FACULTY OF VETERINARY MEDICINE
accredited by EAEVE

Novel insights in commercial *in vitro* embryo production in cattle

Maaike Catteeuw

Promoter: Prof. dr. Ann Van Soom

Copromoters: Prof. dr. Joris Vermeesch, Dr. Katrien Smits

Dissertation submitted to Ghent University in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences

2018

Department of Reproduction,

Obstetrics and Herd Health,

Faculty of Veterinary Medicine

GHENT UNIVERSITY

Members of the examination committee

Prof. dr. Herman Favoreel

Chairman – Faculty of Veterinary Medicine, Ghent University, Belgium

Prof. dr. Bjorn Heindryckx

Faculty of Medicine and Health Sciences, Ghent University, Belgium

Prof. dr. Luc Peelman

Faculty of Veterinary Medicine, Ghent University, Belgium

Prof. dr. Geert Opsomer

Faculty of Veterinary Medicine, Ghent University, Belgium

Dr. Erik Mullaart

CRV BV, the Netherlands

Dr. Karen Goossens

Research institute for Agriculture, Fisheries and Food (ILVO), Belgium

Funding

Project G039214N: Chromosomal instability during early embryonic development: elucidating key mechanisms in a bovine model

Research Foundation Flanders
Opening new horizons

Printed by University Press, Zelzate

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AC	Apoptotic cell
ACR	Apoptotic cell ratio
AI	Artificial insemination
AMH	Anti-Muellerian Hormone
ART	Assisted reproductive technologies
BAF	B allele frequency
BMP15	Bone morphogenetic protein 15
BoHV1	Bovine Herpes Virus 1
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CIDR	Controlled internal drug release
CIN	Chromosomal instability
CN	Copy number
COCs	Cumulus oocyte complexes
CR1aa	Charles Rosenkrans 1 amino acids
dpi	Days post insemination
eCG	Equine chorionic gonadotropin
EGA	Embryonic genome activation
EGF	Epidermal growth factor
EHM	Embryo holding medium
FCS	Fetal calf serum
FISH	Fluorescent in situ hybridization
FSH	Follicle stimulating hormone
GC	Guanine Cytosine
GDF9	Growth differentiation factor 9
GEO	Gene Expression Omnibus
GnRH	Gonadotrophin releasing hormone
h	Hours
i.m.	Intramuscularly
ICM	Inner cell mass
IETS	International Embryo Technology Society
ITS	Insulin-Transferrin-Selenium
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
LH	Luteinizing hormone
LogR	Log R ratio
LOS	Large offspring syndrome
LSM	Least square means
MDA	Multiple displacement amplification
MEM	Minimal essential medium

MET	Maternal-embryonic transition
MOET	Multiple ovulation and embryo transfer
MPF	Maturation promoting factor
NA	Not available
NGS	Next generation sequencing
OCS	Oestrus cow serum
OMIA	Online Mendelian Inheritance in Animals
OPU	Ovum pick-up
OR	Odds ratio
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGD	Preimplantation genetic diagnosis
PGF	Prostaglandin
PGS	Preimplantation genetic screening
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrrolidone
qPCR	Quantitative polymerase chain reaction
rob	Robertsonian translocation
RT	Room temperature
SE	Standard error
SEM	Standard error of the mean
SNP	Single polynucleotide polymorphism
SOF	Synthetic oviductal fluid
TALP	Tyrode's albumin-pyruvate-lactate
TCM-199	Tissue Culture Medium 199
TCN	Total cell number
TE	Trophectoderm
TLC	Time-lapse cinematography
WGA	Whole genome amplification
WOW	Well-of-the-well

CHAPTER 1

GENERAL INTRODUCTION

1.1 Assisted reproductive technologies in cattle

Today, assisted reproductive technology (ART) refers to all fertility treatments in which human or animals' eggs, semen and embryos are being manipulated. Primarily, ART is used to overcome infertility and to help couples to fulfil their desire to have children. In cattle, ART is mainly performed to improve reproductive results but it is also intensively used to improve genetic selection in valuable individuals or herds. Furthermore, ART can also be a part of the preservation of endangered species. ART can be as simple as artificial insemination (AI) in which sperm cells from a male animal of interest is manually deposited in the reproductive tract of the female. This allows the use of genetic material of superior males, the import of semen to introduce new genetic material without the need to transport live animals, the use of frozen semen long after the animals' dead and the risk reduction of spreading transmittable sexual diseases (Foote 2002). Generally, AI techniques have been standardized for many species and AI is performed globally in more than 100 million cattle every year (Boa-Amponsem and Minozzi 2006). Pregnancy rates after AI however can vary between 30 and 70% depending on several factors such as timing (Lamb *et al.* 2010), parity (Pursley *et al.* 1997) and number of AI performed (Chebel *et al.* 2004). Due to its massive worldwide application, the genetic impact from the male side increased enormously. Some bulls have over hundred thousands of descendants and have therefore a remarkable impact on the genetic pool of a breed (Thibier 2005; Goovaerts *et al.* 2007). Less evident is however producing more offspring from genetically valuable females to increase their genetic impact on the selection process.

Starting from the mid-seventies, female donors are routinely treated with hormones to induce superovulation and are subsequently inseminated to recover embryos by uterine flushing. These *in vivo* derived embryos are then transferred to synchronized recipients (multiple ovulation and embryo transfer – MOET). Although annually more than 500,000 *in vivo* derived embryos are transferred worldwide to increase the genetic impact from the female side, it is not successful at all times due to the unpredictable response to the hormonal treatment between cows (Hasler *et al.* 1995; Van Wagtendonk-de Leeuw 2006). Nonetheless, the embryo transfer technique proved to be very useful in the transfer of *in vitro* produced embryos (IVP) (Goovaerts *et al.* 2007), in which immature oocytes are removed from the ovaries and are fertilized with semen in a dish. During culture, the fertilized oocytes or zygotes will start cleaving and when the blastocyst stage is reached, transfer of the embryo to the uterus of a recipient donor is possible. As some disadvantages of MOET, such as the unreliable hormonal response, can be overcome by IVP, it gained worldwide interest and nowadays more than 660,000 IVP embryos are produced each year. Since the first *in vitro* produced calf, including *in vitro* maturation of the oocyte, was born in 1990 (Fukuda *et al.* 1990), tremendous progress has been made

not only in the oocyte retrieval process (ovum pick-up – OPU) but also in the *in vitro* procedures (maturation, fertilization and culture) and cryopreservation techniques.

Other ART are part of fundamental or biomedical research, such as the production of cloned embryos by somatic cell nuclear transfer or the production of transgenic embryos. However, cloned and genetically engineered animals have already raised interest in the meat and dairy industry, for application in breeding programs, such as the production of hornless offspring or improved food products from animals (Foote 2005). Until now, European legislation only recommends cloning or producing genetically modified animals by novel techniques such as Crispr-Cas for research purposes as the European Group on Ethics is still in doubt whether using cloned or genetically modified animals for food supply is ethically justified (European legislation COM/2010/0585).

1.2 Commercial *in vitro* embryo production

In order to produce bovine embryos *in vitro* from follicular oocytes, it is required to perform a series of essential techniques following a strict timing. After oocyte collection, which can be performed in live animals by ovum pick-up (OPU) or by follicular aspiration of slaughterhouse ovaries, three subsequent phases can be distinguished: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) (**Figure 1**). When all consecutive steps are carried out punctually, viable embryos will be the result. In commercial settings, this will be the higher goal of IVP as these viable embryos will be transferred to recipient animals which will hopefully become pregnant and deliver a healthy calf.

1.2.1 Oocyte collection

Ovum pick-up in live donor animals

Repeated oocyte collection by transvaginal ultrasound-guided follicular puncture (OPU) followed by routine IVP has become an important alternative to MOET in cattle. Adapted from human reproduction, OPU in cattle was performed for the first time in 1988 (Pieterse *et al.* 1988). It can be considered to be more advantageous than MOET as it can also be used in acyclic or pregnant cows, in cows with oviduct or genital tract problems and in cows insensitive to hormonal treatment (Boni 2012). Moreover, OPU can already be performed in animals of young age, i.e. nine months. Since these young heifers already have a genomic breeding value nowadays, they can be selected as a donor animal in a reliable way. Hereby, the generation interval is reduced significantly which in turn increases the genetic gain compared to MOET. Today, cattle breeders want as many offspring as possible from genetically superior cows, therefore, OPU/IVP is more and more being used in commercial practice because it does not interfere with the reproductive cycle or the milk production. In 2015, more than 660,000 embryos were produced by *in vitro* techniques, which is a 10% increase compared to the previous year (IETS 2016).

On the one hand, technical modifications were made to optimize the OPU technique over the years. Several studies investigated aspiration pressure, needle bevels and longitudes and ultrasound transducers to optimize the OPU technique itself, with the intention to retrieve not only as many oocytes as possible but also to maintain high quality oocytes (Bols *et al.* 1996b; Bols *et al.* 1997; Bols *et al.* 2004; Van Wagtendonk-de Leeuw 2006). In addition, the OPU operator also has an important influence as years of experience will lead to a higher oocyte collection rate (Scott *et al.* 1994). On the other hand, the oocyte yield is also influenced by biological factors which are less definable and controllable than the technical aspects of OPU. As in MOET programs, the donor itself is responsible

for approximately 20% of the variation seen in OPU oocyte and embryo yield (Van Wagtendonk-de Leeuw 2006). Other factors should also be accounted for such as the frequency of follicle aspiration and the use of hormonal stimulation.

Since the success of OPU is irreversibly connected with the number of oocytes retrieved per puncture session, increasing this number has been a major goal. Although many reports investigated the effect of hormonal pretreatment of the donor animals prior to OPU (Fry *et al.* 1994; Looney *et al.* 1994; Paul *et al.* 1995; Bols *et al.* 1996a), optimizing hormonal treatment protocols showed however inconsistent results, mainly due to the variable response to the stimulation by the donors (Boland *et al.* 1991; Kohram *et al.* 1998; Durocher *et al.* 2006), contamination of pharmaceutical products with interfering hormonal components (Touati *et al.* 1991) and a poor halflife time which is either too long for equine chorionic gonadotropin (eCG) or too short in the case of follicle stimulating hormone (FSH) (Boland *et al.* 1991; Lovie *et al.* 1994). In general, a hormonal treatment protocol is based upon the removal of large follicles via transvaginal aspiration, followed by administration of gonadotropins. By dominant follicle ablation, an endogenous rise of FSH is created (Ooe *et al.* 1997; Baracaldo *et al.* 2000) and this follicular wave is sustained by giving the animals multiple injections of f.e. FSH. The best results are obtained when OPU is performed 48 hours after the last FSH injection. This period, which is also called the coasting period, mimics the *in vivo* process of early follicular dominance (Blondin *et al.* 2002). When however stimulating hormones are given to the donor animals, the frequency of aspiration is dropped to one OPU per two weeks, in comparison with the once or twice weekly OPU schedule when no stimulating hormones are given. This twice weekly schedule is even preferred over the once weekly, as it results in an increased follicular wave frequency and in an arrest of the estrous cycle, follicle maturation and ovulation (Boni 2012). Nonetheless, FSH treatment before OPU resulted in more high quality oocytes and subsequently, in more embryo transfers and pregnancies (Vieira *et al.* 2014). In both cases, with or without hormonal stimulation, OPU can be performed continuously for over several months without harming the donor animal's health and fertility (Boni 2012).

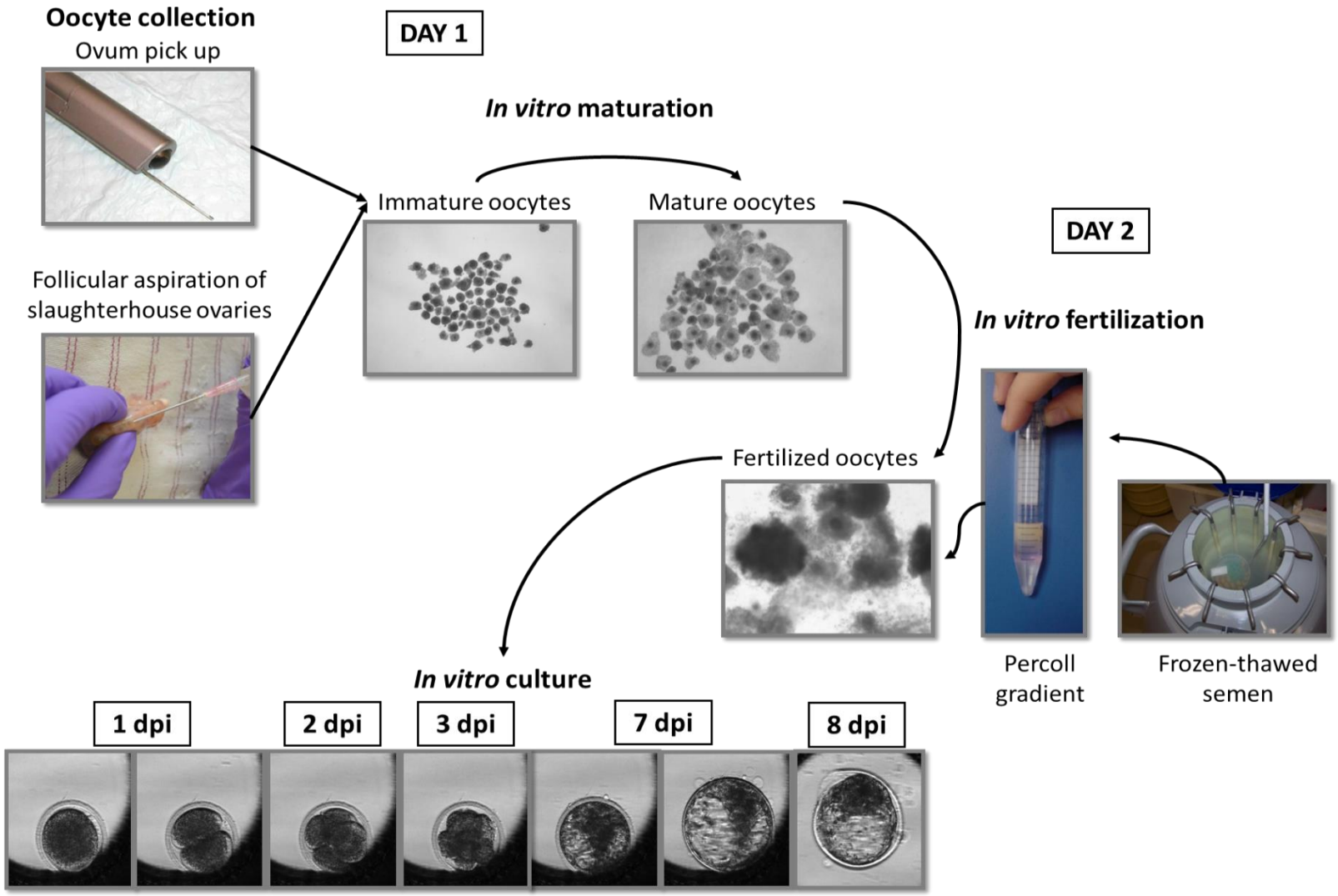


Figure 1 Schematic overview of bovine *in vitro* embryo production. Oocyte collection is followed by *in vitro* maturation and fertilization of the oocytes and subsequently zygotes are cultured until 8 days post insemination (dpi) (Pictures by Maaike Catteeuw)

Despite refinement of the OPU collection method, the number of oocytes retrieved per donor is still disappointingly low; on average, 4 to 11 oocytes per aspiration session are collected in Bos Taurus (Blondin *et al.* 2002; De Roover *et al.* 2005; Chaubal *et al.* 2006; Vieira *et al.* 2014). A study by O'Doherty *et al.* (1997) indicated that the number of oocytes grouped has a major impact on the developmental capacity of the oocytes (**Table 1**). Small groups of 10 oocytes have decreased blastocyst rates compared to larger groups of at least 20 oocytes. From this point on, we are referring to "small donor groups" as groups of 10 oocytes or less coming from the same donor animal. Moreover, depending on the oocyte quality (only one third is of good quality), between 1.5 to 5 embryos can be produced per OPU session (Goovaerts *et al.* 2007). Nonetheless, OPU-IVP can yield 80 to 100 calves per donor per year, while only 20 to 25 and only 1 calf per year using MOET and AI, respectively (Van Wagtendonk-de Leeuw 2006).

Table 1 The effect of oocyte group size on the developmental outcome (O'Doherty et al. 1997).

Group size	Cleavage rate (%)	Blastocyst rate (%)
1	66.8	13.1 a
5	78.3	20.8 b
10	78.0	22.3 b
20	81.5	32.5 c
40	77.6	32.8 c

Follicular aspiration of abattoir ovaries

Oocyte collection from slaughterhouse ovaries results in a onetime recovery of a very limited number of gametes, which strongly contrasts the OPU-technique. It can be offered as a final resource when a cow needs to be slaughtered, but mostly it is done for research purposes. The recovery of oocytes from slaughterhouse ovaries is low budget, as only a syringe and needle are needed and the learning curve is much faster compared to OPU. Furthermore, the recovery rate of oocytes is higher when aspirating ovaries from slaughtered animals compared to OPU (Hashimoto *et al.* 1999). For research, the aspirated follicle fluid, containing cumulus oocyte complexes (COCs), will be left to precipitate in a tube. Subsequently, a rigorous selection of high quality COCs using a stereomicroscope can be done due to the high number of collected COCs. Although the donor's identity will get lost, the high yield of COCs collected makes it possible to create large groups for maturation, with correlated higher success rates (Khurana and Niemann 2000; Fujita *et al.* 2006; Hoelker *et al.* 2010).

1.2.2 Oocyte maturation

In vivo and *in vitro* maturation

In vivo, the preovulatory or immature oocyte in several species, such as human, cattle and mouse, is surrounded by cells, such as granulosa and cumulus cells and follicular fluid. There is a close interaction between the follicular environment and the oocyte. Gap junctions penetrating the zona pellucida ensure a close contact between the oocyte's membrane and the inner layer of surrounding cumulus cells or corona radiata cells (Atef *et al.* 2005). Furthermore, there is a bidirectional communication between the oocyte and granulosa and cumulus cells. On the one hand, oocyte secreted factors, such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), will control the differentiation of cumulus cell through their receptors, such as ALK 5 and ALK 6. Subsequently, SMAD intracellular transducers are activated and will regulate a variation of cumulus cell functions, such as proliferation and expansion (Gilchrist 2011). On the other hand, cumulus cells are crucial for oocyte development as they contribute to oocyte growth and development via paracrine and gap-junction-mediated pathways. Moreover, the follicular environment is regulating both the meiotic arrest of the oocyte at the prophase I stage, as well as the meiotic resumption. An important key player herein is cyclic AMP (cAMP), not only synthesized by the oocyte itself but also delivered by mural granulosa cells and adjacent cumulus cells via gap-junctions (Gilchrist 2011). As long as a high cAMP concentration is maintained inside the oocyte, the oocyte will be kept in meiotic arrest at the prophase I stage. To induce meiotic resumption, the preovulatory luteinizing hormone (LH) surge will act through the receptor, localized at the somatic cells. Through a secondary cascade of epidermal growth factor (EGF) like peptides, ERK1/2 in the cumulus cells are inducing a cAMP downregulation and concomitantly an upregulation of maturation promoting factor (MPF). In the 24 hours following the LH-surge, the primary oocyte undergoes maturation and ovulation is induced (Gordon 2003).

When bovine oocytes are being removed from their follicle, the meiotic-inhibiting influence is ceased resulting in a spontaneous resumption and completion of meiosis (Pincus and Enzmann 1935; Gilchrist 2011). Oocytes will undergo maturation even without the presence of endocrine and paracrine signals inducing maturation *in vivo* (Norris *et al.* 2009). Although an average of 90% of the oocytes resume meiosis up to metaphase II stage, only 30-40% will reach the blastocyst stage in cattle. Similar results are found in sheep and goat (Paramio and Izquierdo 2014). In contrast, only 70% of human immature oocytes reach the MII phase after IVM and less than 40% will develop into a transferable blastocyst, resulting in a pregnancy rate of 25%, which is only half of the pregnancy rate achieved after conventional IVF (Walls *et al.* 2015). Although IVM offers advantages for women having polycystic ovarian syndrome or diagnosed with cancer, higher miscarriage rates have also been reported

(Basatemur and Sutcliffe 2011). The non-physiological resumption of meiosis *in vitro* can compromise the subsequent oocyte developmental potential (Gilchrist and Thompson 2007). Moreover, the oocyte's competence *in vitro* is also affected by an inadequate cytoplasmic maturation, which can be evidenced by a defective distribution and location of mitochondria (Gordon 2003). *In vitro* maturation of bovine oocytes does not only imply an appropriate segregation of the chromosomes but also an adequate cytoplasmic maturation to retrieve full competence. Although some factors can be taken into account when producing *in vitro* embryos, such as oocyte quality and *in vitro* maturation systems, it is clear that the *in vitro* maturation itself has an enormous impact on the developmental outcome of the oocytes. A study by Rizos *et al.* (2002) determined in detail the effect of each sequential step of the bovine embryo production process *in vivo* and *in vitro*. The IVM has a major impact on the further developmental capacities of the oocytes as only 38.9% blastocysts were formed after IVM, compared to 78% when *in vivo* matured oocytes were fertilized and cultured *in vitro*. More insights into bovine *in vivo* and *in vitro* maturation can therefore increase the effectiveness of the embryo development.

Due to the immediate meiotic resumption that is taking place when oocytes are removed from the follicle, these precious gametes require careful handling in commercial settings. Moreover, there are often large time gaps between oocyte collection in different cows, implying not only different timings for onset of oocytes' maturation but also the need for laborious scheduling of the later manipulations. Different methods have been investigated to keep the oocytes in the germinal vesicle stage (Alm *et al.* 2008). Delaying oocyte maturation can be achieved by cAMP modulators by inducing cAMP production or preventing its breakdown. Three types of cAMP modulators can be applied; cAMP analogues, adenylate cyclase activators and phosphodiesterase inhibitors. In cattle, studies have investigated the effect of these cAMP modulators, showing oocyte maturation regulation, increased cAMP levels, reversibility of the inhibitory effects and delay of meiotic resumption. Nonetheless, results regarding oocyte maturation and embryo development were often inconsistent between different studies (Sato *et al.* 1990; Sanbuissho *et al.* 1992; Atkas *et al.* 1995a; Atkas *et al*. 1995b; Guixue *et al*. 2000; Mayes and Sirard 2002; Bilodeau-Goeseels 2003; Luciano *et al.* 2005; Barretto *et al*. 2007). Inhibition of M-phase promoting factor (MPF) kinase activity by roscovitine, which is a purine, has also shown to be successful at maintaining bovine oocytes at the germinal vesicle stage. However, MPF inhibitors also have deleterious effects such as maturation kinetics alterations and a decrease in blastocyst development (Lonergan *et al.* 2000; Ponderato *et al.* 2001; Lagutina *et al.* 2002). Application of pharmacological inhibitors demands a cautious approach as these products are toxic. An easy, safe and coherent method to transport oocytes from different herds, enabling pausing of meiotic resumption, would definitely facilitate logistics for commercial practice.

Oocyte quality

The oocyte's quality is defined by its competence to develop into a viable blastocyst in *in vitro* conditions, which is influenced by the cumulus oocyte complex (COC) itself but also by laboratory conditions (Merton *et al.* 2003). First, biological factors have a major impact on oocyte quality. Nuclear maturation in mammalian oocytes involves the breakdown of the germinal vesicle, chromosome condensation and segregation, extrusion of the first polar body and resumption of the meiotic division up to the metaphase II stage (Roth and Hansen 2005). Also the redistribution of multiple cytoplasmic organelles defined as cytoplasmic maturation, such as the alignment of cortical granules just beneath the oolemma (Gilchrist and Thompson 2007), storage of mRNA, protein and transcription factors are essential for the oocyte to take the control of the first cleavage divisions until embryonic genome activation (Fair *et al.* 1995; Ferreira *et al.* 2009). Incomplete cytoplasmic maturation lowers the oocyte's potential, even if the nuclear maturation is fully completed. An adequate coherence between nuclear and cytoplasmic maturation is therefore necessary for successful fertilization and embryo development.

Other factors, such as follicle and oocyte diameter (Lonergan *et al.* 1994; Fair *et al.* 1995), oocyte and cumulus morphology (Yuan *et al.* 2005) (**Figure 2**), have been associated with the oocyte's quality and developmental competences in cattle. By aspirating antral follicles with sizes ranging from >2 to 8 mm, oocytes have a better developmental potential compared to those collected from follicles <2 mm. Furthermore, selecting cumulus oocyte complexes having a homogeneous cytoplasm and multiple compact layers of cumulus cells has proven to be crucial as the presence of a tight cumulus cell-oocyte contact is essential for maturation (Hashimoto 2009). Both oocyte and cumulus cells are capable of producing regulatory factors which facilitate a bidirectional communication, essential for fertility (Gilchrist *et al.* 2008).

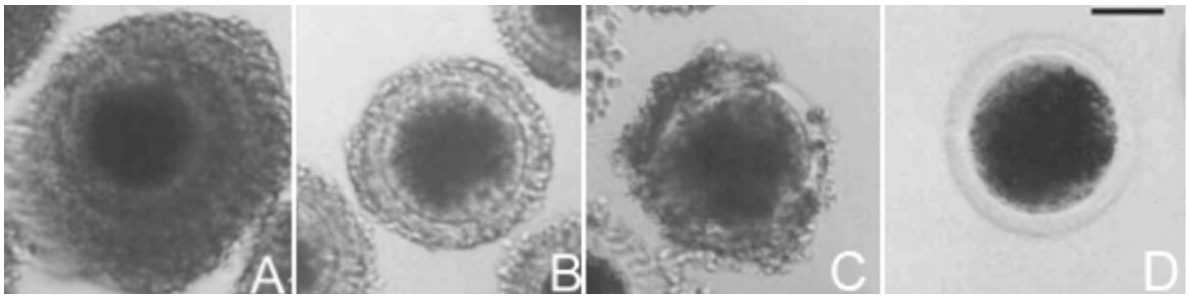


Figure 2 Categories of oocyte quality. (A) high, (B) good, (C) low and (D) bad quality oocytes. (Pictures by Maaike Catteeuw)

Second, some technical aspects in connection with bovine commercial IVP, may result in an impaired oocyte quality and developmental competence. In commercial practice, it is routine that oocytes and resulting embryos are grouped per donor, to prevent identity loss of the originating donor and to limit

possible disease transmission. Due to the low oocyte yield per donor, no strict oocyte selection, is performed. The low numbers of variable quality oocytes grouped together decrease as such the overall embryo developmental capacity (Marquant-Leguienne and Humblot 1998).

In vitro maturation culture systems

In vivo, the oocyte is stimulated by gonadotrophins, FSH and LH and the composition of follicular fluid is changed over time to fully support the progression of the oocyte's maturation. In contrast, the *in vitro* maturation environment is often a static system composed of complex media, such as TCM-199, hormones (FSH, LH), serum or serum replacements and growth factors. In our lab, maturation medium is protein-free and composed of TCM-199 and EGF (Epidermal Growth Factor), which stimulates cumulus cell expansion and improves the percentage of oocytes undergoing nuclear maturation as well as the proportion of embryos attaining the blastocyst stage (Lonergan *et al.* 1996). Generally, *in vitro* maturation of bovine COCs is performed for 22 to 24 hours in specialized medium in order to achieve full developmental competence. In cattle, OPU oocytes can be matured *in vitro* for 16 to 28 hours without compromising the embryo production (Merton *et al.* 2012).

In general, bovine COCs are matured in a large volume of medium as this facilitates oocyte handling compared to maturation medium droplets under mineral oil. Since only small numbers of oocytes are retrieved per donor resulting in maturation in small groups of COCs, low oocyte densities (number of oocytes per volume unit of medium (µl)) are therefore common in commercial practice. The group size or number of COCs matured together is playing an important role, therefore, maturing large groups of COCs together is still preferred since a higher blastocyst yield is achieved compared to the maturation of individual COCs or small groups, even when similar oocyte densities are maintained (Carolan *et al.* 1996). Grouping more COCs in a way that the oocytes do not lose their identity would benefit the commercial OPU-IVP practice as oocytes could possibly retrieve higher developmental capacities.

1.2.3 Oocyte fertilization

In vivo and *in vitro* fertilization

In vivo, fertilization is a complex process which involves the activation of the oocyte by the spermatozoon. Once the spermatozoon passes the cumulus oophorus and contacts the zona pellucida, it undergoes the acrosome reaction and penetrates the zona due to its hyperactivated state. Subsequently the spermatozoon binds to the oolemma and fuses. The oocyte is activated and various events are initiated such as cortical granule release and cytoplasm rearrangement. The oocyte completes the second meiosis and subsequently, the second polar body is extruded. The penetrating

sperm cell undergoes morphological changes when transforming into the male pronucleus. After only a few hours, both male and female pronuclei are formed and will migrate to each other to form a diploid zygote nucleus. Shortly after, the cell division starts (in detail cfr. 1.4.1) and a first division towards a two cell embryo occurs.

Routinely, *in vitro* fertilization is performed by co-incubating capacitated spermatozoa with mature oocytes. Prior to the incubation, semen of a previously IVF tested bull is separated over a discontinuous density gradient, in order to purify motile sperm from dead cells, thereby increasing the fertilization rate. Bovine IVF is performed with cumulus-enclosed oocytes. When oocytes are devoid of cumulus cells, which is sometimes the case in OPU settings, the fertilization rate decreases. Furthermore, these cumulus cells participate in the mechanisms of sperm capacitation and acrosome reaction, which directly affects polyspermy rate (Tanghe *et al.* 2002a). Polyspermy or penetration of more than one spermatozoon, has been reported to be between 2.9% and 22.0% in bovine *in vitro* fertilization (Parrish *et al.* 1986; Xu and Greve 1988; Verberckmoes *et al.* 2005; Destouni *et al.* 2016). Normally, when a spermatozoon has entered, the zona pellucida hardens which is also called 'zona block' or 'zona hardening' and prevents other spermatozoa from entering. The exocytosis of cortical granules is thought to be the major role player in this hardening process. However, polyspermy appears to be not only related to failure of exocytosis (Coy *et al.* 2008), but also to the oocytes' morphology (Hosoe *et al.* 2014) and the maturation status (Sugimura *et al.* 2017).

In vitro fertilization culture systems

In commercial settings, the oocytes are fertilized in groups per donor animal. In theory, it would be possible to fertilize each oocyte separately with semen of a different bull to obtain a higher variation in genetic combinations, compared to MOET. Nonetheless, a good fertilisation capacity of a bull's semen *in vivo* does not mean that this is also the case *in vitro*. It is therefore indicated to assess the *in vitro* fertilizing ability of a specific bull. The best way to evaluate this ability is to perform IVF and assess embryo development, which is a rather expensive and time-consuming method. However, semen quality parameters will already give a good indication of the bull's *in vitro* fertilization capacity, such as sperm motility, progressive motility, normal morphology after thawing and life-dead ratio after fertilization (Tanghe *et al.* 2002b).

Fertilization media comprise capacitating agents to increase the fertilization rate. One of the main agents used is heparin as it induces acrosome reaction of bovine spermatozoa, resulting in an improved frequency and quality of IVF (Fukui *et al.* 1990). However, increasing the heparin concentration results in an increased incidence of polyspermy (Fukui *et al.* 1990; Marquant-Le Guienne *et al.* 1990). Other

chemical agents to stimulate sperm motility can be used as well, such as caffeine and PHE (penicillamine, hypotaurine and epinephrine) (Izquierdo *et al.* 1998). Routinely, mature oocytes are co-incubated with 1 million spermatozoa per ml to achieve a proper fertilization rate.

1.2.4 Embryo culture

In vivo and *in vitro* embryonic development

In vivo, the fertilized oocyte or zygote starts its first cleavage about 20 hours after the ovulation has occurred (Laurincik *et al.* 1994). The oviduct provides the optimal environment for early embryo development. There is a dynamic relationship as various endocrine and paracrine secretions are providing the appropriate environment to support development by producing oviduct-specific molecules (Hunter 1994). These oviduct proteins are playing a role in fertilization and can also act as stabilizers, carrier molecules or immunosuppressive agents and they might influence the embryonic development. Studying embryo development inside the oviduct is not routinely done as more invasive techniques, such as surgery and slaughter, are required to retrieve the *in vivo* embryos. However, a novel technique has been developed which makes it possible to flush the oviduct, bringing the embryos in the uterus where they can be retrieved by uterine flush (Besenfelder *et al.* 2010). *In vivo*, the bovine embryo reaches the uterine horn between day 4 and 6 of pregnancy and comprises 8 to 16 cells. The subsequent embryo development takes place in the uterus, where compact morula and blastocyst stages are reached by day 6 and day 7 of the pregnancy, respectively. During blastocyst formation, the first two embryonic cell lines are generated. These cell lines are the undifferentiated inner cell mass (ICM) and the outer epithelial trophectoderm cells (TE). The ICM will give rise to the hypoblast and epiblast, resulting in the foetal tissue and extraembryonic endoderm and extraembryonic mesoderm while the TE will develop into placental tissue. After hatching of the blastocyst, the bovine embryo will start elongating on day 14 and reach 20 to 30 cm length on day 20, just before implantation occurs (Ushizawa *et al.* 2004).

In vitro produced embryos differ in various ways compared to their *in vivo* counterparts. Abnormalities such as polyspermy, polygyny (two or more female pronuclei) and asynchronous development of male and female pronucleus are more prominent in IVP embryos (Gordon 2003). Morphological differences are more noticed between *in vitro* produced embryos and the *in vivo* derived ones when serum was applied during IVC; blastomeres have a darker cytoplasm, blastomeres are more permeable and the zona is more sensitive to pronase digestion. Furthermore, there are clear and measurable differences in the inner cell mass, in the compaction process and in the lower metabolic activity (Gordon 2003).

More importantly for commercial purposes, the *in vitro* embryos are more sensitive to freezing-thawing procedures, resulting in lower embryo survival (Gordon 2003).

The first cell cleavages are an indication of the initial oocyte quality as the embryo is still dependent on stored maternal RNA and proteins present in the oocyte's cytoplasm. After a few cleavages, the embryo takes over the development. The activation of the embryo genome is a multiple-step event with minor transcription of a small number of genes during early embryogenesis (Kues *et al.* 2008). This maternal-embryonic-transition (MET) corresponds to a shift from the maternal control to the embryonic control of development. The major genome activation is mainly occurring in the fourth cell cycle in bovine embryos. Previously, it was generally accepted that the embryonic genome activation (EGA) was happening during the 8-16 cell stage in cattle, while it has been reported to occur earlier, i.e. at the 4-8 cell stage (Meirelles *et al.* 2004). Maternal transcripts are degraded fast during MET, whereas embryonic transcripts are rapidly synthesized (Kues *et al.* 2008). When embryos fail to transcribe their own genome, embryonic development is arrested. This developmental block is most likely correlated with the cytoplasmic quality of the oocyte and the inability to activate transcription of important developmental genes. The maternal reserves necessary for early embryogenesis are accumulating in the oocyte during follicular growth. Therefore blastocyst development is mainly determined by the intrinsic quality of the oocyte, while culture conditions have an impact on the blastocyst quality (Rizos *et al.* 2001).

In vitro embryo culture systems

Up until the late nineties, the most popular method to culture bovine *in vitro* embryos was co-culture with somatic cells. Although the interaction between the somatic cells and the embryos stimulated metabolic pathways, important deficits in enzyme and mRNA were found. Moreover, the concern about disease transmission between somatic cells and embryos has led to *in vitro* culture systems without these cell layers (Ménézo *et al*. 1998). Subsequently, sequential media systems, consisting of macromolecules such as serum albumin and serum fractions, were used as the metabolic needs are different between precompaction and postcompaction stage embryos (Lane and Gardner 2007). However, as oxygen tension has been decreased from 20% to 5% and synthetic oviductal fluid (SOF) with bovine serum albumin (BSA) medium is used, it is possible to culture the embryo from zygote to blastocyst without extra interventions (Galli *et al.*, 2003). This method of culturing bovine *in vitro* embryos is therefore amongst many preferred.

Traditionally, embryos are cultured together in groups in a droplet of medium. This is typically done for research purposes as the identity of the embryos is not important in this case. Moreover, large

group culture is advantageous as not only the blastocyst yield is higher, but also the quality of those embryos is higher compared to individual embryo or small group culture (O'Doherty *et al.* 1997; Cebrian-Serrano *et al.* 2014). Single or small embryo culture which is linked to the commercial embryo production from oocytes collected by OPU (Carolan *et al.* 1996; Vajta *et al.* 2000; Ward *et al.* 2000; Goovaerts *et al.* 2009), has led to the evolution of new embryo culture dishes, such as the Corral® dish and Well-of-the-well (WOW) and embryo culture systems, such as microfluidics, trying to achieve a higher embryonic development while the embryo's identity can be preserved.

On the one hand, novel dishes were designed to keep track of the embryos' identities while embryos were grouped because it is generally known that embryos cultured in group have better developmental competences compared to embryos cultured singly or in small groups. The Corral® dish, existing of central wells divided into quadrants by a semi-permeable wall, was designed for human embryo culture, and one embryo was placed per quadrant (Ebner *et al.* 2010). The culture medium is connecting the quadrants whereby exchange of nutrients and embryotrophic factors is possible, while embryos stay put. The design of the WOW-dish is quite similar, one droplet of medium is covering a small group of embryos. However, the embryos are placed in a narrow well inside the droplet well, referring to the well of the well principle. Embryos are closer to each other compared to the Corral® dish and higher blastocyst formation has been reported in a comparative study in cattle (Wydooghe *et al.* 2014b). Both dishes would definitely offer an opportunity for commercial IVP as oocytes can be allocated per donor in these dishes. Embryo identification throughout the culture is possible and as more embryos can be grouped, it would be possible to benefit from these larger group cultures.

On the other hand, emerging new *in vitro* technologies, such as microfluidics, are trying to simulate the oviduct and uterine environments by adjusting the medium composition or fluid flow, according to the specific needs of the embryo during each stage of the development (Feugang *et al.* 2009). *In vivo*, the female tract does not only remove harmful substances from the environment, also a variety of nutrients are provided that are necessary for the embryonic regulation and development. These novel culture systems may produce higher quality embryos resulting in more successful pregnancies after transfer (Absalón-Medina *et al.* 2014). Unfortunately, these systems are often still very expensive and therefore not commonly used in commercial embryo industries.

In vitro embryo culture conditions

Culturing fertilized oocytes under strict conditions is necessary to obtain high quality embryos capable of developing into viable blastocysts which will subsequently be transferred and in ideal circumstances implant and result in life birth (Ménézo *et al.* 1998). These *in vitro* environmental conditions are trying

to mimic the *in vivo* micro-environment of the maternal tract necessary for early embryonic development. The culture media, which are complex mixtures of nutrient supplements, will inevitably have an impact on the growth and function of the embryonic cells (Mather and Roberts 1998). Historically, serum was an integrated component of the embryo culture system. However, embryos are not exposed to serum *in vivo*. Moreover, studies revealed that serum has various negative effects on *in vitro* embryo production such as morphological, metabolic, genetic and ultrastructural changes (Gardner 1994; Gardner *et al.* 1994; Thompson *et al.* 1995; Khosla *et al.* 2001). Serum is also associated with 'large offspring syndrome' (LOS) in cattle and sheep which is characterized by longer gestation length, increased perinatal loss, abnormally high birth weight and more breathing difficulties in the neonates (Young *et al.* 1998). Today, serum is more and more excluded from the culture medium and chemically semi-defined or even defined media are being used. These media are used to simplify and to control the culture conditions better and moreover, to prevent the transmission of pathogens affecting the embryonic development. The exact composition of fetal calf serum (FCS) is unknown and FCS supplementation can introduce pathogens into the medium (Blondin 2017). Finally, endless choices are offered to establish the best *in vitro* culture conditions regarding media, gas, temperature and oil to obtain high blastocyst and transfer results (Vajta *et al.* 2010).

Embryo density and embryotrophic factors

In many mammalian species, including cattle (Donnay *et al.* 1997; O'Doherty *et al.* 1997; Goovaerts *et al.* 2009; Salvador *et al.* 2011; Wydooghe *et al.* 2014a), mice (Paria and Dey 1990; Canseco *et al.* 1992; Lane and Gardner 1992), pigs (Stokes *et al.* 2005), cats (Spindler and Wildt 2002) and humans (Ebner *et al.* 2010), embryos cultured in large groups have better developmental competences compared to embryos cultured individually or in small groups. The beneficial effect of embryo group culture can be ascribed to the production and secretion of autocrine factors by preimplantation embryos. These embryotrophic factors include a wide range of biochemical messengers. Cytokines and growth factors, such as interleukin-1, insulin-like growth factor-1, survivin and peptide preimplantation factor, but also lipids, such as platelet activating factor, miRNAs, hyaluronic acid and nucleotides are signalling molecules beneficial for embryo development (Spanos *et al.* 2000; O'Neill 2005). These autocrine factors can be secreted through different mechanisms; active secretion, passive outflow, binding to a carrier molecule or transport within extracellular vesicles. By activating PI3K, MAPK and PPAR pathways, embryo survival and anti-apoptotic functions are enabled (Wydooghe *et al.* 2017). These autocrine factors stimulate development of the embryo itself and these of the neighbouring embryos cultured *in vitro* (Paria and Dey 1990; O'Neill 2008). The production of these factors is related to embryo density and that is why embryo density, expressed as the number of COCs or embryos

incubated in a defined volume of medium, is an important parameter during the process of *in vitro* embryo production (Vajta *et al.* 2000; Fujita *et al.* 2006; Feng *et al.* 2007; Hoelker *et al.* 2010; Reed 2012). A low embryo density, by using a large volume of incubation medium or by grouping only a few embryos, is not preferred since the developmental competence is remarkably decreasing under these circumstances (Paria and Dey 1990; Lane and Gardner 1992; Gardner *et al.* 1994), probably because embryotrophic ligands secreted by preimplantation embryos are more diluted with concurrent loss of potency to stimulate embryo growth. Nevertheless, it has been suspected that when embryo density is very high, the risk is growing that waste materials, including ammonium, are accumulating and as a result, embryonic development is reduced also (Lane *et al.* 2002; Lane and Gardner 2003; Dai *et al.* 2012). Different studies state that an embryo density of 1:1 to 1:3 (number of embryos per µL medium) yields optimal results (Ferry *et al.* 1994; Carolan *et al.* 1995; Donnay *et al.* 1997). Ideally, an embryo density of 1:2 is maintained in mice and cattle (Kato and Tsunoda 1994; Palasz *et al.* 2010).

For commercial purposes, such as use in OPU, oocytes and embryos from one donor are being grouped together (Chaubal *et al.* 2006), because it is necessary to keep track of the embryo's identity. Due to low oocyte retrieval, however, only small embryo groups can be maintained resulting in a lower blastocyst formation (16-18%) compared to large group culture (30-40%). Since many commercial laboratories do not adjust the volume of medium to the number of oocytes and embryos collected per donor and use large volumes instead, the embryo density stays low, meaning that the concentration of autocrine factors is probably also too low to stimulate the embryonic development (De Roover *et al.* 2005; Machado *et al.* 2006; Merton *et al.* 2012; Merton *et al.* 2013). Despite the progress made in bovine IVP, its use in cattle breeding remains therefore quite limited within the commercial sector.

1.2.5 Cryopreservation and embryo transfer

In breeding programs, cryopreservation of both *in vivo* derived embryos via MOET or *in vitro* produced embryos is routinely used to preserve spare embryos when no recipient animals are available. It does not only permit unlimited storage of genetically valuable gametes, it also permits long distance transportation. When cryopreserving embryos, two techniques can be applied; slow freezing and vitrification. The freezing process itself causes damage by the formation of ice crystals and changes in the intracellular solute concentrations. Nonetheless, cellular damage can be reduced by controlling the temperature reduction and by using cryoprotectants. However, only by vitrifying the formation of extracellular ice crystals is avoided because of the high viscosity vitrification medium (Mandawala *et al.* 2016). Several studies have investigated the efficacy of both cryopreserving techniques regarding embryo survival after thawing and pregnancy rates after transfer (Van Wagtendonk-De Leeuw *et al.* 1995; Martinez *et al.* 2002; Nedambale *et al.* 2004). In a general field study, it was reported that

vitrification and subsequent dilution using a one-step procedure yields similar pregnancy rates as the conventional slow freezing protocol, i.e. over 50% in case of high quality embryos(Van Wagtendonk-De Leeuw *et al.* 1995; Van Wagtendonk-de Leeuw *et al.* 1997).

When fresh good quality *in vitro* produced embryos are being transferred, only 45-55% of them will give rise to a pregnancy and result in the birth of a healthy calf. Highest pregnancy rates are achieved by transfer of fresh day 7 blastocysts to recipient animals, synchronized correctly (Hasler *et al.* 1995). Earlier stages than morula stage transfer is impossible in contrast to human, where routinely cleavage stage embryos are transferred to minimize the exposure to suboptimal *in vitro* conditions. Even transferring day 8 blastocysts is associated with a decreased pregnancy rate. Furthermore, no matter which blastocyst is transferred (day 7 or day 8), when it has been cryopreserved, the pregnancy rates will drop with another 10% (Hasler 1998). Unfortunately, it is not possible to predict the outcome when transferring a blastocyst. There are however a few emerging technologies that are trying to anticipate the embryo developmental potential to achieve the best results.

1.3 Quality assessment of embryonic development

1.3.1 Embryo morphokinetics by time-lapse imaging

Up until today, parameters such as timing of embryonic cleavages and embryo morphology are still the most used criteria to predict developmental competence and quality, as they can be assessed in an easy and non-invasive manner. Bovine embryos are normally selected for transfer at the end of the culture period (day 7-8) according to their morphology and developmental stage. However, early cell division anomalies such as the formation of fragments, the presence of unevenly sized blastomeres or cleavage from one cell to three blastomeres, could be missed by these current static embryo visualization approaches. Importantly, the appearance of these anomalies is often associated with impaired developmental competence (Somfai *et al.* 2010). Moreover, different *in vitro* produced embryos show substantial differences regarding timing events. Whereas fast cleaving embryos end the first cleavage before 24 hours post insemination, others need even an extra day to reach the two cell stage (Van Soom *et al.* 1992). Previously, it was thought that these fast cleavers were more similar regarding developmental capacity and quality as the *in vivo* ones (Gutierrez-Adan *et al.* 2015). However, there is an ongoing debate on which embryos should be selected for transfer because slow cleaving embryos mostly lack further development, while in mice the fast cleaving embryos with more than 8 cells at 24 hpi show genomic imprinting loss (Market Velker *et al.* 2012). At the moment, the intermediate cleaving embryos are considered to resemble *in vivo* embryos more closely (Gutierrez-Adan *et al.* 2015), but data defining this category are lacking in cattle.

Time-lapse imaging can not only reveal anomalies which should be considered while selecting embryos for transfer, but it can also avoid environmental stressors occurring during routine static observations using microscopy, such as pH and temperature fluctuations. Commercially available systems offer the unique opportunity to monitor continuously embryonic morphokinetics inside the incubator. As microscopic light can compromise embryonic development and viability by ROS generation, disturbed ATP production and ultimately DNA damage (Lane and Gardner 2005; Ottosen *et al.* 2007; Frigault *et al.* 2009). Potential phototoxicity of time-lapse systems has been investigated in human, murine and bovine embryo studies. However, no study has described any significant deleterious effects of time-lapse imaging compared with conventional *in vitro* embryo production (Grisart *et al.* 1994; Yoshioka *et al.* 2000; Nakahara *et al.* 2010; Wong *et al.* 2010). Due to its innovative and accessible application, it is used frequently in preimplantation embryo research to elucidate various developmental insights. Numerous studies have been investigating the morphological events during early embryo development. It has been reported in humans (Fenwick *et al.* 2002; Lemmen *et al.* 2008; Wong *et al.* 2010), mice (Pribenszky *et al.* 2010) and cattle (Van Soom *et al.* 1997; Sugimura *et al.* 2010; Beck 2014) that the development of a zygote into a blastocyst is largely established as early as the first mitotic cleavage. Timing and synchronization of cell cleavage can be considered indicative for increased developmental potential. In cattle, a delay is observed in the onset of the first, second and third mitotic cleavage in non-viable embryos compared to viable embryos (Somfai *et al.* 2010). Moreover, the length of the one cell stage and the duration of the second mitotic cleavage is significantly shorter in viable embryos.

1.3.2 Fluorescent staining techniques

A first differentiation process, segregation between inner cell mass (ICM) and trophectoderm (TE), takes place at the morula stage and it continues throughout the blastocyst formation. By that time, different signs of apoptosis may arise too, such as caspase activation and DNA fragmentation. A differential staining where total cell number (TCN), ICM, TE and apoptotic cells can be distinguished, provides additional information regarding embryo quality (Fouladi-Nashta *et al.* 2005). In our lab (Wydooghe *et al*. 2011), a double-immunofluorescent staining was used, directed against CDX2, a transcription factor only expressed in the trophectoderm cells and against active caspase 3, which plays a central role in all apoptotic pathways. For CDX2 Texas Red was the fluorescent label, which stains the TE cells in red, and for active caspase 3 it was FITC, making apoptotic cells turn green. This is further combined with Hoechst nuclear stain, making all nuclei blue. When three stainings are combined, it is possible to discriminate the ICM as it is only stained blue (with green spots if apoptosis is present) (**Figure 3**). However, the main disadvantage of this invasive technique is that the embryo

cannot be transferred anymore. This technique is therefore not used in commercial embryo industries, but for research purposes.

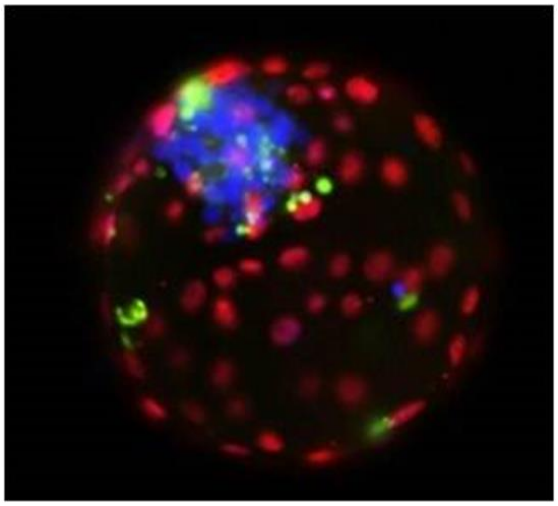


Figure 3 Differential apoptotic staining overlay picture of blastocyst. Blue: Inner cell mass by Hoechst 33342, Red: Trophectoderm by anti-CDX2 immunofluorescent staining, Green: apoptotic cells by anti-Caspase 3 immunofluorescent staining (Picture by Eline Wydooghe).

1.3.3 Cytogenetic analysis and chromosomal instability

By removing cells from the embryo and analysing these cells by cytogenetic techniques, it is possible to screen the embryos' genetic profiles in general (preimplantation genetic screening - PGS) or for specific disorders (preimplantation genetic diagnosis - PGD) before embryos are transferred to a recipient animal. Although it is more frequently used in human embryos, performing genetic analysis in bovine embryos can provide information about the embryo's sex and about genetically inherited and important traits, such as milk production. It can also provide insights in the overall genetic constitution of the embryo as chromosomal abnormalities are associated with infertility and pregnancy loss. In cattle breeding industries, cytogenetic analyses can be performed on both *in vivo* embryos derived after uterine flush and on *in vitro* produced embryos. Different protocols regarding the embryo stage when the biopsy is taken, the method of biopsy and the methods for genetic analysis are used worldwide.

Embryo biopsy

Embryos can be biopsied by drilling the zona pellucida and aspirating one or more blastomeres at the cleavage stage or by cutting off a piece of trophectoderm with a microscalpel at the blastocyst stage since blastocysts comprise many blastomeres which are closely connected by a compact network of tight junctions and are therefore more difficult to aspirate (Herr and Reed 1991). In all procedures, the zona pellucida has been penetrated which makes it no longer intact, this however does not affect the further developmental capacity or pregnancy rate (de Sousa *et al.* 2017).

Embryos will not always develop into high quality transferrable blastocysts. As such unnecessary costs are made if genetic analysis has been done in early cleavage stage embryos that cease development. Moreover, if one blastomere was analysed, the result does not always reflect the exact genetic constitution of the embryo. Analysis performed on all blastomeres of an embryo showed different abnormalities in different blastomeres (Destouni *et al.* 2016). Also when TE is biopsied in a blastocyst, a completely normal ICM can be present while chromosomal abnormalities may be found in the genetic analysis of the TE. A tendency exists for allocation of chromosomally abnormal cells to the trophectoderm rather than the ICM (Iwasaki *et al.* 1992).

