* gene, the C to G substitution at position c.128, showed a trend in respect to the protein yield (kg). This effect could be underlined in the subset including data related to classes of lactation number from 2 to 7. Interestingly, the lowest average protein yields were recorded for the heterozygous animals so, also in this case, an overdominance effect may be hypothesized. In fact, since the CC genotype class at this *locus* resulted highly under-represented in the analyzed population, we can hypothesize that the negative performances associated to the heterozygous animals have led to a selection against the TC genotype, even if the effect was not statistically significant with Bonferroni correction.

The trends observed in present work for milk protein (kg and %) and fat (kg) content, in respect to polymorphisms found within *STAT5A* sequence, highlighted an influence of this gene on dairy traits in buffalo species. On the other hand, in the present research no effects were detected for the fertility parameter of calving interval in buffalo. The *STAT5A* gene has been studied in bovine species for its influence on both reproduction and milk production traits. However, the only phenotype made available for the study of fertility in present research was calving interval. This is just an aspect of the problem and it is difficult to find correct parameters to capture the complexity of fertility, which could be also sufficiently accurate. This is a big issue above all in buffalo species, where artificial insemination is rarely practiced and very little parentage information is available.

The associations found, that could be tested in a larger sample, may offer useful indications for the genetic improvement of fertility, seasonality and production traits in buffalo species. The candidate genes considered in present study are involved in part of the complexity inherent in fertility and seasonality of reproduction. Further researches including other genes should be performed to extend the knowledge about the molecular basis underlying the relationships between production and reproduction and the complex mechanisms behind seasonality and response to photoperiod.

These perspectives could be further enhanced by the next commercial availability of a high-throughput SNP platform for the genotyping of tens thousands of SNP markers for this species and even whole sequence. However, an accurate recording activity of phenotypic parameters, as well as genealogies registration are critical elements in buffalo, and must be improved to enhance the genetic progress in this species. This could also allow obtaining further relevant results from the association analyses performed in present study.

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