Genetic analysis

Different analysing techniques are available, such as karyotyping, fluorescent in situ hybridization (FISH), polymerase chain reaction (PCR), arrays and sequencing techniques (**Table 2**). Until recently, analysing both frequency and nature of chromosomal aberrations was done by conventional karyotyping. In this technique, cells are arrested in cell division as chromosomes are stained in the (pro)metaphase stage by f.e. Giemsa staining as it attaches to DNA. In this way, all types of aneuploidies are revealed. However, the procedure is rather inefficient since many embryonic cells cannot be analysed due to the difficulties obtaining metaphase preparations. Furthermore, the identification of missing or supernumerary chromosomes is challenging. In addition, the obtained result is not really reliable as chromosomal abnormalities will be mostly overestimated (Van Soom and Boerjan 2002). Furthermore, determination of embryo sex was only possible in 58.8% of the embryos (Hasler *et al.* 2002). FISH is a molecular technique based upon the binding of fluorescent probes to the chromosome or parts of the chromosome with a high complementarity in sequence. A major disadvantage, however, is that it is not possible to identify all chromosomal aberrations. Hybridization of differently labelled probes to different chromosomes is necessary to distinguish aneuploidy from polyploidy and haploidy from hybridization errors (Van Soom and Boerjan 2002). A more sensitive and accurate method for embryo sexing and screening specific genes is PCR, which is often used for PGD. Here, only a limited amount of DNA is necessary to conduct this technique as PCR amplifies the regions of DNA that it targets. Agarose gel electrophoresis may follow for size separation of PCR products which makes analysis possible. Moreover, quantitatively determining levels of gene expression is also possible by using quantitative PCR (qPCR) which measures the accumulation of a PCR product after each amplification. When there is no specific gene of interest that is targeted, other techniques are possible if it is necessary to know the complete DNA integrity of the embryo. DNA microarrays or molecular karyotyping methods can be used: these DNA chips comprise probes consisting of specific DNA sequences which will hybridize a DNA sample. Various regions of a genome can be genotyped and

evaluation of SNPs (single nucleotide polymorphisms) can be used in commercial practice to determine functional variants referenced to a general population. Furthermore, whole genome sequencing is a technique to determine the DNA sequence of an individual's genome. As this is an expensive tool, it is mostly used in research. Next generation sequencing (NGS) will sequence millions of small fragments of DNA simultaneously, resulting in accurate data and insight into DNA variations (Behjati and Tarpey 2013). Submicroscopic chromosomal changes such as microdeletions can be detected by NGS data, however, some regions are difficult to sequence such as regions with low/high guanine/cytosine (GC) content (Treangen and Salzberg 2011). The main disadvantage of these emerging genetic analysing methods is that expensive equipment is necessary and that, the amount of data that needs to be managed is enormous, requiring experienced personnel to analyse and interpret the data (Behjati and Tarpey 2013).

When single cells are used for analysis, a whole genome amplification (WGA) is necessary as the amount of DNA is too little. Unfortunately, WGA can alter the frequency and composition of the cell's alleles resulting in artefacts, making it difficult to interpret the results (Esteki *et al.* 2015). Recently, a novel analysis method has been developed which can overcome these issues. Moreover, it can be used for both PGD and PGS since it does not need a case specific design. Haplotypes, a group of genes inherited together from one parent, can be reconstructed genome wide as well as the copy number and segregational origin of those haplotypes. This method, called haplarithmisis (Esteki *et al.* 2015) can be used on single cells to diagnose disease alleles genome wide as well as both numeral and structural aberrations (**Figure 4**). Even a determination between meiotic and mitotic errors can be made. As this technique applies phasing of parental genotypes, grandparents or siblings are necessary. Haplarithmisis is based upon bioinformatics analysis of SNParrays and consists of 8 steps; (1) parental genotype phasing, (2) identification of informative SNP loci, (3) categorizing informative SNPs as paternal or maternal, (4) subcategorization on basis of phased parental SNP genotype combinations, (5) distribution of SNP BAF values (B allele frequency), (6) mirroring of BAF values, (7) segmentation of single cell BAF values for consecutive SNPs which define the haplotype blocks, (8) visualisation of segments and underlying processed SNP BAF values into separate plots. These plots display both parental haplotypes and sites of homologous recombination (**Figure 5**). Importantly, chromosomal imbalances can be deduced and parental and mechanistic origin are revealed. Although this method is still used for research purposes, it would offer new possibilities in genetic testing in cattle as it is a sensitive method to identify chromosomal anomalies (Destouni *et al.* 2016). In this thesis, this technique is also used to study chromosomal instability in bovine *in vivo* derived and *in vitro* produced cleavage stage embryos.

Table 2 Overview of cytogenetic screening tests and the type of genetic lesions that can be detected. (Vermeesch *et al.* 2016)

	Screening test					
Type of genetic lesion	**Karyotyping**	**FISH**	**qPCR**	**Microsequencing**	**SNP array**	**Genome-wide haplotyping**
Balanced chromosomal rearrangements	+/-	-	-	-	-	+
Combination of monogenic and chromosomal disorders	-	-	-	-	-	+
Complex rearrangements	-	+/-*	+/-	-	+	+
De novo mutations	-	+	+	+	-	-
Mechanistic origin of trisomies (mitotic vs meiotic)	-	-	-	-	-	+
Monogenetic disorders	-	-	-	+	-	+
Segmental chromosomal aneuploidy	+/-	+	+/-	-	+	+
Submicroscopic deletions	+/-	+	-	-	+	+
Submicroscopic duplications	+/-	-	-	-	+	+
Unbalanced translocations	+/-	+	+/-	-	-	+
Whole chromosome aneuploidy	+	+/-*	+	-	+	+

+: possible; -: not possible; +/-: possible but specific requirements; FISH: fluorescent in situ hybridization; qPCR: qualitative polymerase chain reaction; *:Limited by the number of fluorochromes.

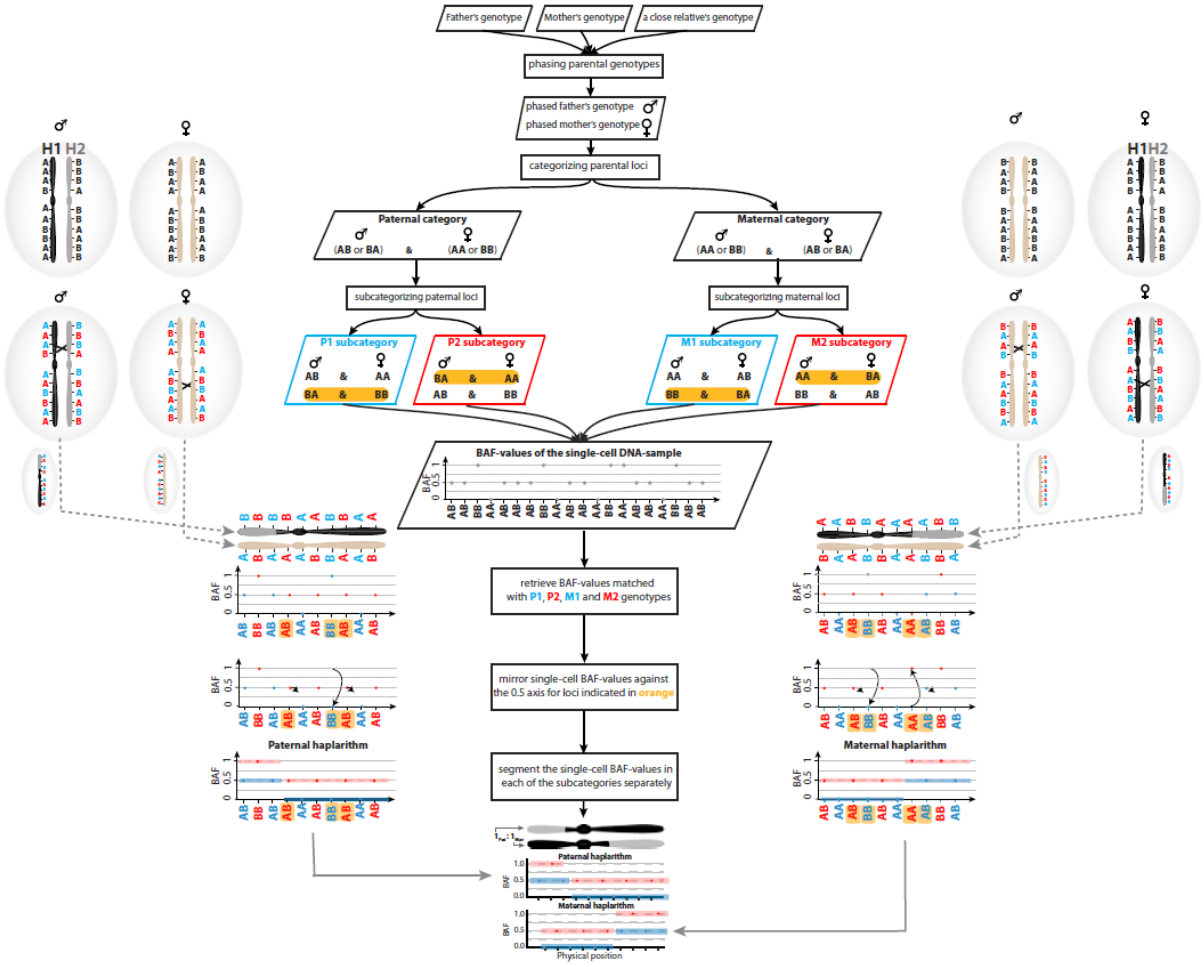


Figure 4 The basis of haplarithmisis. The figure shows how maternal and paternal haplarithm plots can be obtained from a single cell displaying disomy and one homologous recombination on both inherited chromosomes (Esteki *et al.* 2015).

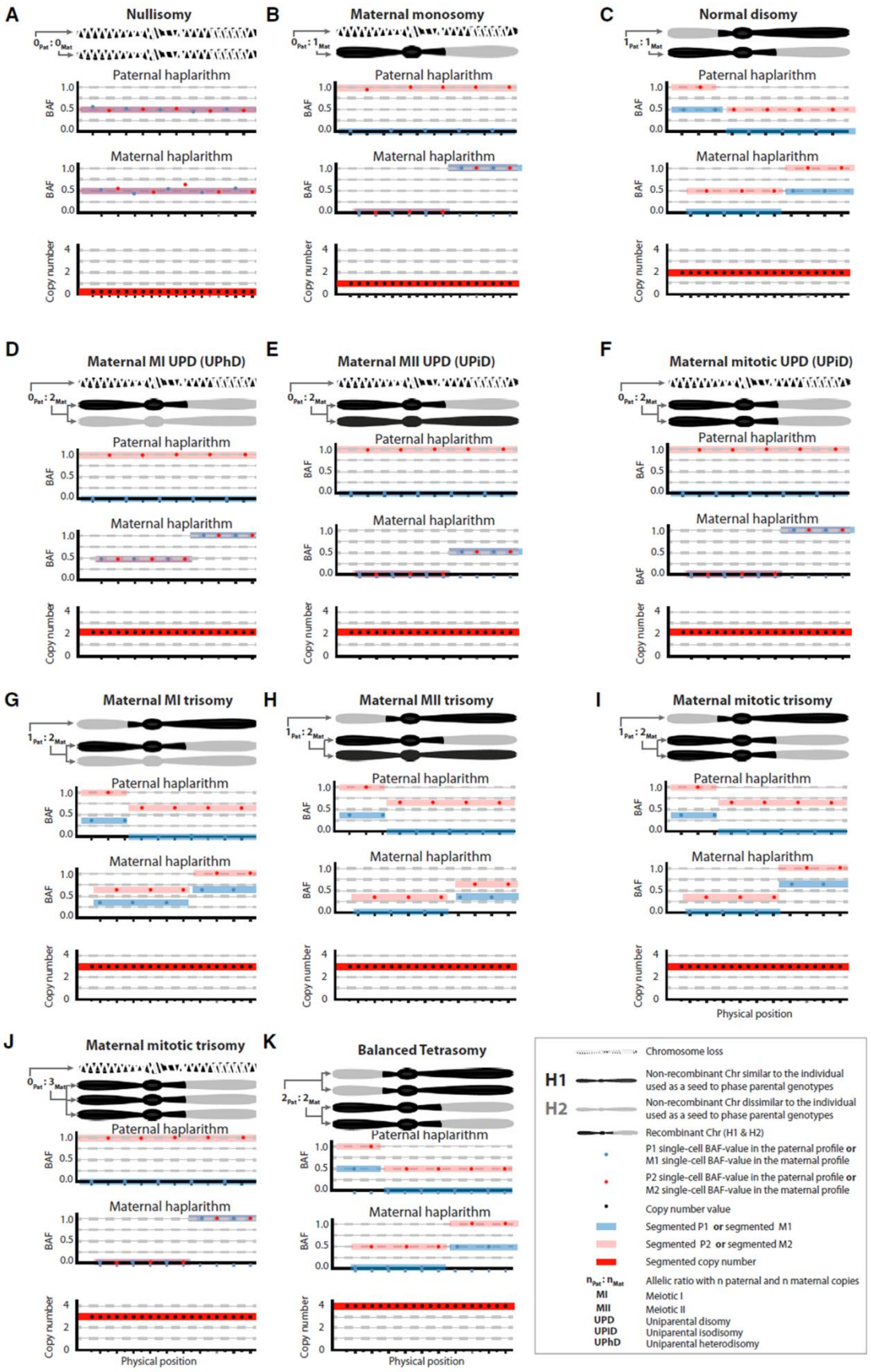


Figure 5 Haplarithm plots for different chromosomal abnormalities (Esteki *et al.* 2015).

Chromosomal aberrations

Chromosomal abnormalities comprise an atypical number of chromosomes and structural abnormalities in one or more chromosomes (**Figure 6**). Numerical disorders are called aneuploidies and refer to the loss of a chromosome from a pair (monosomy) or the presence of more than two chromosomes per pair (trisomy, tetrasomy…). A typical example of a numerical anomaly in human is trisomy 21 or Down Syndrome. In cattle, X monosomy and trisomy have been widely reported (Raudsepp and Chowdhary 2016). Structural anomalies can be either stable or unstable. Unstable anomalies, such as fragments, dicentric and ring chromosomes, will get lost due to anaphase lag or bridge-breakage-fusion. Stable abnormalities can be further divided into balanced or unbalanced abnormalities. When there is no loss or gain in genetic material, such as inversions, this is called a balanced anomaly. Deletions and duplications imply loss and gain respectively and are therefore considered as unbalanced. Translocations can be either balanced or unbalanced. When a part of a chromosome is transferred to another chromosome, this is called a translocation. There are different types of translocations. In a reciprocal translocation, there is an exchange of segments between two non-homologous chromosomes. Whereas in Robertsonian translocations, an entire chromosome is attached to another giving rise to one large metacentric chromosome. Normally, balanced aberrations will not affect the phenotype, unless the abnormality occurs inside a gene, which will then get disrupted or dysregulated resulting in a changed phenotype. Unbalanced translocations are mostly lethal resulting in embryo loss during early embryogenesis. Although the phenotype appears to be normal in individuals affected with balanced chromosomal abnormalities, they are prone to fertility disorders.

Chromosomal anomalies can be of meiotic origin when an altered meiosis anaphase I or II takes place during gametogenesis leading to an unbalanced chromosome segregation. After fertilization of these unbalanced gametes, all embryonic cells will contain the same anomaly and due to the abnormal chromosome number, the majority of affected embryos will die during early development (Szczerbal and Switonski 2016). On the other hand, unbalanced sister chromosome segregation during the mitotic anaphase will lead to an embryo comprising normal diploid blastomeres while other blastomeres reveal abnormalities. When the affected embryo contains two or more cell lines with different chromosome constitutions, it is classified as mosaicism.

Deciding whether or not an embryo should be transferred depends therefore not only on cytogenetic analysis results but also on the embryo stage, the number of cells analysed, where the biopsy was taken, the intrinsic embryo quality regarding morphokinetics…

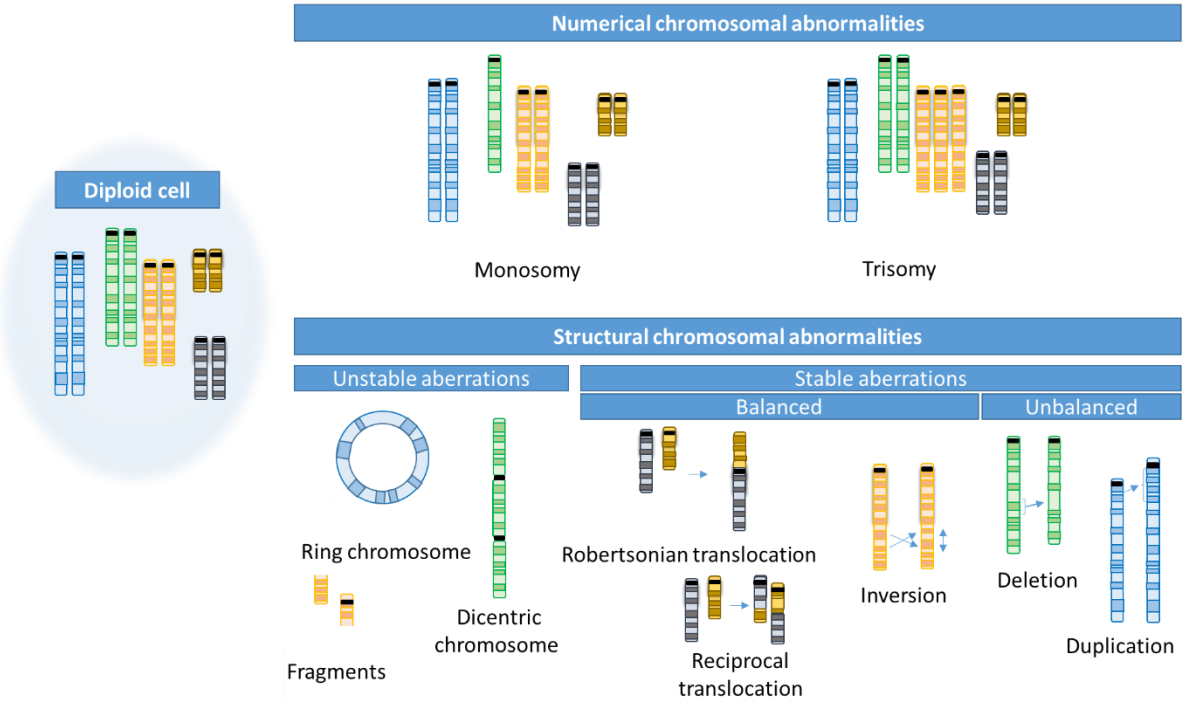


Figure 6 Overview of numerical and structural chromosomal aberrations. A diploid cell can have numerical abnormalities leading to the loss or gain of more than two chromosomes and/or it can have structural abnormalities. These latter can be categorized as unstable which will get lost during the cell cycle or stable aberrations. Compared to unbalanced abnormalities, balanced abnormalities show no gains or losses in genetic material.

1.4 Genetic disorders and chromosomal abnormalities in cattle

Most studies investigating chromosomal anomalies in bovine embryos focussed on polyploidy and mixoploidy. Using FISH, 30% of bovine MII oocytes showed aneuploidy (Nicodemo *et al.* 2010) and mixoploidy has been reported in 25–90% of bovine embryos (Viuff *et al.* 1999; Garcia-Herreros *et al.* 2010). A higher incidence of chromosomal abnormalities has been found in *in vitro* produced bovine embryos than in *in vivo* derived ones (Viuff *et al.* 1999; Hornak *et al.* 2016), indicating that the suboptimal *in vitro* conditions can even induce aneuploidy at this early preimplantation stage (Demyda-Peyrás *et al.* 2013). However, it seems that there might be a repair mechanism by allocating affected blastomeres to the trophectoderm when differentiating (Viuff *et al.* 2002) and pregnancy can be achieved with an incidence of 25% tetraploid trophoblast cells (Hare *et al.* 1980). These studies often use low-coverage cytogenetic analysing methods, reporting only the numerical chromosomal abnormalities. However, since the discovery of a Robertsonian translocation in cattle, more attention has been paid (Gustavsson 1979). The rob(1;29) translocation is the most frequent aberration detected, leading to a 5-10% reduction in fertility (Udroiu and Sgura 2017). By extensive cytogenetic screening programs, the import of affected cattle or semen can be detected. Affected animals have been dismissed from reproductive programs. This cytogenetic monitoring already resulted in an almost full eradication of rob(1;29) in Swedish carrier bulls (Udroiu and Sgura 2017). Beside the numerical and

structural chromosomal abnormalities, knowing whether the origin of the aberration is meiotic or mitotic can have a further influence on the decision making whether an embryo should be transferred or not. Meiotic errors will be present in all blastomeres and will therefore have a higher impact on embryonic development, mostly leading to early embryo death, while mitotic disorders take place in the later preimplantation embryonic development and do not affect all cells.

In cattle, there are already over 500 inherited disorders described which can be consulted in the OMIA database (Online Mendelian Inheritance in Animals) (Nicholas and Hobbs 2012). Moreover, the highest number of these described traits, 58 until now, have been associated with abortion. This does not only imply the influence of infertility and subfertility in cattle breeding programs but also the importance of preimplantation genetic screening within an OPU/IVP or MOET program. The application of PGD an PGS would not only allow us to select embryos having the desired traits, but also to detect genetic abnormalities associated with early embryo loss and infertility in later life, as both have an enormous economic impact.

1.5 Chromosomal abnormalities in human embryos and the bovine model

Over six million children have been conceived via reproductive techniques and fertility treatments (Maheshwari *et al.* 2016). Despite progress made, the pregnancy rate per embryo transfer is still 30 to 50% (Dyer *et al.* 2016; European IVF-monitoring Consortium, 2017). Embryo implanting failure and spontaneous miscarriages can explain the low pregnancy rates. Chromosomal aneuploidy can play a major role herein, as 70-90% of human cleavage-stage embryos have at least one aneuploidy cell (Vanneste *et al.* 2009). The chance is probably very low that highly affected embryos will lead to full term pregnancy. The impact of *in vitro* technologies is however strongly disputed. Although it was previously reported that the artificial reproductive techniques, such as IVF and ICSI, were linked with chromosomal birth defects (Hansen *et al.* 2002), taking parental factors into account made the increased risk for birth defects associated with IVF no longer significant (Davies *et al.* 2012). Nonetheless, associations of IVF with imprinting disorders such as Beckwith-Wiedemann syndrome and Angelman syndrome are widely reported (DeBaun *et al.* 2003; Gicquel et al. 2003; Maher et al. 2003; Ørstavik et al. 2003; Sutcliffe et al. 2005; Johnson *et al.* 2018). As IVM is upraising due to the numerous patients with polycystic ovary syndrome or hormone sensitive cancers, concerns about the impact of *in vitro* embryo techniques remains high and widely investigated.

Due to ethical constraints concerning human *in vivo* embryos, animal models are used to investigate chromosomal integrity. To date, the mouse model is still very popular for embryo manipulation and understanding biochemical and physiological processes. However, the differences between mice and human embryos are substantial, i.e. mice are polyovulatory animals having an accelerated life cycle.

Although it is a cheap model, the importance of the bovine model for human studies must not be underestimated (Ménézo and Hérubel 2002). Bovine and human embryos seem quite similar regarding biochemical and parental regulatory mechanisms. As chromosomal abnormalities are a well-known problem in both bovine and human embryos, the bovine embryo is an appropriate model to investigate chromosomal integrity as an example for human.

1.6 References

Absalón-Medina, V., Butler, W., and Gilbert, R. (2014) Preimplantation embryo metabolism and culture systems: experience from domestic animals and clinical implications. *Journal of Assisted Reproduction and Genetics* **31**(4), 393-409

Alm, H., Choi, Y., Love, L., Heleil, B., Torner, H., and Hinrichs, K. (2008) Holding bovine oocytes in the absence of maturation inhibitors: kinetics of in vitro maturation and effect on blastocyst development after in vitro fertilization. *Theriogenology* **70**(7), 1024-1029

Atef, A., François, P., Christian, V., and Marc-André, S. (2005) The potential role of gap junction communication between cumulus cells and bovine oocytes during in vitro maturation. *Molecular Reproduction and Development* **71**(3), 358-367

Aktas, H., Wheeler, M. B., Rosenkrans, C. F., First, N. L., and Leibfried-Rutledge, M. L. (1995a) Maintenance of bovine oocytes in prophase of meiosis I by high [cAMP]i. *Journal of Reproduction and Fertility* **105**(2), 227-235

Aktas, H., Wheeler, M. B., First, N. L., and Leibfried-Rutledge, M. L. (1995b) Maintenance of meiotic arrest by increasing [cAMP] i may have physiological relevance in bovine oocytes. *Journal of Reproduction and Fertility* **105**(2), 237-245

Baracaldo, M., Martinez, M., Adams, G., and Mapletoft, R. (2000) Superovulatory response following transvaginal follicle ablation in cattle. *Theriogenology* **53**(6), 1239-1250

Barretto, L. S. S., Castro, V. C., Garcia, J. M., and Mingoti, G. Z. (2007) Role of roscovitine and IBMX on kinetics of nuclear and cytoplasmic maturation of bovine oocytes in vitro. *Animal Reproduction Science* **99**(1-2), 202-207

Basatemur, E., and Sutcliffe, A. (2011) Health of IVM children. *Journal of Assisted Reproduction and Genetics* **28**(6), 489–493

Beck, A. (2014) Analysis of early bovine embryogenesis after in vitro and in vivo oocyte maturation by time-lapse imaging and 3-D confocal microscopy. Doctoral dissertation Thesis, IMU

Behjati, S., and Tarpey, P.S. (2013) What is next generation sequencing? *Archives of Disease in Childhood-Education and Practice* **98**(6), 236-238

Besenfelder, U., Havlicek, V., Kuzmany, A., and Brem, G. (2010) Endoscopic approaches to manage in vitro and in vivo embryo development: Use of the bovine oviduct. *Theriogenology* **73**(6), 768-776

Bilodeau-Goeseels, S. (2003) Effects of phosphodiesterase inhibitors on spontaneous nuclear maturation and cAMP concentrations in bovine oocytes. *Theriogenology* **60**(9), 1679-1690

Blondin, P. (2017) Logistics of large scale commercial IVF embryo production. *Reproduction Fertility and Development* **29**(1), 32-36

Blondin, P., Bousquet, D., Twagiramungu, H., Barnes, F., and Sirard, M.-A. (2002) Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of Reproduction* **66**(1), 38-43

Boa-Amponsem, K., and Minozzi, G. (2006) Commission on genetic resources for food and agriculture: The state of development of biotechnologies as they relate to the management of animal genetic resources and their potential application in developing countries. *Background study paper* **33**

Boland, M., Goulding, D., and Roche, J. (1991) Alternative gonadotrophins for superovulation in cattle. *Theriogenology* **35**(1), 5-17

Bols, P.E.J., Leroy, J., Vanholder, T., and Van Soom, A. (2004) A comparison of a mechanical sector and a linear array transducer for ultrasound-guided transvaginal oocyte retrieval (OPU) in the cow. *Theriogenology* **62**(5), 906-914

Bols, P.E.J., VanSoom, A., and deKruif, A. (1996a) The use of transvaginal oocyte pick-up (OPU): First OPU calves born in Belgium. *Vlaams Diergeneeskundig Tijdschrift* **65**(2), 86-91

Bols, P.E.J., VanSoom, A., Ysebaert, M.T., Vandenheede, J.M.M., and deKruif, A. (1996b) Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes. *Theriogenology* **45**(5), 1001-1014

Bols, P.E.J., Ysebaert, M.T., VanSoom, A., and deKruif, A. (1997) Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact cumulus oocyte complexes. *Theriogenology* **47**(6), 1221-1236

Boni, R. (2012) Ovum pick-up in cattle: a 25 yr retrospective analysis. *Animal Reproduction* **9**(3), 362-369

Canseco, R.S., Sparks, A.E.T., Pearson, R.E., and Gwazdauskas, F.C. (1992) Embryo density and medium volume effects on early murine embryo development. *Journal of Assisted Reproduction and Genetics* **9**(5), 454-457

Carolan, C., Lonergan, P., Khatir, H., and Mermillod, P. (1996) In vitro production of bovine embryos using individual oocytes. *Molecular Reproduction and Development* **45**(2), 145-150

Carolan, C., Lonergan, P., Vanlangendonckt, A., and Mermillod, P. (1995) Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in-vitro. *Theriogenology* **43**(6), 1115-1128

Cebrian-Serrano, A., Salvador, I., and Silvestre, M.A. (2014) Beneficial Effect of Two Culture Systems with Small Groups of Embryos on the Development and Quality of In Vitro-Produced Bovine Embryos. *Anatomia Histologia Embryologia* **43**(1), 22-30

Chaubal, S.A., Molina, J.A., Ohlrichs, C.L., Ferre, L.B., Faber, D.C., Bols, P.E.J., Riesen, J.W., Tian, X., and Yang, X. (2006) Comparison of different transvaginal ovum pick-up protocols to optimise oocyte retrieval and embryo production over a 10-week period in cows. *Theriogenology* **65**(8), 1631-1648

Chebel, R.C., Santos, J.E., Reynolds, J.P., Cerri, R.L., Juchem, S.O., and Overton, M. (2004) Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. *Animal Reproduction Science* **84**(3), 239-255

Coy, P., Grullon, L., Canovas, S., Romar, R., Matas, C., and Avilesl, M. (2008) Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction* **135**(1), 19-27

Dai, S.-J., Xu, C.-L., Wang, J., Sun, Y.-P., and Chian, R.-C. (2012) Effect of culture medium volume and embryo density on early mouse embryonic development: Tracking the development of the individual embryo. *Journal of Assisted Reproduction and Genetics* **29**(7), 617-623

Davies, M.J., Moore, V.M., Willson, K.J., Van Essen, P., Priest, K., Scott, H., Haan, E.A., and Chan, A. (2012) Reproductive technologies and the risk of birth defects. *New England Journal of Medicine* **366**(19), 1803-1813

De Roover, R., Genicot, G., Leonard, S., Bols, P., and Dessy, F. (2005) Ovum pick up and in vitro embryo production in cows superstimulated with an individually adapted superstimulation protocol. *Animal Reproduction Science* **86**(1-2), 13-25

de Sousa, R.V., da Silva Cardoso, C.R., Butzke, G., Dode, M.A.N., Rumpf, R., and Franco, M.M. (2017) Biopsy of bovine embryos produced in vivo and in vitro does not affect pregnancy rates. *Theriogenology* **90**, 25-31

DeBaun, M.R., Niemitz, E.L., and Feinberg, A.P. (2003) Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *The American Journal of Human Genetics* **72**(1), 156-160

Demyda-Peyrás, S., Dorado, J., Hidalgo, M., Anter, J., De Luca, L., Genero, E., and Moreno-Millán, M. (2013) Effects of oocyte quality, incubation time and maturation environment on the number of chromosomal abnormalities in IVF-derived early bovine embryos. *Reproduction, Fertility and Development* **25**(7), 1077-1084

Destouni, A., Esteki, M.Z., Catteeuw, M., Tsuiko, O., Dimitriadou, E., Smits, K., Kurg, A., Salumets, A., Van Soom, A., Voet, T., and Vermeesch, J.R. (2016) Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage-stage chimerism and mixoploidy. *Genome Research* **26**(5), 567-578

Donnay, I., VanLangendonckt, A., Auquier, P., Grisart, B., Vansteenbrugge, A., Massip, A., and Dessy, F. (1997) Effects of co-culture and embryo number on the in vitro development of bovine embryos. *Theriogenology* **47**(8), 1549-1561

Durocher, J., Morin, N., and Blondin, P. (2006) Effect of hormonal stimulation on bovine follicular response and oocyte developmental competence in a commercial operation. *Theriogenology* **65**(1), 102-115

Dyer, S., Chambers, G.M., de Mouzon, J., Nygren, K.G., Zegers-Hochschild, F., Mansour, R., Ishihara, O., Banker, M., and Adamson, G.D. (2016) International Committee for Monitoring Assisted Reproductive Technologies world report: assisted reproductive technology 2008, 2009 and 2010. *Human Reproduction* **31**(7), 1588-1609

Ebner, T., Shebl, O., Moser, M., Mayer, R.B., Arzt, W., and Tews, G. (2010) Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. *Reproductive Biomedicine Online* **21**(6), 762-768

Esteki, M.Z., Dimitriadou, E., Mateiu, L., Melotte, C., Van der Aa, N., Kumar, P., Das, R., Theunis, K., Cheng, J.Q., Legius, E., Moreau, Y., Debrock, S., D'Hooghe, T., Verdyck, P., De Rycke, M., Sermon, K., Vermeesch, J.R., and Voet, T. (2015) Concurrent Whole-Genome Haplotyping and Copy-Number Profiling of Single Cells. *American Journal of Human Genetics* **96**(6), 894-912

European IVF-monitoring Consortium (EIM), European Society of Human Reproduction and Embryology (ESHRE), Calhaz-Jorge, C., De Geyter, C., Kupka, M. S., de Mouzon, J., Erb, K., Mocanu, E., Motrenko, T., Wyns, C., and Goossens, V., (2017) Assisted reproductive technology in Europe, 2013: results generated from European registers by ESHRE

Fair, T., Hyttel, P., and Greve, T. (1995) Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Molecular Reproduction and Development* **42**(4), 437-442

Feng, W.G., Sui, H.S., Han, Z.B., Chang, Z.L., Zhou, P., Liu, D.J., Bao, S., and Tan, J.H. (2007) Effects of follicular atresia and size on the developmental competence of bovine oocytes: A study using the well-in-drop culture system. *Theriogenology* **67**(8), 1339-1350

Fenwick, J., Platteau, P., Murdoch, A., and Herbert, M. (2002) Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. *Human Reproduction* **17**(2), 407-412

Ferreira, E., Vireque, A., Adona, P., Meirelles, F., Ferriani, R., and Navarro, P. (2009) Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **71**(5), 836-848

Ferry, L., Mermillod, P., Massip, A., and Dessy, F. (1994) Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. *Theriogenology* **42**(3), 445-453

Feugang, J.M., Camargo-Rodríguez, O., and Memili, E. (2009) Culture systems for bovine embryos. *Livestock Science* **121**(2), 141-149

Foote, R.H. (2002) The history of artificial insemination: Selected notes and notables. *Journal of Animal Science* **80**, 1-10

Foote, R.H. (2005) 'Highlights in Dairy Cattle Reproduction in the Last 100 Years.' (Internet-First University Press)

Fouladi-Nashta, A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K., and Webb, R. (2005) Differential staining combined with TUNEL labelling to detect apoptosis in preimplantation bovine embryos. *Reproductive Biomedicine Online* **10**(4), 497-502

Frigault, M.M., Lacoste, J., Swift, J.L., and Brown, C.M. (2009) Live-cell microscopy–tips and tools. *Journal of Cell Science* **122**(6), 753-767

Fry, R., Simpson, T., Squires, T., Parr, R., and Damanik, R. (1994) Factors affecting transvaginal oocyte pick up in heifers. *Theriogenology* **41**(1), 197

Fujita, T., Umeki, H., Shimura, H., Kugumiya, K., and Shiga, K. (2006) Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. *Journal of Reproduction and Development* **52**(1), 137-142

Fukuda, Y., Ichikawa, M., Naito, K., and Toyoda, Y. (1990) Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells in vitro up to the blastocyst stage. *Biology of Reproduction* **42**(1), 114-119

Fukui, Y., Sonoyama, T., Mochizuki, H., and Ono, H. (1990) Effects of heparin dosage and sperm capacitation time on in vitro fertilization and cleavage of bovine oocytes matured in vitro. *Theriogenology* **34**(3), 579-591

Galli, C., Duchi, R., Crotti, G., Turini, P., Ponderato, N., Colleoni, S., Lagutina, I., and Lazzari, G. (2003) Bovine embryo technologies. *Theriogenology* **59**(2), 599-616

Garcia-Herreros, M., Carter, T., Villagómez, D., MacAulay, A., Rath, D., King, W., and Lonergan, P. (2010) Incidence of chromosomal abnormalities in bovine blastocysts derived from unsorted and sex-sorted spermatozoa. *Reproduction, Fertility and Development* **22**(8), 1272-1278

Gardner, D.K. (1994) Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biology International* **18**(12), 1163-1179

Gardner, D.K., Lane, M., Spitzer, A., and Batt, P.A. (1994) Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells, amino acids, vitamins, and culturing embryos in groups stimulate development. *Biology of Reproduction* **50**(2), 390-400

Gicquel, C., Gaston, V., Mandelbaum, J., Siffroi, J. P., Flahault, A., and Le Bouc, Y. (2003) In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCNQ1OT gene. *The American Journal of Human Genetics* **72**(5), 1338-1341

Gilchrist, R.B. (2011) Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. *Reproduction Fertility and Development* **23**(1), 23-31

Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Human Reproduction Update* **14**(2), 159-177

Gilchrist, R.B., and Thompson, J.G. (2007) Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology* **67**(1), 6-15

Goovaerts, I.G.F., Leroy, J.L.M.R., Merton, J.S., Van Soom, A., and Bols, P.E.J. (2007) In vitro productie van runderembryo's: een stand van zaken 10 jaar na de eerste OPU/IVP-kalveren in België. *Vlaams Diergeneeskundig Tijdschrift* **76**, 283-292

Goovaerts, I.G.F., Leroy, J.L.M.R., Van Soom, A., De Clercq, J.B.P., Andries, S., and Bols, P.E.J. (2009) Effect of cumulus cell coculture and oxygen tension on the in vitro developmental competence of bovine zygotes cultured singly. *Theriogenology* **71**(5), 729-738

Gordon, I. (2003) 'Laboratory production of cattle embryos.' (CABI)

Grisart, B., Massip, A., and Dessy, F. (1994) Cinematographic analysis of bovine embryo development in serum-free oviduct-conditioned medium. *Journal of Reproduction and Fertility* **101**(2), 257-264

Guixue, Z., Luciano, A. M., Coenen, K., Gandolfi, F., and Sirard, M. A. (2001) The influence of cAMP before or during bovine oocyte maturation on embryonic developmental competence. *Theriogenology* **55**(8), 1733-1743

Gustavsson, I. (1979) Distribution and effects of the 1/29 Robertsonian translocation in cattle. *Journal of Dairy Science* **62**(5), 825-835

Gutierrez-Adan, A., White, C.R., Van Soom, A., and Mann, M.R. (2015) Why we should not select the faster embryo: lessons from mice and cattle. *Reproduction, Fertility and Development* **27**(5), 765-775

Hansen, M., Kurinczuk, J.J., Bower, C., and Webb, S. (2002) The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. *New England Journal of Medicine* **346**(10), 725-730

Hare, W., Singh, E.L., Betteridge, K., Eaglesome, M., Randall, G., Mitchell, D., Bilton, R., and Trounson, A. (1980) Chromosomal analysis of 159 bovine embryos collected 12 to 18 days after estrus. *Canadian Journal of Genetics and Cytology* **22**(4), 615-626

Hashimoto, S. (2009) Application of In Vitro Maturation to Assisted Reproductive Technology. *Journal of Reproduction and Development* **55**(1), 1-10

Hashimoto, S., Takakura, R., Kishi, M., Sudo, T., Minami, N., and Yamada, M. (1999) Ultrasound-guided follicle aspiration: The collection of bovine cumulus-oocyte complexes from ovaries of slaughtered or live cows. *Theriogenology* **51**(4), 757-765

Hasler, J.F. (1998) The current status of oocyte recovery, in vitro embryo production and embryo transfer in domestic animals, with an emphasis on the bovine. *Journal of Animal Science* **76**, 52-74

Hasler, J.F., Cardey, E., Stokes, J., and Bredbacka, P. (2002) Nonelectrophoretic PCR-sexing of bovine embryos in a commercial environment. *Theriogenology* **58**(8), 1457-1469

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E., and Trimmer, S.A. (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43**(1), 141-152

Herr, C., and Reed, K. (1991) Micronanipulation of bovine embryos for sex determination. *Theriogenology* **35**(1), 45-54

Hoelker, M., Rings, F., Lund, Q., Phatsara, C., Schellander, K., and Tesfaye, D. (2010) Effect of Embryo Density on In vitro Developmental Characteristics of Bovine Preimplantative Embryos with Respect to Micro and Macroenvironments. *Reproduction in Domestic Animals* **45**(5), E138-E145

Hornak, M., Kubicek, D., Broz, P., Hulinska, P., Hanzalova, K., Griffin, D., Machatkova, M., and Rubes, J. (2016) Aneuploidy Detection and mtDNA Quantification in Bovine Embryos with Different Cleavage Onset Using a Next-Generation Sequencing-Based Protocol. *Cytogenetic and Genome Research* **150**(1), 60-67

Hosoe, M., Yoshida, N., Hashiyada, Y., Teramoto, H., Takahashi, T., and Niimura, S. (2014) Sericin accelerates the production of hyaluronan and decreases the incidence of polyspermy fertilization in bovine oocytes during in vitro maturation. *Journal of Reproduction and Development* **60**(4), 268-273

Hunter, R. (1994) Modulation of gamete and embryonic microenvironments by oviduct glycoproteins. *Molecular Reproduction and Development* **39**(2), 176-181

IETS (2016) Embryo technology newsletter. In Vol. 34.

Iwasaki, S., Hamano, S., Kuwayama, M., Yamashita, M., Ushijima, H., Nagaoka, S., and Nakahara, T. (1992) Developmental changes in the incidence of chromosome anomalies of bovine embryos fertilized in vitro. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **261**(1), 79-85

Izquierdo, D., Villamediana, P., Palomo, M., Mogas, T., and Paramio, M. (1998) Effect of sperm capacitation and fertilization media on IVF and early embryo development of prepubertal goat oocytes. *Theriogenology* **49**(8), 1501-1513

Johnson, J.P., Schoof, J., Beischel, L., Schwancke, C., Goldberg, J., Black, L., Ross, L., and Bhatt, S. (2018) Detection of a case of Angelman syndrome caused by an imprinting error in 949 pregnancies analyzed for AS following IVF *Journal of Assisted Reproduction and Genetics*, 1-4

Kato, Y., and Tsunoda, Y. (1994) Effects of the culture densisty of mouse zygotes on the development in vitro and in vivo. *Theriogenology* **41**(6), 1315-1322

Khosla, S., Dean, W., Brown, D., Reik, W., and Feil, R. (2001) Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biology of Reproduction* **64**(3), 918-926

Khurana, N.K., and Niemann, H. (2000) Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology* **54**(5), 741-756

Kohram, H., Bousquet, D., Durocher, J., and Guilbault, L.A. (1998) Alteration of follicular dynamics and superovulatory responses by gonadotropin releasing hormone and follicular structure in cattle: A field trial. *Theriogenology* **49**(6), 1165-1174

Kues, W., Sudheer, S., Herrmann, D., Carnwath, J., Havlicek, V., Besenfelder, U., Lehrach, H., Adjaye, J., and Niemann, H. (2008) Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo. *Proceedings of the National Academy of Sciences* **105**(50), 19768-19773

Lagutina, I., Ponderato, N., Lazzari, G., and Galli, C. (2002) Kinetics of oocyte maturation and subsequent development of IVF, parthenogenetic, and NT bovine embryos after meiotic inhibition with roscovitine. *Cloning and Stem Cells* **4**(2), 113-119

Lamb, G., Dahlen, C., Larson, J., Marquezini, G., and Stevenson, J. (2010) Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: a review. *Journal of animal science* **88**(13), E181-E192

Lane, M., and Gardner, D.K. (1992) Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. *Human Reproduction* **7**(4), 558-562

Lane, M., and Gardner, D.K. (2003) Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biology of Reproduction* **69**(4), 1109-1117

Lane, M., and Gardner, D.K. (2005) Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reproduction, Fertility and Development* **17**(3), 371-378

Lane, M., and Gardner, D.K. (2007) Embryo culture medium: which is the best?. *Best Practice & Research Clinical Obstetrics & Gynaecology* **21**(1), 83-100

Lane, M., Maybach, J.M., and Gardner, D.K. (2002) Ammonium affects ICM development, metabolism, intracellular pH, and fetal growth rates. *Biology of Reproduction* **66**, 104-104

Laurincik, J., Kopecny, V., and Hyttel, P. (1994) Pronucleus development and DNA synthesis in bovine zygotes in vivo. *Theriogenology* **42**(8), 1285-1293

Lemmen, J., Agerholm, I., and Ziebe, S. (2008) Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. *Reproductive Biomedicine Online* **17**(3), 385-391

Lonergan, P., Carolan, C., VanLangendonckt, A., Donnay, I., Khatir, H., and Mermillod, P. (1996) Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro. *Biology of Reproduction* **54**(6), 1420-1429

Lonergan, P., Dinnyes, A., Fair, T., Yang, X., and Boland, M. (2000) Bovine oocyte and embryo development following meiotic inhibition with butyrolactone I. *Molecular Reproduction and Development* **57**(2), 204-209

Lonergan, P., Monaghan, P., Rizos, D., Boland, M., and Gordon, I. (1994) Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. *Molecular Reproduction and Development* **37**(1), 48-53

Looney, C., Lindsey, B., Gonseth, C., and Johnson, D. (1994) Commercial aspects of oocyte retrieval and in vitro fertilization (IVF) for embryo production in problem cows. *Theriogenology* **41**(1), 67-72

Lovie, M., Garcia, A., Hackett, A., and Mapletoft, R. (1994) The effect of dose schedule and route of administration on superovulatory response to Folltropin in Holstein cows. *Theriogenology* **41**(1), 241

Luciano, A. M., Lodde, V., Beretta, M. S., Colleoni, S., Lauria, A., and Modina, S. (2005) Developmental capability of denuded bovine oocyte in a Co-culture system with intact cumulus-oocyte complexes: Role of cumulus cells, cyclic adenosine 3′, 5′-monophosphate, and glutathione. *Molecular Reproduction and Development* **71**(3), 389-397

Machado, S.A., Reichenbach, H.D., Weppert, M., Wolf, E., and Goncalves, P.B.D. (2006) The variability of ovum pick-up response and in vitro embryo production from monozygotic twin cows. *Theriogenology* **65**(3), 573-583

Maher, E.R., Brueton, L.A., Bowdin, S.C., Luharia, A., Cooper, W., Cole, T.R., MacDonald, F., Sampson, J.R., Barratt, C.L., Reik, W., and Hawkins, M. M. (2003) Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *Journal of Medical Genetics* **40**(1), 62-64

Maheshwari, A., Raja, E.A., and Bhattacharya, S. (2016) Obstetric and perinatal outcomes after either fresh or thawed frozen embryo transfer: an analysis of 112,432 singleton pregnancies recorded in the Human Fertilisation and Embryology Authority anonymized dataset. *Fertility and Sterility* **106**(7), 1703-1708

Mandawala, A., Harvey, S., Roy, T., and Fowler, K. (2016) Cryopreservation of animal oocytes and embryos: Current progress and future prospects. *Theriogenology* **86**(7), 1637-1644

Market Velker, B.A., Denomme, M.M., and Mann, M.R. (2012) Loss of genomic imprinting in mouse embryos with fast rates of preimplantation development in culture. *Biology of Reproduction* **86**(5), 143, 1-16

Marquant-Le Guienne, B., Humblot, P., Thibier, M., and Thibault, C. (1990) Evaluation of bull semen fertility by homologous in vitro fertilization tests. *Reproduction Nutrition Development* **30**(2), 259-266

Marquant-Leguienne, B., and Humblot, P. (1998) Practical measures to improve in vitro blastocyst production in the bovine. *Theriogenology* **49**(1), 3-11

Martinez, A.G., Brogliatti, G.M., Valcarcel, A., and de las Heras, M.A. (2002) Pregnancy rates after transfer of frozen bovine embryos: a field trial. *Theriogenology* **58**(5), 963-972

Mather, J.P., and Roberts, P.E. (1998) 'Introduction to cell and tissue culture: theory and technique.' (Springer Science and Business Media)

Mayes, M. A., and Sirard, M. A. (2002) Effect of type 3 and type 4 phosphodiesterase inhibitors on the maintenance of bovine oocytes in meiotic arrest. *Biology of Reproduction* **66**(1), 180-184

Meirelles, F., Caetano, A., Watanabe, Y., Ripamonte, P., Carambula, S., Merighe, G., and Garcia, S. (2004) Genome activation and developmental block in bovine embryos. *Animal Reproduction science* **82**, 13-20

Ménézo, Y., Veiga, A., and Benkhalifa, M. (1998) Improved methods for blastocyst formation and culture. *Human Reproduction* **13**(suppl_4), 256-265

Ménézo, Y.J., and Hérubel, F. (2002) Mouse and bovine models for human IVF. *Reproductive Biomedicine Online* **4**(2), 170-175

Merton, J., de Roos, A.P.W., Mullaart, E., de Ruigh, L., Kaal, L., Vos, P., and Dieleman, S.J. (2003) Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* **59**(2), 651-674

Merton, J.S., de Roos, A.P.W., Koenen, E.P.C., Roelen, B.A.J., Vos, P.L.A.M., Mullaart, E., and Knijn, H.M. (2012) Bovine OPU-Derived Oocytes can be Matured In Vitro for 16-28 h with Similar Developmental Capacity. *Reproduction in Domestic Animals* **47**(6), 1037-1042

Merton, J.S., Knijn, H.M., Flapper, H., Dotinga, F., Roelen, B.A.J., Vos, P.L.A.M., and Mullaart, E. (2013) Cysteamine supplementation during in vitro maturation of slaughterhouse- and opu-derived bovine oocytes improves embryonic development without affecting cryotolerance, pregnancy rate, and calf characteristics. *Theriogenology* **80**(4), 365-371

Nakahara, T., Iwase, A., Goto, M., Harata, T., Suzuki, M., Ienaga, M., Kobayashi, H., Takikawa, S., Manabe, S., and Kikkawa, F. (2010) Evaluation of the safety of time-lapse observations for human embryos. *Journal of assisted Reproduction and genetics* **27**(2-3), 93-96

Nedambale, T., Dinnyes, A., Groen, W., Dobrinsky, J., Tian, X., and Yang, X. (2004) Comparison on in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. *Theriogenology* **62**(3), 437-449

Nicholas, F.W., and Hobbs, M. (2012) 'Online Mendelian Inheritance in Animals (OMIA).' (Frank Nicholas)

Nicodemo, D., Pauciullo, A., Cosenza, G., Peretti, V., Perucatti, A., Di Meo, G., Ramunno, L., Iannuzzi, L., Rubes, J., and Di Berardino, D. (2010) Frequency of aneuploidy in in vitro–matured MII oocytes and corresponding first polar bodies in two dairy cattle (Bos taurus) breeds as determined by dual-color fluorescent in situ hybridization. *Theriogenology* **73**(4), 523-529

Norris, R.P., Ratzan, W.J., Freudzon, M., Mehlmann, L.M., Krall, J., Movsesian, M.A., Wang, H., Ke, H., Nikolaev, V.O., and Jaffe, L.A. (2009) Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* **136**(11), 1869-1878

Nussbaum, R.L., McInnes, R.R., and Willard, H.F. (2015) Thompson and Thompson Genetics in Medicine*. Elsevier Health Services.*

O'Doherty, E.M., Wade, M.G., Hill, J.L., and Boland, M.P. (1997) Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* **48**(1), 161-9

O'Neill, C. (2005) The role of paf in embryo physiology. *Human Reproduction Update* **11**(3), 215-228

O'Neill, C. (2008) The potential roles for embryotrophic ligands in preimplantation embryo development. *Human Reproduction Update* **14**(3), 275-288

Ooe, M., Rajamahendran, R., Boediono, A., and Suzuki, T. (1997) Ultrasound-guided follicle aspiration and IVF in dairy cows treated with FSH after removal of the dominant follicle at different stages of the estrous cycle. *Journal of Veterinary Medical Science* **59**(5), 371-376

Ørstavik, K.H., Eiklid, K., Van Der Hagen, C.B., Spetalen, S., Kierulf, K., Skjeldal, O., and Buiting, K. (2003) Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic sperm injection. *American Journal of Human Genetics* **72**(1), 218

Ottosen, L.D., Hindkjær, J., and Ingerslev, J. (2007) Light exposure of the ovum and preimplantation embryo during ART procedures. *Journal of Assisted Reproduction and Genetics* **24**(2-3), 99-103

Palasz, A.T., Brena, P.B., De la Fuente, J., and Gutierrez-Adan, A. (2010) The effect of bovine embryo culture without proteins supplements until day 4 on transcription level of hyaluronan synthases, receptors and mtDNA content. *Zygote* **18**(2), 121-129

Paramio, M. T., and Izquierdo, D. (2014) Current status of in vitro embryo production in sheep and goats. *Reproduction in Domestic Animals* **49**(s4), 37-48

Paria, B.C., and Dey, S.K. (1990) Preimplantation embryo development in vitro - cooperatice interactions among embryos and role of growth factors. *Proceedings of the National Academy of Sciences of the United States of America* **87**(12), 4756-4760

Parrish, J.J., Suskoparrish, J.L., Leibfriedrutledge, M.L., Critser, E.S., Eyestone, W.H., and First, N.L. (1986) Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology* **25**(4), 591-600

Paul, J., Looney, C., Lindsay, B., and Godke, R. (1995) Gonadotropin stimulation of cattle donors at estrus for transvaginal oocyte collection. *Theriogenology* **1**(43), 294

Pieterse, M.C., Kappen, K.A., Kruip, T.A.M., and Taverne, M.A.M. (1988) Aspiration of bovine oocytes during trans-vaginal ultrasound scanning of the ovaries. *Theriogenology* **30**(4), 751-762

Pincus, G., and Enzmann, E.V. (1935) The comparative behavior of mammalian eggs in vivo and in vitro. *The Journal of Experimental Medicine* **62**(5), 665-75

Ponderato, N., Lagutina, I., Crotti, G., Turini, P., Galli, C., and Lazzari, G. (2001) Bovine oocytes treated prior to in vitro maturation with a combination of butyrolactone I and roscovitine at low doses maintain a normal developmental capacity. *Molecular Reproduction and Development* **60**(4), 579-585

Pribenszky, C., Losonczi, E., Molnár, M., Lang, Z., Mátyás, S., Rajczy, K., Molnár, K., Kovács, P., Nagy, P., and Conceicao, J. (2010) Prediction of in-vitro developmental competence of early cleavage-stage mouse embryos with compact time-lapse equipment. *Reproductive Biomedicine Online* **20**(3), 371-379

Pursley, J., Wiltbank, M., Stevenson, J., Ottobre, J., Garverick, H., and Anderson, L. (1997) Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus1. *Journal of Dairy Science* **80**(2), 295-300

Raudsepp, T., and Chowdhary, B.P. (2016) Chromosome aberrations and fertility disorders in domestic animals. *Annual Review of Animal Biosciences* **4**, 15-43

Reed, M.L. (2012) Culture systems: embryo density. *Methods in Molecular Biology* **912**, 273-312

Rizos, D., Ward, F., Boland, M., and Lonergan, P. (2001) Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification. *Theriogenology* **56**(1), 1-16

Rizos, D., Ward, F., Duffy, P., Boland, M. P., and Lonergan, P. (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development* **61**(2), 234-248

Roth, Z., and Hansen, P. (2005) Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction* **129**(2), 235-244

Salvador, I., Cebrian-Serrano, A., Salamone, D., and Silvestre, M.A. (2011) Effect of number of oocytes and embryos on in vitro oocyte maturation, fertilization and embryo development in bovine. *Spanish Journal of Agricultural Research* **9**(3), 744-752

Sanbuissho, A., Coskun, S., and Lin, Y. C. (1992) Role of cyclic adenosine monophosphate (cAMP) in vitro on bovine oocyte maturation. *Theriogenology* **38**(1), 153-163

Sato, E., Matsuo, M., and Miyamoto, H. (1990) Meiotic maturation of bovine oocytes in vitro: improvement of meiotic competence by dibutyryl cyclic adenosine 3', 5'-monophosphate. *Journal of Animal Science* **68**(4), 1182-1187

Scott, C. A., Robertson, L., Paterson, C., and Boyd, J. S. (1994) Technical aspects of transvaginal ultrasound-guided follicular aspiration in cows. *The Veterinary Record* **134**(17), 440-443

Somfai, T., Inaba, Y., Aikawa, Y., Ohtake, M., Kobayashi, S., Konishi, K., and Imai, K. (2010) Relationship Between the Length of Cell Cycles, Cleavage Pattern and Developmental Competence in Bovine Embryos Generated by In Vitro Fertilization or Parthenogenesis. *Journal of Reproduction and Development* **56**(2), 200-207

Spanos, S., Becker, D.L., Winston, R.M.L., and Hardy, R. (2000) Anti-apoptotic action of insulin-like growth factor-I during human preimplantation embryo development. *Biology of Reproduction* **63**(5), 1413-1420

Spindler, R.E., and Wildt, D.E. (2002) Quality and age of companion felid embryos modulate enhanced development by group culture. *Biology of Reproduction* **66**(1), 167-173

Stokes, P.J., Abeydeera, L.R., and Leese, H.J. (2005) Development of porcine embryos in vivo and in vitro; evidence for embryo 'cross talk' in vitro. *Developmental biology* **284**(1), 62-71

Sugimura, S., AKAI, T., and IMAI, K. (2017) Selection of viable in vitro-fertilized bovine embryos using time-lapse monitoring in microwell culture dishes. *Journal of Reproduction and Development* **63**(4), 353-357

Sugimura, S., Akai, T., Somfai, T., Hirayama, M., Aikawa, Y., Ohtake, M., Hattori, H., Kobayashi, S., Hashiyada, Y., Konishi, K., and Imai, K. (2010) Time-Lapse Cinematography-Compatible Polystyrene-Based Microwell Culture System: A Novel Tool for Tracking the Development of Individual Bovine Embryos. *Biology of Reproduction* **83**(6), 970-978

Sutcliffe, A.G., Peters, C.J., Bowdin, S., Temple, K., Reardon, W., Wilson, L., Clayton-Smith, J., Brueto, L.A., Bannister, W., and Maher, E.R. (2005) Assisted reproductive therapies and imprinting disorders—a preliminary British survey. *Human Reproduction* **21**(4), 1009-1011

Szczerbal, I., and Switonski, M. (2016) Chromosome abnormalities in domestic animals as causes of disorders of sex development or impaired fertility. In 'Insights from Animal Reproduction.' (InTech)

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., and De Kruif, A. (2002a) Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Molecular Reproduction and Development* **61**(3), 414-424

Tanghe, S., Van Soom, A., Sterckx, V., Maes, D., and De Kruif, A. (2002b) Assessment of different sperm quality parameters to predict in vitro fertility of bulls. *Reproduction in Domestic Animals* **37**(3), 127-132

Thibier, M. (2005) The zootechnical applications of biotechnology in animal reproduction: current methods and perspectives. *Reproduction Nutrition Development* **45**(3), 235-242

Thompson, J.G., Gardner, D.K., Pugh, P.A., McMillan, W.H., and Tervit, H.R. (1995) Lamb birth-weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biology of Reproduction* **53**(6), 1385-1391

Tortora, G., and Derrickson, B. (2008) 'Principles of Anatomy and Physiology' (John Wiley and Sons)

Touati, K., Beckers, J., and Ectors, F. (1991) Hormonal control of folliculogenesis in the bovine: better superovulatory responses after pure FSH administration preceding the classical treatment. *Theriogenology* **35**(1), 285

Treangen, T.J., and Salzberg, S.L. (2011) Repetitive DNA and next-generation sequencing: computational challenges and solutions. *Nature Reviews Genetics* **13**(1), 3117

Udroiu, I., and Sgura, A. (2017) Cytogenetic tests for animal production: state of the art and perspectives. *Animal Genetics*

Ushizawa, K., Herath, C.B., Kaneyama, K., Shiojima, S., Hirasawa, A., Takahashi, T., Imai, K., Ochiai, K., Tokunaga, T., and Tsunoda, Y. (2004) cDNA microarray analysis of bovine embryo gene expression profiles during the pre-implantation period. *Reproductive Biology and Endocrinology* **2**(1), 77

Vajta, G., Peura, T.T., Holm, P., Paldi, K., Greve, T., Trounson, A.O., and Callesen, H. (2000) New method for culture of zona-included or zona-free embryos: The Well of the Well (WOW) system. *Molecular Reproduction and Development* **55**(3), 256-264

Vajta, G., Rienzi, L., Cobo, A., and Yovich, J. (2010) Embryo culture: can we perform better than nature? *Reproductive Biomedicine Online* **20**(4), 453-469

Van Soom, A., and Boerjan, M. (2002) 'Assessment of mammalian embryo quality.' (Springer)

Van Soom, A., Van Vlaenderen, I., Mahmoudzadeh, A., Deluyker, H., and de Kruif, A. (1992) Compaction rate of in vitro fertilized bovine embryos related to the interval from insemination to first cleavage. *Theriogenology* **38**(5), 905-919

Van Soom, A., Ysebaert, M.T., and de Kruif, A. (1997) Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Molecular Reproduction and Development* **47**(1), 47-56

Van Wagtendonk-De Leeuw, A., Den Daas, J., Kruip, T.A., and Rall, W. (1995) Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. *Cryobiology* **32**(2), 157-167

Van Wagtendonk-de Leeuw, A., Den Daas, J., and Rall, W. (1997) Field trial to compare pregnancy rates of bovine embryo cryopreservation methods: vitrification and one-step dilution versus slow freezing and three-step dilution. *Theriogenology* **48**(7), 1071-1084

Van Wagtendonk-de Leeuw, A.M. (2006) Ovum pick up and in vitro production in the bovine after use in several generations: a 2005 status. *Theriogenology* **65**(5), 914-925

Vanneste, E., Voet, T., Melotte, C., Debrock, S., Sermon, K., Staessen, C., Liebaers, I., Fryns, J.P., D'Hooghe, T., and Vermeesch, J. R. (2009) What next for preimplantation genetic screening? High mitotic chromosome instability rate provides the biological basis for the low success rate. *Human Reproduction* **24**(11), 2679-2682

Verberckmoes, S., Van Soom, A., Dewulf, J., and de Kruif, A. (2005) Comparison of three diluents for the storage of fresh bovine semen. *Theriogenology* **63**(3), 912-922

Vermeesch, J.R., Voet, T., and Devriendt, K. (2016) Prenatal and pre-implantation genetic diagnosis. *Nature Reviews Genetics* **17**(10), 643-656

Vieira, L.M., Rodrigues, C.A., Netto, A.C., Guerreiro, B.M., Silveira, C.R.A., Moreira, R.J.C., Sa, M.F., Bo, G.A., Mapletoft, R.J., and Baruselli, P.S. (2014) Superstimulation prior to the ovum pick-up to improve in vitro embryo production in lactating and non-lactating Holstein cows. *Theriogenology* **82**(2), 318-324

Viuff, D., Palsgaard, A., Rickords, L., Lawson, L.G., Greve, T., Schmidt, M., Avery, B., Hyttel, P., and Thomsen, P.D. (2002) Bovine embryos contain a higher proportion of polyploid cells in the trophectoderm than in the embryonic disc. *Molecular Reproduction and Development* **62**(4), 483-488

Viuff, D., Rickords, L., Offenberg, H., Hyttel, P., Avery, B., Greve, T., Olsaker, I., Williams, J.L., Callesen, H., and Thomsen, P.D. (1999) A high proportion of bovine blastocysts produced in vitro are mixoploid. *Biology of Reproduction* **60**(6), 1273-1278

Walls, M. L., Hunter, T., Ryan, J. P., Keelan, J. A., Nathan, E., and Hart, R. J. (2014) In vitro maturation as an alternative to standard in vitro fertilization for patients diagnosed with polycystic ovaries: a comparative analysis of fresh, frozen and cumulative cycle outcomes. *Human Reproduction* **30**(1), 88-96

Ward, F.A., Lonergan, P., Enright, B.P., and Boland, M.P. (2000) Factors affecting recovery and quality of oocytes for bovine embryo production in vitro using ovum pick-up technology. *Theriogenology* **54**(3), 433-446

Wong, C.C., Loewke, K.E., Bossert, N.L., Behr, B., De Jonge, C.J., Baer, T.M., and Pera, R.A.R. (2010) Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nature biotechnology* **28**(10), 1115-1121

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E., De Sutter, P., Vandaele, L., and Van Soom, A. (2014a) Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reproduction Fertility and Development* **26**(5), 717-724

Wydooghe, E., Vandaele, L., Beek, J., Favoreel, H., Heindryckx, B., De Sutter, P., and Van Soom, A. (2011) Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Analytical Biochemistry* **416**(2), 228-230

Wydooghe, E., Vandaele, L., Piepers, S., Dewulf, J., Van den Abbeel, E., De Sutter, P., and Van Soom, A. (2014b) Individual commitment to a group effect: strengths and weaknessess of bovine embryo group culture. *Reproduction* **148**(5), 519-529

Wydooghe, E., Vandaele, L., Heras, S., De Sutter, P., Deforce, D., Peelman, L., De Schauwer, C., and Van Soom, A. (2017) Autocrine embryotropins revisited: how do embryos communicate with each other in vitro when cultured in groups? *Biological Reviews* **92**(1), 505-520

Xu, K.P., and Greve, T. (1988) A detailed analysis of early events during in vitro fertilization of bovine follicular oocytes. *Journal of Reproduction and Fertility* **82**(1), 127-134

Yoshioka, K., Suzuki, C., and Iwamura, S. (2000) Effects of activin A and follistatin on developmental kinetics of bovine embryos: cinematographic analysis in a chemically defined medium. *Journal of Reproduction and Fertility* **118**(1), 119-125

Young, L.E., Sinclair, K.D., and Wilmut, I. (1998) Large offspring syndrome in cattle and sheep. *Reviews of Reproduction* **3**(3), 155-163

Yuan, Y.Q., Van Soom, A., Leroy, J., Dewulf, J., Van Zeveren, A., de Kruif, A., and Peelman, L. (2005) Apoptosis in cumulus cells, but not in oocytes, may influence bovine embryonic developmental competence. *Theriogenology* **63**(8), 2147-2163

CHAPTER 2

AIMS OF THE STUDY

In commercial settings, bovine *in vitro* embryo production still yields low blastocyst rates. There are two main factors responsible for low embryo development. First, donors are often yielding low oocyte numbers at OPU sessions and moreover, these oocytes have a variable, mostly minor quality. Second, it is necessary to know at all times the embryo's identity, as from which donor-semen combination the embryo originates. In the end, only small groups are made during the complete *in vitro* production process. The general aim of this thesis is to establish a more efficient embryo production protocol and gain insights in kinetics and genetics of early embryo development explaining the overall low efficiency in commercial settings.

The specific objectives of the present thesis were formulated as follows:

1. To simplify the transportation protocol by using a commercial available holding medium which keeps the oocytes in meiotic arrest during storage (**Chapter 3**),
2. To improve developmental capacities of the donors' oocytes and embryos by using the embryo Corral® dish during *in vitro* maturation and *in vitro* culture (**Chapter 4**),
3. To assess the predictive value of early developmental events and define 'early', 'intermediate' and 'late' cleaving in serum-free conditions using Well-of-the-Well dishes and time-lapse imaging during *in vitro* culture (**Chapter 5**),
4. To investigate the chromosomal instability in early cleavage stage embryos, not only in *in vivo* derived but also in *in vitro* produced embryos (**Chapter 6**).

CHAPTER 3

HOLDING IMMATURE BOVINE OOCYTES IN A COMMERCIAL EMBRYO HOLDING MEDIUM

Modified from

Holding immature bovine oocytes in a commercial embryo holding medium: high developmental competence for up to 10 hours at room temperature

Bogado Pascottini O*, Catteeuw M*, Van Soom A, Opsomer G

* These authors contributed equally to this work.

Theriogenology, 2018, 107, 63-69

Abstract

Bovine *in vitro* embryo production (IVP) following Ovum Pick-up (OPU) is all too often hampered by a large time gap between the harvest of oocytes of the first and last OPU session of the day. Immediately after retrieval, oocyte maturation is initiated, resulting in oocytes maturing at different time points which necessitates laborious scheduling of the IVP process. In this study, the potential of a commercial embryo holding medium (EHM; Syngro, Bioniche Inc.) to hold immature bovine oocytes was validated. We assessed the effect of holding time and temperature on (1) oocytes' maturation; (2) blastocyst development and quality at day 8 post insemination; and (3) blastocyst yield in small groups of oocytes/zygotes simulating OPU settings. Oocytes, harvested from slaughterhouse ovaries, were held for 6 h (either at 4°C, room temperature [RT; 22 to 25°C], or 38.5°C), for 10 h (at 4°C or RT), and for 14 h (only at RT) in 1 mL sterile glass osmometer tubes filled with EHM prior to standard maturation (22h at 38.5°C) and subsequent IVP. Results were compared with controls in which no prior holding was applied. Differences between the treated and control groups were assessed by generalized mixed-effects models and considered significant at $P < 0.05$. Generally, oocytes held up to 14 h in EHM at different temperatures remained at the germinal vesicle stage. Holding immature oocytes in EHM for 6 h at 38.5°C and for 10 h at 4°C significantly decreased maturation (57.1±4.1% VS 80.9±3.2% and 68.6±3.5% VS 80.7±2.9%; respectively), and development (11.0 ± 1.8% VS 36.2 ± 2.8% and 20.1 ± 3.3% VS 40.6 ± 4.6%) ($P < 0.05$). However, holding in EHM for both 6 and 10 h at RT, did not affect the maturation rates (83.2±2.9% and 78.9±3.2%) nor day 8 blastocyst rates (35.2±2.7% and 40.2±4.5%). Prolonging holding time to 14 h in RT decreased maturation and day 8 blastocyst yield (71.9±3.5% VS 84.5±2.7% and 25.7±2.5% VS 39.5±2.8%, respectively) ($P < 0.05$). Holding oocytes in EHM did not significantly affect embryonic quality as assessed by differential apoptotic staining in any of the time points. To simulate OPU-settings, small groups of 10 oocytes were held in EHM for 6 or 10 h at RT. When subsequently matured, fertilized and cultured per 8 zygotes, day 8 blastocyst rate was not affected (19.8±3.5% VS 20.6±3.6% and 18.8±3.6% VS 18.3±3.4%). In conclusion, immature bovine oocytes can be successfully conserved in EHM at RT for up to 10 h without compromising their embryonic developmental competence nor quality.

Introduction

Over the last couple of years, ovum pick-up (OPU) followed by *in vitro* embryo production (IVP) has gained popularity and is regarded as a more efficient alternative to bovine multiple ovulation and embryo transfer programs, as it can be applied irrespective of the donor's reproductive status (Boni 2012). The demand for these OPU-IVP embryos is therefore increasing, resulting in farmers all over the world interested in applying OPU in their breeding programs (Boni 2012). While OPU sessions are performed under field conditions, the harvested oocytes need to be transported to specialized laboratories in order to generate embryos under a controlled environment. These valuable gametes should therefore be handled with care; however, transportation remains a major challenge. Usually, immature oocytes are transported in maturation media in large portable incubators, keeping the temperature and atmosphere under control (Alm *et al.* 2008). However, this results in oocytes arriving at the laboratory at very different maturation stages significantly impairing the scheduling of the subsequent IVP procedure.

In small follicles, granulosa and cumulus cells are activating the *in vivo* production of maturation inhibiting factors, such as cyclic adenosine monophosphate (cAMP), which are transferred through gap junctions to the oocytes, keeping the oocytes in the prophase of meiosis I (Tanghe *et al.* 2002). When mammalian oocytes are removed from the follicle, the connection with these follicular cells and the contact with the follicular fluid containing the inhibitors, is lost. Typically, the meiotic progression is therefore initiated immediately after oocyte collection (Bilodeau-Goeseels 2011). Final IVP results are however dependent on the strict timing between oocyte maturation and fertilization (Blondin 2017). Due to the often large time span between the first and last oocyte collection of an OPU session, oocyte maturation is ending at widely different time points. Because the subsequent lab work, such as fertilization, has to take place at strict times, this asynchrony in maturation leads to late evening or night work. Therefore, the rising demand for IVP bovine embryos necessitates further simplification of the applied protocols. Holding immature bovine oocytes after OPU in handy transporting tubes containing a commercially available medium that is able to keep the oocytes in meiotic arrest while fully maintaining developmental competences, will not only facilitate logistics but also reduce the costs and allow more convenient scheduling for further manipulations in the laboratory. In horses, holding immature oocytes during long lasting shipping is already common practice (so that oocytes may be transported to distantly located labs). In this regard, the potential holding capacity of distinct media for transport of immature equine oocytes was evaluated at different temperatures and for different periods of time (Choi *et al.* 2006; Foss *et al.* 2013; Galli *et al.* 2014; Martino *et al.* 2014; Dini *et al.* 2016).

In cattle, media containing meiotic inhibitors were evaluated to hold immature oocytes to improve their developmental competence (Lonergan *et al.* 2000; Ponderato *et al.* 2001; Lagutina *et al.* 2002; Adona *et al.* 2008). Meiotic inhibitors are however not recommended due to their limited availability and potential toxicity allowing only specialized personnel to work with these compounds. Alm *et al.* (2008) demonstrated that immature bovine oocytes held for 16 to 18 h at room temperature (RT) in the absence of meiotic inhibitors developed into blastocysts, but oocytes' kinetics were changed as more oocytes were matured after a reduced maturation time following the holding period. However, it is still unknown what the effects of holding immature bovine oocytes are on maturation kinetics and developmental capacity for different time spans, at distinct temperatures, and in various group sizes. Therefore, the present study aimed to evaluate timing and temperature of a commercially available embryo holding medium (EHM) to hold immature bovine oocytes by determining (1) oocytes' maturation status, (2) embryo development and quality and (3) blastocyst development in small groups of zygotes, simulating OPU procedures.

Methods

Media and reagents

Basic Eagle's Medium amino acids, Minimal Essential Medium (MEM) non-essential amino acids (100 ×), TCM-199-medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium) and all other components were obtained from Sigma (Schnelldorf, Germany), unless otherwise stated. All the media were filter-sterilized using a 0.22 µm filter (Pall Corporation, Ann Arbor, MI, USA) before use.

In vitro embryo production protocol

The ovaries were collected at the local slaughterhouse and processed within 2 h. Only follicles between 2 and 8 mm were punctured, and follicular fluid containing the oocytes was collected in 2.5 mL of Hepes-Tyrode's albumin-pyruvate-lactate (TALP). Embryos were produced as previously described by Wydooghe *et al.* (Wydooghe *et al.* 2014). Briefly, cumulus oocyte complexes (COCs) with uniformly granulated cytoplasm and surrounded by at least 3 compact layers of cumulus cells were grouped per 60 and placed in 500 µL maturation medium, consisting of modified bicarbonate-buffered TCM-199 supplemented with 50 µg/mL gentamycin and 20 ng/mL epidermal growth factor (EGF) for 22 h at 38.5°C in 5% CO_2 in humidified air. After maturation, frozen-thawed semen of a previously tested bull was used. Spermatozoa were separated over a discontinuous Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden) and sperm concentration was adjusted to 1×10^6

spermatozoa/mL using IVF-TALP, supplemented with 6 mg/ml BSA (Sigma A8806) and 25 µg/ml heparin. Matured oocytes were incubated in 500 µL IVF-TALP with spermatozoa for 21 h at 38.5°C in 5% CO_2 in humidified air. After removal of excess spermatozoa and cumulus cells, presumed zygotes were transferred to synthetic oviductal fluid supplemented with essential and non-essential amino acids (SOFaa), 0.4% BSA (Sigma A9647) and ITS (5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium) and placed in groups of 25 in a 50 µL droplet of culture medium. Each droplet was covered by mineral oil and incubated at 38.5°C for 7 days in 5% CO_2, 5% O_2 and 90% N_2.

Holding medium usage

We tested the ability of the Syngro EHM (Bioniche Inc., WA, USA) to hold oocytes before maturation. The EHM was always kept at 4°C when not used. Two hours before the onset of each experiment, the EHM was placed in the incubator to warm up (38.5°C) to prevent cold shock when transferring the oocytes to the EHM. Oocytes were held in 1 mL sterile glass osmometer tubes (Novolab, Geraardsbergen, Belgium), filled to the top with the EHM to limit the amount of air. Caps and parafilm were put on the tip of the vials to ensure a tight seal and prevent leakage.

Experiment 1: Effect of holding time and temperature of EHM on oocytes' maturation

These trials were designed to find out at which temperature and for how long immature bovine oocytes can be stored in EHM without compromising meiotic progression. Nuclear maturation of oocytes was assessed in three separate experiments, each of them replicated three times. COCs were stored in EHM for 6, 10, and 14 h, respectively. The experimental set-up is summarized in **Table 1**. Briefly, immature oocytes were grouped per 60, and each group was transferred into glass osmometer tubes containing 1 mL of EHM. Each oocyte group was randomly assigned to different temperatures. In the first trial, COCs were stored in EHM during 6 h at 4°C, RT (22 to 25°C) and 38.5°C. Oocytes to be held at 38.5°C were placed in the incubator (38.5°C). The vials with oocytes to be stored at RT and 4°C, were put in 50 mL centrifuge tubes containing water at 25°C. The oocytes to be held at 4°C were placed in an Equitainer (Hamilton Res Inc., Ipswich, MA, USA), originally designated for transporting equine semen. The centrifuge tubes containing the RT group oocytes were placed in a Styrofoam container for light insulation. After holding in EHM for 6 h, oocytes were directly fixed (immature holding) or they were fixed after subsequent 22 h of maturation (mature holding). Before fixation, oocytes were denuded by 8 min vortex in 2.5 mL of Hepes-TALP supplemented with 0.01% hyaluronidase. Fixation was performed in 2% paraformaldehyde during 20 minutes. Because maturation rates were substantially lower after holding at 38.5°C, this temperature was ruled out in the following trials. In the second trial, COCs were stored in EHM during 10 h at 4°C and RT, and fixed immediately after

(immature holding) or fixed after subsequent 22 h of maturation (mature holding). Due to the substantial low embryo development at 4°C, a third trial with COCs in holding for 14 h was only done at RT; and then proceeded as previously described. During all three trials, an immature control group consisting of oocytes fixed immediately after aspiration and a mature control group consisting of oocytes placed for 22 h in maturation medium without prior EHM storage were included each time.

All oocytes were stained for 10 minutes with 0.1% Hoechst 33342, and standardly mounted on a microscope slide. The stained oocytes were evaluated using a 400x magnification fluorescence microscope (Leica DM 5500 B). Oocytes' maturation stages were classified as: 1) germinal vesicle stage 2) meiotic progression (diakinesis, metaphase I, or anaphase I), 3) matured (telophase I or metaphase II), and 4) degenerated (degraded chromatin) (**Figure 1**).

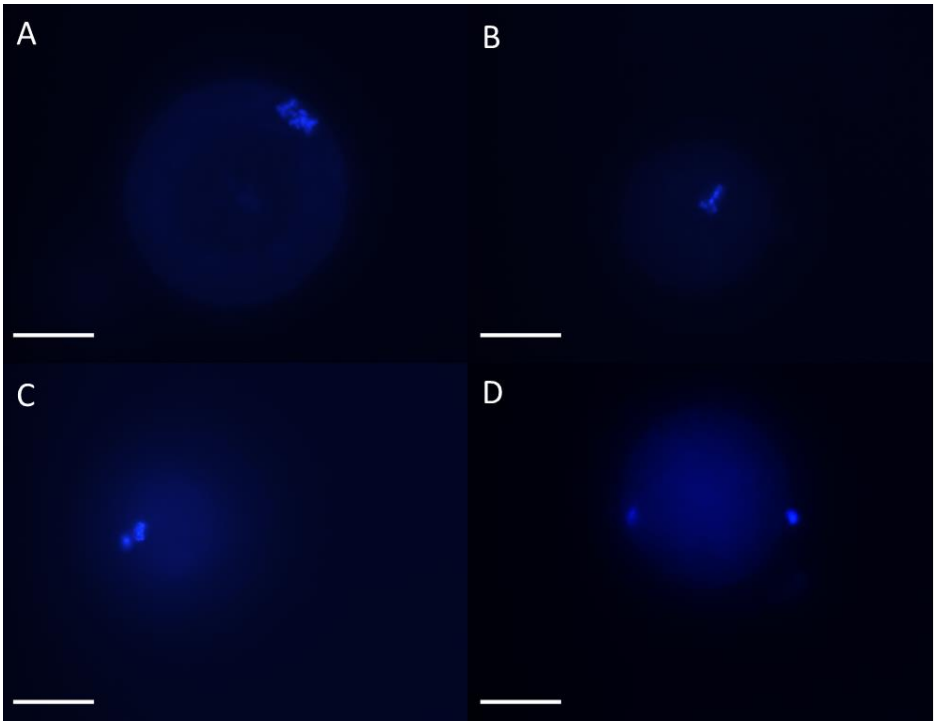


Figure 1 Fluorescent images of bovine oocytes after Hoechst 33342 staining. (A) germinal vesicle stage (immature), (B) metaphase I (meiotic progression), (C) telophase I (matured), and (D) metaphase II (matured). Scale bar: 50 µm.

Experiment 2: Effect of holding time and temperature of EHM on embryo development and quality

The experimental design of experiment 2 was similar to the one described in experiment 1 (**Table 1**): In three separate trials (each of them replicated three times), immature oocytes were grouped per 60 and stored in EHM for 6 h at 4°C, RT and 38.5°C; for 10 h at 4°C and RT; and for 14 h at RT. After holding, oocytes were washed with Hepes-TALP (38.5°C) and transferred to maturation medium as described in the embryo production protocol. Subsequently, oocytes were fertilized and zygotes were cultured

until 8 days post insemination (dpi). During all three trials, a control group was included each time. This group consisted of 60 COCs immediately transferred to maturation medium without prior holding, followed by the standard embryo production protocol. To evaluate embryo development, cleavage (45 h post insemination) and blastocyst rates (8 dpi) were assessed.

Table 1. Overview of storage time and temperature of the immature cumulus oocyte complexes (COCs) in a commercial embryo holding medium (EHM).

Experiment	Holding time	Temperature		
		Room temperature	4°C	38.5°C
Exp. 1.1	6 h	X	X	X
Exp. 1.2	10 h	X	X	
Exp. 1.3	14 h	X		

The maximum storage time for immature COCs in EHM was determined in three experiments. The temperature at which the COCs were held is indicated with an X for each experiment.

In order to assess the embryonic quality, a random subgroup (n = 8-12) of day 8 blastocysts per experiment (and replicate) were fixed in 2% paraformaldehyde for 20 min at RT and stored in phosphate-buffered saline (PBS) containing 0.5% BSA, at 4°C. To assess the blastocyst quality, a differential apoptotic staining was performed as described by Wydooghe *et al.* (Wydooghe *et al.* 2011). The assessment of the number of trophectoderm (TE) cells, inner cell mass number (ICM), total cells number (TCN; TE+ICM), ICM/TCN ratio and total number of apoptotic cells (AC) and apoptotic cells ratio (ACR; AC/TCN) was done by fluorescence microscopy (Leica DM 5500 B) using a triple bandpass filter for DAPI, FITC and Texas Red; by a single observer.

Experiment 3: Effect of time and temperature of EHM on blastocyst development in small groups of oocytes/zygotes, simulating OPU procedures

In two trials (each with three replicates), the commercial settings of OPU were simulated. These trials were performed to evaluate day 8 blastocyst development in individual donor groups. Only RT holding (6 and 10 h) was assessed because of results gained in the "experiments 1 and *2*". First, ovaries (collected at the local slaughterhouse) were punctured separately from each other, considering each ovary as an individual donor. For each replicate, 16 donors were used. From each donor, the first 8 to 10 oocytes were randomly selected. All quality oocytes were used, but denuded oocytes were excluded. If fewer than 8 oocytes were collected, the donor was excluded from the experiment. Half

of the donor groups were randomly assigned as control. These oocytes were immediately transferred to maturation medium, followed by conventional IVF. Then, 8 zygotes per donor were randomly chosen and cultured in 20 µL drops of SOF + 0.4% BSA + ITS. During the whole procedure, the oocytes and embryos were constantly grouped per donor and there was no physical contact between different donor groups as different dishes and medium was used per donor. The other half of the donor groups were assigned to the treatments to determine maximal storage time in EHM at RT. Oocytes were grouped per donor in 1 mL EHM for 6 h at RT. After storage in EHM, the oocytes were handled per donor, washed (Hepes-TALP) and transferred to maturation medium and fertilized. Subsequently, only 8 zygotes per donor were randomly cultured in 20 µL drops of SOF + 0.4% BSA + ITS. For the second trial, the same procedure was followed, but treatment groups were held in EHM for 10 h at RT. To evaluate embryo development, blastocyst rates were evaluated at 8 dpi.

Statistical analysis

For all statistical analyses, the oocyte/zygote/embryo was set as the unit of interest. Replicates were included as the random effect. Generalized mixed effect models were computed using the function *glmer* of the package lme4 via R Studio version 3.3.0, 2016 (R inc., Boston, USA). The statistical significance was set at $P < 0.05$, via Tukey test. Results are expressed as least square means (LSM) with their respective standard error (SE).

In experiment 1, to test the effect of holding oocytes on the nuclear maturation outcome, the responsive variable was set as immature, meiotic progression, matured, and degenerated. Fixed effects tested were; holding 6 h (4°C vs. RT vs. 38.5°C vs. control), 10 h (4°C vs. RT vs. control) and 14 h (RT vs. control). In experiment 2, to assess the effect of time and temperature of holding immature oocytes on the embryonic development, the cleavage and day 8 blastocyst outcomes were set as the responsive variables. Each of the treatments, being held for 6 h (4°C vs. RT vs. 38.5°C vs. control), 10 h (4°C vs. RT vs. control) and 14 h (RT vs. control); were tested as fixed effects. To determine the day 8 embryo quality, the TE, ICM, TCN, TCN/ICM ratio, and ACR outcomes were stated as the responsive variable and holding 6 h (4°C vs. RT vs. 38.5°C vs. control), 10 h (4°C vs. RT vs. control) and 14 h (RT vs. control); as fixed effect. In experiment 3, the average of the day 8 embryo outcome of the 16 donors in each replicate was set as the responsive variable. Tested fixed effects were; holding 6 h (RT vs. control) and 10 h (RT vs. control).

Results

A total of 3.043 oocytes were used for experiment 1 (results summarized in **Table 2** and **3**). **Table 2** shows that the maturation rates were not affected by the holding time (6, 10 and 14 h) nor by the holding temperature (4°C, RT and 38.5°C) ($P < 0.05$), as none of the oocytes matured while held in EHM (without subsequent maturation). However, significant differences were noticed when oocytes were matured after EHM storage (**Table 3**). When oocytes were held for 6 h and subsequently matured, the number of matured oocytes was significantly lower in oocytes held at 38.5°C in comparison to the other groups (control, RT and 4°C). When held for 10 h, the oocytes' maturation rate was similar between the control and RT groups ($P > 0.05$), but it was significantly lower in oocytes held at 4°C. Lastly, when compared to oocytes held at RT for 14 h, the maturation rate was higher in the control group ($P < 0.05$).

Table 2. Kinetics of cumulus oocyte complexes (COCs) nuclear status after holding in embryo holding medium (EHM) for different times and at different temperatures (without subsequent maturation). Controls represent no previous holding of COCs in EHM. No statistical differences (*P* > 0.05) were found between groups.

Holding 6 h (without maturation)					
Group	**n**	**Immature**	**Meiotic progression**	**Matured**	**Degenerated**
Control	165	92.1±2.1[a]	6.6±1.9[a]	0.0±0.0[a]	1.2±0.8[a]
RT	152	92.7±2.1[a]	4.6±1.7[a]	0.0±0.0[a]	2.6±1.2[a]
4°C	162	90.7±2.3[a]	8.0±2.1[a]	0.0±0.0[a]	1.2±0.3[a]
38.5°C	160	91.8±2.2[a]	5.6±1.8[a]	0.0±0.0[a]	2.5±1.2[a]
Holding 10 h (without maturation)					
Group	**n**	**Immature**	**Meiotic progression**	**Matured**	**Degenerated**
Control	171	91.4±2.3[a]	7.4±2.1[a]	0.0±0.0[a]	1.1±0.8[a]
RT	173	92.0±2.2[a]	4.5±1.6[a]	0.0±0.0[a]	3.4±1.3[a]
4°C	170	90.7±2.4[a]	5.7±1.9[a]	0.0±0.0[a]	3.5±1.4[a]
Holding 14 h (without maturation)					
Group	**n**	**Immature**	**Meiotic progression**	**Matured**	**Degenerated**
Control	175	93.7±1.8[a]	5.1±1.2[a]	0.5±0.5[a]	0.5±0.5[a]
RT	173	94.2±1.7[a]	2.8±1.2[a]	0.0±0.0[a]	2.8±1.2[a]

Results are expressed as least square mean ± standard error (LSM±SE)

Table 3. Kinetics of cumulus oocyte complexes (COCs) nuclear status after holding in embryo holding medium (EHM) for different times and at different temperatures and subsequent 22 h maturation. Controls represent no previous holding of COCs in EHM. Different superscripts (a and b) represents statistical differences ($P < 0.05$) between groups.

Holding 6 h (+22 h maturation)					
Group	**n**	**Immature**	**Meiotic progression**	**Matured**	**Degenerated**
Control	168	8.9±2.1[a]	7.7±2.0[a]	80.9±3.2[a]	2.3±1.1[a]
RT	179	7.2±1.9[a]	4.4±1.5[a]	83.2±2.9[a]	4.9±1.7[a,b]
4°C	154	8.4±2.2[a]	7.1±2.0[a]	81.2±3.3[a]	3.1±1.4[a,b]
38.5°C	170	24.7±3.3[b]	8.2±2.1[a]	57.1±4.1[b]	9.7±2.6[b]
Holding 10 h (+22 h maturation)					
Group	**n**	**Immature**	**Meiotic progression**	**Matured**	**Degenerated**
Control	177	3.8±1.5[a]	13.8±2.8[a]	80.7±2.9[a]	1.0±0.8[a]
RT	166	5.2±1.8[a]	13.7±2.9[a]	78.9±3.1[a]	1.7±1.0[a]
4°C	169	1.4±3.2[b]	13.4±2.8[a]	68.6±3.5[b]	1.7±1.1[a]
Holding 14 h (+22 h maturation)					
Group	**n**	**Immature**	**Meiotic progression**	**Matured**	**Degenerated**
Control	181	5.6±1.7[a]	8.1±2.2[a]	84.5±2.7[a]	1.0±0.7[a]
RT	178	6.0±1.8[a]	17.2±3.5[b]	71.9±3.5[b]	3.9±1.5[a]

Results are expressed as least square mean ± standard error (LSM±SE)

A total of 2.682 presumed zygotes were evaluated in experiment 2. **Table 4** represents the overall results of the effect of holding immature oocytes in EHM at different temperatures for 6, 10 and 14 h on the cleavage and day 8 blastocyst rates. When oocytes were held for 6 h, the cleavage rate was lower at 38.5°C compared to the other groups (control, RT and 4°C) ($P < 0.05$). At 8 dpi, blastocyst rates were higher in the control and RT groups compared to 4°C and 38.5°C groups ($P < 0.05$). When oocytes were held for 10 h, the cleavage and blastocyst rates were significantly higher in the control and RT compared to the 4°C group. However, cleavage and blastocyst rates were significantly lower when oocytes were held for 14 h at RT compared to the control group. No differential staining parameters (TCN, ICM, ICM/TCN, AC, and AC/TCN) were found to be significantly different regardless holding times or temperature of the EHM (**Figures 1** and **2**).

Table 4. Cleavage and blastocyst day 8 rate expressed as percentage from presumed zygotes (least square mean ± standard error (LSM±SE)) performed in three different experiments (three replicates for each experiment). In each experiment, immature cumulus oocyte complexes (COCs) were held in embryo holding medium (EHM) at different temperatures for 6, 10 and 14 h prior to *in vitro* maturation. Controls represent no previous holding of COCs in EHM.

Holding 6 h			
Group	**N° of presumed zygotes**	**Cleavage**	**Blastocysts at day 8**
Control	298	78.0±3.0[a]	36.2±2.8[a]
RT	298	75.9±3.4[a]	35.2±2.7[a]
4°C	297	71.5±3.1[a]	23.2±2.4[b]
38°C	298	50.0±3.9[b]	11.0±1.8[c]
Holding 10 h			
Group	**N° of presumed zygotes**	**Cleavage**	**Blastocysts at day 8**
Control	299	74.9±2.5[a]	40.6±4.6[a]
RT	297	73.7±2.6[a]	40.2±4.5[a]
4°C	298	46.9±2.9[b]	20.1±3.3[b]
Holding 14 h			
Group	**N° of presumed zygotes**	**Cleavage**	**Blastocysts day 8**
Control	299	77.5±2.4[a]	39.5±2.8[a]
RT	298	60.8±2.8[b]	25.7±2.5[b]

Different superscripts (a, b, and c) represents statistical differences ($P < 0.05$) between groups. Results are expressed as least square mean ± standard error (LSM±SE)

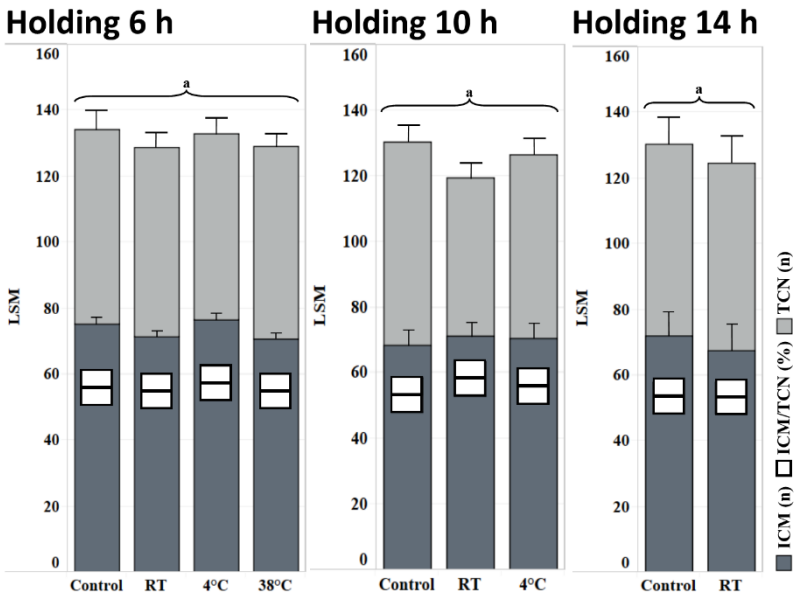


Figure 2. Total cells number (TCN), inner cell mass (ICM) and ICM/TCN of day 8 blastocyst (n = 315) differentially stained. No significant differences ($P < 0.05$) were found between groups in three different experiments. In each experiment, cumulus oocyte complexes (COCs) were held in embryo holding medium (EHM) at different temperatures for 6 h (n = 140), 10 h (n = 105) and 14 h (n = 70). Controls represent no previous holding of COCs in EHM. Results are expressed as least square means (LSM) and standard error.

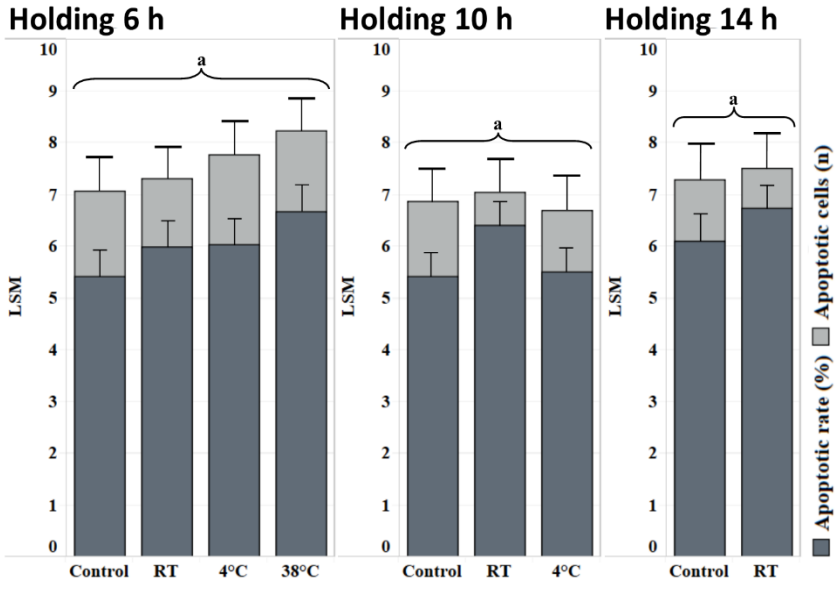


Figure 3. Total apoptotic cells and apoptotic rate (total apoptotic cells/total cells number) of day 8 blastocyst (n = 315) differentially stained. In each experiment, cumulus oocyte complexes (COCs) were held in embryo holding medium (EHM) at different temperatures for 6 h (n = 140), 10 h (n = 105) and 14 h (n = 70). Controls represent no previous holding of COCs in EHM. No significant differences ($P < 0.05$) were found between groups. Results are expressed as least square means (LSM) with their respective standard error.

In experiment 3, OPU conditions were simulated by culturing oocytes in small groups (n = 380). No significant differences were found between the day 8 blastocyst rate of the control and RT groups, for both treatments where oocytes were held for 6 or 10 h **(Table 5)**.

Table 5. **This table represents experiments which aimed to mimic the commercial settings of bovine ovum pick-up (OPU).** In two extra trials (each with three replicates), small number of cumulus oocyte complexes (COCs) (n= 10) were either immediately matured (control) or held in embryo holding medium (EHM) for 6 or 10 h. No significant differences ($P < 0.05$) were found between groups (control vs. RT).

	Control		RT	
Holding time	**N° of presumed zygotes**	**Blastocysts at day 8**	**N° of presumed zygotes**	**Blastocysts at day 8**
6 h	189	20.6±3.6[a]	189	19.8±3.5[a]
10 h	190	18.3±3.4[a]	191	18.8±3.6[a]

Results are expressed as least square mean ± standard error (LSM±SE)

Discussion

The fact that we demonstrated that bovine oocytes can be held for several hours at RT in a commercial holding medium opens new perspectives for commercial IVP industries in cattle. To obtain consistent results in OPU, collected oocytes are routinely placed in maturation media and allocated to a controlled environment (5% CO_2 at 38.5°C) as soon as possible (Blondin 2017). These steps do not only require special equipment (transportable incubator), but also fresh maturation media which are necessary to achieve consistent results. From a practical point of view, when several OPU sessions are performed within the same day, the maturation gap may widely differ between the first and the last session. Since oocytes are already maturing during transportation, this may be translated to tedious manipulations schedules at the IVF lab. In this regard, we considered it to be essential for commercial practice to establish an easily applicable alternative by storing immature oocytes in EHM during transport, in order to prevent the start of maturation while keeping the oocyte alive and to plan further IVP steps at the best suitable time.

Elevated intra-oocyte levels of cAMP are vital for the maintenance of meiotic arrest (prophase I) *in vivo* (Bilodeau-Goeseels 2011). In cows, meiotic inhibitors such as roscovitine and butyrolactone were effectively tested to mimic intrafollicular conditions to preserve oocytes in meiotic arrest *in vitro* (Lonergan *et al.* 2000; Mermillod *et al.* 2000; Ponderato *et al.* 2001; Lagutina *et al.* 2002; Adona *et al.* 2008). Since meiotic inhibitors are potentially toxic, the holding ability of a mixture of 1:1 TCM 199 with Hanks' salts and TCM 199 with Earle's salts with 25 mM Hepes and 20% serum (absence of maturation inhibitors) was successfully tested by Alm *et al.* (2008). This enriched phosphate buffered medium made it possible to maintain the pH and osmotic balance in a low CO_2 environment (~5% CO_2), with an extra source of energy (Earle's salts; glucose). In our study, however, we considered it to be essential for practitioners to provide a more easy transport method by using a holding medium. Therefore, we decided to test a ready-to-use commercial synthetic medium (Syngro EHM). This EHM

medium was originally designated to hold bovine and equine preimplantation embryos. Although its exact composition is not reported by the company, EHM does not contain materials of animal origin (such as serum) and is supplemented with hyaluronan, which is involved in the regulation of embryonic gene expression, cell proliferation and differentiation (Lapčík *et al.* 1998). Furthermore, we decided to store oocytes in glass vials, to avoid any type of bias associated with toxicity of plastic polymers (Cruickshank *et al.* 1960; Lithner *et al.* 2011).

We demonstrated that the temperature in which immature oocytes are being held can be detrimental to the further embryonic development. Previous studies identified that a temperature zone between 4 and 0°C significantly affects the oocytes' ability to mature (Wu *et al.* 1999). However, in the present study, the maturation rate after holding oocytes at 4°C for 6 h was not affected, it was only detrimental when held at 4°C for 10 h. Interestingly, the blastocyst yield was significantly lower in oocytes held at 4°C compared to the control and RT groups, no matter if oocytes were held for 6 or 10 h. Chromosomal defects have been reported when oocytes were exposed to low temperatures (Moor and Crosby 1985). This defect may indicate that the spindle reassembly is lacking when immature oocytes are stored at 4°C, which in turn is resulting in a lower blastocyst formation. However, the lowest results with regards to maturation and blastocyst outcomes were noticed when oocytes were held at 38.5°C. Comparable to the study by Hashimoto *et al.* (Hashimoto *et al.* 2003), a deleterious effect of high temperature storage was noted when arresting oocytes at 39°C. The enzymatic activity might be increased in rising temperatures (Rekharsky *et al.* 1986). Eventually, various metabolites can be accumulated inside the oocytes held in EHM at 38.5°C, due to high enzymatic activity (Hashimoto *et al.* 2003), interfering with the oocyte's viability. Still, regardless of the holding time, immature oocytes held in EHM (without subsequent maturation) did not reach metaphase II. Probably, holding for 6 to 14 h was not enough time to induce major meiotic changes in oocytes stored in EHM, as the metaphase II is normally reached between 18 to 24 h after the onset of maturation in normal IVP procedures without EHM use (Ward *et al.* 2002).

Holding immature bovine oocytes in a commercial EHM at RT either for 6 or 10 h achieved comparable results to the control group in terms of oocyte maturation and blastocyst yield. Importantly, the kinetics of maturation were not affected during the EHM storage. In similar studies, where oocytes were held for 18 to 24 h at RT, a reduced maturation time must be taken into account in subsequent IVP steps (Ponderato *et al.* 2001; Hashimoto *et al.* 2002; Lagutina *et al.* 2002; Adona *et al.* 2008; Alm *et al.* 2008). Since we held oocytes for a considerable lower period of time, only standard maturation time (22 h) is necessary to achieve acceptable blastocyst rates. Similarly, Merton at al. (Merton *et al.* 2003) suggested that oocytes can be *in vitro* matured for up to 28 h. Nevertheless, when oocytes were

held for 14 h at RT, their developmental competence was affected. Although oocytes did not mature during storage, their ability to reach metaphase II in the maturation medium decreased significantly. This was also translated to a lower blastocyst yield in comparison to the control group. Additionally, we assessed standard quality parameters of individual blastocysts in the different groups of holding treatments, using an immunofluorescent differential apoptotic staining. These parameters, being TCN, ICM, ICM/TCN, AC and AC/TCN, are very sensitive indicators of individual blastocyst quality (Fouladi-Nashta *et al.* 2005; Wydooghe *et al.* 2011). Surprisingly, neither of these blastocyst quality parameters were affected by the oocytes' storage temperature (RT, 4°C and 38°C) nor time (6, 10 and 14 h). Therefore, we hypothesize that storing oocytes before maturation may imply a natural selection process of the most capable specimen of the oocytes' pool, translated to an uniform quality of blastocysts at 8 dpi.

In experiment 3, we evaluated the effect of time and temperature of the EHM on the blastocyst development in small groups of zygotes (simulating OPU procedures), based on results obtained in experiments 1 and 2 (holding 6 to 10 h at RT). In European conditions, an average of 8 oocytes per donor animal is obtained per OPU session (Merton *et al.* 2012). Moreover, the quality of oocytes obtained by OPU is unequal since some oocytes may loose some cumulus cells due to fluctuations of the vacuum pressure (Bols *et al.* 1996). The number of cumulus cells surrounding the oocyte is directly associated with its quality and embryo predictive developmental potential (Goovaerts *et al.* 2010). This implies that during IVP only a small numbers of oocytes (n = 10) with variable quality are matured and cultured together (Catteeuw *et al.* 2017). Similarly to the control group, also consisting of small numbers of oocytes (n = 10) having a variable quality but without prior holding, blastocyst rates were around 20% when oocytes were held for both 6 and 10 h at RT in small groups of oocytes. This blastocyst outcome is similar to previous publications, where oocytes and embryos were cultured separately per donor (Merton *et al.* 2003; Machado *et al.* 2006). Therefore, independently from the group size, this study demonstrates a high developmental competence after holding immature bovine oocytes for up to 10 h at RT.

Conclusions

In conclusion, using a commercially available EHM to store and/or transport immature bovine oocytes has no detrimental effect on maturation rates, subsequent embryonic development and standard embryo quality parameters. Storing immature oocytes in EHM after collection can delay and therefore synchronize maturation. It offers the opportunity of scheduling laboratory work during more convenient working hours. However, it is recommended to maintain the oocytes in EHM at RT for no longer than 10 h, otherwise an unaffected development can not be guaranteed.

Acknowledgements

The authors wish to thank Isabel Lemahieu and Petra Van Damme for their excellent technical assistance. Also, all personnel involved in helping for the collection of the ovaries at the slaughterhouse are cordially thanked.

References

Adona, P., Pires, P., Quetglas, M., Schwarz, K., and Leal, C. (2008) Nuclear maturation kinetics and in vitro embryo development of cattle oocytes prematured with butyrolactone I combined or not combined with roscovitine. *Animal Reproduction science* **104**(2), 389-397

Alm, H., Choi, Y., Love, L., Heleil, B., Torner, H., and Hinrichs, K. (2008) Holding bovine oocytes in the absence of maturation inhibitors: kinetics of in vitro maturation and effect on blastocyst development after in vitro fertilization. *Theriogenology* **70**(7), 1024-1029

Bilodeau-Goeseels, S. (2011) Cows are not mice: The role of cyclic AMP, phosphodiesterases, and adenosine monophosphate-activated protein kinase in the maintenance of meiotic arrest in bovine oocytes. *Molecular Reproduction and Development* **78**(10-11), 734-743

Blondin, P. (2017) Logistics of large scale commercial IVF embryo production. *Reproduction Fertility and Development* **29**(1), 32-36

Bols, P.E.J., VanSoom, A., Ysebaert, M.T., Vandenheede, J.M.M., and deKruif, A. (1996) Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes. *Theriogenology* **45**(5), 1001-1014

Boni, R. (2012) Ovum pick-up in cattle: a 25 yr retrospective analysis. *Animal Reproduction* **9**(3), 362-369

Catteeuw, M., Wydooghe, E., Mullaart, E., Knijn, H.M., and Soom, A. (2017) In vitro production of bovine embryos derived from individual donors in the Corral® dish. *Acta Veterinaria Scandinavica* **59**(1), 41

Choi, Y., Love, L., Varner, D., and Hinrichs, K. (2006) Holding immature equine oocytes in the absence of meiotic inhibitors: effect on germinal vesicle chromatin and blastocyst development after intracytoplasmic sperm injection. *Theriogenology* **66**(4), 955-963

Cruickshank, C., Hooper, C., Lewis, H., and MacDougall, J. (1960) The toxicity of rubbers and plastics used in transfusion-giving sets. *Journal of clinical pathology* **13**(1), 42-50

Dini, P., Pascottini, O.B., Ducheyne, K., Hostens, M., and Daels, P. (2016) Holding equine oocytes in a commercial embryo-holding medium: New perspective on holding temperature and maturation time. *Theriogenology* **86**(5), 1361-1368

Foss, R., Ortis, H., and Hinrichs, K. (2013) Effect of potential oocyte transport protocols on blastocyst rates after intracytoplasmic sperm injection in the horse. *Equine Veterinary Journal* **45**(S45), 39-43

Fouladi-Nashta, A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K., and Webb, R. (2005) Differential staining combined with TUNEL labelling to detect apoptosis in preimplantation bovine embryos. *Reproductive Biomedicine Online* **10**(4), 497-502

Galli, C., Colleoni, S., Turini, P., Crotti, G., Dieci, C., Lodde, V., Luciano, A.M., and Lazzari, G. (2014) Holding equine oocytes at room temperature for 18 hours prior to in vitro maturation maintains their developmental competence. *Journal of Equine Veterinary Science* **34**(1), 174-175

Goovaerts, I.G.F., Leroy, J.L.M.R., Jorssen, E.P.A., and Bols, P.E.J. (2010) Noninvasive bovine oocyte quality assessment possibilities of a single oocyte culture. *Theriogenology* **74**(9), 1509-1520

Hashimoto, S., Kimura, K., Iwata, H., and Takakura, R. (2003) Oocyte transport: developmental competence of bovine oocytes arrested at germinal vesicle stage by cycloheximide under air. *Journal of Reproduction and Development* **49**(1), 61-66

Hashimoto, S., Minami, N., Takakura, R., and Imai, H. (2002) Bovine immature oocytes acquire developmental competence during meiotic arrest in vitro. *Biology of Reproduction* **66**(6), 1696-1701

Lagutina, I., Ponderato, N., Lazzari, G., and Galli, C. (2002) Kinetics of oocyte maturation and subsequent development of IVF, parthenogenetic, and NT bovine embryos after meiotic inhibition with roscovitine. *Cloning and Stem Cells* **4**(2), 113-119

Lapčík, L., Lapcik, L., De Smedt, S., Demeester, J., and Chabrecek, P. (1998) Hyaluronan: preparation, structure, properties, and applications. *Chemical reviews* **98**(8), 2663-2684

Lithner, D., Larsson, Å., and Dave, G. (2011) Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Science of the Total Environment* **409**(18), 3309-3324

Lonergan, P., Dinnyes, A., Fair, T., Yang, X., and Boland, M. (2000) Bovine oocyte and embryo development following meiotic inhibition with butyrolactone I. *Molecular Reproduction and Development* **57**(2), 204-209

Machado, S.A., Reichenbach, H.D., Weppert, M., Wolf, E., and Goncalves, P.B.D. (2006) The variability of ovum pick-up response and in vitro embryo production from monozygotic twin cows. *Theriogenology* **65**(3), 573-583

Martino, N.A., Dell'Aquila, M.E., Uranio, M.F., Rutigliano, L., Nicassio, M., Lacalandra, G.M., and Hinrichs, K. (2014) Effect of holding equine oocytes in meiosis inhibitor-free medium before in vitro maturation and of holding temperature on meiotic suppression and mitochondrial energy/redox potential. *Reproductive Biology and Endocrinology* **12**(1), 99

Mermillod, P., Tomanek, M., Marchal, R., and Meijer, L. (2000) High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. *Molecular Reproduction and Development* **55**(1), 89-95

Merton, J., de Roos, A.P.W., Mullaart, E., de Ruigh, L., Kaal, L., Vos, P., and Dieleman, S.J. (2003) Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* **59**(2), 651-674

Merton, J.S., de Roos, A.P.W., Koenen, E.P.C., Roelen, B.A.J., Vos, P.L.A.M., Mullaart, E., and Knijn, H.M. (2012) Bovine OPU-Derived Oocytes can be Matured In Vitro for 16-28 h with Similar Developmental Capacity. *Reproduction in Domestic Animals* **47**(6), 1037-1042

Moor, R., and Crosby, I. (1985) Temperature-induced abnormalities in sheep oocytes during maturation. *Journal of Reproduction and Fertility* **75**(2), 467-473

Ponderato, N., Lagutina, I., Crotti, G., Turini, P., Galli, C., and Lazzari, G. (2001) Bovine oocytes treated prior to in vitro maturation with a combination of butyrolactone I and roscovitine at low doses maintain a normal developmental capacity. *Molecular Reproduction and Development* **60**(4), 579-585

Rekharsky, M., Galchenko, G., Egorov, A., and Berenzin, I. (1986) 'Thermodynamics of enzymatic reactions.' (Springer-Verlag: Berlin, Heidelberg)

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., and De Kruif, A. (2002) Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Molecular Reproduction and Development* **61**(3), 414-424

Ward, F., Enright, B., Rizos, D., Boland, M., and Lonergan, P. (2002) Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology* **57**(8), 2105-2117

Wu, B., Tong, J., and Leibo, S. (1999) Effects of cooling germinal vesicle–stage bovine oocytes on meiotic spindle formation following in vitro maturation. *Molecular Reproduction and Development* **54**(4), 388-395

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E., De Sutter, P., Vandaele, L., and Van Soom, A. (2014) Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reproduction Fertility and Development* **26**(5), 717-724

Wydooghe, E., Vandaele, L., Beek, J., Favoreel, H., Heindryckx, B., De Sutter, P., and Van Soom, A. (2011) Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Analytical Biochemistry* **416**(2), 228-230

CHAPTER 4

IN VITRO PRODUCTION OF BOVINE EMBRYOS DERIVED FROM INDIVIDUAL DONORS IN THE CORRAL® DISH

Modified from

In vitro production of bovine embryos derived from individual donors in the Corral® dish.

Catteeuw M, Wydooghe E, Mullaart E, Knijn HM, Van Soom A

Acta Veterinaria Scandinavica 2017; 59:41

Abstract

Since the identity of the embryos is utmost important during commercial *in vitro* embryo production, bovine oocytes and embryos have to be cultured strictly per donor. Due to the rather low yield of oocytes collected after ovum pick-up per individual cow, oocyte maturation and embryo culture take place in small groups, which is often associated with inferior embryo development. The objective of this study was to improve embryonic development in small donor groups by using the Corral® dish. This dish is commercially designed for human embryo production. It contains two central wells that are divided into quadrants by a semi-permeable wall. In human embryo culture, one embryo is placed per quadrant, allowing individual follow-up while embryos were exposed to a common medium. In our study, small groups of oocytes and subsequently embryos of different bovine donors were placed in the Corral® dish, each donor group in a separate quadrant. In two experiments, the Corral® dish was evaluated during *in vitro* maturation (IVM) and/or culture (IVC) by grouping oocytes and embryos of individual bovine donors per quadrant. At day 7, a significantly higher blastocyst rate was noted in the Corral® dish used during IVM and IVC than when only used during IVM (12.9% ± 2.10 versus 22.8% ± 2.67) ($P < 0.05$). However, no significant differences in blastocyst yield were observed anymore between treatment groups at day 8 post insemination. In the present study, the Corral® dish was used for *in vitro* embryo production in cattle; allowing to allocate oocytes and/or embryos per donor. As fresh embryo transfers on day 7 have higher pregnancy outcomes, the Corral® dish offers an added value for commercial OPU/IVP, since a higher blastocyst development at day 7 is obtained when the Corral® dish is used during IVM and IVC.

Introduction

Currently, many bovine embryos are being generated *in vitro* for commercial embryo transfer. In 2013, more than 500.000 embryos have been produced worldwide by ovum pick-up (OPU) and *in vitro* embryo production (IVP) technologies, with South America taking the lead in OPU/IVP. However, during the last decade there has been an almost three-fold rise in OPU/IVP embryos produced in Europe and North America, indicating an increasing interest in this application (George 2014). Since OPU/IVP has become an alternative and highly competitive technique for multiple ovulation and embryo transfer (Boni 2012), much research has been done to optimize the OPU technique because numerous factors can influence the oocyte yield, such as hormone treatment prior to oocyte collection (De Roover *et al.* 2008), OPU equipment (Bols *et al.* 1997) and interval between OPU sessions (Ding *et al.* 2008). However, an average of only eight oocytes per Holstein-Friesian donor are obtained, a breed particularly used in Europe (Merton *et al.* 2003). When these oocytes are being matured, they are grouped per individual donor, since the genetic identity of the OPU/IVP embryo needs to be preserved. This implies that during OPU/IVP, only small numbers (less than 10) of oocytes and embryos are cultured together. Moreover, quality of oocytes derived after OPU is very variable since some oocytes lack cumulus cells due to vacuum pressure (Bols *et al.* 1996). The quality of the oocyte is however crucial and is predictive of the developmental potential of the resulting embryo (Goovaerts *et al.* 2010). It has been demonstrated that *in vitro* production starting from oocytes surrounded by few cumulus cells or denuded oocytes resulted in a lower blastocyst formation compared to IVP starting from oocytes surrounded by compact layers of cumulus cells (Khurana and Niemann 2000; Merton *et al.* 2003). Due to the scarcity of the oocytes retrieved per donor, a strict selection including only the best COCs prior to the *in vitro* process is not always possible. In commercial settings, where oocytes and embryos are cultured separately per donor, an average blastocyst rate of 16 to 18% is obtained (Machado *et al.* 2006; Merton *et al.* 2012). Besides the low blastocyst yield, there are also indications that grouping small numbers of embryos results in a lower total cell number and more apoptosis compared with embryos cultured in large groups (77.16 cells versus 98.48 cells and 24.17% versus 12.14%, respectively) (Cebrian-Serrano *et al.* 2014). In mice (Lane and Gardner 1992; Dai *et al.* 2012), cattle (O'Doherty *et al.* 1997; Gopichandran and Leese 2006) and human (Ebner *et al.* 2010), pooling oocytes and embryos in large groups increases blastocyst yield up to 40%. This beneficial effect of group culture has been related to a higher concentration of embryo secreted factors in the surrounding culture media, such as insulin-like growth factor-I (Spanos *et al.* 2000) or platelet activating factor (O'Neill 2005). These secreted factors act potentially as a survival factor by preventing apoptosis of the embryonic cells or as a mitogenic factor (O'Neill 2008). In addition, during *in vitro* maturation cumulus cells and oocytes are also able to secrete signalling molecules. Oocyte secreted factors, such as bone morphogenetic protein 15 and growth differentiation factor 9, regulate a variety of cumulus cell

functions associated with growth and differentiation, which in turn may regulate and stimulate the developmental competence of the oocyte (Gilchrist and Thompson 2007). These paracrine and autocrine factors require a close interaction between groups of COCs and subsequently embryos, thus creating a supporting microenvironment for development.

The Corral® dish, designed especially for human *in vitro* embryo production, consists of two central wells that are divided into four quadrants by a semi-permeable wall (**Figure 1A**). In human IVP, a single embryo, having a larger diameter than the gaps between the wall, is placed per quadrant, with a maximum of two times four embryos of the same female patient per Corral® dish. This makes individual monitoring of embryo development possible while the medium and embryotrophic factors can flow through the quadrants (**Figure 1B**) (Ebner *et al.* 2010). In human studies, single embryo culture has been applied in a Corral® dish setting (Ebner *et al.* 2010) where only one embryo was placed in one quadrant, but in our study, we chose to allocate 8 embryos belonging to the same donor in one quadrant, since embryo culture in small groups is routinely used in bovine OPU-IVF (O'Doherty *et al.* 1997). We hypothesized that the embryonic development would be stimulated in the common medium by the exchange of putative autocrine factors between the donor cows, while individual allocation of oocytes and subsequently embryos per donor cow still remained possible.

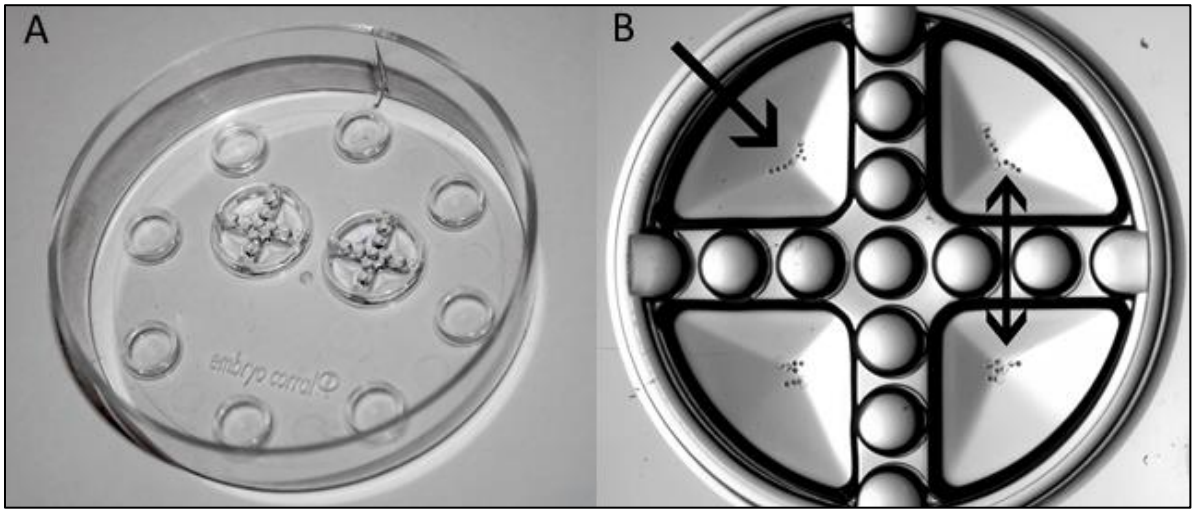


Figure 1. Design of the Corral® dish. (A) The Corral® dish consists of two central wells. (B) These central well is divided into quadrants by a semi-permeable wall, allowing medium and embryotrophic factors to pass (double arrow) but oocytes or embryos remained per individual donor in a quadrant (arrow). Each quadrant of the Corral® dish contains the oocytes or embryos of one specific donor and is filled with 30 µL medium.

Methods

Media and reagents

Basic Eagle's Medium amino acids, Minimal Essential Medium (MEM) non-essential amino acids (100 ×), TCM-199-medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium) and all other components were obtained from Sigma (Schnelldorf, Germany), unless otherwise stated. All the media were filter-sterilized using a 0.22 µm filter (Pall Corporation, Ann Arbor, MI, USA) before use.

In vitro embryo production protocol

Bovine embryos were produced by adapting previously described routine *in vitro* methods (Wydooghe *et al.* 2014). Briefly, ovaries were collected per slaughtered cow in separate plastic bags in a local slaughterhouse and processed within 2 hours. Follicles between 2 and 8 mm diameter were punctured. Subsequently, COCs and embryos were strictly kept per donor cow during the complete procedure. From each donor, the first eight to ten COCs visible in the petri dish were collected, without selection based on the quality of these COCs, only denuded oocytes were discarded. If fewer than eight COCs were available, the donor was excluded from the experiments. The COCs were transferred to maturation medium which consisted of modified bicarbonate-buffered TCM-199 supplemented with 50 µg/mL gentamycin and 20 ng/mL epidermal growth factor (EGF). Subsequently, COCs were matured for 22 h at 38.5°C in 5% CO2 in humidified air.

Fertilization occurred per donor with the semen of the same proven bull. Frozen-thawed spermatozoa were separated over a discontinuous Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden). Sperm concentration was adjusted to 1×10^6 spermatozoa/mL using IVF-Tyrode's Albumin-Pyruvate-Lactate (TALP), which consisted of bicarbonate buffered Tyrode's solution, supplemented with BSA (Sigma A8806; 6 mg/mL) and heparin (25 µg/mL). The mature oocytes were incubated in 500 µL IVF-TALP with spermatozoa for 21 h at 38.5°C in 5% CO2 in humidified air.

After fertilization, excess spermatozoa and cumulus cells were removed by vortexing. Eight presumptive zygotes per donor were transferred to synthetic oviductal fluid (SOF) supplemented with essential and non-essential amino acids (SOFaa), 0.4% BSA (Sigma A9647) and ITS (5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL selenium) and were incubated at 38.5°C in 5% CO2, 5% O2 and 90% N2 till day 8 post insemination. During this culture period, embryos were kept in the same culture dish and no renewal of SOF medium was performed.

Experiment 1: Embryo culture in the Corral® dish

For the first experiment, ovaries from individual donor cows were collected and processed separately. This experiment was conducted 4 times (4 replicates), for each replicate the ovaries of 16 different donor cows were collected. From each donor, eight to ten COCs were matured in 500 µL maturation medium in separate 4-well dishes. Subsequently, the oocytes were fertilized per donor in 4 well dishes. After fertilization, the first eight presumptive zygotes were grouped, without prior selection and cultured per donor. Half of these donor groups were allocated to a droplet and the other half to a quadrant of the Corral® dish, this was chosen completely at random. Culture droplets consisted of 30 µL medium, eight droplets were made per culture dish (IVF Petridish, Nunc®, Thermo Fisher, Denmark) and 8.5 mL mineral oil was covering these droplets (Drop IVC). In the Corral® dish, the two central wells were filled with 120 µL culture medium, each quadrant containing 30 µL (Corral® IVC). A layer of 8.5 mL mineral oil was put on top. Because of the typical structure of the Corral® dish, zygotes of eight different donors were grouped in those two central wells. An overview of COC and embryo distribution is shown in **Figure 2A**.

Experiment 2: Oocyte maturation and embryo culture in the Corral® dish

Comparable to experiment 1, this experiment was conducted 4 times (4 replicates). For each replicate, ovaries from 16 different donors were collected separately. From each donor, ten COCs were matured in a quadrant of the Corral® dish. Each quadrant contains 30 µL maturation medium. In one central well, 40 COCs of four different donors were matured in 120 µL medium, each donor separated by the semi-permeable wall dividing the Corral® dish in quadrants. Both central wells were covered with 8.5 mL mineral oil. Routine fertilization occurred per donor in 4 well dishes. As described in the first experiment, eight presumptive zygotes were cultured per donor either in a 30 µL drop of medium (Corral® IVM) or in a quadrant of the Corral® dish (Corral® IVM/IVC). In the latter, eight different donors were again grouped in the two central wells. An overview of COC and embryo distribution is shown in **Figure 2B**.

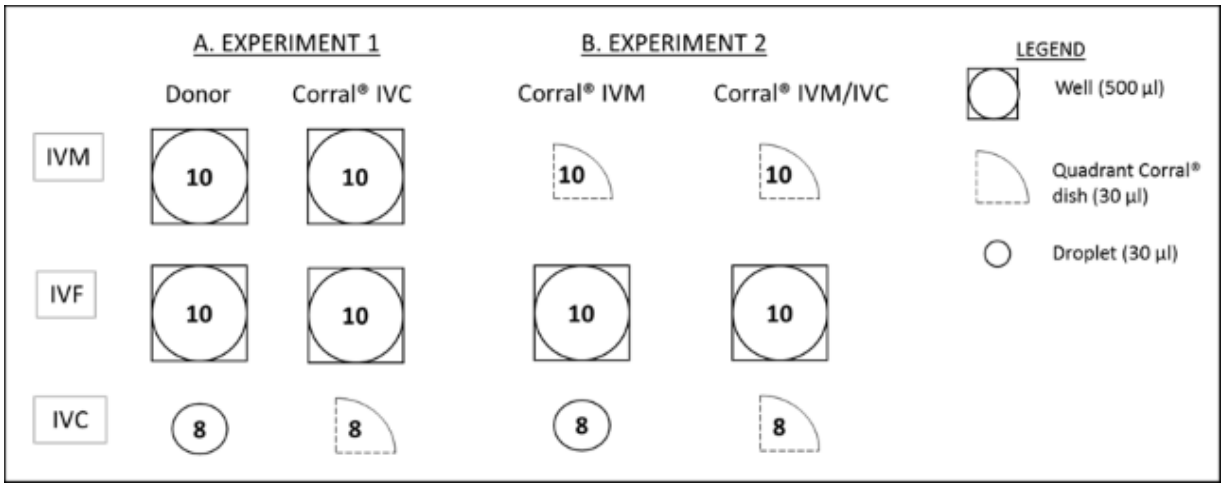


Figure 2. Schematic design of the two experiments. As indicated in the legend, oocytes and embryos were grouped per donor in a 4-well dish, a droplet or in a quadrant of the Corral® dish during the different phases of the *in vitro* embryo production (*in vitro* maturation-IVM, *in vitro* fertilization-IVF, *in vitro* culture-IVC). Furthermore, the number of oocytes and embryos grouped together is indicated in the icons. (A) In experiment 1, embryos were placed per donor in the Corral® dish or in a separate drop during IVC. (B) In experiment 2, oocytes and embryos were assigned per donor to the Corral® dish during IVM or during IVM and IVC.

Evaluation of embryo development and embryo quality

To evaluate the embryo development, the cleavage rate was assessed at 45 hours post insemination (hpi) as the percentage of presumed zygotes that cleaved. Blastocyst stages were evaluated according to the fourth edition IETS manual at 7 and 8 days post insemination (dpi). At 8 dpi, hatching rate was evaluated as the percentage of hatching or hatched blastocysts. Subsequently, total cell number (TCN) of the blastocysts was assessed by Hoechst 33342 staining. Briefly, day 8 blastocysts were fixed in 2% paraformaldehyde during 20 minutes and subsequently stained for 10 minutes with 0.1% Hoechst 33342. The stained blastocysts were evaluated using a 400x magnification fluorescence microscope (Leica DM 5500 B).

Statistical analysis

Statistical analyses were carried out with IBM SPSS Statistics 23. Differences at P-value < 0.05 were considered statistically significant. Cleavage, blastocyst and hatching rates were analysed using a binary logistic regression model with treatment (Drop IVC vs Corral® IVC and Corral® IVM vs Corral® IVM/IVC) and replicate as fixed effects. The effect of replicates was assessed and excluded from the final model if it was not significant. Total cell numbers were analysed using a mixed model analysis of variance, with treatment (Drop IVC vs Corral® IVC and Corral® IVM vs Corral® IVM/IVC) as fixed effect and replicate as random effect and are expressed as means ± standard error of the mean (SEM). If the effect of replicates was not significant, this was excluded from the final model.

Results

Experiment 1: Embryo culture in the Corral® dish

There was no significant difference noted in embryonic development between embryos cultured in individual donor droplets (Drop IVC) or embryos cultured in the Corral® dish (Corral® IVC) (Table 1). Both cleavage and blastocyst rate were similar in both groups. At day 8, a blastocyst rate was reached of 26.9% in Corral® IVC and 24.8% in Drop IVC. Furthermore, no differences were observed in TCN of day 8 blastocysts (Drop IVC: 188.1 ± 10.49; Corral® IVC: 194.2 ± 13.59).

Experiment 2: Oocyte maturation and embryo culture in the Corral® dish

Cleavage rate did not differ between two groups. Significantly more blastocysts were observed in the Corral® IVM/IVC compared to the Corral® IVM, at 7 dpi ($P < 0.05$), respectively 22.8% and 12.9%. This was however no longer the case for blastocyst rate at day 8, 26.7% was reached in the Corral® IVM and 30.1% in Corral® IVM/IVC (Table 1). Furthermore, TCN of day 8 blastocysts in these two groups did not differ (Corral® IVM: 125.0 ± 3.21; Corral® IVM/IVC: 133.9 ± 3.87).

Table 1. Embryonic development in the different treatment groups of experiment 1 and 2.

	Treatment	Number of Oocytes	Cleavage (%)	Blastocysts D7 (%)	Blastocysts D8 (%)	% Hatched
Exp 1	Drop IVC	219	158 (72.1)	41 (18.7)	59 (26.9)	22.0
	Corral® IVC	234	169 (72.2)	38 (16.2)	58 (24.8)	17.2
Exp 2	Corral® IVM	255	186 (72.9)	33 (12.9)*	68 (26.7)	22.1
	Corral® IVM/IVC	246	191 (77.6)	56 (22.8)*	74 (30.1)	28.4

Cleavage (45 hpi), blastocyst (7 dpi and 8 dpi) and hatching rates of embryos produced per donor in droplets (Drop IVC) or in a quadrant of the Corral® dish (Corral® IVC) during *in vitro* culture (experiment 1) and embryos produced per donor in a quadrant of the Corral® dish during *in vitro* maturation (Corral® IVM) or during *in vitro* maturation and culture (Corral® IVM/IVC) (experiment 2). Asterisks (*) in the same column indicate a statistical difference between treatments within the same experiment ($P < 0.05$).

Discussion

The overall aim of this study was to evaluate the efficiency of Corral® dish for commercial purposes. The Corral® dish allows grouping of embryos from different donor groups without losing track of genetic identity by placing the embryos of each donor in a quadrant of the central wells. This allows secreted embryotrophic factors to reach a larger group of embryos (32 instead of 8) since all embryos are exposed to the same surrounding medium. These factors stimulate growth and development which would result in a higher blastocyst yield. In this study, there was no difference found in embryonic development when applying the Corral® dish during culture in comparison with the allocation of embryos per donor in a separate droplet of medium. This was similar to the study of Ebner (Ebner *et al.* 2010) on human embryos, where one embryo was allocated to either a quadrant of the central wells or one embryo allocated to one of the other wells. However, when the Corral® dish was used both during maturation and culture, blastocyst yield was increased at day 7 pi (post insemination) compared to its use only during maturation, but this effect was no longer noticed at day 8 pi. Because more embryos reach the blastocyst stage on day 7 in the Corral® dish, when used during IVM and IVC, it offers the opportunity to transfer more fresh IVP embryos, which may subsequently give rise to more pregnancies and live born calves. It has been reported that pregnancy outcome is the highest when transferring fresh day 7 *in vitro* blastocysts, after transfers with *in vivo* derived embryos (Hasler *et al.* 1995; Hasler 1998).

On the other hand, the Corral® dish, has a specific design which implicates also three main disadvantages. First, the distance between donor groups is over 4 mm (**Figure 3**). Gopichandran and Leese (Gopichandran and Leese 2006) reported that an optimal blastocyst formation occurred when a distance of 165 µm between the embryos was achieved. In the Corral® dish, the distance components in the medium have to cross between two donor groups is probably too large for optimal exchange of autocrine factors. A mathematical model constructed by Matsuura (2014) calculated the concentration of secreted factors by embryos cultured in microwells based upon diffusion coefficients which are dependent on the size of the molecules. Small molecules (<1 kDa), such as waste secretions and reactive oxygen species, are rapidly diffusing away from the embryo and macromolecules (5-200 kDa), such as growth factors, are slowly diluted and remain quite high in the neighbourhood of the embryo. Due to the sloped sides of the quadrants (**Figure 3**), diffusion of secreted factors could therefore be facilitated in a vertical and oblique direction, with growth factors remaining in the neighbourhood of the embryos located in the deepest point of the well.

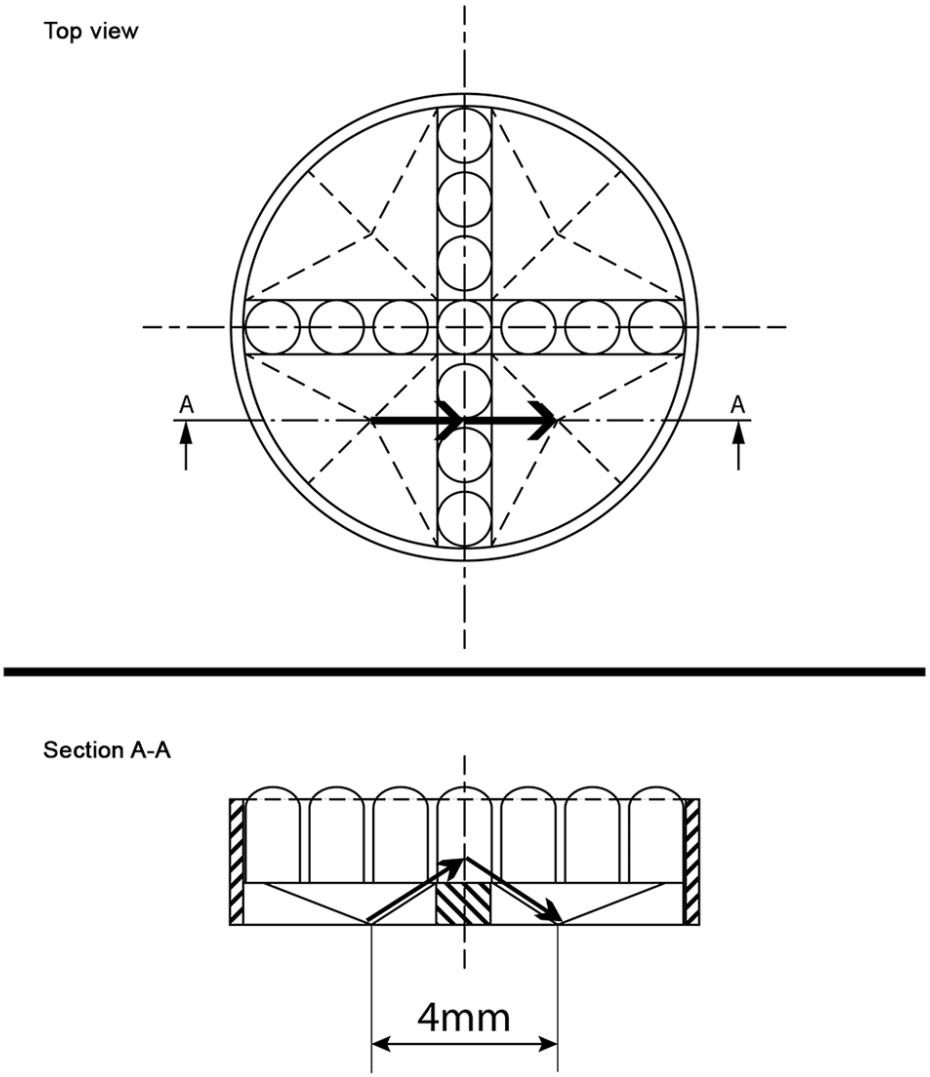


Figure 3. Graphic design of the Corral® dish. This figure is pointing out a distance of 4 mm between the deepest sites of the quadrants. An even larger distance has to be covered by the embryotrophic factors, secreted by the allocated cumulus-oocyte complexes or embryos, to reach another quadrant. The diffusion of secreted factors can only appear in a vertical and oblique direction (bold arrows), due to the well-shaped quadrants and the in-between wall.

Second, each quadrant needs to be filled with 30 µL medium in order to connect the four quadrants. In this way, adjusting the incubation volume to the number of oocytes or embryos is impossible. This static design has therefore a major impact on oocyte/embryo density, which is referring to the number of embryos on a given amount in µl of medium and which is an important parameter during *in vitro* embryo production (IVP) (Vajta *et al.* 2000; Fujita *et al.* 2006; Feng *et al.* 2007; Hoelker *et al.* 2010; Reed 2012). Because of low embryo numbers in commercial practices, the medium volume cannot be decreased to achieve the ideal density of 1:1 to 1:3 (Ferry *et al.* 1994; Carolan *et al.* 1995; Donnay *et*

al. 1997). The design of the Corral® dish could therefore be more suitable for donor cows having large numbers of COCs, since this is the only way to acquire a high embryo density in the Corral® dish. In Nelore cattle, a typical Brazilian breed, 30 or more oocytes can be collected per ovum pick-up session and this breed is therefore an excellent candidate for providing embryos for culture in the Corral dish (Pontes *et al.* 2011). Moreover, the fixed design of the Corral® dish makes the dish also two times more expensive than the traditional four well dish. Finally, from a sanitary point of view, possible transmission of pathogens can be considered as a risk factor between oocytes and embryos of different donors grouped in the Corral® dish. In theory, infection can be present as a consequence of intrafollicular infection or *in vitro* fertilization with infected semen. However, the risk of infection is rather small, since donor cows are carefully selected and tested for the absence of specific viral infections like BoHV1 before entering an *in vitro* embryo program, and also every bull is tested for absence of infectious pathogens before he is allowed to enter an artificial insemination program. The zona pellucida plays a major role in protecting the embryo, and only very small viruses can form a risk for transzonal infection. Furthermore, before embryo transfer, washing embryos in combination with trypsin treatment is advised by sanitary procedures of the IETS (International Embryo Technology Society) (Stringfellow 1998) to inactivate and remove possible viruses. In the end, it should still be advised to group oocytes and embryos of different donor cows in the Corral® dish only when the full health status is known.

Conclusions

A novel aspect of this study was that we used the Corral® dish for grouping only 10 oocytes of bovine oocytes and embryos per donor cow, whereas in other studies embryos have been cultured singly, thereby decreasing a possible beneficial effect of group culture. The dish is easy applicable to group donor's oocytes or embryos together without losing track of the embryo's identity. It is however doubtful whether the embryos can benefit from being grouped in the Corral® dish: the well-shaped quadrants on the one hand, and the distance between the quadrants on the other hand, could make it difficult for the secreted factors to diffuse out of one quadrant and reach the cumulus-oocyte complexes or embryos in the neighbouring quadrant. An improved version of the Corral® dish could be consisting of a dish with smaller wells, at a closer distance from each other and allowing culture in a smaller amount of medium to increase embryo density. In conclusion, the Corral® dish is an easy applicable tool to produce *in vitro* embryos by grouping bovine oocytes and embryos per donor. It may be a particularly interesting tool for donor cows like Nelore with high number of oocyte/embryos. Nevertheless, the Corral dish® increases blastocyst development at day 7 pi, when used during IVM and IVC, and is therefore beneficial for commercial practice regarding embryo transfers as higher pregnancy rates are achieved with fresh day 7 blastocysts.

Acknowledgements

The authors wish to thank Isabel Lemahieu and Petra Van Damme for their excellent technical assistance.

References

Bols, P.E.J., VanSoom, A., Ysebaert, M.T., Vandenheede, J.M.M., and deKruif, A. (1996) Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes. *Theriogenology* **45**(5), 1001-1014

Bols, P.E.J., Ysebaert, M.T., VanSoom, A., and deKruif, A. (1997) Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact cumulus oocyte complexes. *Theriogenology* **47**(6), 1221-1236

Boni, R. (2012) Ovum pick-up in cattle: a 25 yr retrospective analysis. *Animal Reproduction* **9**(3), 362-369

Carolan, C., Lonergan, P., Vanlangendonckt, A., and Mermillod, P. (1995) Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in-vitro. *Theriogenology* **43**(6), 1115-1128

Cebrian-Serrano, A., Salvador, I., and Silvestre, M.A. (2014) Beneficial Effect of Two Culture Systems with Small Groups of Embryos on the Development and Quality of In Vitro-Produced Bovine Embryos. *Anatomia Histologia Embryologia* **43**(1), 22-30

Dai, S.-J., Xu, C.-L., Wang, J., Sun, Y.-P., and Chian, R.-C. (2012) Effect of culture medium volume and embryo density on early mouse embryonic development: Tracking the development of the individual embryo. *Journal of Assisted Reproduction and Genetics* **29**(7), 617-623

De Roover, R., Feugang, J.M.N., Bols, P.E.J., Genicot, G., and Hanzen, C. (2008) Effects of ovum pick-up frequency and FSH stimulation: A retrospective study on seven years of beef cattle in vitro embryo production. *Reproduction in Domestic Animals* **43**(2), 239-245

Ding, L.J., Tian, H.B., Wang, J.J., Chen, J., Sha, H.Y., Chen, J.Q., and Cheng, G.X. (2008) Different Intervals of Ovum Pick-Up Affect the Competence of Oocytes to Support the Preimplantation Development of Cloned Bovine Embryos. *Molecular Reproduction and Development* **75**(12), 1710-1715

Donnay, I., VanLangendonckt, A., Auquier, P., Grisart, B., Vansteenbrugge, A., Massip, A., and Dessy, F. (1997) Effects of co-culture and embryo number on the in vitro development of bovine embryos. *Theriogenology* **47**(8), 1549-1561

Ebner, T., Shebl, O., Moser, M., Mayer, R.B., Arzt, W., and Tews, G. (2010) Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. *Reproductive Biomedicine Online* **21**(6), 762-768

Feng, W.G., Sui, H.S., Han, Z.B., Chang, Z.L., Zhou, P., Liu, D.J., Bao, S., and Tan, J.H. (2007) Effects of follicular atresia and size on the developmental competence of bovine oocytes: A study using the well-in-drop culture system. *Theriogenology* **67**(8), 1339-1350

Ferry, L., Mermillod, P., Massip, A., and Dessy, F. (1994) Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. *Theriogenology* **42**(3), 445-453

Fujita, T., Umeki, H., Shimura, H., Kugumiya, K., and Shiga, K. (2006) Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. *Journal of Reproduction and Development* **52**(1), 137-142

George, P. (2014) 2013 Statistics of Embryo Collection and Transfer in Domestic Farm Animals. *Embryo Transfer Newsletter,* 14-26

Gilchrist, R.B., and Thompson, J.G. (2007) Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology* **67**(1), 6-15

Goovaerts, I.G.F., Leroy, J.L.M.R., Jorssen, E.P.A., and Bols, P.E.J. (2010) Noninvasive bovine oocyte quality assessment possibilities of a single oocyte culture. *Theriogenology* **74**(9), 1509-1520

Gopichandran, N., and Leese, H.J. (2006) The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos. *Reproduction* **131**(2), 269-277

Hasler, J.F. (1998) The current status of oocyte recovery, in vitro embryo production and embryo transfer in domestic animals, with an emphasis on the bovine. *Journal of Animal Science* **76**, 52-74

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E., and Trimmer, S.A. (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43**(1), 141-152

Hoelker, M., Rings, F., Lund, Q., Phatsara, C., Schellander, K., and Tesfaye, D. (2010) Effect of Embryo Density on In vitro Developmental Characteristics of Bovine Preimplantative Embryos with Respect to Micro and Macroenvironments. *Reproduction in Domestic Animals* **45**(5), E138-E145

Khurana, N.K., and Niemann, H. (2000) Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology* **54**(5), 741-756

Lane, M., and Gardner, D.K. (1992) Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. *Human Reproduction* **7**(4), 558-562

Machado, S.A., Reichenbach, H.D., Weppert, M., Wolf, E., and Goncalves, P.B.D. (2006) The variability of ovum pick-up response and in vitro embryo production from monozygotic twin cows. *Theriogenology* **65**(3), 573-583

Matsuura, K. (2014) Numerical calculations for diffusion effects in the well-of-the-well culture system for mammalian embryos. *Reproduction Fertility and Development* **26**(5), 742-751

Merton, J., de Roos, A.P.W., Mullaart, E., de Ruigh, L., Kaal, L., Vos, P., and Dieleman, S.J. (2003) Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology* **59**(2), 651-674

Merton, J.S., de Roos, A.P.W., Koenen, E.P.C., Roelen, B.A.J., Vos, P.L.A.M., Mullaart, E., and Knijn, H.M. (2012) Bovine OPU-Derived Oocytes can be Matured In Vitro for 16-28 h with Similar Developmental Capacity. *Reproduction in Domestic Animals* **47**(6), 1037-1042

O'Doherty, E.M., Wade, M.G., Hill, J.L., and Boland, M.P. (1997) Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* **48**(1), 161-9

O'Neill, C. (2005) The role of paf in embryo physiology. *Human Reproduction Update* **11**(3), 215-228

O'Neill, C. (2008) The potential roles for embryotrophic ligands in preimplantation embryo development. *Human Reproduction Update* **14**(3), 275-288

Pontes, J.H.F., Sterza, F.A.M., Basso, A.C., Ferreira, C.R., Sanches, B.V., Rubin, K.C.P., and Seneda, M.M. (2011) Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. *Theriogenology* **75**(9), 1640-1646

Reed, M.L. (2012) Culture systems: embryo density. *Methods in Molecular Biology* **912**, 273-312

Spanos, S., Becker, D.L., Winston, R.M.L., and Hardy, R. (2000) Anti-apoptotic action of insulin-like growth factor-I during human preimplantation embryo development. *Biology of Reproduction* **63**(5), 1413-1420

Stringfellow, D. (1998) Recommendations for the sanitary handling of in vivo derived embryos. *Manual of the international embryo transfer society*, 79-84

Vajta, G., Peura, T.T., Holm, P., Paldi, K., Greve, T., Trounson, A.O., and Callesen, H. (2000) New method for culture of zona-included or zona-free embryos: The Well of the Well (WOW) system. *Molecular Reproduction and Development* **55**(3), 256-264

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E., De Sutter, P., Vandaele, L., and Van Soom, A. (2014) Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reproduction Fertility and Development* **26**(5), 717-724

CHAPTER 5

DEVELOPMENTAL COMPETENCE OF EARLY, INTERMEDIATE AND LATE CLEAVING EMBRYOS USING TIME-LAPSE IMAGING

Modified from

Time-lapse analysis of early cleavage in bovine embryos produced in serum-free medium.

Catteeuw M, Wydooghe E, Bogado Pascottini O, Piepers S, Smits K, Destouni A, Tšuiko O, Vermeesch JR, Van Soom A.

Presented at the Association Europeenne de Transfer Embryonnaire (AETE).

Ghent, Belgium, September 11-12, 2015.

Abstract

Two decades ago, early cleaving embryos were considered as developmentally more competent than late cleaving embryos. But this theory has been challenged, since it has been shown that murine embryos developing at fast speed display loss in genomic imprinting and embryos developing at slow speed have low developmental rates. For the first time, time-lapse cinematography (TLC) as a non-invasive tool was used to define the developmental competences and quality from bovine intermediate cleaving embryos in comparison with early and late cleaving embryos in a serum-free culture system. Immature oocytes were matured in 500 µL TCM199 supplemented with 20 ng/mL epidermal growth factor (EGF). After *in vitro* fertilization with frozen-thawed semen, presumed zygotes were cultured in Primo Vision^{TM} micro dish (Well-of-the-well type) in 30 µL Synthetic Oviduct Fluid (SOF) supplemented with 0.4% BSA, 5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL selenium (ITS), covered with mineral oil. In total, 63 zygotes were followed with TLC and images were taken every 10 min for up to 90 hours post insemination (hpi). At 192 hpi, blastocyst formation was set as endpoint. At the 2-cell, 3-cell and 4-cell stage, embryos were categorized as early, intermediate or late cleaving depending on their time of cleavage. For both 2-cell and 3-cell stage embryos, there was a significant increase in blastocyst formation in the intermediate cleaving embryos compared to the late cleaving ones. However, no differences were found between the early and intermediate categories. Furthermore, no differences were noted at the 4-cell stage. Nonetheless, when embryos reached the 2-cell stage before 31.1 hours, the odds ratio was 18 times higher to form a blastocyst compared to embryos cleaving after 31.1 hours. The odds ratio increased for embryos at the 4-cell stage at 41.9h (OR=43). In this study, we demonstrated that the bovine embryos can be easily cultured in the WOW-dish under time-lapse imaging. Furthermore, timing of the first cleavages in bovine embryos can be predictive for the further embryonic development, with early and intermediate cleaving embryos having the highest chance of forming a blastocyst.

Introduction

In commercial practice, the *in vitro* production (IVP) of bovine embryos has proven to be a useful method to enhance reproductive performance and genetic gains in cattle (Monteiro *et al.* 2017). The number of offspring from valuable females can be increased because more transferable embryos can be produced by ovum pick-up (OPU) and IVP compared to multiple ovulation and embryo transfer programs (Machado *et al.* 2006). A significant improvement of both OPU and IVP procedures has led to an annual production of more than a million embryos, which is over 40% of the embryos produced worldwide (Perry 2014). The success of the OPU/IVP technique is however highly dependent on individual characteristics of the donor animal (Watanabe *et al.* 2017), since multiple factors are influencing the efficiency of this *in vitro* procedure, such as breed, age and parity (Merton *et al.* 2009). Moreover, there is a high variability in oocyte yield and the intrinsic oocyte quality between donors (Machado *et al.* 2006), even if an identical OPU/IVP protocol is being used, which often results in unpredictable embryo development and blastocyst outcome.

Traditionally, assessing the COCs and embryos' quality is based upon the morphology and developmental progress by inspection of the embryos at specific time points (Mandawala *et al.* 2016). These inspections require that embryos are being removed from the incubator, which may harm the embryos' development due to changes in culture conditions such as altered pH, temperature and humidity inducing additional stress (Campagna *et al.* 2001). Moreover, these single observations at specific time points can result in missing out on events such as abnormal cleavage patterns, which have been associated with a disturbed developmental competence and a decreased blastocyst formation in cattle (Somfai *et al.* 2010b) and in human (Rubio *et al.* 2012). As an alternative to these static observations, time-lapse monitoring systems are gaining more interest since numerous studies have shown that substantially more information can be acquired regarding morphokinetics and embryo viability in both bovine and human studies (Racowsky *et al.* 2015). Moreover, different morphokinetic parameters can be used to improve the prediction of blastocyst formation and embryo selection. Higher implantation and pregnancy rates have been reported after selection of human embryos based on the acquisition of this extra time-lapse information (Armstrong *et al.* 2014). Whereas in the past, it was accepted that early cleaving *in vitro* produced embryos were more similar to their *in vivo* counterparts, studies in mice have shown that early cleaving embryo show loss in genomic imprinting while late cleaving embryos have a decreased blastocyst formation (Market Velker *et al.* 2012). Therefore, the question rises whether intermediate cleaving embryos should be selected for embryo transfer rather than the early cleaving embryos (Gutierrez-Adan *et al.* 2015). Up until today, it is still unclear when embryos can be defined as intermediate cleaving and whether or not these bovine

intermediate cleaving embryos have a higher developmental rate and a better blastocyst quality compared to both early and late cleaving embryos.

Another factor which may affect embryo quality and long-term development is the composition of the culture medium. An important improvement has been the switch from serum supplemented media and/or cell coculture to (semi-)defined media. Due to the link between serum and large offspring syndrome (Young *et al.* 1998), the interest in these chemically (semi-)defined media is increasing. Furthermore, serum has an important influence on the embryos' kinetics as it is inhibiting the first embryo cleavage divisions (Pinyopummintr and Bavister 1991; Van Langendonckt *et al.* 1997). Investigating how bovine embryos are developing in these serum-free conditions can therefore contribute to the commercial OPU/IVP business.

The aim of this present study was to determine bovine embryo kinetics by time-lapse imaging by defining which embryos can be considered as early, intermediate and late cleaving depending on the speed of the first embryonic cleavages in a serum-free environment and to investigate whether the timing of these first embryonic cleavages has a predictive value for the further embryonic development and quality. For the first time, bovine embryos were produced in serum-free conditions and cultured in a well-of-the-well (WOW) dish with time lapse monitoring. This method would allow commercial breeding companies to keep track of the development of each embryo while being cultured in groups and to predict the developmental outcome and potentially improve embryo selection for transfer based upon embryo kinetics.

Methods

Media and reagents

Basic Eagle's Medium amino acids, Minimal Essential Medium (MEM) non-essential amino acids (100 ×), TCM-199-medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium) and all other components were obtained from Sigma (Schnelldorf, Germany), unless otherwise stated. All the media were filter-sterilized using a 0.22 µm filter (Pall Corporation, Ann Arbor, MI, USA) before use.

In vitro embryo production protocol

Bovine embryos were produced as described before (Wydooghe *et al.* 2014a). Bovine ovaries were processed within 2 h of slaughter. Only follicles between 2 and 8 mm were punctured, and follicular fluid containing the oocytes was collected in 2.5 mL of Hepes-Tyrode's albumin-pyruvate-lactate (TALP). Standard, high and good quality cumulus oocyte complexes (COCs) with uniformly granulated

cytoplasm and surrounded by at least 3 compact layers of cumulus cells were grouped per 60 and placed in 500 µL maturation medium, consisting of modified bicarbonate-buffered TCM-199 supplemented with 50 µg/mL gentamycin and 20 ng/mL epidermal growth factor (EGF) for 22 h at 38.5°C in 5% CO2 in humidified air. After maturation, frozen-thawed semen of a previously tested bull was used. Spermatozoa were separated over a discontinuous Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden) and the semen concentration was adjusted to 1 × 106 spermatozoa/mL using IVF-TALP, supplemented with 6 mg/ml BSA (Sigma A8806) and 25 µg/ml heparin. Matured oocytes were incubated in 500 µL IVF-TALP with spermatozoa for 21 h at 38.5°C in 5% CO2 in humidified air. After removal of excess spermatozoa and cumulus cells, presumed zygotes were transferred to synthetic oviductal fluid supplemented with essential and non-essential amino acids (SOFaa), 0.4% BSA (Sigma A9647) and ITS (5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium). Primo VisionTM micro well group culture dishes (Vitrolife, Göteborg, Sweden) were used, consisting of 9 small wells covered by a 30 µL droplet of medium and 2.5 mL mineral oil to prevent evaporation. These dishes are designed by the well-of-the-well (WOW) principle. In each WOW dish, 9 presumed zygotes were placed in individual wells and placed under a time lapse imaging system. WOW dishes were incubated at 38.5°C for 7 days in 5% CO2, 5% O2 and 90% N2.

Time lapse imaging system

A compact, digital inverted microscope (Primo VisionTM; Vitrolife, Göteborg, Sweden) was placed inside a trigas incubator (5% CO2, 5% O2 and 90% N2). The WOW dishes were placed into the sample holder of the microscope. The focus was set mechanically and all embryos were positioned in the field of the view. Every 10 min, a single picture was taken. All images recorded were saved to be analysed later by the software. The embryos were not moved or disturbed during the time lapse analysis.

Determination of early cleavage events in bovine embryos using time-lapse analysis

In 7 replicates, 63 embryos were cultured in WOW dishes for 7 days and time-lapse analysis was performed from the start of culture untill day 3 post insemination (pi). At day 3 pi, the WOW dish including the embryos were placed in the same trigas incubator till day 8 pi but no time-lapse imaging was executed anymore. Only the first cleavages were visualized since the main interest was to determine the timing of the first cell cleavages in order to categorize the embryos depending on their development into early, intermediate or late cleaving embryos.

Analysis

A total of 63 embryos were observed using time-lapse imaging. The time between the fertilization and the cleavage to the 2 cell, 3 cell and 4 cell stage was assessed. Furthermore, the time between the 2 cell and 4 cell stage was noted as the second cell cleavage. An embryo displaying a direct cleavage from one to three or more cells was considered as abnormal first cell cleavage. At day 8 post insemination, total cell number of all blastocysts was evaluated by Hoechst 33342 staining as a criterion for embryo quality (Yuan *et al* 2003). Briefly, blastocysts were individually fixed in 2% paraformaldehyde during 20 min and subsequently stained for 10 min with 0.1% Hoechst. The stained blastocysts were evaluated using a 400x magnification fluorescence microscope (Leica DM 5500B).

Statistical analyses were carried out using RStudio. First, descriptive statistical analyses were executed using quartiles and means. In order to obtain the speed categories, embryos were grouped per cell stage according to the quartile distribution, i.e. embryos present in the first quartile were grouped as early cleaving, embryos present in the second and third quartile as intermediate cleaving and embryos present in the last quartile were grouped as late cleaving embryos. Furthermore, binomial logistic regression was used to assess statistical differences in embryonic development between intermediate versus late and early cleaving embryos. The level of significance was set at P value less than 0.05. The odds ratio was assessed using only two categories, i.e. late and early cleaving embryos. This was based upon the ROC-curve. Embryo quality as assessed by total cell number was evaluated using multilevel regression with timing of cleavage as fixed effect and replicate as random effect.

Results

In total, the morphokinetics of 63 embryos were analysed using time-lapse imaging. From those, 8 did not cleave (12.7%), while 21 embryos reached the blastocyst stage (33.3%). Most of the remaining embryos stopped their development before reaching the 8 cell stage (36.5%). When closely looking at the cleavage time points when embryos reach the 2 cell, 3 cell and 4 cell stage, a rather large distribution is noted (**Figure 1**). The grouping of the embryos into early, intermediate and late cleaving was based upon this distribution; the first quartile was considered as early, the second and third as intermediate and the last quartile as late cleaving. The exact categories can be found in **Table 1**. The average time point when embryos cleaved into 2 cells, 3 cells and 4 cells was 33.06h, 38.66h and 40.39h, respectively. For both 2 cell and 3 cell stages, there was a significant difference between the intermediate cleaving embryos compared to the late cleaving ones, as more embryos reached the blastocyst stage in the intermediate group ($P<0.05$). However, no differences were seen between the early and intermediate cleaving embryos. Also at the 4 cell stage, no differences were noted between the intermediate cleaving embryos and early or late embryos (**Figure 2**). There were 12 embryos

displaying an abnormal first cell cleavage (19.0%) and 4 of these embryos reached the blastocyst stage (33.3%). These embryos were all but one considered as early cleaving since their first cleavage took place before 31.45h, at that moment they displayed three or more blastomeres. Only one abnormal cleaving embryo had an intermediate cleaving pattern.

Table 1 Embryos were classified as early, intermediate and late cleaving depending on the timing of cleavage into 2 cell, 3 cell and 4 cell. (hpi: hours post insemination)

	Embryonic kinetics (hpi)		
	Early	Intermediate	Late
2 cells	< 26.64	26.64 ≤ x ≤ 31.36	> 31.36
3 cells	< 31.45	31.45 ≤ x ≤ 40.90	> 40.90
4 cells	< 36.72	36.72 ≤ x ≤ 43.67	> 43.67

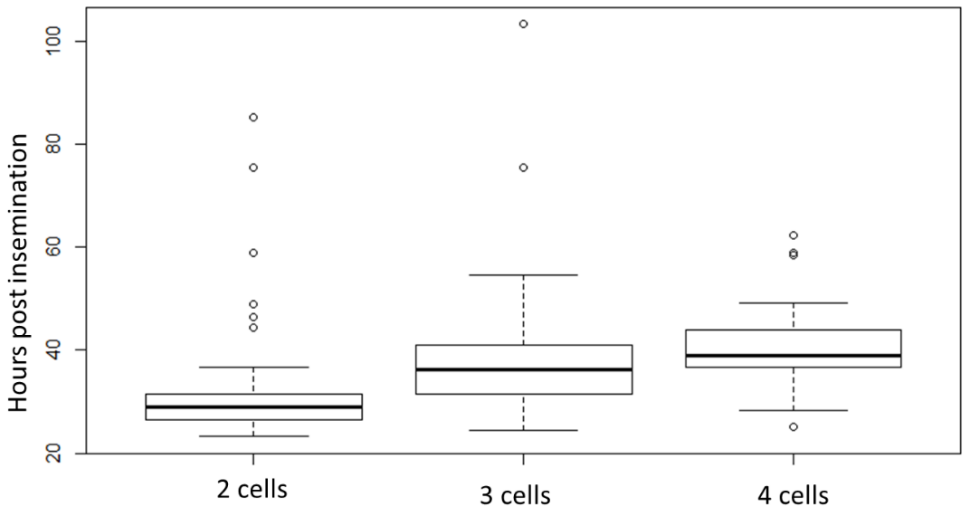


Figure 1 Boxplots show the distribution of embryos cleaving to 2 cell, 3 cell and 4 cell stage embryos. Whiskers represent minimum and maximum, dots represent outliers.

When embryos reach the 2 cell stage before 31.2 hours, the odd ratio is 18 times higher for the embryo to develop into a blastocyst compared to a late cleavage. Moreover, the odds ratio for an embryo to develop into a blastocyst increases even to 43 for embryos reaching the 4 cell stage before 41.9 hours. This is also the case for 2 cell stage embryos that need less than 15.5 hours to reach the 4 cell stage (OR: 43). No differences were found in embryo quality as assessed by the total cell numbers of the blastocysts developing after late, intermediate or early first cleavages (P-values > 0.47) (**Table 2**).

Table 2 Total cell number (TCN) of day 8 blastocysts depending on speed (early, intermediate, late) of cleavage at 2-cell, 3-cell and 4-cell stage. Results are expressed as mean ± standard error.

	Total cell number		
	Early (n)	Intermediate (n)	Late (n)
2 cell	122 ± 14.7 (5)	140 ± 9.4 (11)	84.5 (1)
3 cell	110 ± 19.4 (5)	134 ± 10.7 (11)	84.5 (1)
4 cell	134 ± 4.9 (7)	116 ± 15.5 (11)	84.5 ± 14.0 (2)

Figure 7 The proportion of early (F), intermediate (I) and late (S) cleaving embryos at the 2 cell, 3 cell and 4 cell stage that developed into a blastocyst is indicated in dark grey. The embryos that did not reach the blastocyst stage are visualized in light grey. Intermediate cleaving embryos were compared to early and late cleavers. The asterisk indicates significant differences between groups (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). A trend ($P < 0.1$) was noted at the 4-cell stage for slow compared to fast and intermediate.

Discussion

For the first time, the developmental competences and quality of bovine intermediate cleaving embryos produced in completely serum-free conditions were studied comparing early and late cleaving embryos. A well-of-the-well (WOW) dish under time-lapse monitoring system was applied and mophokinetics were assessed to identify developmental speed depending on the first embryonic cleavages. Furthermore, the predictive values of timing of early cleavage for blastocyst formation and quality were investigated. Although no differences were found between intermediate and early cleaving embryos regarding embryo development rate nor quality, the blastocyst formation was significantly higher in intermediate compared to late cleaving embryos.

It was clear that bovine embryo development was not harmed in these WOW dishes as reported previously (Vajta *et al.* 2000; Sugimura *et al.* 2010; Cebrian-Serrano *et al.* 2014; Wydooghe *et al.* 2014b) since an overall blastocyst rate of 33.3% was observed. Furthermore, a similar embryo development

was reported compared to studies using serum-free conditions (Abe and Hoshi 2003; Wydooghe *et al.* 2014a). Previous time-lapse bovine studies in cattle were performed using culture media containing serum and/or large group (Grisart *et al.* 1994; Somfai *et al.* 2010a; Somfai *et al.* 2010b; Sugimura *et al.* 2010; Beck 2014), while this is the first study using a combination of the WOW principle and serum-free media under time-lapse imaging.

In general, there is a large variation between the timing of the embryos' first divisions, which was also reported in earlier studies using SOF medium without serum supplementation (Van Langendonckt *et al.* 1997; Holm *et al.* 1998). The average time point of cleavage to the 2-cell and 4-cell stage (33.06h and 40.39h), was similar in our study compared to other studies using serum-free conditions (Grisart *et al.* 1994; Yoshioka *et al.* 2000). Whereas serum would inhibit the first embryonic cleavages (Pinyopummintr and Bavister 1991), studies using serum supplementation report an earlier first cleavage than our study (**Table 3**). This can probably be ascribed to the absence of serum during *in vitro* maturation and fertilization in our experimental set-up. It has been reported that serum proteins are important for pronucleus formation but not for cleavage and embryo development (Eckert and Niemann 1995). Moreover, sperm penetration was delayed in a protocol without serum during IVM (Holm *et al.* 2002). However, when comparing morphokinetic studies performed on bovine embryos cultured in the presence or absence of serum serum supplementation does not tend to affect cleavage kinetics to the 4-cell stage and beyond (Table 3). In general, *in vitro* produced bovine embryos have a dark cytoplasm due to lipid content, making it difficult to count the number of cells accurately when development continues and the embryo comprises more blastomeres.

Regarding embryo quality, total cell numbers (TCN) of the blastocysts were assessed. Although there was no significant difference in embryo quality between early, intermediate and late cleaving embryos, these results could be biased due to the low number of embryos analysed. Previous studies investigating bovine embryo development using time-lapse did not observe any differences in blastocysts' TCN either (Somfai *et al.* 2010b; Sugimura *et al.* 2010). Assessment of other parameters of bovine blastocyst quality such as chromosomal constitution, gene expression, proteomic and metabolomic analysis, could indicate differences between early, intermediate and late cleaving embryos. Aneuploidy rate was observed to be significantly higher in late bovine cleaving embryos compared to early cleaving two-cell embryos (Hornak *et al.* 2016). Although a lot of information on early and late cleaving bovine embryos has already been published, there is still a gap in reports focussing on intermediate cleaving embryos.

Table 3 Overview of mean time points of the first bovine embryonic cleavages in our study and in previous time-lapse imaging studies

Reference	IVC medium	Culture system	2-cell (hpi)	3-cell (hpi)	4-cell (hpi)
Our study	SOF + 0.4% BSA +ITS	9 embryos in 30 µL WOW	33.1	38.7	40.4
Van Langendonckt *et al.* 1997	SOF + 1% BSA	28-30 embryos in 27 µL	28.9 ± 4.9	36.9 ± 6.7	
Holm *et al.* 1998	M199 + 5% FCS	30-40 embryos in 400 µL	32 ± 3.9	40.8 ± 1.6	42.8 ± 4.7
Yoshioka *et al.* 2000	SOF + PVA	25 embryos in 40 µL	31.5 ± 0.8	39.5 ± 1.0	NA
Majerus *et al.* 2000	SOF + 5% FCS	Groups (number NA) in 20 µL	25.4 ± 1.58	31.2 ± 4.12	NA
Lequarré *et al.* 2003	SOF + 5% FCS	20-25 embryos in 25 µL	28.6 ± 3.28	37.8 ± 1.33	46.0 ± 1.63
Somfai *et al.* 2010	CR1aa + 5% FCS	15-25 embryos in 50 µL	24.9 ± 0.3	33.5	41.7
Sugimura *et al.* 2013	CR1aa + 5% FCS	15 embryos in 125 µL WOW	25.0 ± 2.3	34.9 ± 3.3	NA
Beck 2014	SOF +5% OCS	16 embryos in 160 µL WOW	29.4 ± 5.8	38.4 ± 6.4	NA

BSA: bovine serum albumin;CR1aa: Charles Rosenkrans 1 amino acids; FCS: fetal calf serum; hpi: hours post insemination; ITS: insulin, transferrin, selenium; IVC: *in vitro* culture; NA: not available; OCS: oestrus cow serum; PVA: polyvinyl alcohol; SOF: synthetic oviductal fluid; WOW: well-of-the-well.

In this study, we noticed a high incidence of direct cleavages in which the embryo cleaved immediately from one to 3-4 blastomeres (19.0%), these could all but one, intermediate cleaving embryo, be considered as early cleaving embryos. Other studies have noted similar high incidences in cattle (Somfai *et al.* 2010b) and human (Yang *et al.* 2015; Zhan *et al.* 2016). In human, studies investigating these first abnormal cleavages events showed no significant differences in euploidy and aneuploidy (Campbell *et al.* 2013), however, it has been reported that abnormal division events may have a negative effect on the embryonic development (Meseguer *et al.* 2011). In contrast, direct cleavages from one to more than three blastomeres seem to have no major impact on further growth in cattle (Somfai *et al.* 2010b), but these bovine embryos have a higher frequency of chromosomal abnormalities (Somfai *et al.* 2010b). Moreover, it has been hypothesized that the chromosomal segregation patterns are aberrant in these direct cleavages and that one paternal genome segregates into a distinct blastomere lineage (Destouni *et al.* 2016). This has been recently investigated in bovine cleavage stage embryos using novel cytogenetical analysis and called 'heterogoneic cell division'. Additionally, we found that the segregation of paternal and maternal genomes into different blastomeres happens at a high frequency in *in vitro* embryos (53.8%) while this was not observed in *in vivo* derived embryos (Tšuiko *et al.* 2017). Nonetheless, we did not notice any negative influence on the further development of these embryos displaying a direct cleavage in this study. Two possible rescue mechanisms can be suggested, apoptosis of a limited number of compromised blastomeres

while healthy blastomeres continue development (Everett and West 1998) and allocation of compromised blastomeres to the trophectoderm at the moment of differentiation (James *et al.* 1995). More research is necessary to elucidate these mechanisms since this direct cleavage and heterogoneic cell division can possibly cause molar pregnancies of androgenetic origin in human (Obeidi *et al.* 2015; Tšuiko *et al.* 2017) and hydroallantois as a result of a dysfunctional placenta in cattle, which is more often observed after transfer of *in vitro* produced embryos (Hasler *et al.* 1995).

In conclusion, intermediate cleaving bovine embryos were defined by time-lapse analysis for the first time and the developmental competences and quality were compared with early and late cleaving embryos. Although there was no difference in quality between these embryos, it is clear that the developmental fate of an embryo produced in serum-free conditions has been set as early as the first embryonic cleavages. Significantly more intermediate cleaving embryos develop into a blastocyst compared to late cleaving embryos. Still, further research is necessary to obtain better insights regarding the impact of the developmental speed since there was no difference between early and intermediate cleaving embryos in developmental outcome. For this, genome, transcriptome and epigenetic analysis on embryos with different kinetic parameters could lead to a better understanding of preimplantation development in bovine embryos. In addition, transfer studies using these *in vitro* produced embryos could provide commercial practices more information whether intermediate cleaving embryos have a higher pregnancy outcome and life birth rate compared to early and late cleaving ones.

Acknowledgements

The authors wish to thank Isabel Lemahieu and Petra Van Damme for their excellent technical assistance. Also, all personnel involved in helping for the collection of the ovaries at the slaughterhouse are cordially thanked.

References

Abe, H., and Hoshi, H. (2003) Evaluation of bovine embryos produced in high performance serum-free media. *Journal of Reproduction and Development* **49**(3), 193-202

Armstrong, S., Vail, A., Mastenbroek, S., Jordan, V., and Farquhar, C. (2014) Time-lapse in the IVF-lab: how should we assess potential benefit? *Human Reproduction* **30**(1), 3-8

Beck, A. (2014) Analysis of early bovine embryogenesis after in vitro and in vivo oocyte maturation by time-lapse imaging and 3-D confocal microscopy. Doctoral dissertation Thesis, IMU

Campagna, C., Sirard, M.-A., Ayotte, P., and Bailey, J.L. (2001) Impaired maturation, fertilization, and embryonic development of porcine oocytes following exposure to an environmentally relevant organochlorine mixture. *Biology of reproduction* **65**(2), 554-560

Campbell, A., Fishel, S., Bowman, N., Duffy, S., Sedler, M., and Hickman, C.F.L. (2013) Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. *Reproductive Biomedicine Online* **26**(5), 477-485

Catteeuw, M., Wydooghe, E., Mullaart, E., Knijn, H.M., and Soom, A. (2017) In vitro production of bovine embryos derived from individual donors in the Corral® dish. *Acta Veterinaria Scandinavica* **59**(1), 41

Cebrian-Serrano, A., Salvador, I., and Silvestre, M.A. (2014) Beneficial Effect of Two Culture Systems with Small Groups of Embryos on the Development and Quality of In Vitro-Produced Bovine Embryos. *Anatomia Histologia Embryologia* **43**(1), 22-30

Destouni, A., Esteki, M.Z., Catteeuw, M., Tsuiko, O., Dimitriadou, E., Smits, K., Kurg, A., Salumets, A., Van Soom, A., Voet, T., and Vermeesch, J.R. (2016) Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage-stage chimerism and mixoploidy. *Genome Research* **26**(5), 567-578

Eckert, J., and Niemann, H. (1995) In vitro maturation, fertilization and culture to blastocysts of bovine oocytes in protein-free media. *Theriogenology* **43**(7), 1211-1225

Everett, C.A., and West, J.D. (1998) Evidence for selection against tetraploid cells in tetraploid [harr] diploid mouse chimaeras before the late blastocyst stage. *Genetics Research* **72**(3), 225-228

Grisart, B., Massip, A., and Dessy, F. (1994) Cinematographic analysis of bovine embryo development in serum-free oviduct-conditioned medium. *Journal of Reproduction and Fertility* **101**(2), 257-264

Gutierrez-Adan, A., White, C.R., Van Soom, A., and Mann, M.R. (2015) Why we should not select the faster embryo: lessons from mice and cattle. *Reproduction, Fertility and Development* **27**(5), 765-775

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E., and Trimmer, S.A. (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43**(1), 141-152

Holm, P., Shukri, N., Vajta, G., Booth, P., Bendixen, C., and Callesen, H. (1998) Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. *Theriogenology* **50**(8), 1285-1299

Holm, P., Booth, P. J., and Callesen, H. (2002) Kinetics of early in vitro development of bovine in vivo- and in vitro-derived zygotes produced and/or cultured in chemically defined or serum-containing media. *Reproduction* **123**(4), 553-565

Hornak, M., Kubicek, D., Broz, P., Hulinska, P., Hanzalova, K., Griffin, D., Machatkova, M., and Rubes, J. (2016) Aneuploidy Detection and mtDNA Quantification in Bovine Embryos with Different Cleavage Onset Using a Next-Generation Sequencing-Based Protocol. *Cytogenetic and genome research* **150**(1), 60-67

James, R.M., Klerkx, A.H., Keighren, M., Flockhart, J.H., and West, J.D. (1995) Restricted distribution of tetraploid cells in mouse tetraploid↔ diploid chimaeras. *Developmental biology* **167**(1), 213-226

Lequarré, A.-S., Marchandise, J., Moreau, B., Massip, A., and Donnay, I. (2003) Cell cycle duration at the time of maternal zygotic transition for in vitro produced bovine embryos: effect of oxygen tension and transcription inhibition. *Biology of reproduction* **69**(5), 1707-1713

Machado, S.A., Reichenbach, H.D., Weppert, M., Wolf, E., and Goncalves, P.B.D. (2006) The variability of ovum pick-up response and in vitro embryo production from monozygotic twin cows. *Theriogenology* **65**(3), 573-583

Majerus, V., Lequarré, A.-S., Ferguson, E., Kaidi, S., Massip, A., Dessy, F., and Donnay, I. (2000) Characterization of embryos derived from calf oocytes: kinetics of cleavage, cell allocation to inner cell mass, and trophectoderm and lipid metabolism. *Molecular Reproduction and Development* **57**(4), 346-52

Mandawala, A., Harvey, S., Roy, T., and Fowler, K. (2016) Time-lapse embryo imaging and morphokinetic profiling: towards a general characterisation of embryogenesis. *Animal reproduction science* **174**, 2-10

Market Velker, B.A., Denomme, M.M., and Mann, M.R. (2012) Loss of genomic imprinting in mouse embryos with fast rates of preimplantation development in culture. *Biology of reproduction* **86**(5), 143, 1-16

Merton, J.S., Ask, B., Onkundi, D.C., Mullaart, E., Colenbrander, B., and Nielen, M. (2009) Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-in vitro production embryo-production program. *Theriogenology* **72**(7), 885-893

Meseguer, M., Herrero, J., Tejera, A., Hilligsøe, K.M., Ramsing, N.B., and Remohí, J. (2011) The use of morphokinetics as a predictor of embryo implantation†. *Human Reproduction* **26**(10), 2658-2671

Monteiro, F., Batista, E., Vieira, L., Bayeux, B., Accorsi, M., Campanholi, S., Dias, E., Souza, A., and Baruselli, P. (2017) Beef donor cows with high number of retrieved COC produce more in vitro embryos compared with cows with low number of COC after repeated ovum pick-up sessions. *Theriogenology* **90**, 54-58

Obeidi, N., Tchrakian, N., Saadeh, F.A., and Mocanu, E. (2015) Suspected ovarian molar pregnancy after assisted reproductive technology conception: a diagnostic challenge. *BMJ case reports* **2015**, bcr2015209353

Perry, G. (2014) Statistics of embryo collection and transfer in domestic farm animals. *Embryo Transfer Newsletter* **32**, 14-26

Pinyopummintr, T., and Bavister, B.D. (1991) In vitro-matured/in vitro-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein-free culture media. *Biology of reproduction* **45**(5), 736-742

Racowsky, C., Kovacs, P., and Martins, W.P. (2015) A critical appraisal of time-lapse imaging for embryo selection: where are we and where do we need to go? *Journal of assisted reproduction and genetics* **32**(7), 1025-1030

Rubio, I., Kuhlmann, R., Agerholm, I., Kirk, J., Herrero, J., Escribá, M.-J., Bellver, J., and Meseguer, M. (2012) Limited implantation success of direct-cleaved human zygotes: a time-lapse study. *Fertility and sterility* **98**(6), 1458-1463

Somfai, T., Inaba, Y., Aikawa, Y., Ohtake, M., Kobayashi, S., Akai, T., Hattori, H., Sugimura, S., Konishi, K., Nagai, T., and Imai, K. (2010a) The effect of oxygen tension on in vitro development of bovine embryos in polydimethylsiloxane-based well of the well dishes prepared under atmospheric or reduced air pressure. *Reproduction Fertility and Development* **22**(1), 235-235

Somfai, T., Inaba, Y., Aikawa, Y., Ohtake, M., Kobayashi, S., Konishi, K., and Imai, K. (2010b) Relationship Between the Length of Cell Cycles, Cleavage Pattern and Developmental Competence in Bovine Embryos Generated by In Vitro Fertilization or Parthenogenesis. *Journal of Reproduction and Development* **56**(2), 200-207

Sugimura, S., Akai, T., Somfai, T., Hirayama, M., Aikawa, Y., Ohtake, M., Hattori, H., Kobayashi, S., Hashiyada, Y., Konishi, K., and Imai, K. (2010) Time-Lapse Cinematography-Compatible Polystyrene-Based Microwell Culture System: A Novel Tool for Tracking the Development of Individual Bovine Embryos. *Biology of Reproduction* **83**(6), 970-978

Sugimura, S., Akai, T., Hashiyada, Y., Aikawa, Y., Ohtake, M., Matsuda, H., Kobayashi, S., Kobayashi, E., Konishi, K., and Imai, K. (2013) Effect of embryo density on in vitro development and gene expression in bovine in vitro-fertilized embryos cultured in a microwell system. *Journal of Reproduction and Development* **59**(2), 115-122

Tšuiko, O., Catteeuw, M., Zamani Esteki, M., Destouni, A., Bogado Pascottini, O., Besenfelder, U., Havlicek, V., Smits, K., Kurg, A., and Salumets, A. (2017) Genome stability of bovine in vivo-conceived cleavage-stage embryos is higher compared to invitro-produced embryos. *Human Reproduction*, 1-10

Vajta, G., Peura, T.T., Holm, P., Paldi, K., Greve, T., Trounson, A.O., and Callesen, H. (2000) New method for culture of zona-included or zona-free embryos: The Well of the Well (WOW) system. *Molecular Reproduction and Development* **55**(3), 256-264

Van Langendonckt, A., Donnay, I., Schuurbiers, N., Auquier, P., Carolan, C., Massip, A., and Dessy, F. (1997) Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. *Journal of Reproduction and Fertility* **109**(1), 87-93

Watanabe, Y.F., de Souza, A.H., Mingoti, R.D., Ferreira, R.M., Batista, E.O.S., Dayan, A., Watanabe, O., Meirelles, F.V., Nogueira, M.F.G., and Ferraz, J.B.S. (2017) Number of oocytes retrieved per donor

during OPU and its relationship with in vitro embryo production and field fertility following embryo transfer. *Animal Reproduction* **14**(3), 635-644

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E., De Sutter, P., Vandaele, L., and Van Soom, A. (2014a) Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reproduction Fertility and Development* **26**(5), 717-724

Wydooghe, E., Vandaele, L., Piepers, S., Dewulf, J., Van den Abbeel, E., De Sutter, P., and Van Soom, A. (2014b) Individual commitment to a group effect: strengths and weaknesses of bovine embryo group culture. *Reproduction* **148**(5), 519-529

Yang, S., Shi, J., Gong, F., Zhang, S., Lu, C., Tan, K., Leng, L., Hao, M., He, H., and Gu, Y. (2015) Cleavage pattern predicts developmental potential of day 3 human embryos produced by IVF. *Reproductive Biomedicine Online* **30**(6), 625-634

Yoshioka, K., Suzuki, C., and Iwamura, S. (2000) Effects of activin A and follistatin on developmental kinetics of bovine embryos: cinematographic analysis in a chemically defined medium. *Journal of Reproduction and Fertility* **118**(1), 119-125

Young, L.E., Sinclair, K.D., and Wilmut, I. (1998) Large offspring syndrome in cattle and sheep. *Reviews of Reproduction* **3**(3), 155-163

Yuan, Y.Q., Van Soom, A., Coopman, F.O.J., Mintiens, K., Boerjan, M.L., Van Zeveren, A., de Kruif, A., and Peelman, L.J. (2003) Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. *Theriogenology* **59**(7), 1585-1596

Zhan, Q., Ye, Z., Clarke, R., Rosenwaks, Z., and Zaninovic, N. (2016) Direct unequal cleavages: embryo developmental competence, genetic constitution and clinical outcome. *PloS one* **11**(12), e0166398

CHAPTER 6

CHROMOSOME INSTABILITY IN *IN VIVO* AND *IN VITRO* BOVINE EMBRYOS

Modified from

Genome stability of bovine *in vivo*-conceived cleavage-stage embryos is higher compared to *in vitro* produced embryos

Tšuiko O*, Catteeuw M*, Zamani Esteki M*, Destouni A, Bogado Pascottini O, Besenfelder U, Havlicek V, Smits K, Kurg A, Salumets A, D'Hooghe T, Voet T, Van Soom A, Vermeesch JR.

* These authors contributed equally to this work.

Human Reproduction, 2017, 1-10.

Abstract

Chromosomal instability (CIN) occurs at high frequency during early embryogenesis and is known to be associated with early embryonic loss, but the stability of *in vivo*-conceived cleavage-stage embryos remains largely unknown. Five young, healthy, cycling Holstein Friesian heifers were used to analyse single blastomeres of *in vivo* embryos, *in vitro* embryos produced by ovum pick-up with ovarian stimulation (OPU-IVF), and *in vitro* embryos produced from *in vitro* matured oocytes retrieved without ovarian stimulation (IVM-IVF). A novel genome wide haplotyping and copy number profiling approach, called haplarithmisis was applied to investigate the allelic architecture of single blastomeres of bovine *in vitro* and *in vivo* produced embryos. The study revealed that the genomic stability of single blastomeres in both of the *in vitro*-cultured embryo cohorts was severely compromised ($P < 0.0001$), and the frequency of whole chromosome or segmental aberrations was higher in embryos produced *in vitro* than in embryos derived *in vivo*. Only 18.8% of *in vivo*-derived embryos contained at least one blastomere with chromosomal anomalies, compared to 69.2% of OPU-IVF embryos ($P < 0.01$) and 84.6% of IVM-IVF embryos ($P < 0.001$). Although CIN is also present in *in vivo*-developed embryos, *in vitro* procedures exacerbate chromosomal abnormalities during early embryo development. Our results encourage to refine and improve *in vitro* culture conditions and assisted reproduction technologies.

Introduction

Up until today, embryonic loss in cattle is still disappointingly high resulting in important economic losses for animal breeders. Whereas the fertilization rate is more than 80% (Hawk and Tanabe 1986), inseminating dairy cows will only lead in 40 to 55% to offspring (Diskin *et al.* 2012). This implies high embryonic and fetal losses, which mostly occur during the first 45 days of gestation (Berg *et al.* 2010). During early embryo development, critical events such as embryonic genome activation, first lineage differentiation and pregnancy recognition by embryonic-maternal signalling are initiated. Failures during these vital phases will lead to embryonic losses in the early stages of pregnancy. Losses can be ascribed to both maternal causes and embryo quality. Pregnancy failures solely due to maternal causes vary between 6 and 16% for heifers and cows, respectively, while 24% of embryo loss is due to the unsatisfactory developmental competence of the embryo itself (Berg *et al.* 2010).

A major cause of embryo loss can be ascribed to chromosomal aberrations in the embryos. Chromosomal instability (CIN) can arise from errors during gametogenesis or around the time of fertilization and subsequent early development (King 1990). There is a progressive embryo loss during early development, but as embryo development continues, the incidence of chromosomally abnormal embryos decreases. Severe aberrations or abnormalities affecting the whole chromosome set are incompatible with development to term. (King 1990; Iwasaki *et al.* 1992; Kawarsky *et al.* 1996). Moreover, aneuploidy has been reported in one-fifth of bovine aborted foetuses and non-viable neonates (Coates *et al.* 1988). Chromosomal abnormalities have been widely investigated in *in vivo* and *in vitro* preimplantation bovine embryos (Iwasaki *et al.* 1992; Hyttel *et al.* 2000; Viuff *et al.* 2000; Viuff *et al.* 2001a; Destouni *et al.* 2016). In *vivo* derived embryos showed less anomalies such as polyploidy immediately after fertilization, compared to *in vitro* produced embryos (Viuff *et al.* 2001b). However, the major limitation of previous studies was the application of low-resolution karyotyping methods, mostly fluorescent in situ hybridization (FISH) with only a few chromosome probes, that can neither detect CIN at the single cell level nor reveal subtle sub-chromosomal aberrations. Thus, the knowledge about the genomic stability of *in vivo*-conceived embryos remained limited, largely due to the lack of robust genome analysis technologies.

To compare *in vitro* versus *in vivo* chromosome instability directly, we used bovine cleavage-stage embryos. A genome-wide single-cell analysis method was applied to enable haplotyping and copy-number profiling, called haplarithmisis (Zamani Esteki *et al.* 2015), on all individual bovine blastomeres obtained from *in vivo* embryos derived from oocytes that were matured and fertilized *in vivo* after ovarian stimulation of donor animals (referred to as *in vivo* embryos). In parallel, we tested *in vitro* produced embryos derived from *in vitro* matured and fertilized oocytes that were retrieved from the

same donor animals using ovarian stimulation and ovum pick-up (referred to as OPU-IVF embryos) and *in vitro* produced embryos derived from *in vitro* matured and fertilized oocytes that were retrieved from these donor animals without ovarian stimulation (referred to as IVM-IVF embryos). All single blastomeres were analysed for the presence of chromosomal aberrations.

Methods

Ethical approval

This study was approved by the Ethical Committee of the Faculty of Veterinary Sciences of Ghent University, Belgium (EC2013/197, EC2015/71).

Study design

The aim of the study was to evaluate chromosome instability in naturally conceived preimplantation embryos. In parallel, we investigated the influence of different IVF procedures on embryo development using bovine as a model for human early embryogenesis (Menezo and Herubel 2002; Destouni *et al.* 2016). Five young, healthy, cycling Holstein Friesian heifers (Bos taurus) between 16 and 27 months of age were used as oocyte and embryo donors. All donor cows were subjected to hormonal stimulation with subsequent ovum pick-up or *in vivo* embryo collection (Besenfelder *et al.* 2008). Blood samples from the donor cows (mothers) and semen from the bull (father) were used to extract bulk DNA (DNeasy Blood and Tissue kit, Qiagen, Germany). Bulk DNA was also obtained from the parents of the bull (paternal grandparents) and the available parents of the cows (maternal grandparents; only for crosses 4757, 8301 and 9617). After hormonal treatments, the cows were left untreated for one month before they were slaughtered. After collection of ovaries, oocytes were retrieved and embryos were produced *in vitro* by routine procedures (Catteeuw *et al.* 2017). Subsequently, single blastomeres were isolated, whole-genome amplified and hybridized on BovineHD BeadChip arrays (Illumina Inc., USA). The acquired array data was used for single-cell genome-wide haplotyping and copy-number profiling (Zamani Esteki *et al.* 2015).

Media and reagents

Basic Eagle's Medium amino acids, Minimal Essential Medium (MEM) non-essential amino acids (100 ×), TCM-199-medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium) and all other components were obtained from Sigma (Schnelldorf, Germany), unless otherwise stated. All the media were filter-sterilized using a 0.22 µm filter (Pall Corporation, Ann Arbor, MI, USA) before use.

Stimulation protocol and ovum pick-up

Stimulation protocol for ovum pick-up was used to generate OPU-IVF embryos and was performed 3 to 6 times in all animals with at least one week interval between OPU sessions. On day 0, heifers were given an epidural anesthesia using 3 mL of Procaine Hydrochloride 2% (VMD, Belgium) to decrease peristalsis and discomfort. An ultrasound probe was inserted in the vagina, and follicles larger than 5 mm were removed by puncturing the ovaries. Animals received dinoprost ($PGF_{2\alpha}$) intramuscularly (i.m.) (Dinolytic®, Zoetis, Belgium), and a CIDR (controlled internal drug release, Progesterone, Zoetis, Belgium) was administered in the vagina. In following days, pFSH injections (Stimufol®, Reprobiol, Belgium) were given i.m. twice a day. The CIDR was removed 40 to 44 hours after the last pFSH injection and OPU was performed. On the day of OPU animals were given an epidural anesthesia using 3 mL of Procaine Hydrochloride 2%. All follicles were aspirated using an ultrasound probe, a 7.5 MHz transducer and a stainless steel guide. Puncturing was performed using disposable 19G needles connected to a 50 mL tube via silicon tubing. Needles were changed between ovaries of the same animal and between animals, further tubing was also renewed between animals. Follicular fluid containing the oocytes was collected in 5 mL HEPES-buffered TCM-199 supplemented with 18 IU/mL heparin, 50 µg/mL gentamicin and 0.1% fetal calf serum (FCS). Immediately following recovery, the collected follicular fluid was filtered through a 75 µm mesh filter with HEPES-buffered TCM-199. For every donor, a new sterile filter was used. Oocytes were grouped per donor and embryos were produced according to the standard *in vitro* embryo production protocol.

In vitro bovine embryo production protocol

Bovine OPU-IVF and IVM-IVF embryos were produced per donor by previously described methods (Catteeuw *et al.* 2017). Briefly, oocytes retrieved via ovum pick-up and oocytes retrieved from ovaries of slaughtered animals were placed per donor in 500 µL maturation medium, consisting of modified bicarbonate-buffered TCM-199 supplemented with 50 µg/mL gentamycin and 20 ng/mL epidermal growth factor (EGF) for 22 h at 38.5°C in 5% CO_2 in humidified air. After maturation, frozen-thawed semen of a previously tested Holstein Friesian bull was used for fertilization. Spermatozoa were separated over a discontinuous Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden) and sperm concentration was adjusted to 1×10^6 spermatozoa/mL using IVF-TALP, which is supplemented with 6 mg/ml BSA (Sigma A8806) and 25 µg/ml heparin. Matured oocytes were incubated per donor in 500 µL IVF-TALP with spermatozoa for 21 h at 38.5°C in 5% CO_2 in humidified air. Presumptive zygotes were transferred to synthetic oviductal fluid (SOF) supplemented with essential and non-essential amino acids (SOFaa), 0.4% BSA (Sigma A9647) and ITS (5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium) and were placed per donor in a droplet of culture medium.

The droplet size differed between donors depending on the number of zygotes, an embryo:medium ratio of 1:2 was maintained with a minimal droplet size of 20 µL. Each droplet was covered by mineral oil and incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2.

Oviductal flush and collection of in vivo embryos

The *in vivo* collection of embryos was performed by oviductal flush as described earlier (Besenfelder *et al.* 2008). First, estrous cycles of the donor animals were pre-synchronized by i.m. administering 2 ml PGF2α (500 µg Cloprostenol, Estrumate, Belgium) twice within 11 days. Forty eight hours after both $PGF_{2\alpha}$ treatments, the animals received i.m. 21 µg Gonadotropin Releasing Hormone (GnRH) (Receptal®, MSD AH, Belgium). Dominant follicles were ablated nine days after heat detection. Thirty six hours later, pFSH was administered in decreasing dosages twice a day for four days (1.5 mL, 1.4 mL, 1.2 mL, 1.1 mL, 0.8 mL, 0.6 mL, 0.5 mL, 0.5 mL), and in total 380 µg follitropine was given. The donor animals received two $PGF_{2\alpha}$ treatments 60 and 72h after the initial pFSH treatment. Finally, 24 h after the last pFSH treatment, 21 µg GnRH was administered to induce ovulation, simultaneously animals were inseminated with frozen-thawed semen. Artificial insemination (AI) was repeated 12 h and 24 h later. Embryos were flushed bilaterally 36 h after the last AI. Briefly, donor animals were given epidural anesthesia using 5 mL of Procaine Hydrochloride 2%. An embryo flushing catheter was directed through the cervix and fixed in the uterine horn. An integrated device consisting of a universal tube, an endoscope and flushing system was inserted through the vaginal wall into the peritoneal cavity, which was passively filled with air. Oviducts were flushed with 40 to 60 mL flushing medium (PBS supplemented with 1% FCS) to pass the embryos through the uterotubal junction. Once in the uterine horn, flushing medium containing the embryos was collected via the uterus flushing catheter into an embryo filter. Finally, the uterine horn was flushed with another 300 to 500 mL medium through the uterine flushing catheter. This procedure was repeated for flushing the other oviduct and uterine horn. The collected medium was transferred to petri dishes and examined for embryos using a stereomicroscope.

Single blastomere isolation and SNP genotyping

IVM-IVF, OPU-IVF and *in vivo*-derived embryos were treated with pronase (0.1% protease for IVM-IVF and OPU-IVF embryos and 1% protease for *in vivo* embryos from S. griseus, Sigma P88110) in Hepes-buffered TCM-199 (Life Technologies Europe, Belgium) to dissolve zona pellucida. The zona-free embryos were then washed in HEPES-buffered TCM-199 with 10% FCS followed by Ca^{+2}/Mg^{+2} free PBS with 0.05% BSA to stimulate blastomere dissociation. Next, each blastomere was washed three times in wash medium (Ca^{+2}/ Mg^{+2} free PBS with 0.1% PVP) and subsequently transferred into a 0.2-

mL PCR tube containing 2µL of PBS and whole-genome amplified (WGA) using a commercial multiple displacement amplification (MDA) kit according to the manufacturer's fast 3 h protocol (REPLI-g Single Cell Kit, Qiagen, Germany). WGA products were purified with SPRI-beads (Beckman Coulter Inc., USA) at 0.8× total reaction volume and SNP genotyped on BovineHD SNP arrays using the Infinium HD whole-genome genotyping assay. Genotyping data obtained in this study has been submitted to NCBI Gene Expression Omnibus (GEO; accession number GSE95358; http://www.ncbi.nlm.nih.gov/geo/).

Single blastomere whole-genome analysis

SNP genotypes, log R Ratio (logR) and B Allele Frequency (BAF) values were obtained for each sample by applying the GenCall algorithm, embedded in the GenomeStudio software Genotyping Module v.3.1 (Illumina Inc.). SNP genotypes were called by setting the GenCall score at 0.75. Next, computational workflow "siCHILD-bovine" was used to acquire genome-wide haplarithm plots for each sample as described previously (Destouni *et al.* 2016). Briefly, the acquired single cell SNP data underwent quality control (QC) using a combination of unsupervised hierarchical clustering on the discrete SNP genotype calls and cumulative chromosome specific standard deviation on the logR values. Substandard samples were excluded from further investigations. The entire process of haplarithmisis was then applied for data analysis as previously described (Zamani Esteki *et al.* 2015). Briefly, haplarithmisis uses single-cell SNP BAF-values and phased parental genotypes to determine genome-wide haplotypes, copy-number state of the haplotypes, as well as the parental and segregational origin of putative haplotype anomalies in the cell. The parental genotypes are phased via SNP genotype calls derived from a close relative, e.g. sibling or the grandparents. In this study we have used paternal and maternal grandparents. Next, specific combinations of phased parental genotypes are retrieved that consequently define single-cell SNP BAF-values. Consequently, these values are plotted on paternal and maternal haplarithms. All the haplarithm plots obtained from this study are provided in Supplementary Figure S3. In parallel with haplarithmisis, genome-wide haplotypes of single blastomeres were also reconstructed. Data were visualized with siCHILD, Circos (Krzywinski *et al.* 2009) and R (https://www.r-project.org/).

Statistical analysis

Statistical calculations were carried out using GraphPad Prism 6 software (GraphPad Software Inc., USA). The prevalence of CIN and the nature of detected chromosomal abnormalities were compared between the three embryo groups and corresponding single blastomeres by two-tailed Fisher's exact test with Bonferroni correction for multiple testing. The differences in the frequencies of CIN between the three embryo cohorts were considered to be statistically significant when the multiple testing

corrected P-value was <0.01. When comparing monospermic embryos, a P-value <0.05 was considered to be statistically significant.

Results

Embryo collection and genome-wide analysis of single bovine blastomeres

Five healthy, cycling Holstein Friesian heifers (*Bos taurus*) were used to produce IVM-IVF, OPU-IVF and *in vivo* embryos (Fig. 1A), and the incidence of CIN was evaluated for all three groups. The use of the same cows and bull seed to study the effect of CIN *in vivo* and *in vitro* reduces potential genetic background confounding effects. First, donor animals were subjected to varying numbers of ovum pick-up sessions depending on the ovarian response to hormonal stimulation and the number of oocytes retrieved per session (Table S1). Overall, 49 oocytes were collected, of which 13 (26.5%, *n* = 49) were good quality oocytes with homogeneous non-granulated cytoplasm and at least three compact layers of cumulus cells. On day-1 post insemination (pi), 28 (57.1%, *n* = 49) of the presumed zygotes cleaved, and subsequently 77 single blastomeres were collected from 10 OPU-IVF embryos on day-2 pi (median 5.0 blastomeres per embryo) and five OPU-IVF embryos on day-3 pi (median 6.0 blastomeres per embryo).

Next, a total of 42 *in vivo* oviductal-stage embryos were retrieved on day-2 pi by oviduct flushing (Besenfelder *et al.* 2008), from which 34 (81.0%) have cleaved. Due to ovarian stimulation and OPU, the cow from cross 4757 developed scar tissue and no flushing of *in vivo* embryos was possible, because of the obstruction of the oviduct. Of all the cleaved *in vivo* embryos, 12 either had indigestible zona pellucida or lysed during washing and single cell collection. As a result, 22 zona-free embryos were collected (median 4.45 blastomeres per embryo) and 18 were hybridized on SNP arrays after successful amplification of at least half of the blastomeres per embryo (*n* = 73).

Following OPU-IVF and *in vivo* embryo collection, donor animals were slaughtered, ovaries were collected, and *in vitro* embryos were produced (Catteeuw *et al.* 2017). Thirty-one oocytes were aspirated, of which 16 (51.6%, *n* = 31) were of good quality. On day-1 pi, 20 (64.5%, *n* = 31) of the presumed zygotes have cleaved. Subsequently, 72 blastomeres were isolated from 13 day-2 pi IVM-IVF embryos (mean 5.54 blastomeres per embryo) and further analyzed.

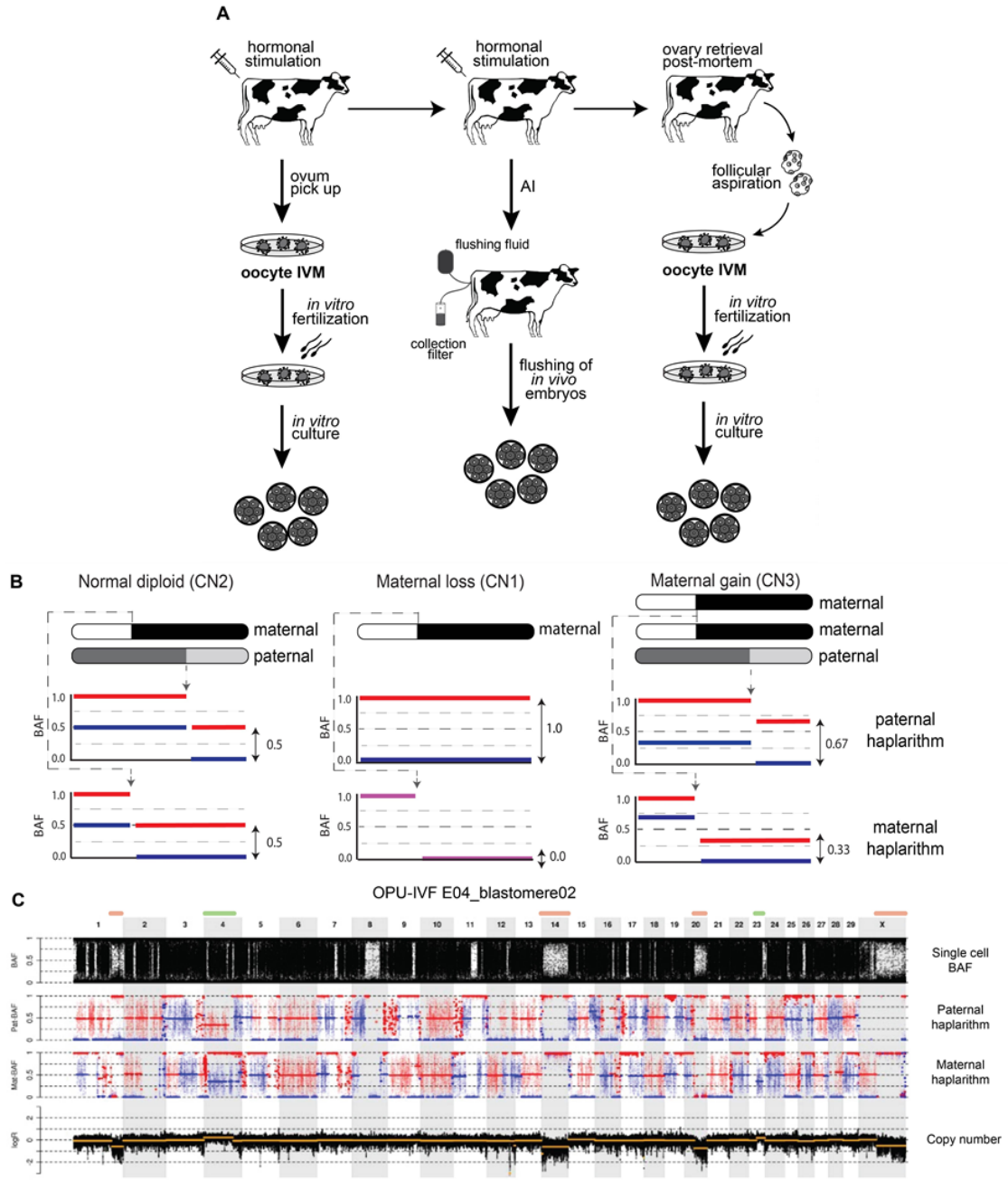


Figure 1. Schematic overview of study design and data analysis. (A) Different protocols used for embryo production. First, five donor cows were used to obtain OPU-IVF embryos (left). Next, *in vivo* embryos were derived from the same donor cows via oviduct flush (middle). Finally, donor cows were slaughtered and IVM-IVF embryos were produced (right). (B) Schematic representation of haplarithm profiles for different genomic rearrangements. During initial parental phasing using informative SNPs, single cell B allele frequency (BAF) values are assigned to P1 or M1 (blue lines) and P2 or M2 (red lines) subcategories (for more detail see (Zamani Esteki *et al.* 2015)). Defined single cell BAF values of the segmented P1, P2, M1 and M2, as well as the distance between the P1-P2 or M1-M2 denote the origin and nature of copy number (CN) alterations. Dashed grey arrows showing the pairwise P1-P2 and M1-M2 breakpoints in the haplarithm profiles signify homologous recombination sites, accompanied by the switch in the haplotype blocks. (C) An example of genome-wide haplarithm profile of single blastomere of OPU-IVF embryo E04 with segmental and full chromosome losses (light red) and gains (light green), corroborated by the corresponding paternal and maternal haplarithm patterns and the normalized logR-values.

In summary, a total of 222 individual bovine blastomeres were collected from 13 IVM-IVF, 15 OPU-IVF and 18 *in vivo*-derived bovine cleavage-stage embryos. Following quality control and initial data analysis, 171 (77.0%, *n* = 222) blastomeres were considered for further data interpretation (66 blastomeres from 13 IVM-IVF embryos, 46 blastomeres from 13 OPU-IVF embryos and 59 blastomeres from 16 *in vivo*-derived embryos, respectively; Table S2). For crosses 4757, 8301 and 9617 we applied haplarithmisis using both maternal and paternal grandparents as seeds for parental genotype phasing to reconstruct haplotypes of single blastomeres (Fig. 1B-C). For crosses 4006 and 4770, lacking maternal grandparental DNA samples, only parents of the bull were used as a seed for creating the paternal haplarithm profile.

Characteristics of CIN in embryos developed in vitro and in vivo

We first aimed to assess the prevalence of chromosome instability in IVM-IVF, OPU-IVF and *in vivo*-derived bovine cleavage-stage embryos. To evaluate the genomic stability of embryos, we investigated chromosome segregation patterns in all analysed blastomeres (*n* = 171, Fig. S1). In this analysis, euploid blastomeres, irrespective of their ploidy, that lacked full chromosome or segmental aberrations were scored as balanced. The genomic integrity of single blastomeres was higher in *in vivo* embryos than in OPU-IVF and IVM-IVF embryos (in both cases $P < 0.0001$, Fisher's exact test; Fig. 2A). At the embryonic level, the number of abnormal embryos carrying at least one blastomere with a full or segmental chromosomal aberration increased from 18.8% in *in vivo* embryos (3/16) up to 84.6% in IVM-IVF embryos (11/13) ($P < 0.001$, Fisher's exact test; Fig. 2B). The CIN rate in OPU-IVF embryos (69.2%, 9/13) was comparable to the CIN rate in IVM-IVF embryos ($P > 0.05$, Fisher's exact test), but was higher than in *in vivo*-derived embryos ($P < 0.01$, Fisher's exact test). Because OPU-IVF group also contained five day-3 pi embryos that may have undergone at least one more cell division that can lead to mitotic error, we decided to analyse day-2 pi OPU-IVF embryos (*n* = 8) separately to obtain more consistency between the groups. Comparison of day-2 OPU-IVF and *in vivo* embryos provided similar results, demonstrating that CIN is higher in day-2 pi OPU-IVF embryos (75%, 6/8) than in *in vivo*-derived day-2 pi embryos ($P = 0.02$, Fisher's exact test). Likewise, OPU-IVF embryos contained a significantly larger proportion of unbalanced blastomeres that can subsequently lead to altered embryonic development (16/26, 61.5%, $P < 0.0001$, Fisher's exact test).

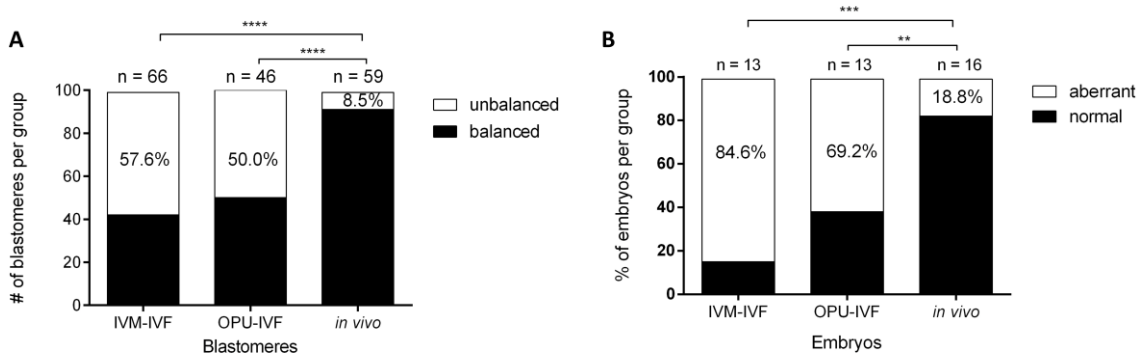


Figure 2. The rate of CIN in IVM-IVF, OPU-IVF and *in vivo* embryos. The numbers above the columns represent the total numbers of blastomeres (A) and embryos (B) included in the study after quality control. (A) The comparison of balanced and unbalanced blastomeres represents the chromosome dynamics of single blastomeres in IVM-IVF (n = 66), OPU-IVF (n = 46) and *in vivo*-derived embryos (n = 59); ****P < 0.0001, two-tailed Fisher's exact test for multiple testing. (B) The proportion of normal diploid embryos and aberrant embryos in IVM-IVF (n = 13), OPU-IVF (n = 13) and *in vivo* group (n = 16); **P < 0.01, ***P < 0.001, two-tailed Fisher's exact test for multiple testing.

Next, we determined the nature of chromosomal aberrations in *in vitro*-produced and *in vivo*-derived embryos. As was expected, aneuploidy was the most prevalent type of error and all abnormal *in vivo*-derived, OPU-IVF and IVM-IVF embryos contained whole chromosome aberrations. Therefore, the number of embryos with aneuploidy was significantly higher in OPU-IVF embryos (69.2%, 9/13) and in IVM-IVF embryos (84.6%, 11/13) than in the *in vivo* group (18.8%, 3/16, $P < 0.01$ and $P < 0.001$, respectively, Fisher's exact test; Fig. 3A). Meiotic errors were observed only in Cross9617, once within the OPU-IVF group and once within the IVM-IVF group, in which case the embryos showed the same chromosomal aneuploidy in all of the sister blastomeres (OPU-IVF E01_Cross9617 and IVM-IVF E09_Cross9617 were monosomic for chromosomes 26 and 24, respectively; Fig. S1). The remaining aberrations were of mitotic origin and resulted in either whole chromosome or segmental imbalances. Segmental imbalances were most prevalent in IVM-IVF embryos (9/13, 69.2%) when compared to OPU-IVF embryos (2/13, 15.4%, $P = 0.01$, Fisher's exact test) and *in vivo* embryos (1/16, 6.3%, $P = 0.001$, Fisher's exact test; Fig. 3A).

We also observed a number of embryos that had at least one blastomere with an abnormal ploidy state (Fig. 3A). Single-cell haplarithm profiles uncovered the presence of only paternal (androgenetic) or only maternal (gynogenetic) genomes in a single blastomere and enabled triploid blastomeres to be classified as diandric or digynic in origin. Upon comparing IVM-OPU, OPU-IVF and *in vivo* embryos, we observed that IVM-IVF embryos were burdened with mixoploidy. In this study, mixoploidy is defined by the presence of cell lineages of different parental origin and/or different genome-wide ploidy states within the same embryo. As such, mixoploid embryos harbour simultaneously haploid, diploid and/or triploid cells (Fig. 3B, Fig. S1). Mixoploidy in IVM-IVF embryos can be attributed to the dispermic

fertilization (69.2%, 9/13) that was identified by the presence of two different paternal haplotypes within the same embryo. In seven IVM-IVF embryos one of the extra paternal genomes segregated into a separate androgenetic cell line carrying only paternal DNA, a phenomenon that was recently discovered in *in vitro* produced bovine cleavage-stage embryos and termed heterogoneic cell division (Destouni *et al.* 2016). For example, a 10-cell IVM-IVF embryo contained eight androgenetic blastomeres, one biparental and one triploid blastomere (E10_Cross4770, Fig. 3B). In addition, we observed amplified shattered paternal chromosomal DNA fragments and no maternal DNA in blastomere Bl005 of E07_Cross4770 (Fig. S1), and in E11_Cross4770 two blastomeres (Bl002 and Bl003) contained residues of paternal DNA that were reciprocal in nature (Fig. S1). Such replication and division of the remnants of sperm genome were also observed in a previous study on bovine *in vitro*-produced embryos (Destouni *et al.* 2016). In contrast, only one OPU-IVF embryo underwent dispermic fertilization resulting in a diandric triploid embryo (E03_Cross4757, Fig. S1). Notably, no abnormal fertilization events occurred in the *in vivo*-derived embryos.

Because dispermy might influence CIN in embryos, we then analysed only those embryos that developed from monospermic zygotes. For this purpose, we combined monospermic IVM-IVF (n = 4) and OPU-IVF (n = 12) embryos into a single group (referred to as *in vitro*) and compared them to *in vivo*-derived embryos (n = 16). The CIN rates confirmed a considerable difference between the *in vitro* produced and cultured embryos, and *in vivo*-derived embryos (10/16, 62.5% vs 3/16, 18.8%, P = 0.03, Fisher's exact test; Fig. S2A). In addition, it was clear that *in vitro* procedures had a highly significant negative impact on CIN, when we compared the low frequency of chromosomal aberrations and aberrant ploidy states in blastomeres of *in vivo*-derived embryos (7/59, 11.9%) with the high frequency chromosomal aberrations and aberrant ploidy states of *in vitro* produced and cultured embryos (27/57, 47.4%, P < 0.0001, Fisher's exact test; Fig. S2B). Similarly, when analysing only day-2 pi embryos, seven *in vitro* embryos were classified as abnormal (7/11, 63.6%, P = 0.04), while the total number of abnormal blastomeres in the *in vitro* group reached up to 45.9% (17/37, P < 0.001, Fisher's exact test). Together, these results strongly suggest that *in vitro* procedures, such as maturation, fertilization and culture, enhance embryonic CIN and consequently impede embryo developmental potential.

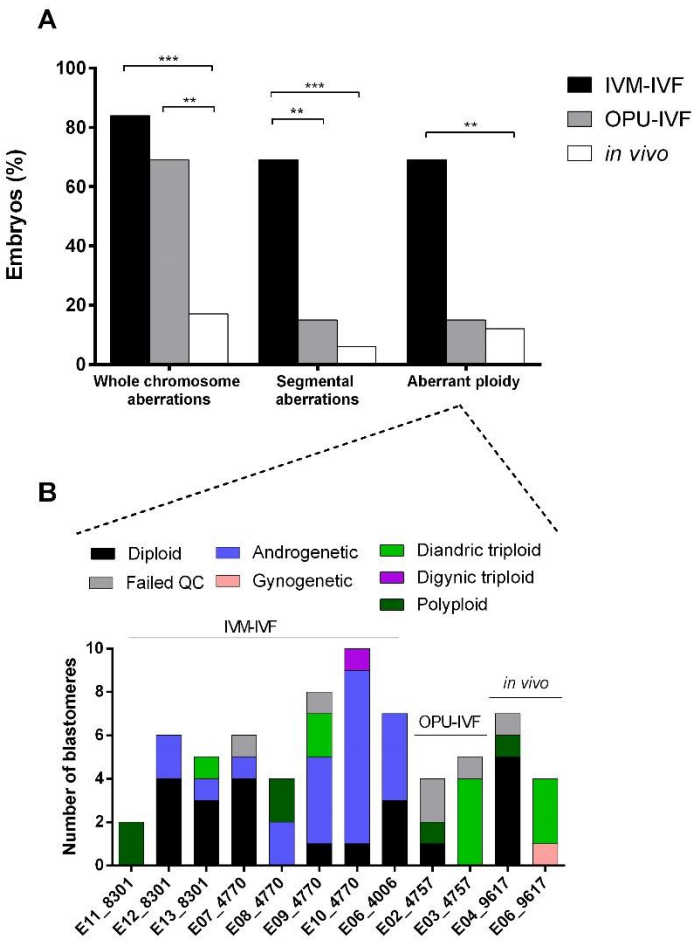


Figure 3. Incidence and nature of CIN in IVM-IVF, OPU-IVF and *in vivo* embryos. (A) The proportion of embryos with different genomic rearrangements. Abnormal ploidy state of blastomeres was not considered in evaluating whole-chromosome aberrations to avoid bias; for whole chromosome aberrations P-values stand as **P < 0.01, ***P < 0.001; for segmental imbalances and aberrant ploidy P-values stand as **P = 0.01, ***P = 0.001, two-tailed Fisher's exact test for multiple testing. (B) Examples of embryos containing at least one blastomere with an abnormal ploidy status. The stacked bar plots depict the number of blastomeres with different genomic anomalies per embryo. Blastomeres with normal karyotype and with single aneuploidies and/or segmental losses or gains that have both maternal and paternal alleles present in their genome (diploid embryos) are depicted in black.

Discussion

Over the last thirty years, commercial embryo production and transfer has become a widely established technology in cattle breeding. Approximately 15% of bovine embryos are being produced via *in vitro* techniques, which is annually more than 500,000 embryos worldwide (Mapletoft and Hasler 2005). Current cytogenetic testing are mostly used for sexing embryos, however, it is likely that these techniques will be applied on a large scale in the future for diagnostic purposes , such as evaluation of chromosome integrity. In this study, we applied an advanced genome analysis method to scrutinize the characteristics of chromosomal aberrations in *in vivo*-derived embryos and to investigate the potential influence of ART treatments on the rate and nature of chromosome instability during early

embryo development. We demonstrated that the genomic stability of *in vivo* embryos is significantly higher compared to OPU-IVF and IVM-IVF embryos.

Only 50% of inseminations or embryo transfers will result in healthy offspring. Infertility and early embryo loss are playing a main role in this low calving rate, and the occurrence of chromosomal abnormalities in early embryos has been implicated as a possible cause (Schmutz *et al.* 1996). Chromosomal instability (CIN) has been reported to occur in both *in vivo* and *in vitro* produced bovine embryos (Viuff *et al.* 1999; Viuff *et al.* 2000; Viuff *et al.* 2001a; Garcia-Herreros *et al.* 2010; Demyda-Peyrás *et al.* 2013; Destouni *et al.* 2016), but the incidence of anomalies varied widely among these studies, possibly due to low-resolution cytogenetic techniques. With this novel genome analysis, CIN was found to be significantly more frequent in *in vitro*-produced rather that *in vivo*-derived bovine embryos. Since CIN was observed in less than 20% of *in vivo* embryos compared to at least 70% in *in vitro* embryos, *in vivo* conceived embryos will most likely be overall more viable. This observation has two major implications: (1) commercial industries should be aware of the fact that ART may compromise embryo quality and (2) improvements to the embryo *in vitro* environment are likely still possible to enhance ART success.

When oocytes are matured *in vivo*, they originate from ovulatory follicles that undergo strongly regulated processes of selection, growth and dominance, until luteinizing hormone (LH) surge induces the meiotic maturation of the fully grown oocytes into fertilizable oocytes (Li and Albertini 2013). In addition, during preimplantation embryo development *in vivo*, the female reproductive tract provides the appropriate environment and the essential nutrition that guide physiological processes of mammalian early embryogenesis (Gardner *et al.* 1996). In contrast, during preimplantation embryo development *in vitro,* even minor alterations during *in vitro* culture and the micromanipulation of oocytes and embryos may negatively impact embryo quality and subsequent fetal development (Wale and Gardner 2016). Indeed, a study in cattle investigated the separate effect of *in vitro* procedures (maturation, fertilization and culture) on embryo developmental potential, and it was clear that *in vivo* oocyte maturation and *in vivo* embryo development show consistently more favourable outcome in terms of embryo quality compared to *in vitro* conditions (Rizos *et al.* 2002). Moreover, the oocyte plays a central role in maintaining genomic integrity before major embryonic genome activation (EGA), as first post-zygotic divisions are highly dependent on the large pool of maternal mRNAs and proteins provided by the oocyte (Braude *et al.* 1988). This view is supported by a time-lapse study, demonstrating that the generation of embryonic aneuploidies precedes the major wave of EGA (Chavez *et al.* 2012), while the inheritance of an aberrant oocyte transcriptome has been associated with abnormal first post-zygotic cleavage (Vera-Rodriguez *et al.* 2015). Therefore, in the current study,

the higher rate of chromosomal abnormalities in *in vitro* embryos may also arise from the defective maternal resources of the oocytes; however more research should target the precise impact of the intrinsic quality of the oocyte on the incidence of chromosomal aberrations in cleavage-stage embryos. Finally, although it was recently demonstrated that mosaic embryos may be viable, as abnormal cells get depleted during embryo development, there needs to be a sufficient proportion of normal cells within the embryo to ensure its survival (Bolton *et al.* 2016).

Ovarian stimulation is widely used to bypass the physiological limitation of one ovulation per oestrus cycle when embryo transfer is performed. Gonadotrophins are administered in order to attain multiple ovulations in the donor animal, which is subsequently inseminated. Resulting embryos can be flushed from the uterus and transferred to recipient cows (Aerts and Bols 2010). Although it is not necessary to use hormonal treatment prior to OPU, since follicles are punctured before ovulation, it is also commonly used in commercial industries. It does not increase the overall recovery rate since less sessions can be performed within the same timeframe compared to OPU without hormonal pre-stimulation, however, the oocytes' quality seemed to be higher after ovarian stimulation (Goodhand *et al.* 1999). Moreover, superstimulation is considered to be a safe procedure and in contrast to ovarian stimulation in women, there is no risk of hyperstimulation syndrome (Van Wagtendonk-de Leeuw 2006). In our study, although both *in vivo* and OPU-IVF embryos were obtained after hormonal stimulation of donor animals, we observed more chromosomally normal diploid embryos and blastomeres in *in vivo*-derived embryos than in OPU-IVF embryos. This indicates that *in vitro* maturation, fertilization and culture are the major causes of embryonic CIN, rather than ovarian stimulation itself. This further suggests that improvements in culture conditions are necessary to increase IVF success rates.

In vivo matured oocytes are after ovulation in contact with the oviductal environment, which enables the oocyte to reach full cytoplasmic maturation, which includes hardening of the zona pellucida (Mondéjar *et al.* 2013). The zona pellucida of IVM oocytes, which lack oviductal contact, may therefore become less resistant to dispermic fertilization under *in vitro* conditions (Xia 2013). Our results seem to corroborate this view as dispermic fertilization was almost exclusively found among IVM-IVF embryos. In addition, we observed that dispermic embryos segregate their extra paternal genome into a separate androgenetic cell lineage (Destouni *et al.* 2016) leading to mixoploidy. These embryos would have a low developmental potential, but due to a highly proliferative cell lineage carrying paternal genome only, they would have a higher implantation capacity and could potentially give rise to molar pregnancies of androgenetic origin. Complete hydatidiform moles of androgenetic origin have been reported in both human (Ibrahim *et al.* 1989; Kwon *et al.* 2002; Sun *et al.* 2012; Obeidi *et al.* 2015) and

in cattle (Meinecke *et al.* 2002). Furthermore, it can be suggested as well that these embryos which segregate into separate parental lineages give rise to hydrallantois pregnancies, since this is a result of a dysfunctional placenta causing an increased production and accumulation of fluid in the allantoic sac. It has already been reported that the frequency of hydrallantois pregnancies is significantly higher among IVF pregnancies compared to natural pregnancies, 1 in 200 vs 1 in 7500 pregnancies respectively (Hasler *et al.* 1995; Farin *et al.* 2006).

A limitation of our study is the small number of embryos analysed, and because some of the cells did not pass the quality control, it was also not possible to determine the chromosomal status of those QC-failed blastomeres. In addition, due to small cohort sizes, we were not able to compare the pedigrees between each other to determine any cow-specific confounding factors, influencing the frequency of aneuploidy in embryos. Thus more studies are warranted to corroborate our findings. Also, *in vivo*-derived embryos do not entirely represent the natural conception, as donor cows underwent hormonal stimulation to increase the number of *in vivo*-derived embryos via oviductal flush. Moreover, the ovarian response to hormonal treatment is unpredictable and can vary from cycle to cycle, and may result in either 'low' or 'high' response to hormone treatment in cattle (De Roover *et al.* 2005; Durocher *et al.* 2006). Although the difference in oocyte and embryo quality after ovarian stimulation between donor animals was also noticed in this study (Table S1), future research is needed to evaluate the impact of hormonal stimulation on CIN in embryos. However, the overall reduced CIN in *in vivo* embryos compared to OPU-IVF embryos suggests that the effect of hormonal stimulation will be minor.

Conclusions

This is the first study to date that compared simultaneously the impact of three different embryo production protocols on subsequent embryo development using single-cell technologies. We showed that *in vitro* environment influence chromosome instability and compromises cleavage-stage embryo development and survival. This highlights the importance of understanding *in vivo* regulation of mammalian oocyte maturation and subsequent embryonic development to refine assisted reproductive technologies.

Acknowledgements

The authors thank the CRV Belgium (www.crv4all.be) team for providing grandparental bovine genotyping data used in this study. We are grateful to Nathalie Sohier, Eftychia Dimitriadou, Isabel Lemahieu and Petra Van Damme for their excellent technical assistance.

References

Aerts, J., and Bols, P. (2010) Ovarian follicular dynamics. A review with emphasis on the bovine species. Part II: Antral development, exogenous influence and future prospects. *Reproduction in domestic animals* **45**(1), 180-187

Berg, D., Van Leeuwen, J., Beaumont, S., Berg, M., and Pfeffer, P. (2010) Embryo loss in cattle between Days 7 and 16 of pregnancy. *Theriogenology* **73**(2), 250-260

Besenfelder, U., Havlicek, V., Moesslacher, G., Gilles, M., Tesfaye, D., Griese, J., Hoelker, M., Hyttel, P.M., Laurincik, J., Brem, G., and Schellander, K. (2008) Endoscopic recovery of early preimplantation bovine embryos: effect of hormonal stimulation, embryo kinetics and repeated collection. *Reproduction in Domestic Animals* **43**(5), 566-72

Bolton, H., Graham, S.J., Van der Aa, N., Kumar, P., Theunis, K., Fernandez Gallardo, E., Voet, T., and Zernicka-Goetz, M. (2016) Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. *Nature Communications* **7**, 11165

Braude, P., Bolton, V., and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332**(6163), 459-61

Catteeuw, M., Wydooghe, E., Mullaart, E., Knijn, H.M., and Van Soom, A. (2017) In vitro production of bovine embryos derived from individual donors in the Corral(R) dish. *Acta Veterinaria Scandinavica* **59**(1), 41

Chavez, S.L., Loewke, K.E., Han, J., Moussavi, F., Colls, P., Munne, S., Behr, B., and Reijo Pera, R.A. (2012) Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. *Nature Communications* **3**, 1251

Coates, J.W., Schmutz, S.M., and Rousseaux, C.G. (1988) A survey of malformed aborted bovine fetuses, stillbirths and nonviable neonates for abnormal karyotypes. *Canadian Journal of Veterinary Research* **52**(2), 258-63

De Roover, R., Bols, P.E., Genicot, G., and Hanzen, C. (2005) Characterisation of low, medium and high responders following FSH stimulation prior to ultrasound-guided transvaginal oocyte retrieval in cows. *Theriogenology* **63**(7), 1902-13

Demyda-Peyrás, S., Dorado, J., Hidalgo, M., Anter, J., De Luca, L., Genero, E., and Moreno-Millán, M. (2013) Effects of oocyte quality, incubation time and maturation environment on the number of chromosomal abnormalities in IVF-derived early bovine embryos. *Reproduction, Fertility and Development* **25**(7), 1077-1084

Destouni, A., Esteki, M.Z., Catteeuw, M., Tsuiko, O., Dimitriadou, E., Smits, K., Kurg, A., Salumets, A., Van Soom, A., Voet, T., and Vermeesch, J.R. (2016a) Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage-stage chimerism and mixoploidy. *Genome Research* **26**(5), 567-578

Diskin, M.G., Parr, M.H., and Morris, D.G. (2012) Embryo death in cattle: an update. *Reproduction, Fertility and Development* **24**(1), 244-51

Durocher, J., Morin, N., and Blondin, P. (2006) Effect of hormonal stimulation on bovine follicular response and oocyte developmental competence in a commercial operation. *Theriogenology* **65**(1), 102-115

Farin, P.W., Piedrahita, J.A., and Farin, C.E. (2006) Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology* **65**(1), 178-191

Garcia-Herreros, M., Carter, T., Villagómez, D., MacAulay, A., Rath, D., King, W., and Lonergan, P. (2010) Incidence of chromosomal abnormalities in bovine blastocysts derived from unsorted and sex-sorted spermatozoa. *Reproduction, Fertility and Development* **22**(8), 1272-1278

Gardner, D.K., Lane, M., Calderon, I., and Leeton, J. (1996) Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertility and Sterility* **65**(2), 349-53

Goodhand, K., Watt, R., Staines, M., Hutchinson, J., and Broadbent, P. (1999) In vivo oocyte recovery and in vitro embryo production from bovine donors aspirated at different frequencies or following FSH treatment. *Theriogenology* **51**(5), 951-961

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E., and Trimmer, S.A. (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43**(1), 141-152

Hawk, H., and Tanabe, T. (1986) Effect of unilateral cornual insemination upon fertilization rate in superovulating and single-ovulating cattle. *Journal of animal science* **63**(2), 551-560

Hyttel, P., Viuff, D., Laurincik, J., Schmidt, M., Thomsen, P.D., Avery, B., Callesen, H., Rath, D., Niemann, H., Rosenkranz, C., Schellander, K., Ochs, R.L., and Greve, T. (2000) Risks of in-vitro production of cattle and swine embryos: aberrations in chromosome numbers, ribosomal RNA gene activation and perinatal physiology. *Human Reproduction* **15**, 87-97

Ibrahim, Z.H., Matson, P.L., Buck, P., Atkinson, A.D., and Lieberman, B.A. (1989) Hydatidiform mole following in-vitro fertilization. Case report. *British Journal of Obstetrics and Gynaecology* **96**(10), 1238-40

Iwasaki, S., Hamano, S., Kuwayama, M., Yamashita, M., Ushijima, H., Nagaoka, S., and Nakahara, T. (1992) Developmental changes in the incidence of chromosome anomalies of bovine embryos fertilized in vitro. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **261**(1), 79-85

Kawarsky, S.J., Basrur, P.K., Stubbings, R.B., Hansen, P.J., and Allan King, W. (1996) Chromosomal abnormalities in bovine embryos and their influence on development. *Biology of reproduction* **54**(1), 53-59

King, W. (1990) Chromosome abnormalities and pregnancy failure in domestic animals. *Advances in veterinary science and comparative medicine* **34**, 229-250

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., and Marra, M.A. (2009) Circos: an information aesthetic for comparative genomics. *Genome Research* **19**(9), 1639-45

Kwon, H.E., Park, E.J., Kim, S.H., Chae, H.D., Won, H.S., Kim, C.H., and Kang, B.M. (2002) A case of twin pregnancy with complete hydatidiform mole and coexisting fetus following IVF-ET. *Journal of Assisted Reproduction and Genetics* **19**(3), 144-8

Li, R., and Albertini, D.F. (2013) The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nature Reviews Molecular Cell Biology* **14**(3), 141-52

Mapletoft, R., and Hasler, J. (2005) Assisted reproductive technologies in cattle: a review. *Revue Scientifique et Technique-Office International des Epizooties* **24**(1), 393

Meinecke, B., Kuiper, H., Drogemuller, C., Leeb, T., and Meinecke-Tillmann, S. (2002) A mola hydatidosa coexistent with a foetus in a bovine freemartin pregnancy. *Placenta* **24**(1), 107-12

Menezo, Y.J., and Herubel, F. (2002) Mouse and bovine models for human IVF. *Reproductive Biomedicine Online* **4**(2), 170-5

Mondéjar, I., Martínez-Martínez, I., Avilés, M., and Coy, P. (2013) Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. *Biology of reproduction* **89**(3)

Obeidi, N., Tchrakian, N., Abu Saadeh, F., and Mocanu, E. (2015) Suspected ovarian molar pregnancy after assisted reproductive technology conception: a diagnostic challenge. *BMJ case reports* **2015**

Rizos, D., Ward, F., Duffy, P., Boland, M.P., and Lonergan, P. (2002) Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development* **61**(2), 234-48

Schmutz, S., Moker, J., Clark, E., and Orr, J. (1996) Chromosomal aneuploidy associated with spontaneous abortions and neonatal losses in cattle. *Journal of Veterinary Diagnostic Investigation* **8**(1), 91-95

Sun, C.J., Zhao, Y.P., Yu, S., Fan, L., Wu, Q.Q., Li, G.H., and Zhang, W.Y. (2012) Twin pregnancy and partial hydatidiform mole following in vitro fertilization and embryos transfer: a novel case of placental mosaicism. *Chinese Medical Journal (Engl)* **125**(24), 4517-9

Van Wagtendonk-de Leeuw, A.M. (2006) Ovum pick up and in vitro production in the bovine after use in several generations: a 2005 status. *Theriogenology* **65**(5), 914-925

Vera-Rodriguez, M., Chavez, S.L., Rubio, C., Reijo Pera, R.A., and Simon, C. (2015) Prediction model for aneuploidy in early human embryo development revealed by single-cell analysis. *Nature Communications* **6**, 7601

Viuff, D., Avery, B., Hyttel, P., Greve, T., and Thomsen, P.D. (1999) Mixoploidy is frequent at day 2-5 in bovine embryos produced in vitro. *Biology of Reproduction* **60**, 267-267

Viuff, D., Greve, T., Avery, B., Hyttel, P., Brockhoff, P.B., and Thomsen, P.D. (2000) Chromosome aberrations in in vitro-produced bovine embryos at days 2-5 post-insemination. *Biology of Reproduction* **63**(4), 1143-1148

Viuff, D., Hendriksen, P.J., Vos, P.L., Dieleman, S.J., Bibby, B.M., Greve, T., Hyttel, P., and Thomsen, P.D. (2001a) Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. *Biology of Reproduction* **65**(1), 204-8

Viuff, D., Hendriksen, P.J.M., Vos, P., Dieleman, S.J., Bibby, B.M., Greve, T., Hyttel, P., and Thomsen, P.D. (2001b) Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. *Biology of Reproduction* **65**(1), 204-208

Wale, P.L., and Gardner, D.K. (2016) The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Human Reproduction Update* **22**(1), 2-22

Xia, P. (2013) Biology of Polyspermy in IVF and its Clinical Indication. *Current Obstetetrics and Gynecology Reports* **2**(4), 226-31

Zamani Esteki, M., Dimitriadou, E., Mateiu, L., Melotte, C., Van der Aa, N., Kumar, P., Das, R., Theunis, K., Cheng, J., Legius, E., Moreau, Y., Debrock, S., D'Hooghe, T., Verdyck, P., De Rycke, M., Sermon, K., Vermeesch, J.R., and Voet, T. (2015) Concurrent whole-genome haplotyping and copy-number profiling of single cells. *American Journal of Human Genetics* **96**(6), 894-912

CHAPTER 7

GENERAL DISCUSSION

In cattle, the commercial *in vitro* embryo production is characterized by the oocyte collection via ovum pick-up (OPU) and *in vitro* maturation, followed by fertilization and culture of the embryos. Currently, the efficiency is still rather low due to two major obstacles, namely the low oocyte yield and the differences in oocyte quality. Typically, oocytes and subsequently embryos are always grouped per donor as it is important to know from which donor animal combination (cow – bull) the embryos are originating. This implies the use of donor groups, consisting of less than 10 oocytes with varying quality, which in turn, results in overall low blastocyst rates. Since the main objective is producing high quality blastocysts to transfer and acquire pregnancy and healthy offspring, the low efficiency in OPU/IVP is a major concern to commercial breeding companies. This research focused on establishing a more efficient embryo development protocol by using commercially available media and dishes. Further, more research was performed on how embryos can be selected better using non-invasive time-lapse analysis and which genetic causes can give rise to the low embryonic development *in vitro*.

The first aim of this thesis was to evaluate a commercially available embryo holding medium to store immature bovine oocytes in order to simplify the commercial protocol. Today, these oocytes are collected by OPU, often at far distanced farms which necessitates expensive containers and adapted medium to transport the oocytes to the laboratory. Secondly, due to the overall low efficiency of *in vitro* embryo production in commercial practice, we aimed to increase the embryo development in these small donor groups by grouping oocytes and embryos in the Corral® dish during *in vitro* maturation and/or culture. We also described the kinetics of embryo development in small groups and semi-defined medium by time-lapse analysis. Finally, cytogenetical analysis was performed on single blastomeres from cleavage-stage embryos produced *in vitro* to evaluate the incidence of chromosomal abnormalities, compared to that of *in vivo* derived embryos.

The gained insights have led to a better understanding of bovine embryo development in small donor groups. In this final chapter, we will discuss how the donor can influence intrinsic oocyte quality and embryo development, and how *in vitro* fertilization and culture can induce chromosomal abnormalities (**Figure 1**).

7.1 Influence of the donor animal

Since the technology of *in vitro* embryo production became feasible in cattle, the demand for *in vitro* embryos has been increasing as a stricter selection is possible from both male and female side. In commercial practice, female animals of high economic interest are used in embryo production programs. Unfortunately, not all cows are ideal oocyte donors due to different reasons. We noticed a high variation between different donors regarding both quantity and quality of the collected oocytes. Beside management factors, such as diet, which can be controlled before including animals as an oocyte donor, there are many biological factors we cannot influence.

First, the breed has an impact on the success rate of the oocyte collection; it is known that Nelore cattle can be considered as better donors because an oocyte collection rate of over 30 is no exception (Pontes *et al.* 2011) while often less than 10 oocytes are collected in Holstein Friesians and Belgian Blue (Goovaerts *et al.* 2007). Second, the age is another important biological variable. Young calves of 2-3 months old have on average 16 oocytes per OPU session, compared to only 9 for heifers or cows. However, the developmental capacity of oocytes from pre-pubertal animals is low (Galli *et al.* 2001). The older the donor animal, the lower the ovarian reserve and the less oocytes are collected. Determining anti-muellerian hormone (AMH) in blood plasma can therefore be a useful predictive parameter for OPU and whether or not an animal will be a good oocyte donor (Vernunft *et al.* 2015). The AMH concentration reflects the total number of follicles and oocytes in the ovary or the ovarian reserve. Older animals have typically low AMH levels and also limited follicles and oocytes (Guerreiro *et al* 2014). Besides determining AMH concentration as an indication for ovarian reserve, donor's plasma hormones can be measured such as progesterone and oestradiol to determine the oocyte developmental capacities. Since age is related to different hormone balances, oocytes collected from heifers develop significantly more into high quality blastocysts compared to oocytes from older cows (more than 15 years) (Su *et al.* 2012). The age-related decline in fertility has been better investigated in women than in cattle because older animals are rarely oocyte donors in commercial breeding industries. Numerous studies have reported more oxidative stress, mitotic arrest and aneuploidy with increasing donor age and differences in transcriptional levels of genes involved in oocyte development (Huang *et al*. 2008; Keefe and Liu 2009; Grøndahl *et al.* 2010).

Finally, the use of hormonal stimulation prior to the OPU has an impact on the success rate of the embryo development. There are several studies reporting a positive effect of FSH administration on the oocytes' developmental competence (Blondin *et al.* 1997; Merton *et al.* 2009). Although the number of oocytes retrieved per session is not increasing by these hormonal treatments, it is indeed noticed that the quality of these oocytes is higher (Boni 2012). The efficiency rate increased to an

average of 3 embryos per OPU session after FSH stimulation with two week interval compared to only 1 embryo per session in a twice weekly OPU scheme (Galli *et al.* 2014). On a weekly basis, this implicates that 1.5 embryos are produced by OPU after FSH prestimulation and 2 embryos by twice weekly OPU without hormonal treatment. Although it remains difficult to predict whether a donor animal will be sensitive to the hormonal treatment or not, insensitive animals are perfectly usable in the twice weekly OPU scheme as this does not require any hormones (De Roover *et al.* 2005). The aspect of responders and non-responders is also typically seen in human (Broekmans *et al.* 2014).

In **Chapter 6**, we have used Holstein Friesian heifers when performing OPU. Holstein Friesian are less muscled which makes it easier to manipulate the animals' ovaries. In addition, the Holstein Friesian breed is a typical European dairy breed and CRV, the company assisting the OPU sessions, has the most experience with this breed. Although our animals were quite uniform regarding age and origin, there was a remarkable difference between the donors as the collection rate varied from one oocyte to 15 oocytes per OPU session (**Supp table S1**). Moreover, there was also a large variation in collection rate between different OPU sessions in the same donor, as some donors had a high collection rate in one OPU session and a low rate in the subsequent OPU session, or the other way around. The lack of consistency in collection rate makes it even more difficult to predict whether the animal will be an excellent oocyte donor in OPU/IVP programs. Inflammation to the ovaries was also observed in one donor cow due to the frequency of needle puncturing when performing OPU. This resulted in scar tissue which made the animal unsuitable for following OPU sessions and *in vivo* oviduct flushing. Although OPU is generally considered as a harmless technique, accumulation of fibrous tissue around the ovaries and hardening of the ovaries have been reported (Gibbons *et al.* 1994; Boni *et al.* 1997; Petyim *et al.* 2001; Chastant-Maillard *et al.* 2003; McEvoy *et al.* 2006). At the end of our study, cows were slaughtered after a period of at least 6 weeks without hormonal stimulation and ovaries were punctured. Slightly more oocytes per donor were recovered than during OPU sessions. This can be more likely ascribed to the way of collection (OPU technique versus follicular aspiration *ex vivo*) than to the effect of hormonal prestimulation.

Nowadays, breeding companies are guided to a selection of donor animals based on a combination of reproductive efficiency and the herd's genetic gains (Watanabe *et al.* 2017). Female animals having a genomic breeding value are selected as oocyte donor, which has been resulting in a more efficient OPU-IVP program. More research can still increase and maximize the number of oocytes that can be collected per OPU session. More insights into the natural cycle's follicular dynamics of the individual donor animals can lead to an improvement of the oocyte quality (Sirard *et al*. 2018). Moreover, instead

of a generalized protocol for OPU, individualized approaches, taking into account all biological factors, can optimize oocyte collection rates.

7.2 The intrinsic oocyte quality

The oocyte quality can be determined by its ability to be fertilized, develop into a blastocyst and establish a pregnancy resulting in healthy offspring. Although it is impossible to predict the initial oocyte's competences, some factors can be taken into account when producing bovine *in vitro* embryos.

Collection technique: OPU vs follicular aspiration of slaughterhouse ovaries

Often less than 10 oocytes having all sort of qualities are being collected by follicular aspiration during OPU sessions. In our experiments, slaughterhouse derived oocytes were also collected by follicular aspiration and not by ovary slicing. Small numbers of oocytes (10 per donor), having different qualities, were grouped to simulate grouping per donor as in OPU settings.

We noticed that oocytes collected from slaughterhouse ovaries were often of better quality compared to those collected after OPU (**Chapter 6**), as more OPU-oocytes were lacking cumulus cells. On the one hand, post-mortem changes induce loosening of the cumulus oocyte complex (COC) from the follicular wall which makes it easier to collect the oocyte, surrounded by more compact layers of cumulus cells. This is not the case when performing OPU in live animals. Furthermore, due to the needle guiding system and the vacuum pressure during OPU, cumulus cells are often stripped from the oocyte (Bols *et al.* 1996; Bols *et al.* 1997). On the other hand, others have reported the opposite as they noted a more heterogeneous quality in oocytes collected from slaughterhouse ovaries (Manjunatha *et al.* 2008). Oocytes harvested from follicles with a diameter larger than 2 mm show a better developmental competence than those of smaller diameters. Nonetheless, follicles are still being punctured in different stages of growth and atresia. Repeating OPU twice a week results in more homogenously sized follicles and reduced follicular atresia as follicles are aspirated before they become atretic (Gasparrini 2002), resulting in a collection rate of 46% good quality oocytes (Saini *et al.* 2015). In our OPU protocol (**Chapter 6**), a follicular ablation followed by a period of hormonal stimulation with FSH and subsequently OPU was preferred above the twice weekly OPU scheme, as it has been reported to be a more efficient and cost-effective protocol (Blondin *et al.* 2002; Chaubal *et al.* 2006). In total, we collected 49 oocytes by OPU of which 26.5% were of good quality, i.e. oocytes having homogeneous cytoplasm and surrounded by multiple layers of compact cumulus cells. This was lower than the 35.2% rate in the original study (Blondin *et al.* 2002), reporting a high developmental rate of 60% as well. Different circumstances should be taken into account, such as technical experience and materials used

for performing OPU, clarifying this rather small decrease in good quality oocytes in our study. In **Chapter 6**, we did not culture the oocytes until the blastocyst stage as they were collected at early cleavage stage for cytogenetical analysis. For this reason, we cannot conclude whether 60 % of these oocytes had the competence to develop into blastocysts, as in the original study (Blondin *et al.* 2002).

In vitro maturation systems

During *in vitro* maturation, it is necessary for immature oocytes to complete both nuclear and cytoplasmic maturation in order to become fertilized. This is established by multiple pathways signalled by paracrine and autocrine factors, produced by both cumulus cells and the oocyte (Gilchrist 2011). There is a close interaction between the oocyte and cumulus cells, essential in the oocyte's maturation process. It is important to find the best COC density to profit as much as possible from these secretions.

In research settings, 50-60 high quality cumulus oocyte complexes are pooled and grouped together, resulting in an overall blastocyst rate of 30 to 40%. However, when only limited numbers of oocytes are being grouped, the blastocyst rate is reduced with almost 50% (**Chapter 3**). In general commercial settings, donor's oocytes are matured in large volumes of maturation medium resulting in a low oocyte density, implying also low concentrations of paracrine and autocrine factors. A simple solution would be to adjust the volume of medium to the number of oocytes to achieve a higher density. This is more difficult to attain than in embryo culture, since each COC is composed of a different number of cumulus cells. In addition, maturing large numbers of COCs has been reported to be more effective than low numbers (Hashimoto *et al.* 1998). For this reason, the specially designed Corral® dish was applied in **Chapter 4** (Ebner *et al.* 2010). It offers the opportunity to group COCs of four different donor groups in the central wells. Due to semi-permeable walls, oocytes stayed inside the initial quadrant while maturation medium could flow through all four quadrants. With regard to the special design, our hypothesis was that grouping different donor groups in the Corral® dish would result in higher embryo development due to the larger number of oocytes grouped together and therefore resulting in more secreted factors that stimulate growth. As a matter of fact, when the Corral® dish was used for grouping four donor oocyte groups together during both IVM and IVC, a positive effect was noticed on the blastocyst formation. This emphasises the importance of grouping oocytes and embryos during the complete embryo production process.

Nuclear maturation and chromosomal integrity

Oocytes that reach the metaphase of meiosis II at the end of IVM can be considered as mature oocytes and are able to be fertilized. In general, 90 % of bovine immature oocytes will reach this phase

(Lonergan *et al.* 1996) depending on the laboratory settings such as medium supplements, oxygen tension, temperature, time period of maturation. Assessing the nuclear maturation of oocytes was necessary when applying the commercial embryo holding medium (EHM) to get a good indication of the effect of EHM on the oocytes' developmental capacities (**Chapter 3**). First, we did not see any meiotic resumption when holding immature bovine oocytes in the commercial embryo holding medium (EHM), as oocytes were immediately fixed after storage and subsequently stained with Hoechst 33342 DNA stain. Second, only EHM oocytes stored at room temperature were able to reach the MII after a subsequent IVM step when the storage time did not exceed 10 hours. In contrast, significantly less EHM oocytes stored at 38.5°C for 6h and at 4°C for 10h were mature after the subsequent IVM step. It is clear that the temperature at which the immature oocytes are stored in EHM has an effect on the oocytes' ability to resume meiosis and the developmental competences. It has been reported that keeping oocytes at temperatures lower than 4°C is causing chromosomal defects as the spindle reassembly is lacking (Moor and Crosby 1985). We can only assume that this is the case when immature oocytes are stored at 4°C in EHM because we only performed a Hoechst DNA staining to observe the maturation status. It is not possible to deduce from this staining whether there were any chromosomal abnormalities responsible for the meiotic arrest. It appeared that holding oocytes at 38.5°C had also a detrimental effect on the oocytes' maturation and subsequent embryo development. Possibly high enzymatic activity is causing metabolite accumulation and consequently, perturbing the oocytes viability (Rekharsky *et al.* 1986; Hashimoto *et al.* 2003). Finally, the exact composition of EHM is not known but the instructions for using EHM indicate "room temperature storage".

In **Chapter 6**, the cytogenetic analysis done by haplarithmisis on embryos derived by different techniques (OPU, IVP, *in vivo*), showed that only two embryos were burdened by a meiotic error, i.e. monosomy for chromosome 24 and 26, respectively. Typically, meiotic errors are maternal in origin (Tšuiko et al. 2018). Female spindle assembly checkpoint mechanisms are not able to block meiotic progression when only one chromosome is affected (Nagaoka *et al.* 2011), which was also the case in both bovine embryos. Also here, from the genome-wide haplarithm plots can be deduced that this meiotic error was maternal in origin, since only one copy was present from paternal origin. Moreover, these meiotic errors were both observed in embryos from the same donor animal.

Oocytes that display chromosomal aberrations can be a major cause of fertilization disorders and embryonic, fetal and neonatal loss (Plachot and Popescu 1993). Although systematic genetic analysis of oocytes is not done, anomaly rates for metaphase II ranging from 3 up to 58.7% have been described in human (Pellestor 1991; Almeida and Bolton 1993). In cattle, the frequency of chromosomally

abnormal oocytes has been reported to vary from 1.9 to 16.5% (King *et al.* 1986; King 1990; Yadav *et al.* 1991; Lechniak *et al.* 1996). In **Chapter 6**, 4.8% (2/42) of the analysed embryos displayed chromosomal abnormalities due to abnormal oocytes. Despite the low numbers of samples analysed, haplarithmisis can reveal even mutations in small chromosomal segments. In contrast, previous studies could not reveal very small abnormalities because karyotyping techniques were used such as chromosome spread to reveal the presence of diploid oocytes.

7.3 Embryo developmental competences

Within the reproductive tract of the cow, the embryo is not only constantly provided with all necessary nutrients for early development, also harmful substances and waste are being removed. In the final phase of *in vitro* embryo production, the fertilized oocyte or zygote is cultured to the blastocyst stage, at which time it can be transferred to a recipient animal or cryopreserved. Although a huge number of studies have been investigating the culture of bovine embryos, it is still a suboptimal environment because only 30 to 40% of inseminated oocytes will develop into a viable blastocyst. In this thesis, we noticed that there is even more room for improvement for embryos cultured in small donor groups, since the blastocyst development is even lower than when embryos are being cultured for research purposes. Moreover, different elements play a substantial role herein and will be elucidated further.

In vitro embryo culture per donor

In commercial settings, donor's embryos are often cultured in large volumes of culture medium. Comparable to COCs, embryos are also producing and secreting embryotrophic factors (Gopichandran and Leese 2006). Various studies have investigated the influence of embryo density on the developmental yield and noticed that a good equilibrium between the number of embryos and the medium volume should be attained. In ideal circumstances, an embryo density of 1:2 (meaning 1 embryo per 2 µl of medium) showed the highest embryo development. In **Chapter 4** and **Chapter 5**, we obtained an embryo density of 1:3 to 1:4 by using commercially available dishes, such as the Corral® dish or WOW dish or by culturing embryos per donor in droplets of medium. Although the embryo density was still too low, it is generally accepted that culturing large groups of embryos together (as was done by using the different quadrants, resulting in total embryo number of 36) is still preferred to individual embryo culture or even small donor groups, due to the secretion of these autocrine factors (Fujita *et al.* 2006). For this reason, the Corral® dish was applied during IVC. Although more blastocyst development was observed when implementing this dish during both IVM and IVC, no significant improvement was noticed when the Corral® dish was only used during IVC. In this dish, the donor groups are located 4 mm from each other during IVC. When the distance is larger than 165 µm, embryos do not have a direct effect on each other anymore (Gopichandran and Leese 2006). Grouping

the donors' embryos in the Corral® dish during IVC did therefore not improve the blastocyst development because autocrine factors need to cross too much distance. In the WOW dish used in **Chapter 5**, when morphokinetics were investigated, 9 to 16 embryos can be monitored, depending on its design. An advantage of the PrimoVision WOW dish compared to the Corral dish is that the embryos are much closer together. In this manner, it is more likely that embryotrophic factors secreted by one embryo can reach its neighbouring embryos and support their development. In addition, applying these dishes did not improve embryo development compared to standard group culture in cattle (Wydooghe *et al.* 2014b). Due to the specific design, embryos stay in place which allows perfect individual follow-up. However, as stated before, only 9 to 16 embryos can be cultured together. The static design of the dish can again be considered as a disadvantage in commercial practice as there will be routinely more or less embryos per donor. This results in grouping embryos from different donors to fill all wells in the dish or to leave wells unused when the dish can only contain embryos from one donor. In the last case, cross-contaminations are being avoided, however, the dish is not used to the fullest which may question its positive effect on embryo development. In the end, both Corral® and WOW dishes have their advantages and disadvantages. The preference is however still grouping oocytes and embryos per donor in a droplet of medium, because it requires no special designed dishes or handlings, it avoids cross-contamination and it allows adapting the volume of medium to retrieve the ideal density.

Regardless of the use of different culture dish systems (**Chapter 4** and **Chapter 5**), the composition of the used culture medium remained unchanged and consisted of SOF, BSA and ITS in all experimental designs. First, semi- or even defined medium is preferred to avoid possible contamination coming from other cells than those of the donor itself. Even the use of oil is often avoided because it is possible that nutrients are migrating from the media to the oil and chemical oil compounds the other way around, affecting the embryo development (Gasperin *et al.* 2010). Second, SOF medium supplemented with BSA and ITS is not only reported to be an ideal replacement for serum, as serum has been implicated as a possible causes of large offspring syndrome. Finally, our culture medium was optimized for individual embryo culture, resulting in similar blastocyst rates as in standard group culture (Wydooghe *et al.* 2014a). Although this medium was used for donor groups, a lower blastocyst formation was noted. Since we were also using oocytes from variable qualities, these oocytes were possibly not fully capable of secreting embryotrophic factors. Identifying embryotrophic factors would make it possible to supplement these into the culture medium and support the embryos' development to the fullest (Fujita *et al.* 2006).

Developmental kinetics and chromosomal integrity

The earliest embryonic cleavages are regulated by maternally inherited components such as mRNA and proteins, which are stored inside the oocyte. Typically, the first cycle is rather long (Barnes and Eyestone 1990) and a high number of embryos reach the 2-cell stage but fail to develop into blastocyst. Usually, there is a developmental block at the 8-cell stage in cattle (Telford *et al.* 1990, Graf *et al.* 2014). This phenomenon has been related to the transition from maternal to embryonic control. Embryos reaching the blastocyst stage regularly display an early or intermediate cleavage pattern, a synchronous division and a successful switch to transcribe the embryonic genome, compared to those embryos that fail to develop. In **Chapter 5**, there was a lot of variation in embryonic development between the different donors, but embryos that reach beyond the 4-cell stage tend to have better chances of becoming a blastocyst, possibly due to a successful embryo genome activation. These embryos displayed an early to intermediate cleavage pattern. The average timings for the early cleavage events were similar to the timings reported by other time-lapse studies. Different conditions, such as laboratory environments and medium supplements, may cause the small variations in between these studies.

It is likely that the overall low blastocyst formation in these donor groups is partially caused by chromosomal abnormalities (**Chapter 6**). High incidences were observed in the *in vitro* produced embryos compared to the *in vivo* derived ones, suggesting that the *in vitro* procedures are causing these aberrations. In addition, the *in vitro* produced embryos were burdened with over 70% of mixoploidy, defined by embryos having simultaneously haploid, diploid and/or triploid blastomeres. In other studies, chromosomal analyses have shown that the majority of bovine, porcine and ovine embryos are mixoploid (52.4%) (King 1990). These previous reports were set back by cytogenetic methods such as karyotyping and FISH, as only a minor fraction of the total cell number were analysed. Because of this, the frequency of chromosomal aberrations in embryos from these older studies is only an estimation. In **Chapter 6**, each blastomere from all cleavage stage embryos was analysed using a novel technique, called haplarithmisis which is a genome-wide single-cell analysis method that enables haplotyping and copy-number profiling (Esteki *et al.* 2015). Instead of earlier low-resolution karyotyping methods, chromosomal instability (CIN) was detected at single cell level and sub-chromosomal aberrations were revealed. Although our CIN results are perhaps more accurate, it still reflects a moment in time since embryos were analysed at a certain point making it impossible to know how embryos will be evolving when affected with CIN. High rates of CIN do not mean that these embryos will definitely cease development. Previous studies using FISH showed that the level of polyploidy decreases when development continues, since 13% of day 3 *in vitro* produced embryos

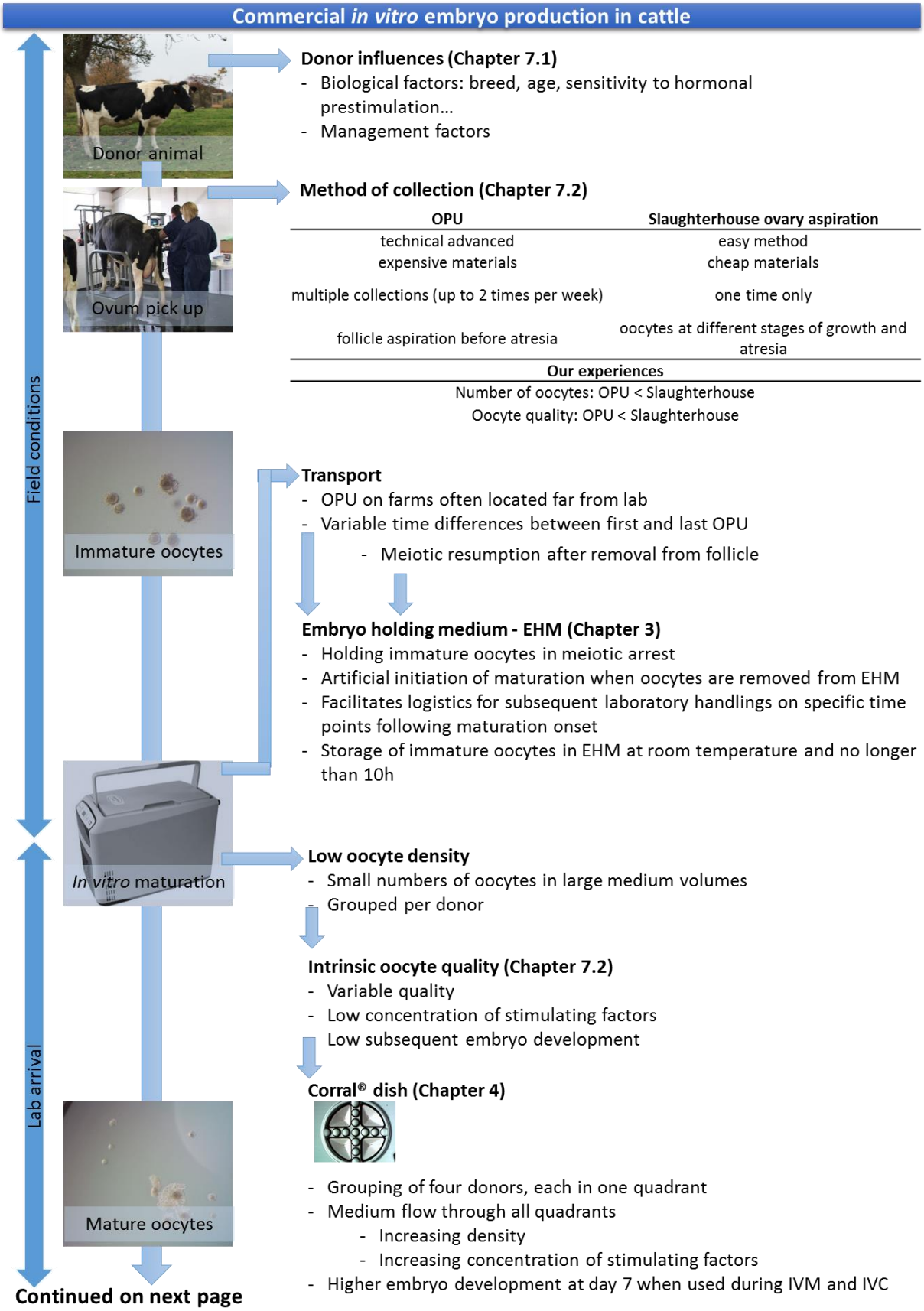
were polyploid while at day 5 no polyploidy was observed (Viuff *et al.* 2000). This was also observed when analysing trophectoderm (TE) and inner cell mass (ICM) from day 7 IVP blastocysts and TE and ICM from day 7 IVP blastocysts that were subsequently transferred and flushed at day 12; a two-fold (ICM) and seven-fold (TE) more polyploidy was observed in day 7 IVP blastocysts compared to day 12 flushed embryos (Viuff *et al.* 2002). Furthermore, 25% of tetraploidy in trophectoderm is still compatible with pregnancy (Viuff *et al.* 1999). On the other hand, mixoploidy appears to increase while *in vitro* embryos develop, going from 16% in day 4 embryos up to 72% in day 7/8 blastocysts (Viuff *et al.* 2000). Since over 50% of transferred blastocysts result in live birth, it means that mixoploidy is probably of minor importance for establishing a pregnancy. There are two important mechanisms in which affected embryos still develop into a normal foetus when only limited numbers of cells are affected. Compromised blastomeres can go in apoptosis while diploid blastomeres continue development (Jurisicova *et al.* 1996; Everett and West 1998). Furthermore, chromosomal abnormal cells can be allocated to the trophectoderm when differentiating (James *et al.* 1995).

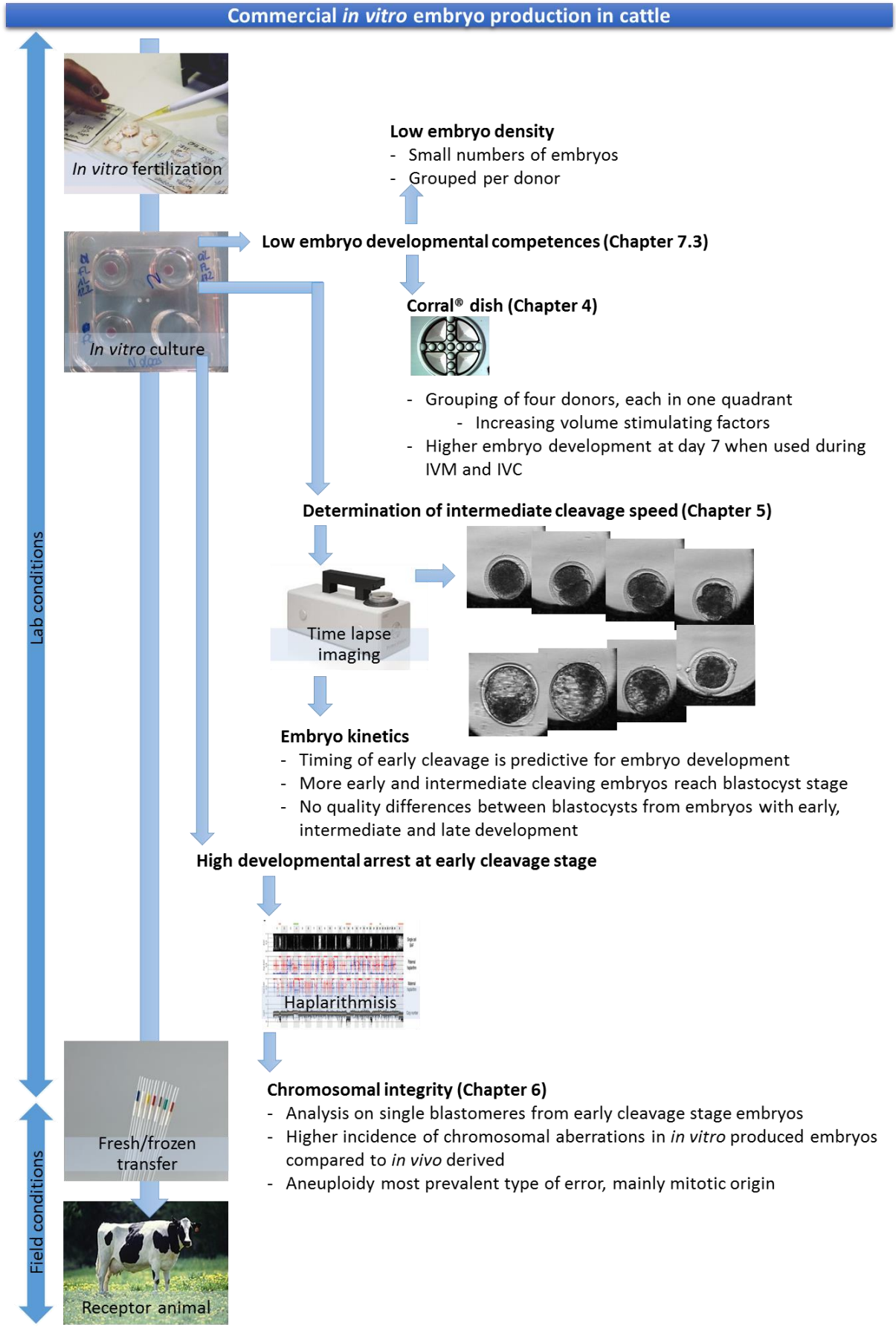
Haplarithmisis showed that there was a remarkably high incidence of polyspermy in embryos produced *in vitro* (12/28; 42%) compared to no polyspermy in *in vivo* embryos. It is possible that the polyspermy rate is overestimated due to the limited number of available embryos which were analysed, as other reports showed polyspermy incidence in *in vitro* produced bovine embryos ranging from 5 to 45% (Wang *et al.* 1997; Viuff *et al.* 2000; Roh *et al.* 2002; Coy *et al.* 2005; Iwata *et al.* 2008; Hosoe *et al.* 2014; Destouni *et al.* 2016). Studies in our lab showed polyspermy rates ranging from 7 to 17% (Tanghe *et al.* 2004; Verberckmoes *et al.* 2005) based upon the presence of more than 2 pronuclei using Hoechst staining, while we found 22% polyspermic zygotes in a more recent study using the same innovative haplarthmisis technique (Destouni *et al.* 2016). There is also a bull factor as some bulls tend to have more polyspermy than others in IVF (Parrish *et al.* 1986). Nonetheless, in line with our findings, there was also significantly less polyspermy in *in vivo* derived embryos or even in *in vivo* matured oocytes (Leibfriedrutledge *et al.* 1989). There are however many different factors that have been associated with a higher incidence of polyspermy in cattle, such as a small perivitelline space of the *in vitro* oocyte as a result of a deficient hyaluronic acid accumulation whereby large amounts of hyaluronic acid, as it occurs *in vivo*, prevent the fusion of extra sperm and oocytes (Hosoe *et al.* 2014), an inappropriate oocyte maturation (Viuff *et al.* 2001; Sugimura *et al.* 2017), a delayed cortical granule release (Wang *et al.* 1997)… Due to the novel insights in **Chapter 6**, this high rate of polyspermy can probably be ascribed to the *in vitro* procedures. Important during IVF is the concentration of sperm, since a high concentration (10^7 sperm cells per ml) resulted in polyspermy while a low concentration (10^4 sperm cells per ml) resulted in a low fertilization rate (Saeki *et al.* 1995). We use standardly 1 million spermatozoa per ml to fertilize mature oocytes *in vitro.* Although we did not test this

beforehand, a possible solution for this high polyspermy rate could have been to lower the concentration of sperm during IVF. *In vivo*, there is a natural selection process of sperm residing in the female tract, decreasing the chance of polyspermy. In a recent 3D-printed oviduct-on-a-chip, *in vivo* conditions were mimicked as bovine epithelial cells were cultured in a 3D chamber in which oocytes and sperm were introduced. While fertilization occurred, this system prevented polyspermy, even with the same sperm concentration of 10^6 sperm cells per ml. (Ferraz *et al.* 2017). Oviductal secretions can induce modifications to the zona pellucida, resulting in a hardening of the zona and preventing polyspermy (Coy *et al.* 2008). Identification of these molecules and introducing these in the *in vitro* procedure could reduce polyspermy by modifying and hardening the zona pellucida.

Polyspermy has also been associated with abnormal first cleavage patterns (Sugimura *et al.* 2017). Aberrant cleavage patterns are often missed unless time-lapse monitoring systems are used. It has been reported that the abnormal event of direct cleavage from 1 to 3-4 cells has no impact on embryo development; however, these embryos are prone to more chromosome errors (Somfai *et al.* 2010). In **Chapter 6**, in 7 out of 9 dispermic embryos, one of the extra paternal genomes segregated into a separate cell lineage, consisting of only paternal DNA, meaning that these embryos had an abnormal genome constitution. This phenomenon, which has been called heterogoneic cell division, was recently discovered in *in vitro* produced bovine embryos (Destouni *et al.* 2016). Since no time-lapse imaging was applied during that study, it is not sure whether these embryos had an abnormal first cleavage. In the future, we plan to investigate this by specific studies combining cytogenetic analysis and time-lapse imaging.

Figure 1 Schematic overview of the commercial *in vitro* embryo production process in cattle, elucidating important aspects influencing the efficiency and key findings of this dissertation.




Commercial *in vitro* embryo production in cattle
Lab conditions
Field conditions
In vitro fertilization
In vitro culture
Low embryo density
- Small numbers of embryos
- Grouped per donor
Low embryo developmental competences (Chapter 7.3)
Corral® dish (Chapter 4)
- Grouping of four donors, each in one quadrant
- Increasing volume stimulating factors
- Higher embryo development at day 7 when used during IVM and IVC
Determination of intermediate cleavage speed (Chapter 5)
Time lapse imaging
Embryo kinetics
- Timing of early cleavage is predictive for embryo development
- More early and intermediate cleaving embryos reach blastocyst stage
- No quality differences between blastocysts from embryos with early, intermediate and late development
High developmental arrest at early cleavage stage
Haplarithmisis
Chromosomal integrity (Chapter 6)
- Analysis on single blastomeres from early cleavage stage embryos
- Higher incidence of chromosomal aberrations in *in vitro* produced embryos compared to *in vivo* derived
- Aneuploidy most prevalent type of error, mainly mitotic origin
Fresh/frozen transfer
Receptor animal

7.4 General conclusions

Following take home messages can be concluded from this research providing novel insights in the commercial *in vitro* embryo production practice:

1. Commercially available embryo holding medium (EHM) can be safely used to store and transport immature bovine oocytes at room temperature without harming the embryonic development and quality when the storage time does not exceed 10 hours.
2. Laboratory handling schedules can be facilitated by storage of immature oocytes in EHM due to the possibility of artificially initiating the oocyte maturation when oocytes are removed from the EHM.
3. The Corral dish® is easily applicable for grouping oocytes and embryos from different donors together without losing the donor specific identity.
4. When the Corral dish® is used during both IVM and IVC, a higher blastocyst yield can be achieved at 7dpi, which makes it possible to transfer more fresh embryos on day 7.
5. Timing of cleavage is predictive for embryonic development as more intermediate cleavers develop into blastocysts compared to late cleaving embryos.
6. Cleavage stage embryos produced by *in vitro* techniques vs *in vivo* reveal a high incidence of chromosomal abnormalities which can be related to the *in vitro* embryo production procedure.
7. Aneuploidy is the most prevalent type of chromosomal error in early cleavage stage embryos and chromosomal aberrations are mainly of mitotic origin resulting in whole chromosome or segmental imbalances.

7.5 Perspectives for future research

Apart from the simplification and optimization of the embryo production procedure per donor, further attention should be payed to the high rates of chromosomal instabilities observed in the *in vitro* produced bovine embryos compared to the *in vivo* counterparts. Clarifying the origin of these chromosomal aberrations and their consequences on the further embryonic development, pregnancy and offspring should be addressed in future research. Furthermore, it would be interesting to investigate whether it is possible to link morphokinetics to chromosomal abnormalities, since these can be studied using non-invasive techniques such as time-lapse analysis.

Non-invasive techniques to identify predictive parameters for high quality embryo development and even establishing pregnancy are uprising. Today, no unique morphokinetic or combinations of parameters have been found to predict the oocyte and embryo's competence. Recent developments in life-cell time-lapse analysis made it possible to track nuclei from zygote to blastocyst stage by implementing fluorescent markers (Strnad *et al.* 2016). Artificial intelligence is fine-tuned as automated annotation tools are necessary to avoid subjectivity in data analysis since many parameters are scored by persons. Finally, the 3D constructions to mimic *in vivo* conditions are very promising. As many 3D lab-on-a-chips are still in their infancy, more research is necessary to elucidate the opportunities and investigate the impact on both pregnancy and birth rates in cattle breeding industries.

In the end, not only improving the *in vitro* embryo production protocol and investigating embryo predictive parameters are necessary in cattle breeding industries. All begins with the choice of donor animal. A change of mind-set is therefore fundamental. Instead of selecting only those donor animals having high genetic and economic values, reproductive parameters should also be taken into account. There are important factors such as breed, age, responsiveness to hormones, oocyte yield and quality, that need to be considered even more in the future to lift the bovine *in vitro* embryo production to the next level.

7.6 References

Almeida, P.A., and Bolton, V.N. (1993) Immaturity and chromosomal abnormalities in oocytes that fail to develop pronuclei following insemination in vitro. *Human Reproduction* **8**(2), 229-232

Barnes, F., and Eyestone, W. (1990) Early cleavage and the maternal zygotic transition in bovine embryos. *Theriogenology* **33**(1), 141-152

Blondin, P., Bousquet, D., Twagiramungu, H., Barnes, F., and Sirard, M.-A. (2002) Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of Reproduction* **66**(1), 38-43

Blondin, P., Guilbault, L., and Sirard, M.-A. (1997) The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. *Theriogenology* **48**(5), 803-813

Bols, P.E.J., VanSoom, A., Ysebaert, M.T., Vandenheede, J.M.M., and deKruif, A. (1996) Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes. *Theriogenology* **45**(5), 1001-1014

Bols, P.E.J., Ysebaert, M.T., VanSoom, A., and deKruif, A. (1997) Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact cumulus oocyte complexes. *Theriogenology* **47**(6), 1221-1236

Boni, R. (2012) Ovum pick-up in cattle: a 25 yr retrospective analysis. *Animal Reproduction* **9**(3), 362-369

Boni, R., Roelofsen, M., Pieterse, M., Kogut, J., and Kruip, T. (1997) Follicular dynamics, repeatability and predictability of follicular recruitment in cows undergoing repeated follicular puncture. *Theriogenology* **48**(2), 277-289

Broekmans, F.J., Verweij, P.J., Eijkemans, M.J., Mannaerts, B.M., and Witjes, H. (2014) Prognostic models for high and low ovarian responses in controlled ovarian stimulation using a GnRH antagonist protocol. *Human Reproduction* **29**(8), 1688-1697

Chastant-Maillard, S., Quinton, H., Lauffenburger, J., Cordonnier-Lefort, N., Richard, C., Marchal, J., Mormede, P., and Renard, J. (2003) Consequences of transvaginal follicular puncture on well-being in cows. *Reproduction* **125**(4), 555-563

Chaubal, S.A., Molina, J.A., Ohlrichs, C.L., Ferre, L.B., Faber, D.C., Bols, P.E.J., Riesen, J.W., Tian, X., and Yang, X. (2006) Comparison of different transvaginal ovum pick-up protocols to optimise oocyte retrieval and embryo production over a 10-week period in cows. *Theriogenology* **65**(8), 1631-1648

Coy, P., Grullon, L., Canovas, S., Romar, R., Matas, C., and Avilesl, M. (2008) Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction* **135**(1), 19-27

Coy, P., Romar, R., Payton, R.R., McCann, L., Saxton, A.M., and Edwards, J.L. (2005) Maintenance of meiotic arrest in bovine oocytes using the S-enantiomer of roscovitine: effects on maturation, fertilization and subsequent embryo development in vitro. *Reproduction* **129**(1), 19-26

De Roover, R., Bols, P.E.J., Genicot, G., and Hanzen, C. (2005) Characterisation of low, medium and high responders following FSH stimulation prior to ultrasound-guided transvaginal oocyte retrieval in cows. *Theriogenology* **63**(7), 1902-1913

Destouni, A., Esteki, M.Z., Catteeuw, M., Tsuiko, O., Dimitriadou, E., Smits, K., Kurg, A., Salumets, A., Van Soom, A., Voet, T., and Vermeesch, J.R. (2016) Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage-stage chimerism and mixoploidy. *Genome Research* **26**(5), 567-578

Ebner, T., Shebl, O., Moser, M., Mayer, R.B., Arzt, W., and Tews, G. (2010) Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. *Reproductive Biomedicine Online* **21**(6), 762-768

Esteki, M.Z., Dimitriadou, E., Mateiu, L., Melotte, C., Van der Aa, N., Kumar, P., Das, R., Theunis, K., Cheng, J.Q., Legius, E., Moreau, Y., Debrock, S., D'Hooghe, T., Verdyck, P., De Rycke, M., Sermon, K., Vermeesch, J.R., and Voet, T. (2015) Concurrent Whole-Genome Haplotyping and Copy-Number Profiling of Single Cells. *American Journal of Human Genetics* **96**(6), 894-912

Everett, C.A., and West, J.D. (1998) Evidence for selection against tetraploid cells in tetraploid [harr] diploid mouse chimaeras before the late blastocyst stage. *Genetics Research* **72**(3), 225-228

Ferraz, M.A., Henning, H.H., Costa, P.F., Malda, J., Melchels, F.P., Wubbolts, R., Stout, T.A., Vos, P.L., and Gadella, B.M. (2017) Improved bovine embryo production in an oviduct-on-a-chip system: prevention of poly-spermic fertilization and parthenogenic activation. *Lab on a Chip* **17**(5), 905-916

Fujita, T., Umeki, H., Shimura, H., Kugumiya, K., and Shiga, K. (2006) Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. *Journal of Reproduction and Development* **52**(1), 137-142

Galli, C., Crotti, G., Notari, C., Turini, P., Duchi, R., and Lazzari, G. (2001) Embryo production by ovum pick up from live donors. *Theriogenology* **55**(6), 1341-1357

Galli, C., Duchi, R., Colleoni, S., Lagutina, I., and Lazzari, G. (2014) Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology* **81**(1), 138-151

Gasparrini, B. (2002) In vitro embryo production in buffalo species: state of the art. *Theriogenology* **57**(1), 237-256

Gasperin, B.G., Barreta, M.H., Santos, J.T., Ferreira, R., Neves, J.P., Oliveira, J.F., and Gonçalves, P.B. (2010) Oil-free culture system for in vitro bovine embryo production. *Italian Journal of Animal Science* **9**(2), e32

Gibbons, J., Beal, W., Krisher, R., Faber, E., Pearson, R., and Gwazdauskas, F. (1994) Effects of once- versus twice-weekly transvaginal follicular aspiration on bovine oocyte recovery and embryo development. *Theriogenology* **42**(3), 405-419

Gilchrist, R.B. (2011) Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. *Reproduction Fertility and Development* **23**(1), 23-31

Goovaerts, I.G.F., Leroy, J.L.M.R., Merton, J.S., Van Soom, A., and Bols, P.E.J. (2007) In vitro productie van runderembryo's: een stand van zaken 10 jaar na de eerste OPU/IVP-kalveren in België. *Vlaams Diergeneeskundig Tijdschrift* **76**, 283-292

Gopichandran, N., and Leese, H.J. (2006) The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos. *Reproduction* **131**(2), 269-277

Graf, A., Krebs, S., Heininen-Brown, M., Zakhartchenko, V., Blum, H., and Wolf, E. (2014) Genome activation in bovine embryos: Review of the literature and new insights from RNA sequencing experiments. *Animal Reproduction Science* **149**(1-2), 46-58

Grøndahl, M. L., Yding Andersen, C., Bogstad, J., Nielsen, F. C., Meinertz, H., and Borup, R. (2010) Gene expression profiles of single human mature oocytes in relation to age. *Human Reproduction* **25**(4), 957-968.

Guerreiro, B. M., Batista, E. O. S., Vieira, L. M., Sá Filho, M. F., Rodrigues, C. A., Netto, A. C., ... and Accorsi, M. (2014) Plasma anti-mullerian hormone: an endocrine marker for in vitro embryo production from Bos taurus and Bos indicus donors. *Domestic Animal Endocrinology*, ***49***, 96-104

Hashimoto, S., Kimura, K., Iwata, H., and Takakura, R. (2003) Oocyte transport: developmental competence of bovine oocytes arrested at germinal vesicle stage by cycloheximide under air. *Journal of Reproduction and Development* **49**(1), 61-66

Hashimoto, S., Saeki, K., Nagao, Y., Minami, N., Yamada, M., and Utsumi, K. (1998) Effects of cumulus cell density during in vitro maturation on the developmental competence of bovine oocytes. *Theriogenology* **49**(8), 1451-1463

Hosoe, M., Yoshida, N., Hashiyada, Y., Teramoto, H., Takahashi, T., and Niimura, S. (2014) Sericin accelerates the production of hyaluronan and decreases the incidence of polyspermy fertilization in bovine oocytes during in vitro maturation. *Journal of Reproduction and Development* **60**(4), 268-273

Huang, X., Andreu-Vieyra, C. V., York, J. P., Hatcher, R., Lu, T., Matzuk, M. M., and Zhang, P. (2008) Inhibitory phosphorylation of separase is essential for genome stability and viability of murine embryonic germ cells. *PLoS Biology* **6**(1), e15

Iwata, H., Shiono, H., Kon, Y., Matsubara, K., Kimura, K., Kuwayama, T., and Monji, Y. (2008) Effects of modification of in vitro fertilization techniques on the sex ratio of the resultant bovine embryos. *Animal reproduction science* **105**(3-4), 234-244

James, R.M., Klerkx, A.H., Keighren, M., Flockhart, J.H., and West, J.D. (1995) Restricted distribution of tetraploid cells in mouse tetraploid↔ diploid chimaeras. *Developmental biology* **167**(1), 213-226

Jurisicova, A., Varmuza, S., and Casper, R. (1996) Programmed cell death and human embryo fragmentation. *MHR: Basic Science of Reproductive Medicine* **2**(2), 93-98

Keefe, D. L., and Liu, L. (2008) Telomeres and reproductive aging. *Reproduction, Fertility and Development* **21**(1), 10-14

King, W. (1990) Chromosome abnormalities and pregnancy failure in domestic animals. *Advances in Veterinary Science and Comparative Medicine* **34**, 229-250

King, W., Bousquet, D., Greve, T., and Goff, A. (1986) Meiosis in bovine oocytes matured in vitro and in vivo. *Acta Veterinaria Scandinavica* **27**, 267-279

Lechniak, D., Świtoński, M., and Sosnowski, M. (1996) The incidence of bovine diploid oocytes matured in vitro. *Theriogenology* **46**(2), 267-277

Leibfriedrutledge, M.L., Critser, E.S., Parrish, J.J., and First, N.L. (1989) In vitro maturation and fertilization of bovine oocytes. *Theriogenology* **31**(1), 61-74

Lonergan, P., Carolan, C., VanLangendonckt, A., Donnay, I., Khatir, H., and Mermillod, P. (1996) Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro. *Biology of Reproduction* **54**(6), 1420-1429

Manjunatha, B., Gupta, P., Ravindra, J., Devaraj, M., and Nandi, S. (2008) In vitro embryo development and blastocyst hatching rates following vitrification of river buffalo embryos produced from oocytes recovered from slaughterhouse ovaries or live animals by ovum pick-up. *Animal Reproduction Science* **104**(2), 419-426

McEvoy, T., Alink, F., Moreira, V., Watt, R., and Powell, K. (2006) Embryo technologies and animal health–consequences for the animal following ovum pick-up, in vitro embryo production and somatic cell nuclear transfer. *Theriogenology* **65**(5), 926-942

Merton, J.S., Ask, B., Onkundi, D.C., Mullaart, E., Colenbrander, B., and Nielen, M. (2009) Genetic parameters for oocyte number and embryo production within a bovine ovum pick-up-in vitro production embryo-production program. *Theriogenology* **72**(7), 885-893

Moor, R., and Crosby, I. (1985) Temperature-induced abnormalities in sheep oocytes during maturation. *Journal of Reproduction and Fertility* **75**(2), 467-473

Nagaoka, S.I., Hodges, C.A., Albertini, D.F., and Hunt, P.A. (2011) Oocyte-specific differences in cell-cycle control create an innate susceptibility to meiotic errors. *Current Biology* **21**(8), 651-657

Parrish, J.J., Suskoparrish, J.L., Leibfriedrutledge, M.L., Critser, E.S., Eyestone, W.H., and First, N.L. (1986) Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology* **25**(4), 591-600

Pellestor, F. (1991) Differential distribution of aneuploidy in human gametes according to their sex. *Human Reproduction* **6**(9), 1252-1258

Petyim, S., Båge, R., Forsberg, M., Rodríguez-Martínez, H., and Larsson, B. (2001) Effects of repeated follicular punctures on ovarian morphology and endocrine parameters in dairy heifers. *Transboundary and Emerging Diseases* **48**(8), 449-463

Plachot, M., and Popescu, P. (1993) Chromosome and gene anomalies: their consequences on reproduction and embryonic development. *Reproduction in Mammals and Man*, 715-730

Pontes, J.H.F., Sterza, F.A.M., Basso, A.C., Ferreira, C.R., Sanches, B.V., Rubin, K.C.P., and Seneda, M.M. (2011) Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. *Theriogenology* **75**(9), 1640-1646

Rekharsky, M., Galchenko, G., Egorov, A., and Berenzin, I. (1986) 'Thermodynamics of enzymatic reactions.' (Springer-Verlag: Berlin, Heidelberg)

Roh, S., Hwang, W., Lee, B., Lim, J., and Lee, E. (2002) Improved monospermic fertilization and subsequent blastocyst formation of bovine oocytes fertilized in vitro in a medium containing NaCl of decreased concentration. *Journal of Veterinary Medical Science* **64**(8), 667-671

Saeki, K., Nagao, Y., Hoshi, M., and Nagai, M. (1995) Effects of heparin, sperm concentration and bull variation on in vitro fertilization of bovine oocytes in a protein-free medium. *Theriogenology* **43**(4), 751-759

Saini, N., Singh, M., Shah, S., Singh, K., Kaushik, R., Manik, R., Singla, S., Palta, P., and Chauhan, M. (2015) Developmental competence of different quality bovine oocytes retrieved through ovum pick-up following in vitro maturation and fertilization. *Animal* **9**(12), 1979-1985

Sirard, M. A., Grand, F. X., Labrecque, R., Vigneault, C., and Blondin, P. (2018) The use of natural cycle's follicular dynamic to improve oocyte quality in dairy cows and heifers. *Journal of animal science*.

Somfai, T., Inaba, Y., Aikawa, Y., Ohtake, M., Kobayashi, S., Konishi, K., and Imai, K. (2010) Relationship Between the Length of Cell Cycles, Cleavage Pattern and Developmental Competence in Bovine Embryos Generated by In Vitro Fertilization or Parthenogenesis. *Journal of Reproduction and Development* **56**(2), 200-207

Strnad, P., Gunther, S., Reichmann, J., Krzic, U., Balazs, B., De Medeiros, G., Norlin, N., Hiiragi, T., Hufnagel, L., and Ellenberg, J. (2016) Inverted light-sheet microscope for imaging mouse pre-implantation development. *Nature methods* **13**(2), 139

Su, L., Yang, S., He, X., Li, X., Ma, J., Wang, Y., Presicce, G.A., and Ji, W. (2012) Effect of donor age on the developmental competence of bovine oocytes retrieved by ovum pick up. *Reproduction in domestic animals* **47**(2), 184-189

Sugimura, S., AKAI, T., and IMAI, K. (2017) Selection of viable in vitro-fertilized bovine embryos using time-lapse monitoring in microwell culture dishes. *Journal of Reproduction and Development*, 2017-041

Tanghe, S., Van Soom, A., Duchateau, L., Nauwynck, H., and de Kruif, A. (2004) Carbohydrates and glycoproteins involved in bovine fertilization in vitro. *Molecular Reproduction and Development* **68**(4), 492-499

Telford, N.A., Watson, A.J., and Schultz, G.A. (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Molecular Reproduction and Development* **26**(1), 90-100

Tšuiko, O., Jatsenko, T., Grace, L.K.P., Kurg, A., Vermeesch, J.R., Lanner, F., Altmäe, S., and Salumets, A. (2018) A speculative outlook on embryonic aneuploidy: can molecular pathways be involved? *Developmental biology*

Verberckmoes, S., Van Soom, A., Dewulf, J., and de Kruif, A. (2005) Comparison of three diluents for the storage of fresh bovine semen. *Theriogenology* **63**(3), 912-922

Vernunft, A., Schwerhoff, M., Viergutz, T., Diederich, M., and Kuwer, A. (2015) Anti-Muellerian hormone levels in plasma of Holstein-Friesian heifers as a predictive parameter for ovum pick-up and embryo production outcomes. *Journal of Reproduction and Development*,***61***(1), 74-79

Viuff, D., Avery, B., Hyttel, P., Greve, T., and Thomsen, P.D. (1999) Mixoploidy is frequent at day 2-5 in bovine embryos produced in vitro. *Biology of Reproduction* **60**, 267-267

Viuff, D., Greve, T., Avery, B., Hyttel, P., Brockhoff, P.B., and Thomsen, P.D. (2000) Chromosome aberrations in in vitro-produced bovine embryos at days 2-5 post-insemination. *Biology of Reproduction* **63**(4), 1143-1148

Viuff, D., Hendriksen, P.J.M., Vos, P., Dieleman, S.J., Bibby, B.M., Greve, T., Hyttel, P., and Thomsen, P.D. (2001) Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. *Biology of Reproduction* **65**(1), 204-208

Viuff, D., Palsgaard, A., Rickords, L., Lawson, L.G., Greve, T., Schmidt, M., Avery, B., Hyttel, P., and Thomsen, P.D. (2002) Bovine embryos contain a higher proportion of polyploid cells in the trophectoderm than in the embryonic disc. *Molecular Reproduction and Development* **62**(4), 483-488

Wang, W., Hosoe, M., Li, R., and Shioya, Y. (1997) Development of the competence of bovine oocytes to release cortical granules and block polyspermy after meiotic maturation. *Development, Growth and Differentiation* **39**(5), 607-615

Watanabe, Y.F., de Souza, A.H., Mingoti, R.D., Ferreira, R.M., Batista, E.O.S., Dayan, A., Watanabe, O., Meirelles, F.V., Nogueira, M.F.G., and Ferraz, J.B.S. (2017) Number of oocytes retrieved per donor during OPU and its relationship with in vitro embryo production and field fertility following embryo transfer. *Animal Reproduction* **14**(3), 635-644

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van den Abbeel, E., De Sutter, P., Vandaele, L., and Van Soom, A. (2014a) Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reproduction Fertility and Development* **26**(5), 717-724

Wydooghe, E., Vandaele, L., Piepers, S., Dewulf, J., Van den Abbeel, E., De Sutter, P., and Van Soom, A. (2014b) Individual commitment to a group effect: strengths and weaknesses of bovine embryo group culture. *Reproduction* **148**(5), 519-529

Yadav, B., King, W., Xu, K., Pollard, J., and Plante, L. (1991) Chromosome analysis of bovine oocytes cultured in vitro. *Genetics Selection Evolution* **23**(3), 191

CHAPTER 8

SUMMARY – SAMENVATTING

Over the years, bovine assisted reproductive technologies have gained more and more interest and ovum pick-up (OPU) and *in vitro* embryo production (IVP) has become a technique that can be applied in practice (**Chapter 1.1**). Today, bovine *in vitro* embryos, with their own specific needs and demands, are being produced for commercial purposes via several sequential culture steps (**Chapter 1.2**). Although years of refinement have passed, there is still a lot of room for improvement as the embryo yield per donor cow is still quite low. This can be ascribed to two important aspects; only limited numbers of oocytes are retrieved per donor which implies that embryos are cultured in small groups since embryo identification is necessary at all times and moreover, these oocytes often have variable qualities. Due to the OPU technique, such as vacuum aspiration, cumulus cells are stripped from the oocytes. In general, oocytes lacking cumulus cells or denuded oocytes have a lower developmental competence compared to high quality oocytes, i.e. oocytes with homogeneous cytoplasm surrounded by multiple layers of compact cumulus cells. Oocytes and resulting embryos are carefully evaluated throughout and at the end of the developmental process, since preferably high quality embryos will be chosen for transfer which increases the chance of establishing a pregnancy followed by the birth of a healthy calf (**Chapter 1.3**). As in multiple ovulation and embryo transfer (MOET) programs, genetic analysis can be performed on *in vitro* produced embryos, exposing not only the chromosomal constitution associated with genetic disorders, but also revealing genetically inherited economic traits by embryo biopsy before transfer (**Chapter 1.4**). Nonetheless there is a low efficiency, OPU-IVP has numerous advantages as OPU can be applied to retrieve oocytes from prepubertal animals, cows pregnant during the first three months and cows having oviduct pathologies. OPU can be performed up to two times a week for a longer period without harming the donor's health. Furthermore, hormonal stimulation can be done but it is not necessary prior to OPU. Moreover, each oocyte can be fertilized with the semen of a different bull, resulting in a higher variation of genetic combinations. These benefits outweigh the often low efficiency of the *in vitro* production, resulting in a further raising demand for *in vitro* produced bovine embryos.

The general aim of this thesis was to establish a more efficient IVP protocol (**Chapter 2**). Furthermore, more research was performed on how embryos can be selected better using non-invasive time-lapse analysis and which genetic causes can give rise to the low embryonic development *in vitro*.

Initially, the focus was set on the simplification of a first aspect of the commercial IVP procedure, namely the transport of freshly collected immature oocytes (**Chapter 3**). The maturation process such as the meiotic resumption, is initiated immediately after the oocytes are removed from their follicles. Since there can be a lot of time between different oocyte collections on different farms, the initiation of the maturation process has a wide range. This complicates further laboratory handlings needed on

specific time points following the onset of oocyte maturation, such as fertilization and culture of the embryos to blastocyst stage. By applying a commercially available transportation medium that keeps the immature oocytes in meiotic arrest, synchronized scheduling of subsequent laboratory manipulations is made possible. Previously used meiotic inhibitors did not have the desired effect because the subsequent embryonic development was compromised. We introduced non-toxic embryo holding medium for oocyte transport and we determined the ideal temperature at which the oocytes should be held as well as the time span during which the immature oocytes could be stored without harming the further embryo growth. The use of commercial holding medium was found to be successful in arresting the immature oocytes at room temperature without disturbance of blastocyst formation when storage was no longer than 10 hours. This can render logistics more easy and low-budget for practitioners who need to transport the immature oocytes from farms to the laboratory.

The goal of commercial IVP is to obtain as many transferable blastocysts of high quality as possible resulting in more pregnancies and more offspring. With this in mind, we focussed on improving the low blastocyst yield up to a level that is easily reached when large numbers of oocytes and embryos are pooled together as routinely done for research purposes. Knowing that embryo development benefits from culture in large groups, a specially designed dish was used which would expose the embryos to autocrine factors secreted by their neighbours, in order to stimulate growth (**Chapter 4**). In the Corral® dish, two central wells are divided into quadrants by a semi-permeable wall allowing medium to flow through while oocytes or embryos cannot pass this wall. Four donor groups were grouped together in this Corral® dish, making it possible to keep track of each separate donor group due the specific design. This dish was easily applicable during both maturation and culture periods. Our results showed an improvement of the day 7 blastocyst rate when the Corral® dish was applied during both IVM and IVC. Importantly, this day is the elected moment for fresh embryo transfer as this corresponds to the highest pregnancy rates. However, some questions can arise concerning sanitary risks and the possibility of disease contamination when oocytes and embryos of different donors are grouped together.

To improve the embryo selection method, time-lapse analysis was used as a non-invasive method to evaluate embryonic development and kinetics as a predictive value for blastocyst formation (**Chapter 5**). For the first time, categories ‘early’, ‘intermediate’ and ‘late’ cleaving embryos were defined in the well-of-the-well (WOW) dish in serum-free media. Recently, it has been discussed that these intermediate cleaving embryos would have a higher quality and therefore be more similar to their *in vivo* counterparts. In our observations, it was clear that fast cleaving embryos have higher odds to become a blastocyst compared to slow cleaving ones (Odds ratio 43 at 4-cell stage for 41.9h).

Furthermore, there was a significant increase in blastocyst formation in the intermediate cleaving embryos compared to the slow cleaving ones at the 2-cell and 3-cell stage.

A large proportion of *in vitro* produced embryos are facing developmental arrest during early cleavage. In **Chapter 6**, we investigated the chromosomal constitution of these early cleavage stage embryos by a novel technique called haplarithmisis. Five donor animals were repeatedly used to obtain embryos by (1) *in vivo* oviductal flush, (2) by IVP procedures after hormonal treatment and OPU (OPU-IVF) and (3) by IVP after slaughter without prior hormonal treatment (IVM-IVF). We did not only determine the incidence but also the origin of the chromosomal abnormalities. Overall, aneuploidy was the most prevalent type of error and a significantly higher rate of abnormalities was noted for IVP embryos when compared to embryos that developed *in vivo*. Analyses showed that 18.8% of *in vivo* derived embryos contained at least one blastomere with chromosomal anomalies, compared to 69.2% of OPU-IVF embryos and 84.6% of IVM-IVF embryos. Meiotic errors were only observed in two embryos (OPU-IVF and IVM-IVF group) produced from the same donor. Segmental imbalances were more present in IVM-IVF embryos (69.2%) compared to OPU-IVF (15.4%) and *in vivo* embryos (6.3%). Remarkably, a high number of dispermic fertilization was noted in IVM-IVF embryos (69.2%), while this was only 7.7% in OPU-IVF and no abnormal fertilization occurred in *in vivo* embryos. Heterogoneic cell divisions, which have been recently described as the spontaneous segregation of entire parental genomes into different cell lineages during cell cleavage, were observed in 7 IVM-IVF embryos (53.8%). It is clear that the *in vitro* procedure itself exacerbates the frequency of chromosomal aberrations, which can be a cause for this high developmental arrest in early cleavage embryos.

In **chapter 7**, the general discussion and conclusions are presented. The following take home messages are listed below:

1. Commercially available embryo holding medium (EHM) can be safely used to store and transport immature bovine oocytes at room temperature without harming the embryonic development and quality when the storage time does not exceed 10 hours.
2. Laboratory handling schedules can be facilitated by the storage of immature oocytes in EHM due to the possibility of artificially initiating the oocyte maturation when oocytes are removed from the EHM.
3. The Corral dish® is easily applicable for grouping oocytes and embryos from different donors together without losing the donor specific identity.

4. When the Corral dish® is used during both IVM and IVC, a higher blastocyst yield can be achieved at 7dpi, which makes it possible to transfer more fresh embryos on day 7.
5. Timing of cleavage is predictive for embryonic development as fast and intermediate cleaving embryos have a higher chance to develop into blastocysts compared to late cleaving embryos.
6. Cleavage stage embryos produced by *in vitro* techniques vs *in vivo* reveal a high incidence of chromosomal abnormalities which can be related to the *in vitro* embryo production procedure.
7. Aneuploidy is the most prevalent type of chromosomal error in early cleavage stage embryos and chromosomal aberrations are mainly of mitotic origin resulting in whole chromosome or segmental imbalances.

Doorheen de jaren nam de belangstelling voor geassisteerde reproductieve technieken bij rundvee toe waardoor ovum pick-up en *in vitro* embryo productie (OPU-IVP) ook commercieel uitgebaat worden (**Hoofdstuk 1.1**). Deze runderembryo's kunnen *in vitro* geproduceerd worden via verschillende opeenvolgende cultuurstappen waarbij rekening gehouden wordt met specifieke noden van het ontwikkelende embryo (**Hoofdstuk 1.2**). Ondanks de vele optimalisaties aan de techniek is er nog veel ruimte voor verbetering, aangezien de embryo-opbrengst per donorkoe nog steeds vrij laag is. Dit kan toegewezen worden aan twee belangrijke aspecten. Enerzijds wordt slechts een gelimiteerd aantal eicellen gecollecteerd per donor en omdat de identiteit van de embryo's op elk moment moet gegarandeerd worden, betekent dit dat de embryo's in kleine groepen per donor worden geproduceerd. Anderzijds zijn deze eicellen vaak van een variabele kwaliteit. Om alsnog de embryo's met de hoogste kwaliteit voor transfer te selecteren, wordt de ontwikkeling van deze eicellen en embryo's doorheen de *in vitro* productie nauwkeurig opgevolgd. Dit verhoogt namelijk de kansen op een dracht en bijgevolg ook op de geboorte van een gezond kalf (**Hoofdstuk 1.3**). Zoals in 'superovulatie en transfer' (MOET) programma's kunnen ook genetische analyses uitgevoerd worden op deze pre-implantatie embryo's. Hierdoor kan nog voor de transplantatie informatie verkregen worden over zowel de chromosomale constitutie, die genetische afwijkingen kan onthullen, als over interessante economische eigenschappen die genetisch overdraagbaar zijn (**Hoofdstuk 1.4**). Ondanks de lage efficiëntie kent OPU-IVP vele mogelijke toepassingen, waaronder het verzamelen van eicellen bij prepuberale dieren, bij koeien drachtig in de eerste drie maanden en bij koeien met eileiderpathologieën. OPU kan tot twee keer per week uitgevoerd worden, dit gedurende een lange periode zonder dat de gezondheid van de donor in het gedrang komt. Bovendien kunnen donoren hormonaal gestimuleerd worden vooraleer OPU wordt uitgevoerd, maar dit is niet noodzakelijk. In principe kan elke eicel met sperma van een verschillende stier bevrucht worden, waardoor er meer genetische combinaties gemaakt kunnen worden. Deze belangrijke voordelen wegen dan ook op tegen de lage efficiëntie van de *in vitro* embryo productie, waardoor de vraag naar IVP runderembryo's alleen maar verder toeneemt.

De algemene doelstelling van dit onderzoek was het ontwikkelen van een efficiënter IVP protocol (**Hoofdstuk 2**). Tevens werd onderzocht hoe de selectie van embryo's kan verbeterd worden en welke mogelijke genetische oorzaken aan de basis kunnen liggen van een minder efficiënte embryo-opbrengst *in vitro*.

Initieel werd de focus gelegd op het vereenvoudigen van een eerste aspect, namelijk het transport van onrijpe eicellen na OPU (**Hoofdstuk 3**). Het rijpingsproces wordt onmiddellijk geïnitieerd wanneer eicellen uit de follikels verwijderd worden. Er kan echter veel tijd zitten tussen verschillende

eicelcollecties en dus ook tussen de start van het rijpingsproces van de verschillende groepen eicellen. Dit bemoeilijkt de timing voor handelingen, die op specifieke tijdstippen na de start van de eicelrijping moeten gebeuren, zoals de bevruchting. Door tijdens het transport gebruik te maken van een commercieel beschikbaar embryo holding medium (EHM) kunnen eicellen in meiotisch arrest gehouden worden, wat het plannen van de bevruchting vereenvoudigt. Voorheen werden reeds meiotische inhibitoren gebruikt, maar deze hadden niet het gewenste effect doordat de embryonale ontwikkeling in het gedrang kwam. Wij hebben niet alleen de ideale temperatuur van het transport in EHM bepaald, maar ook hoe lang de eicellen in het medium konden bewaard worden zonder dat een negatieve invloed op de embryo-ontwikkeling werd waargenomen. Het EHM werd daarbij succesvol bevonden voor het bewaren van onrijpe eicellen wanneer deze bij kamertemperatuur en niet langer dan 10 uur bewaard werden. Hierdoor kan het transport van de onrijpe eicellen van de verafgelegen boerderijen naar het labo vereenvoudigd worden op een goedkopere manier.

Het doel van commerciële IVP is het verkrijgen van zoveel mogelijk overplantbare blastocysten van hoge kwaliteit om zo meer drachten en nakomelingen te produceren. Met dit in gedachte werd de focus gezet op het verhogen van de lage blastocystopbrengst. Embryo's in grote groepen ontwikkelen namelijk beter door de productie van autocriene factoren die de groei stimuleren. Daarom werd een speciaal ontwikkeld schaaltje gebruikt (**Hoofdstuk 4**). De twee centrale kuipjes in deze Corral® dish worden opgedeeld in kwadranten door middel van een semipermeabele wand. Hierdoor kunnen vier groepen per donor apart gehouden worden, terwijl alle eicellen en embryo's toch blootgesteld worden aan hetzelfde medium. Dit impliceert dat een groter aantal eicellen en embryo's gegroepeerd wordt dan wanneer de eicellen volledig apart per donor gekweekt worden. Het gebruik van de Corral® dish tijdens de *in vitro* maturatie en/of cultuur werd geëvalueerd en hierbij werd een hogere blastocystontwikkeling op dag 7 vastgesteld wanneer de Corral® dish werd gebruikt tijdens zowel maturatie- als de cultuurperiode. Bovendien wordt de transplantatie van dag 7 blastocysten verkozen, omdat hiermee de hoogste drachtpercentages worden verkregen. Ondanks het eenvoudig gebruik van de Corral® dish kunnen er vragen gesteld worden omtrent sanitaire risico's en de mogelijkheid van ziekteoverdracht wanneer eicellen en embryo's van verschillende donoren samen gegroepeerd worden.

Om de embryoselectie te verbeteren, werd gebruik gemaakt van time-lapse analyse als een niet-invasieve methode om de kinetiek van de embryo-ontwikkeling als voorspellende parameter voor de verdere blastocystvorming te evalueren (**Hoofdstuk 5**). Hierbij werden de categorieën 'vroeg', 'intermediair' en 'laat' delende embryo's voor het eerst gedefinieerd in well-of-the-well (WOW) plaatjes in serumvrij medium. Intermediair delende embryo's zouden van een hogere kwaliteit zijn en

aldus het meest lijken op de *in vivo* embryo's. Uit onze observaties werd duidelijk dat snel delende embryo's een hogere waarschijnlijkheid hebben om tot blastocyst te ontwikkelen in vergelijking met traag delende embryo's (Odds ratio 43 op 4-cellig stadium op 41.9h). Bovendien was er een significante stijging in blastocystontwikkeling bij de intermediair delende embryo's in vergelijking met traag delende, op zowel het 2-cellig als het 3-cellig stadium.

De ontwikkeling in een aanzienlijk deel van de *in vitro* geproduceerde embryo's stopt tijdens de vroege delingen. In **Hoofdstuk 6** werd de chromosomale samenstelling van deze vroeg delende embryo's onderzocht met behulp van een recente techniek die 'haplarithmisis' wordt genoemd. Vijf donorkoeien werden herhaaldelijk gebruikt om embryo's te verkrijgen via (1) *in vivo* spoeling van de eileider, (2) met behulp van IVP procedures na hormoonbehandeling en OPU (OPU-IVF) en (3) door IVP na het slachten zonder hormoontherapie (IVM-IVF). In het algemeen was aneuploidie de meest waargenomen afwijking. Analyses toonden aan dat 18.8% van de *in vivo* verkregen embryo's minstens één blastomeer met chromosomale afwijkingen hadden, terwijl dit 69.2% was bij de OPU-IVF embryo's en 84.6% bij de IVM-IVF embryo's. Afwijkingen door fouten in de meiose werden maar bij twee embryo's (OPU-IVF and IVM-IVF groep) afkomstig van hetzelfde donordier waargenomen. Afwijkingen van chromosoomsegmenten waren meer aanwezig in IVM-IVF embryo's (69.2%) vergeleken met OPU-IVF embryo's (15.4%). Opmerkelijk was het hoge aandeel dispermie bij de IVM-IVF embryo's (69.2%). Daarentegen werd enkel bij 7.7% van de OPU-IVF embryo's dispermie gedetecteerd en bij 0% van de *in vivo* embryo's. Heterogoneïsche celdelingen, welke recent werden beschreven als spontane segregatie van volledige ouderlijke genomen in verschillende cellijnen tijdens de embryonale delingen, werden geobserveerd bij 7 IVM-IVF embryo's (53.8%). Het is duidelijk dat de toepassing van *in vitro* technieken de incidentie aan chromosomale afwijkingen doet toenemen, wat aldus een oorzaak kan zijn voor het hoge aantal embryo's dat in hun vroege ontwikkeling stoppen met groeien.

In **Hoofdstuk 7** werden de algemene discussie en conclusies beschreven. De volgende take home messages werden hieronder opgesomd:

1. Commercieel verkrijgbaar embryo holding medium (EHM) kan veilig gebruikt worden om onrijpe rundereicellen te bewaren zonder een nadelig effect op de verdere embryonale ontwikkeling wanneer dit op kamertemperatuur gebeurt en wanneer de opslagtijd niet langer dan 10 uur bedraagt.
2. Labohandelingen kunnen eenvoudiger ingepland worden dankzij het gebruik van EHM om onrijpe eicellen te bewaren doordat de eicelrijping artificieel geïnitieerd kan worden wanneer deze uit het EHM gehaald worden.

3. De Corral® dish is een gebruiksvriendelijk schaaltje waarbij eicellen en embryo's van verschillende donoren eenvoudig gegroepeerd kunnen worden zonder verlies van de specifieke donoridentiteit.
4. Wanneer de Corral® dish gebruikt wordt zowel tijdens *in vitro* maturatie als tijdens de cultuurperiode, dan wordt een hogere blastocystopbrengst waargenomen op dag 7 na inseminatie, waardoor meer transfers met verse blastocysten kunnen uitgevoerd worden op diezelfde dag.
5. Het tijdstip waarop embryo's delen kan als voorspellende parameter voor blastocystontwikkeling aanschouwd worden doordat snelle en intermediaire delers meer tot blastocyst ontwikkelen dan traag delende embryo's.
6. Embryo's in het delingsstadium die geproduceerd worden via *in vitro* technieken vertonen een hogere incidentie chromosomale abnormaliteiten in vergelijking met *in vivo* embryo's. Dit kan gerelateerd worden aan de *in vitro* procedure op zich.
7. Aneuploidie is het meest voorkomende type chromosomale afwijking in embryo's in het delingsstadium en de chromosomale abnormaliteiten bij deze embryo's zijn voornamelijk van mitotische origine.

DANKWOORD

Almost Ph.iniseD!

Het heeft me bloed (follikels maar soms ook duimen werden gepuncteerd), zweet (koppige koeien laten zich niet zomaar verplaatsen) en tranen (frustraties waren onvermijdelijk wanneer de gewenste resultaten uit bleven) gekost, maar dankzij de hulp van velen kan ik trots zijn op wat ik heb bereikt.

Allereerst gaat mijn dank uit naar Prof. dr. Ann Van Soom. Zonder het openstellen van uw lab had ik nooit de kans gehad om deel uit te maken van het RBU-team. Ook toen er een abrupt einde kwam aan mijn beurs, stond u klaar met een plan B. De afgelopen vier en een half jaar heb ik ontzettend veel bijgeleerd. Bedankt voor alle kansen die mij u gaf! U bent dan ook een groot voorbeeld, hoe u een carrière kan combineren met vier kinderen, een hond en katten, ik heb er alleen maar bewondering voor. Bedankt om altijd begripvol te zijn en me het pad te wijzen wanneer ik het bos door de bomen niet meer zag.

Katrien, als co-promotor stond je steeds met raad en daad klaar. Ook pepte je me op als het even wat moeilijker ging. Jouw enthousiasme en positivisme zijn aanstekelijk. Bedankt om alles na te lezen en me elke keer op de goede weg verder te zetten.

Prof. dr. Joris Vermeesch, bedankt dat u samen met de RBU een prachtig project uitschreef waar ik drie jaar heb op mogen werken. Dankzij alle nieuwe technologie die u ter beschikking heeft, hebben we er iets moois van kunnen maken.

Ook gaat een welgemeende danku uit naar de van de examen- en leescommissie. Prof. dr. Favoreel, bedankt om de rol van voorzitter op u te nemen. Prof. dr. Peelman, bedankt om te willen zetelen in mijn examencommissie en vooral om mijn proefschrift grondig na te lezen. Prof. dr. Opsomer, dankjewel om naast lid van de examencommissie, mij vertrouwde om met Osvaldo IVF proeven op te starten. Prof. dr. Heindryckx, bedankt om deel uit te willen maken van de commissie en om mij af en toe eens verder te doen kijken dan het rund. Dr. Goossens en dr. Mullaart, bedankt voor het erg grondig nalezen van deze thesis en het geven van feedback. Bedankt ook dr. Mullaart dat u mij samen met dr. Knijn wegwijs bracht in de commerciële embryoproductie bij het CRV. Bedankt dat ik 2 fantastische dagen mocht meelopen in het bedrijf bij Femmie Dotinga.

Dan zijn er mijn bureaugenootjes waarmee ik uren werkend doorbracht (lees: kletsend - oepsie). Onze bureau of de bureau vol progesteron, toen we met maar liefst 4/6 (66.6%) zwanger waren in 2016.

Fantastische momenten heb ik er beleefd, met elk van jullie! Eline, jij was het die me vanaf het eerste uur begeleidde met mijn twee masterproeven. Jij leerde me hoe je van een eicel een embryo maakte. Jij was het die de differentieel apoptotische kleuring ontwikkelde. Dé kleuring bij uitstek waardoor we uren en dagen in het donker zaten en 's nachts - als onze ogen dicht waren - nog blastomeren aan het tellen waren, bedankt hoor ;-). Maar jij was het ook waar ik mijn proefopzetten (en meer) mee besprak. Jouw ervaring en kennis zorgde er voor dat alles efficiënter kon verlopen. Bedankt voor alle babbels tijdens de pauzes en daarbuiten! Catharina, onze post-doc, altijd op de hoogte van alles, echt, alles. Zelf al zat je op congres in Zweden, je wist de spannende weetjes nog vroeger dan wij. Jouw vechtlust en doorzettingsvermogen waren een inspiratie. Je stond dag en nacht klaar met wijze raad. Bedankt om me onder jouw vleugels te nemen wanneer ik het nodig had. Margot, ook al zat je meer in de kliniek dan op onze bureau, we konden het goed met elkaar vinden. Jouw werklust, steeds gepaard met een vriendelijke lach, daar kunnen vele nog iets van leren! Ik weet nu al dat ik nooit meer zulke leuke collega's ga hebben. Bij jullie kon ik mezelf zijn en mijn hart luchten, jullie wisten soms iets te veel (mondje dicht!). Ilse, het afgelopen jaar zat je wat vaker achter je computer, waardoor we meer tijd samen doorbrachten. Bedankt voor alle grappige studentenverhalen! Leen, als nieuwe doctoraatsstudente burgerde je jou al vlug in en leerde je al gauw dat een onderzoeksvraag alleen maar meer vragen met zich meebrengt – tot grote frustratie. Eén goede raad, gewoon blijven gaan en je komt er, daar twijfel ik niet aan. Bedankt voor jouw heerlijke eerlijkheid. Valérie en Féline, ook al maakten jullie plaats voor anderen, bedankt voor de fijne momenten en gesprekken die we samen hadden! Féline, samen gingen we de strijd met het IWT aan en samen spraken we elkaar moed in. Gelukkig kon jij overwinnen en haalde je de beurs binnen, victory! Nu zit je al even bij het Vlaams Paardenloket, maar toch blijf je werken aan je doctoraat. Binnenkort komen ook de laatste loodjes bij jou langs! Ik duim!

To the former Little Spain of downstairs, Bart and Sonia, thanks for the nice lunch breaks. Nerea and Lynn, thanks for all fun during puncturing and help with the experiments. Ruth, bedankt om de sfeer er steeds in te brengen! To the international office and its visitors, Nuria, Krishna, Gretania, Adriana, Kasia, Xiaoyuan, thanks for all happy lab moments. I wish you all the best, in Ghent or abroad!

Osvaldo, thanks for all help with the experimental cows. Sometimes with hangover (or was it always?), but I could always count on you, even to inseminate five cows at 6 am. It was a nice surprise to meet again in Bangkok. I wish you the best in Canada and maybe someday having your own IVF/lab/farm in Paraguay!

There are some people in Leuven I need to thank as well. First, Aspasia, crazy Aspasia, in our lab it has been said that Greek people are the most friendly to work with. After meeting and working with you,

I have to confirm. Every time you passed by, you bought cookies for the whole lab. Everyone enjoyed it, you being in our lab, except maybe our cholesterol level. You are brilliant (and a bit chaotic), thanks for introducing me the basics of genetics. Olga, I have to be honest, you seem to be even more crazy than Aspasia ☺. I enjoyed working with you, as you explained everything and answered all my genetics questions. Beside producing bovine embryos, we had a good time. Thanks for taking me to Florence and the Machine – with a pipetor in your bag – during terrorism level 4. Luckily you were escorted to put your bag in a locker and we could enjoy the show. Thanks to Heleen, Elia and Masoud for all KULeuven genetic magic you are performing.

Petra en Isabel, lab ladies to the rescue! Isabel, bedankt voor alle fijne momenten tijdens het puncteren en daarbuiten. We hebben heel wat gelachen want je wist al op voorhand waar ik aan dacht en omgekeerd. Ik heb enorm veel respect voor jouw doorzettingsvermogen. Als bezige bij leerde je ons naast de liefde voor IVF de liefde voor de natuur en z'n vogels. Je volgde vanop de webcam de torenvalk (als ik het me goed herinner) die haar eieren aan het uitbroeden was, terwijl je voor iedereen in de weer was. Als ik ooit een rare vogel zie, kom ik bij jou langs ;-)! Petra, bedankt voor elke keer dat je mij een berichtje stuurde toen ik weer iets vergeten was. Je denkt veel verder dan de dagelijkse media voor iedereen, je houdt uren in de gaten, je bestelt de producten nog voor we door hebben dat ze op zijn. Je bent de moederkloek met haar kiekskes, die ze stuk voor stuk bijstaat tot ze het nest uit vliegen. Bedankt voor alle fijne babbels en voor zoveel meer!

Bedankt aan Euro Meat Group Moeskroen en Flanders Meat Group Zele, in het bijzonder Kurt, Adam, Mohammed, om ons elke week van een nieuwe lading eierstokken te voorzien!

Een jaar lang stonden er 3 tot 5 koeien in de stallen, net iets langer dan we hadden voorzien. Daarom bedankt Jan, dat ik zo lang de stal in kon nemen. Bedankt Dirk en Willy om dagelijks de koeien te verzorgen. Bedankt Marnik om alle logistiek op je te nemen en voor alle bestellingen droog ijs. Bedankt Véronique om het OPU materiaal te wassen en te steriliseren. Bedankt Els en Ria voor alle medicatie die ik bij jullie kon bestellen.

Bedankt aan Sandra, je stond niet alleen klaar om al mijn administratieve rompslomp overzichtelijk te maken, je kwam ook met allerlei naald-en-draad-tips paraat! Ooit hoop ik zo goed overweg te kunnen met de naaimachine als jij! Bedankt, Leïla, voor alle bestelbonnen, facturen, kilometervergoedingen en zoveel meer dan financiële zaken. Zonder jullie zijn we allemaal verloren!

Ook gaat een speciaal bedankje naar Eline Claes, want jij bent het die me op de hoogte bracht van de FAVV vacatures. Net op de valreep kon ik me nog aanmelden, en nog geen 5 maanden later ging ik er al aan de slag. Zonder meer is dit een volledige carrièreswitch geweest, maar ik heb er nog geen minuut

spijt van. Bedankt aan al mijn nieuwe collega's, dat jullie begripvol waren dat ik hier en daar nog wat doctoraatszaken moest afwerken.

Aan alle vrienden en familie die vol enthousiasme bleven vragen hoe het met mijn doctoraat ging, bedankt om te luisteren naar weliswaar bescheiden uiteenzettingen voor embryo's, bedankt om het te willen begrijpen of toch om te doen alsof ☺. Ook al bleven sommige koppig geloven dat ik dino's kweekte en niet runderembryo's, nietwaar Pieter?, ik hoop dat ik jullie niet te veel teleurstel met mijn werk over koeien, echt waar, koeien, nee geen codewoord voor dino's, zucht… ☺

Een speciale dankuwel aan Ingrid en André, dankzij Nils kreeg ik er twee fantastische schoonouders bij. Meer nog, twee fantastische grootouders die elke keer staan te springen om Lotte op te vangen. Bedankt André voor al jouw interesse in de wetenschap en mijn doctoraat! Bedankt Ingrid voor alle woensdagnamiddagen, waarbij je niet enkel Lotte entertainde maar zoveel meer voor ons deed!

Liefste mama en papa, alles heb ik aan jullie te danken! De liefde waarmee ik ben opgevoed en opgegroeid heeft me gevormd tot wie ik nu ben. Belangrijke beslissingen werden steeds in onderling overleg genomen en elke keer kreeg ik het extra duwtje in de rug om alles tot een goed einde te brengen. Nog steeds staan jullie dag en nacht klaar, niet alleen voor mijzelf, maar ook voor Nils en Lotte. Woorden kunnen niet omschrijven hoe dankbaar ik jullie ben voor álles!

Nils, lieve schat, ik heb het je de laatste weken niet gemakkelijk gemaakt (doe ik eigenlijk nooit ;-)) maar je was steeds begripvol. Jij was het die zei dat ik dit moest afmaken, dat dit de bekroning zou zijn van mijn onderzoek, dat je trots op me was. Bedankt, om te helpen met nalezen, figuren maken, ons huis op orde te houden in alle chaos, om met Lotte nog maar eens op stap te gaan zodat ik mij in alle stilte kon voorbereiden, om kritisch te zijn, maar vooral om in mij te geloven en me moed in te spreken. Bedankt dat jij er altijd bent! Lotte en jij zijn mijn alles!

Lotte, mijn allerliefste Lotje, Sprotje, klein Marmotje, jij bent de bekroning op ons leven!

BIBLIOGRAPHY

Publications

Destouni A, Zamani Esteki M, **Catteeuw M**, Tsuiko O, Dimitriadou E, Kurg A, Van Soom A, Voet T, Vermeesch JR. *Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage stage chimaerism and mixoploidy.* Genome research, 2016, 26, 567-578.

Pavani KC, Alminana C, Wydooghe E, **Catteeuw M**, Ramírez MA, Mermillod P, Rizos D, Van Soom A. *Emerging role of extracellular vesicles in communication of preimplantation embryos in vitro.* Reproduction, Fertility and Development, 2016, 29, 66-83.

Catteeuw M, Wydooghe E, Mullaart E, Knijn HM, Van Soom A. *In vitro production of bovine embryos derived from individual donors in the Corral® dish.* Acta veterinaria scandinavica, 2017, 59:41.

Tšuiko O*, **Catteeuw M***, Zamani Esteki M*, Destouni A, Bogado Pascottini O, Besenfelder U, Havlicek V, Smits K, Kurg A, Salumets A, D'Hooghe T, Voet T, Van Soom A, Vermeesch JR. *Genome stability of in vivo-conceived cleavage-stage embryos is higher compared to in vitro produced embryos.* Human Reproduction, 2017, 1-10. * These authors contributed equally to this work.

Bogado Pascottini O*, **Catteeuw M***, Van Soom A, Opsomer G. *Holding immature bovine oocytes in a commercial embryo holding medium: High developmental competence for up to 10 h at room temperature.* Theriogenology, 2018, 107, 63-69. * These authors contributed equally to this work.

Wydooghe E, Van Steendam K, Vandaele L, **Catteeuw M**, Heras S, De Schauwer C, De Sutter P, Peelman L, Deforce D, Van Soom A. *Secretome analysis of define medium conditioned by bovine embryos revealed cathepsin-L as embryotrophic factor.* Under revision.

Oral presentations at national and international conferences

Catteeuw M, Wydooghe E, Mullaart E, Knijn HM, Van Soom A. *Runderembryo's gecultiveerd per donor in de Corral dish*. Vereniging voor fertiliteitsstudie (VFS). Maastricht, The Netherlands, November 29, 2013.

Destouni A, Zamani-Estiki M, **Catteeuw M**, Tšuiko O, Smits K, Van Soom A, Voet T, Vermeesch JR. *The cleavage stage bovine embryo is a valuable model for studying chromosome instability in early mammalian embryogenesis*. Preimplantation Genetic Diagnosis International Society (PGDIS). Chicago, United States of America, May 11-13, 2015.

Catteeuw M, Wydooghe E, Piepers S, Smits K, Destouni A, Tšuiko O, Vermeesch JR, Van Soom A. *Time-lapse analysis of early cleavage in bovine embryos produced in serum-free medium.* Association Europeenne de Transfer Embryonnaire (AETE). Ghent, Belgium, September 11-12, 2015.

Catteeuw M, Wydooghe E, Smits K, Van Soom A. *Time-lapse analyse van runderembryo's bij hoge en lage zuurstofspanning.* Vereniging voor fertiliteitsstudie (VFS). Antwerpen, Belgium, December 11, 2015.

Zamani Esteki M, Destouni A, **Catteeuw M**, Tsuiko O, Dimitriadou E, Smits K, Kurg A, Salumets A, Van Soom A, Voet T, Vermeesch J. *Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage-stage chimerism and mixoploidy.* American Society of Human Genetics 66th Annual Meeting. Vancouver, Canada, 18-22 October, 2016.

Destouni A, Zamani Esteki M, **Catteeuw M**, Tšuiko O, Dimitriadou E, Smits K, Kurg A, Salumets A, Van Soom A, Voet T, Vermeesch JR. *Zygote mitosis revisited: How can zygotes segregate entire parental genomes into distinct blastomeres?* EMBO workshop. Dresden, Germany, April 23-26, 2017.

Tšuiko O, **Catteeuw M**, Zamani Esteki M, Destouni A, Bogado Pascottini O, Besenfelder U, Havlicek V, Smits K, KurgA, Salumets A, D'Hooghe T, Voet T, Van Soom A, Vermeesch JR. *In vitro procedures exacerbate chromosome instability in cleavage stage embryos.* Belgian Society of Human Genetics (BeSHG). Louvain-La-Neuve, Belgium, February 17, 2017.

Tšuiko O, **Catteeuw M**, Zamani Esteki M, Destouni A, Bogado Pascottini O, Besenfelder U, Havlicek V, Smits K, Kurg A, Salumets A, D'Hooghe T, Voet T, Van Soom A, Vermeesch JR. *Unravelling chromosome instability in mammalian preimplantation embryos.* International summer school of H2020-TWINN-2015. Tartu, Estonia, June 13-14, 2017.

Posters at national and international conferences

Catteeuw M, Wydooghe E, Mullaart E, Knijn HM, Van Soom A. *Culture of bovine embryos per donor using the Corral dish does not improve blastocyst development.* Association Europeenne de Transfer Embryonnaire (AETE). Istanbul, Turkey, September 6-7, 2013.

Catteeuw M, Wydooghe E, Van Soom A. *Culture and maturation in the Corral dish: effect on bovine embryo development.* European Society for Domestic Animal Reproduction (ESDAR). Helsinki, Finland, September 12-13, 2014.

Destouni A, Zamani-Estiki M, **Catteeuw M**, Smits K, Tšuiko O, Dimitriadou E, Voet T, Van Soom A, Vermeesch JR. *The cleavage stage bovine embryo is a valuable model for chromosome instability in early mammalian embryogenesis*. Belgian Society of Human Genetics (BeSHG). Charleroi, Belgium, March 6, 2015.

Tšuiko O, Zamani-Estiki M, Destouni A, **Catteeuw M**, Smits K, Dimitriadou E, Nõmm M, Jaakma U, Kurg A, Salumets A, Van Soom A, Voet T, Vermeesch JR. *The cleavage-stage bovine embryo is a valuable model to study chromosome instability in early mammalian embryogenesis.* European Society of Human Genetics (ESHG). Glasgow, United Kingdom, June 6-9, 2015.

Catteeuw M, Wydooghe E, Piepers S, Smits K, Destouni A, Tšuiko O, Vermeesch JR, Van Soom A. Time-lapse analysis of early cleavage in bovine embryos produced in serum-free medium. Association Europeenne de Transfer Embryonnaire (AETE). Ghent, Belgium, September 11-12, 2015.

Catteeuw M, Tšuiko O, Zamani Esteki M, Smits K, Vermeesch JR, Van Soom A. *The influence of in vitro procedures on chromosomal instability in cleavage embryos.* International IVI congress. Bilbao, Spain, May 11-13, 2017.

Catteeuw M, Bogado Pascottini O, Opsomer G, Van Soom A. *Effect of commercial embryo holding medium on development and quality of immature bovine oocytes.* International Embryo Technology Society (IETS). Bangkok, Thailand, January 13-16, 2018.

Bogado Pascottini O, **Catteeuw M**, Opsomer G, Van Soom A. *Effect of storage time and temperature of a commercial embryo holding medium on the maturation kinetics of immature bovine oocytes.* International Embryo Technology Society (IETS). Bangkok, Thailand, January 13-16, 2018.

CURRICULUM VITAE

Maaike Catteeuw werd geboren op 18 november 1989 te Kortrijk. Na het behalen van het diploma hoger secundair onderwijs (Latijn-Wiskunde) aan het Sint Aloysius college te Menen, begon ze in 2007 met de studie Diergeneeskunde aan de Universiteit Gent. Ze behaalde het diploma van Dierenarts afstudeerrichting onderzoek, met onderscheiding in 2013.

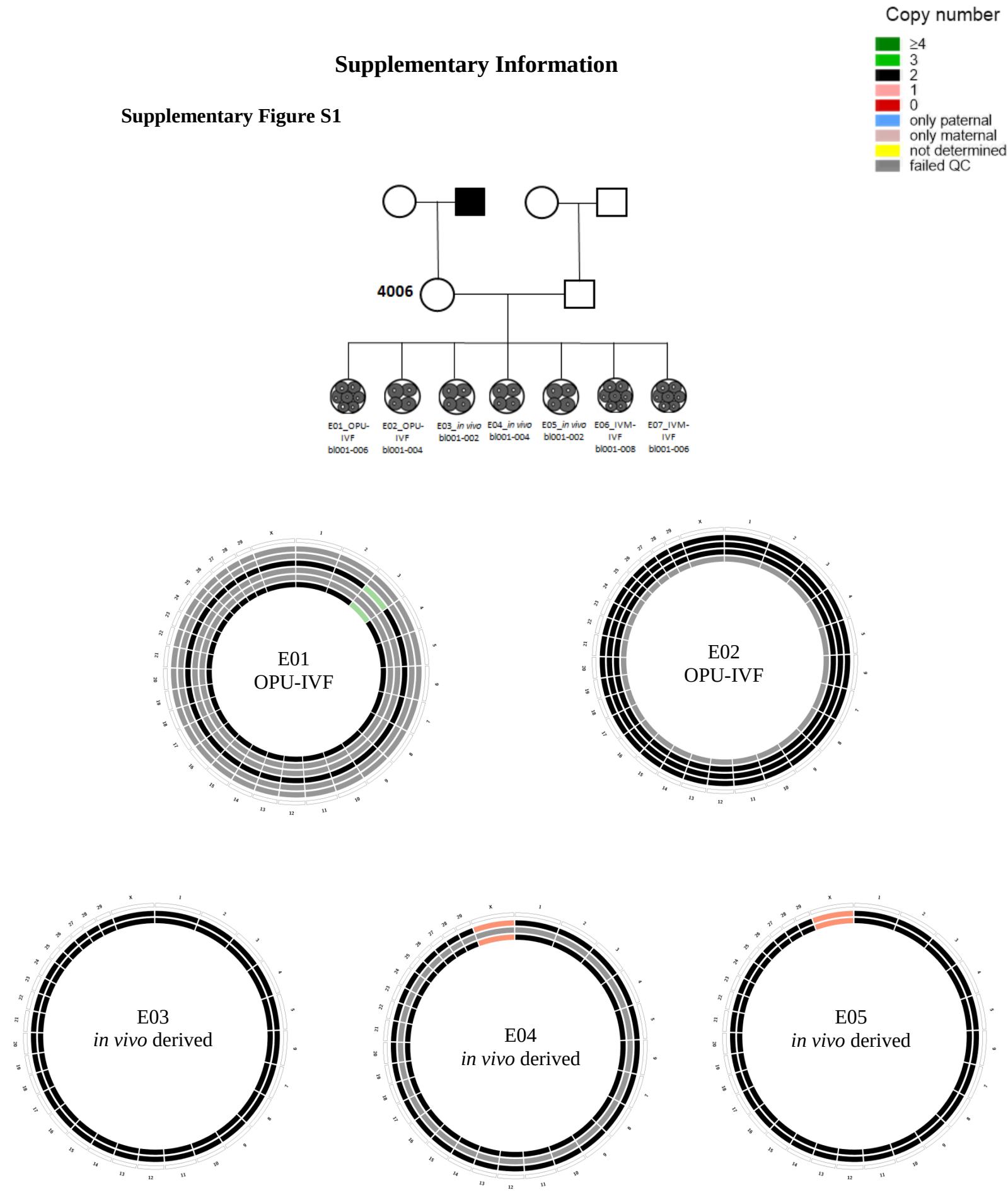
Op 1 oktober 2013 startte ze haar doctoraatsonderzoek, eerst ondersteund door een Dehousse beurs, vervolgens op een FWO project, aan de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Binnen de Reproductive Biology Unit deed ze onderzoek op de *in vitro* embryo productie bij het rund, met focus op de optimalisatie ervan per donor. Naast haar doctoraatsonderzoek participeerde ze ook in de kliniek Voortplanting en Verloskunde Gezelschapsdieren, waar ze demo's gaf aan de studenten Diergeneeskunde. In 2018 voltooide zij de doctoraatsopleiding aan de Doctoral Schools of Life Sciences and Medicine. Sinds 1 april 2018 is ze tewerkgesteld als inspecteur-dierenarts bij het Federaal Agentschap voor Veiligheid van de Voedselketen (FAVV).

Maaike Catteeuw is auteur of medeauteur van verschillende publicaties in internationale wetenschappelijke tijdschriften en nam actief deel aan diverse nationale en internationale congressen.

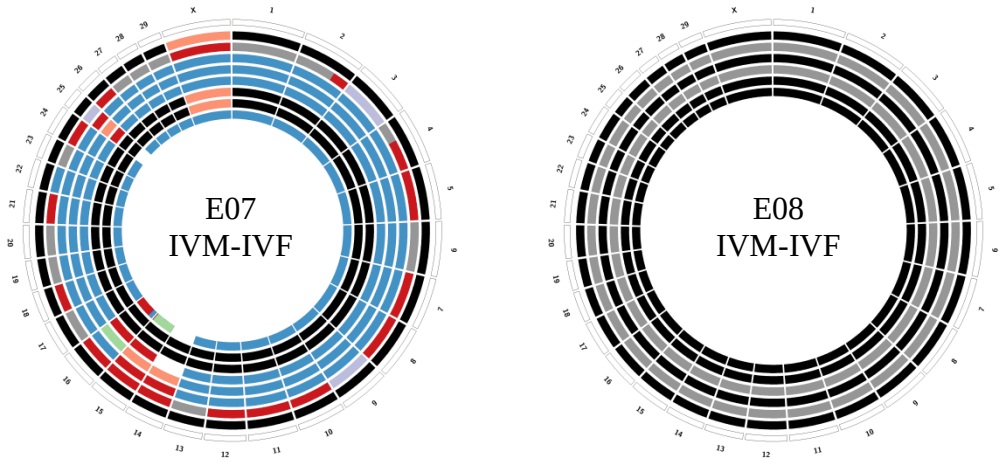
APPENDIX

Supplementary Information

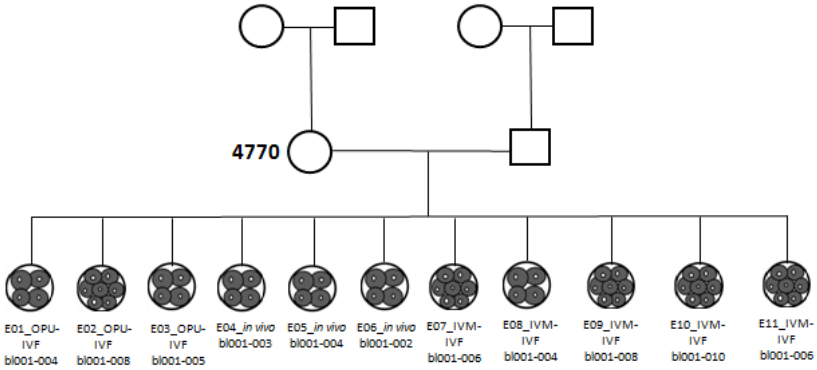
Supplementary Figure S1

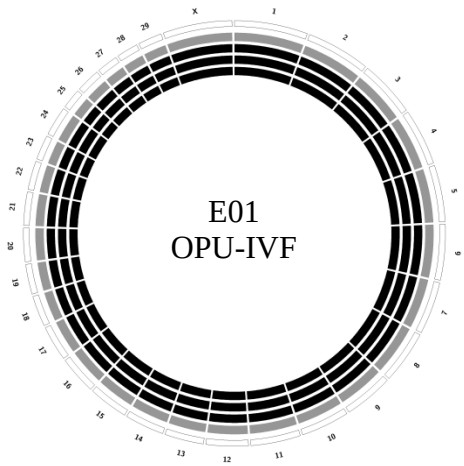


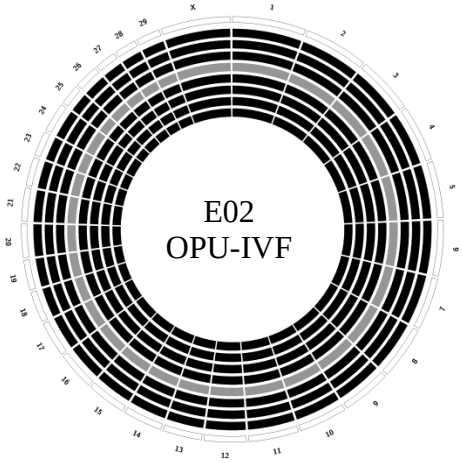
Supplementary Figure S1 (continued)

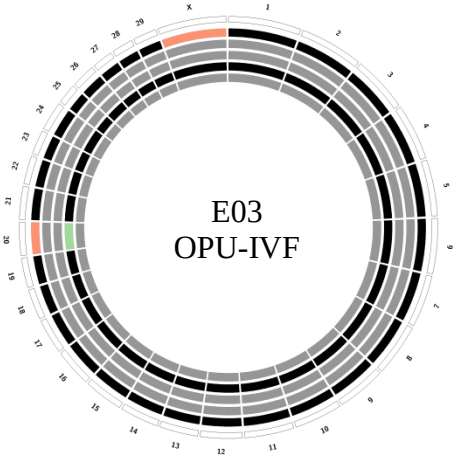


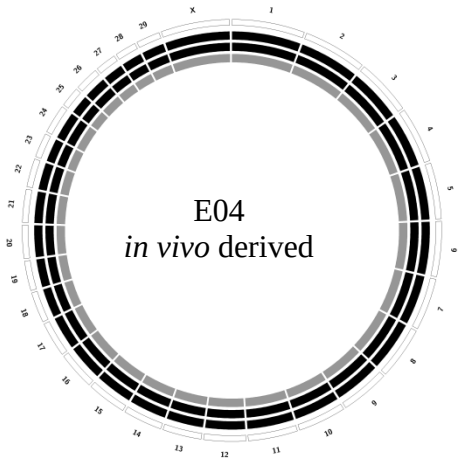
Supplementary Figure S1 (continued)

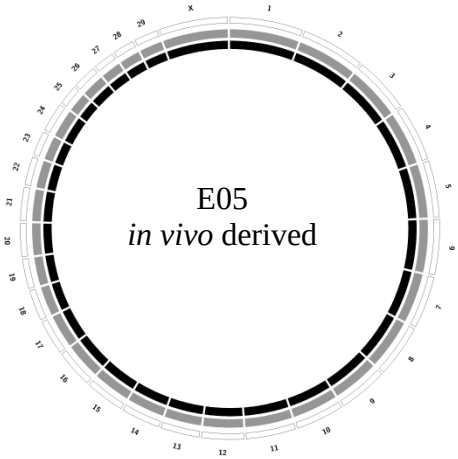


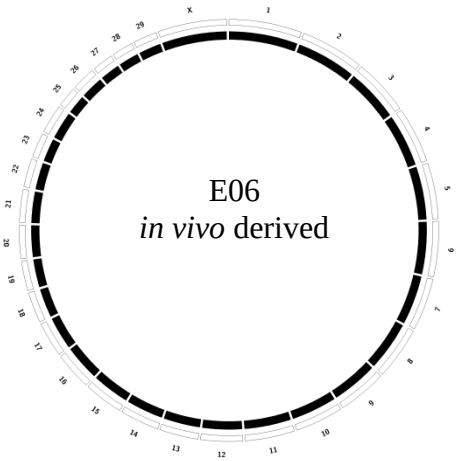




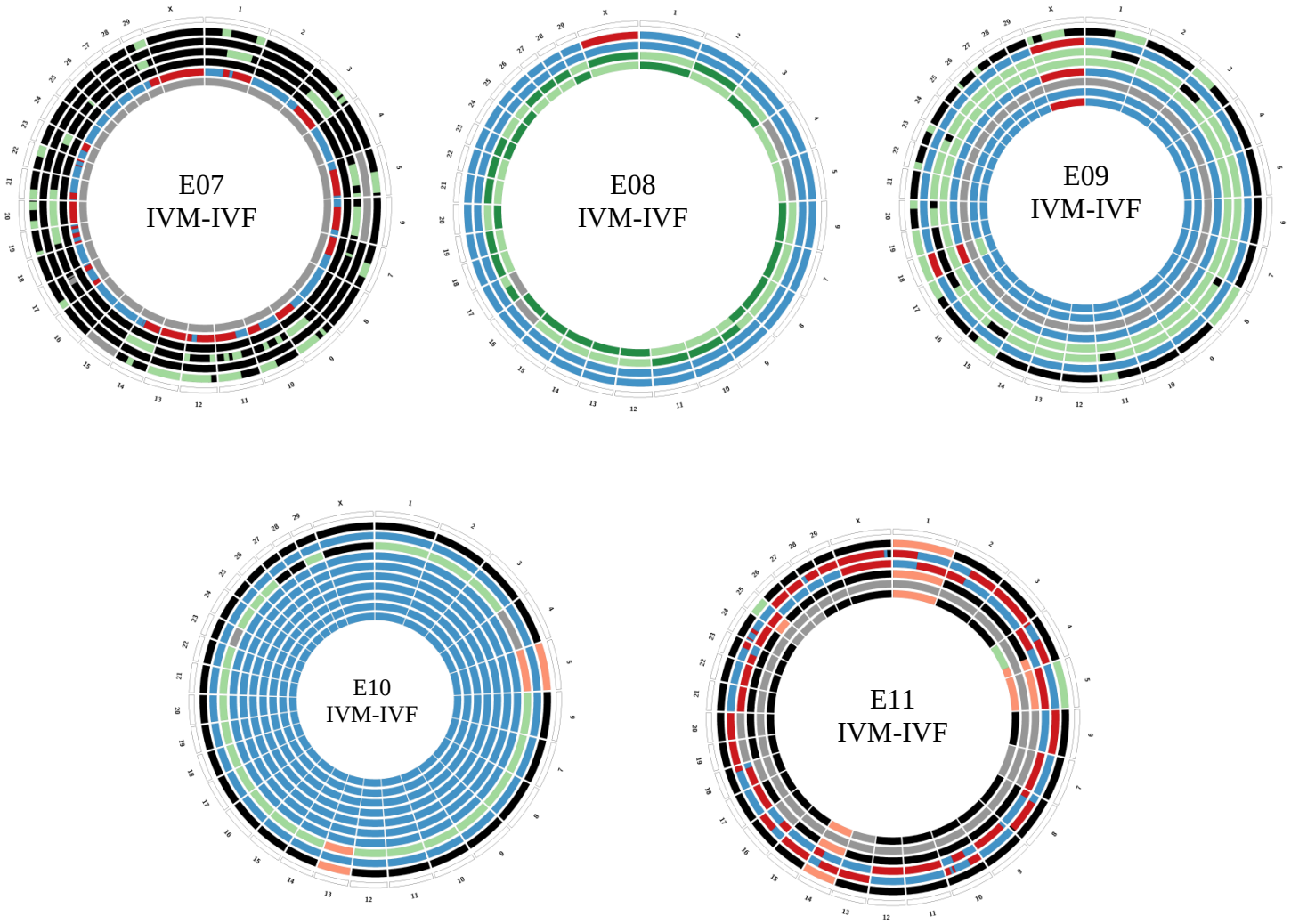




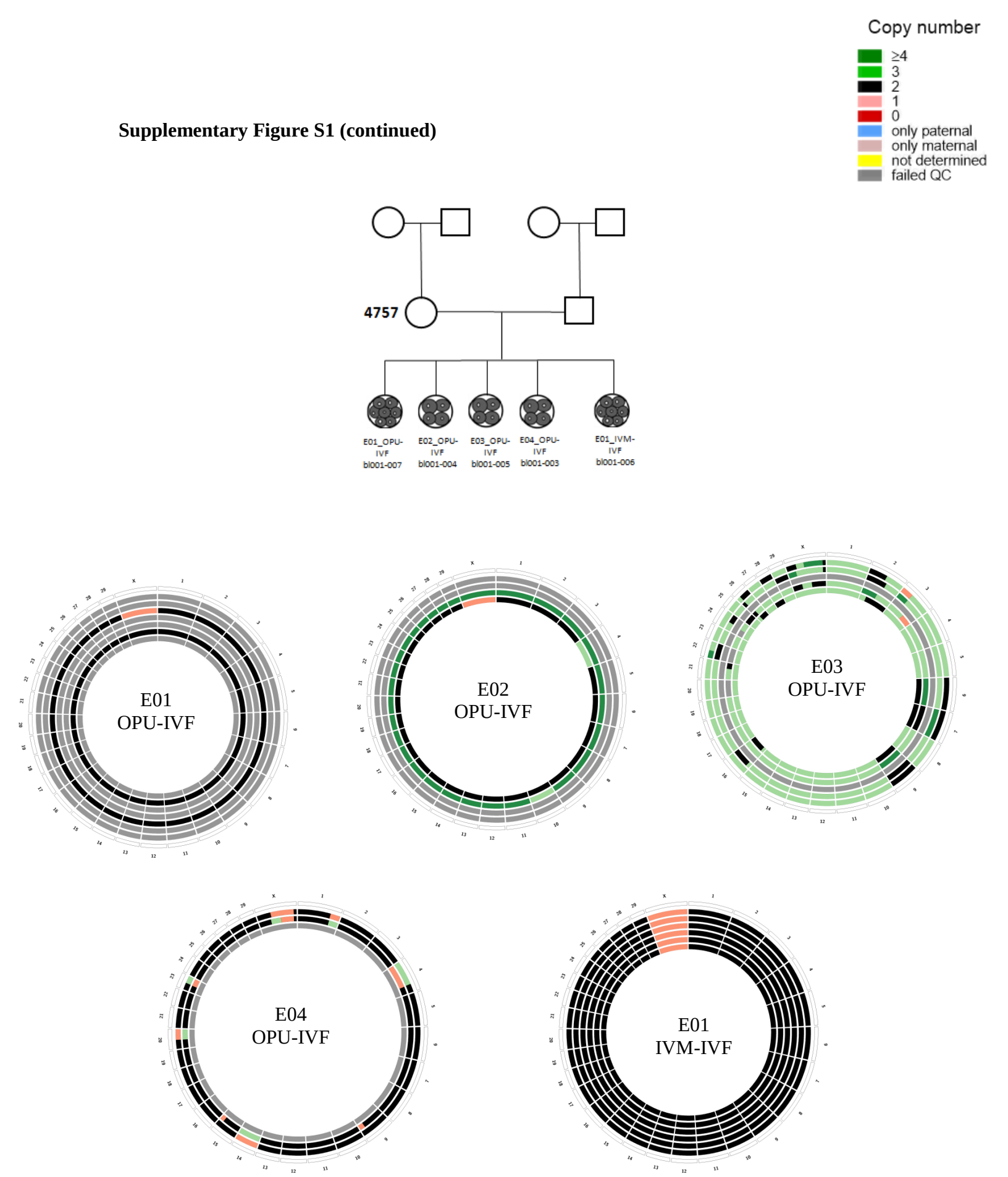




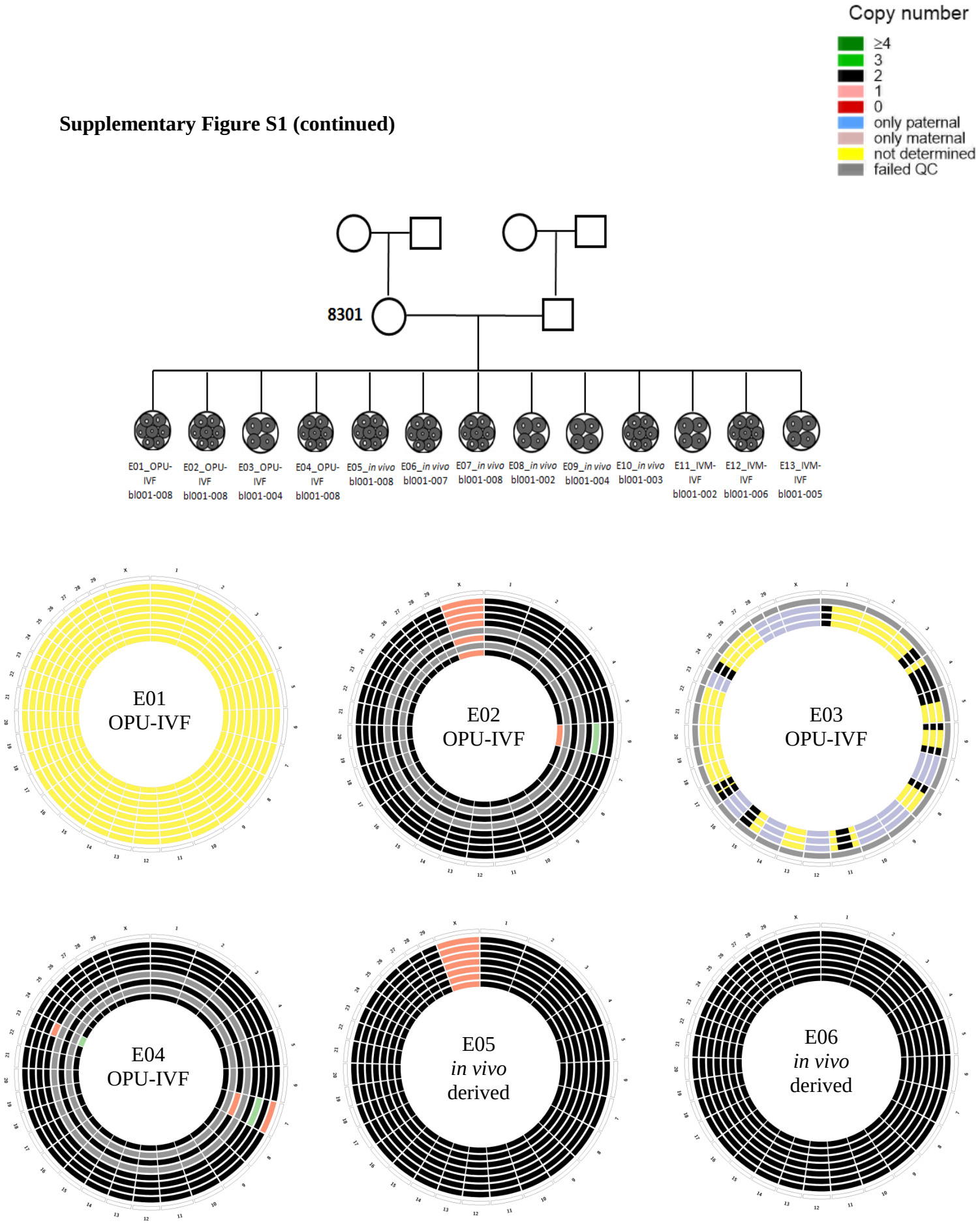
Supplementary Figure S1 (continued)



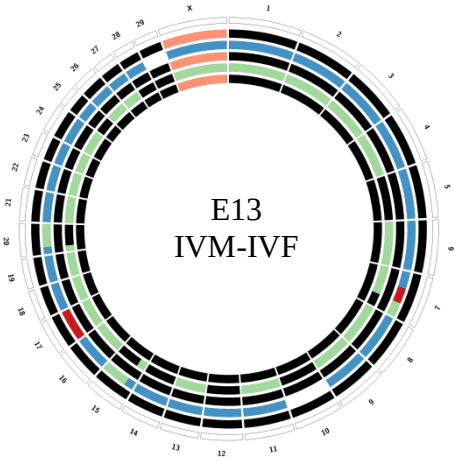
Supplementary Figure S1 (continued)



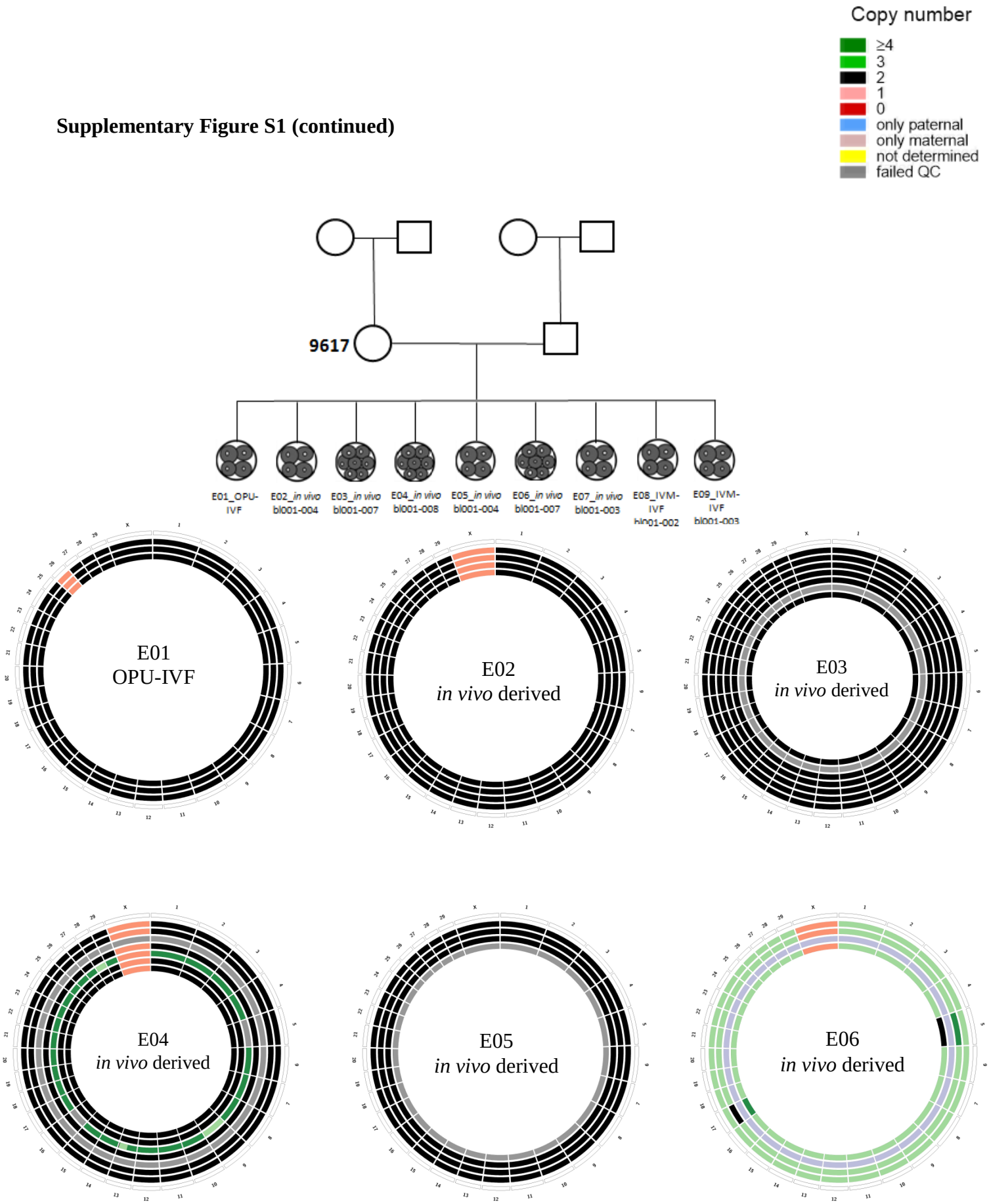
Supplementary Figure S1 (continued)



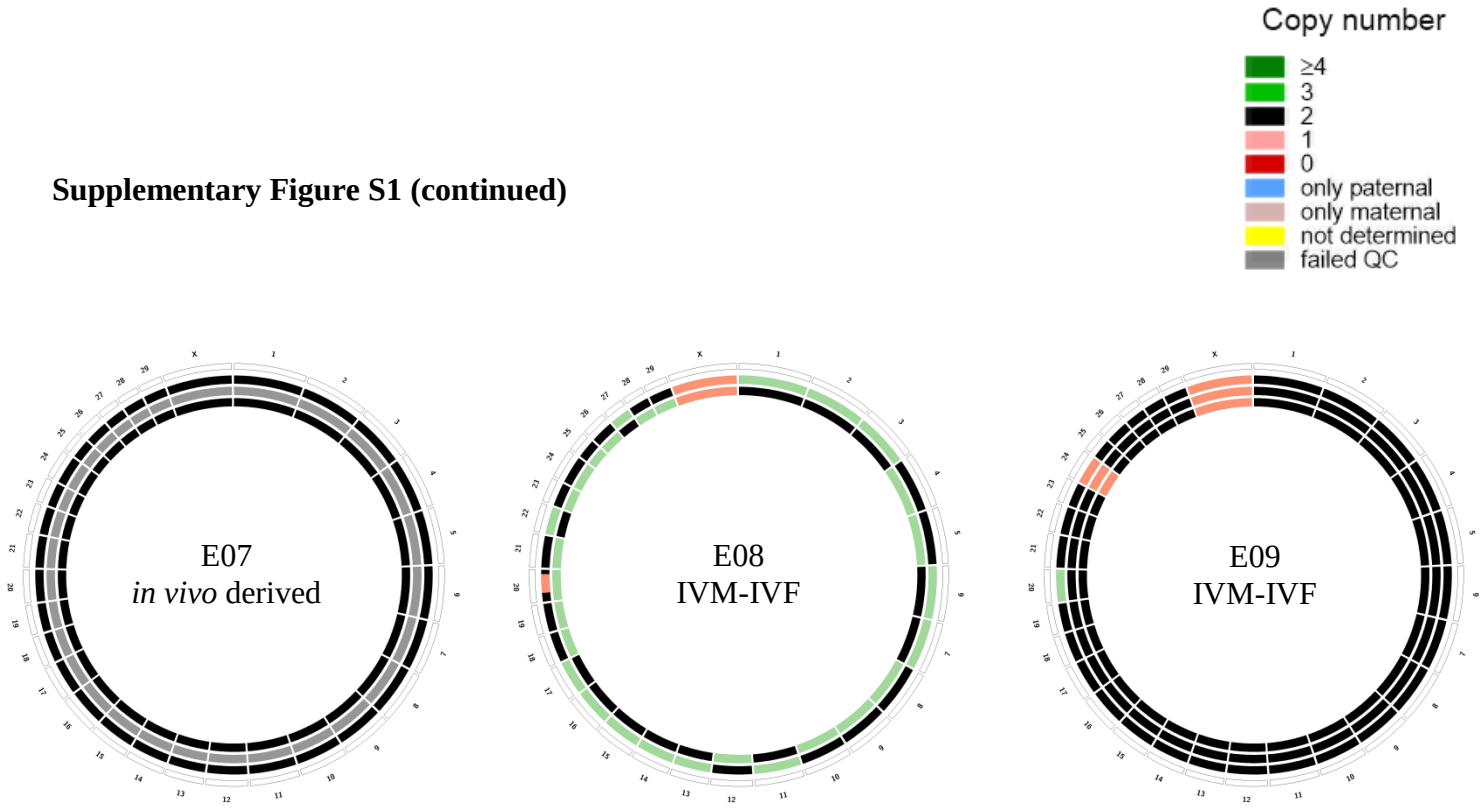
Supplementary Figure S1(continued)


E13
IVM-IVF

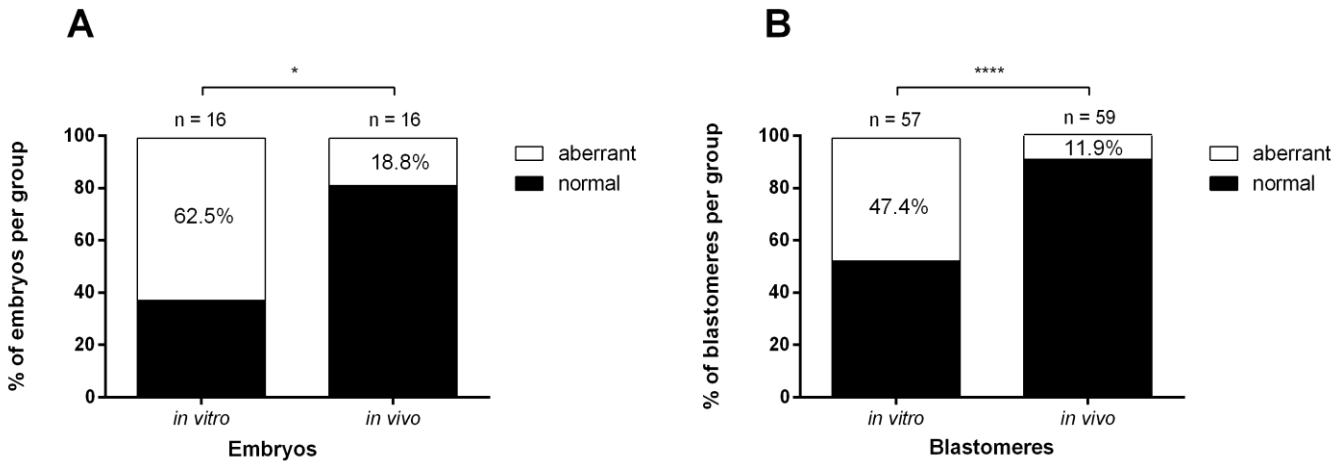
Supplementary Figure S1 (continued)



Supplementary Figure S1 (continued)



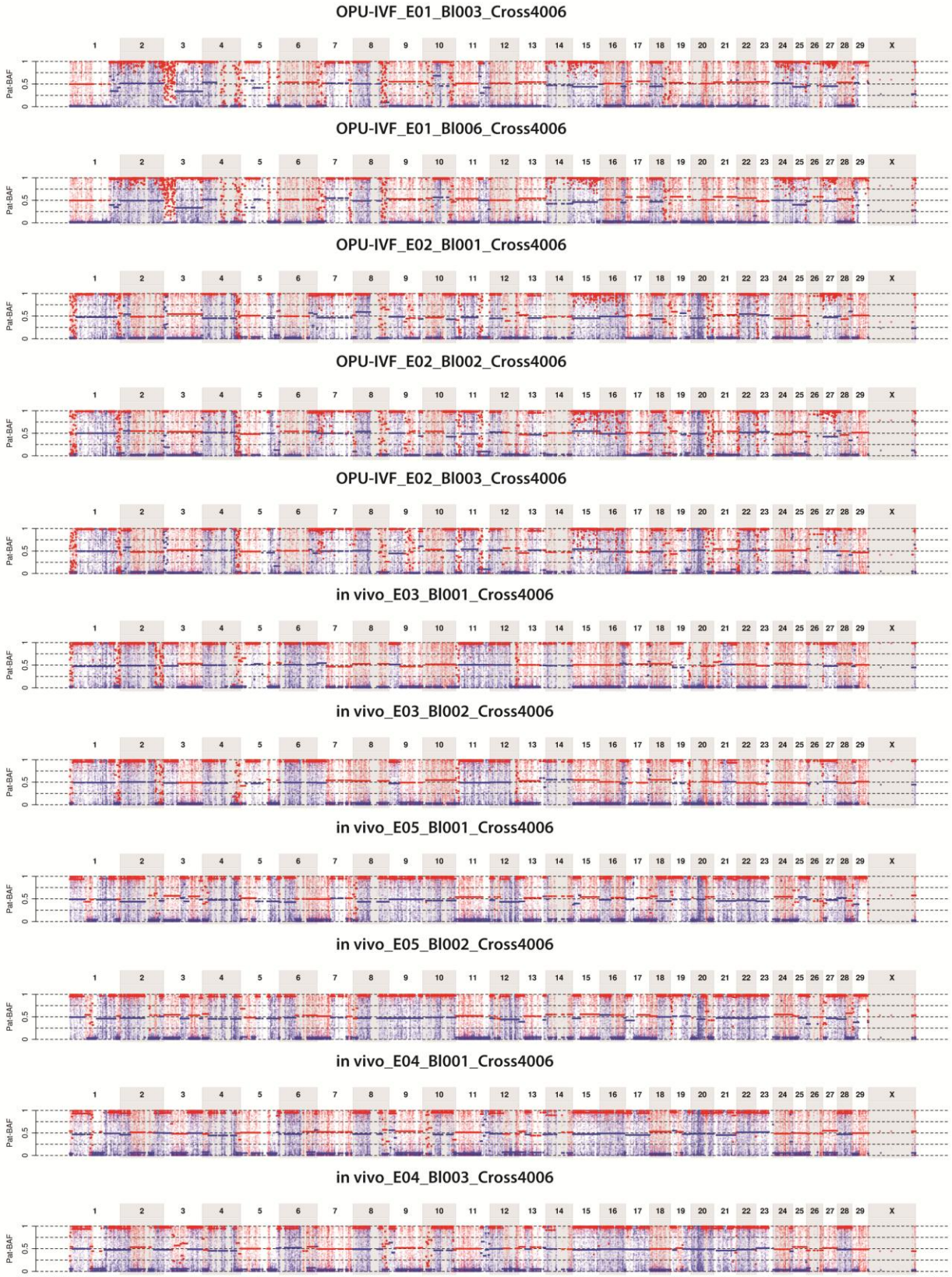
Supplementary Figure S1. Circos plots representing genomic constitution of IVM-IVF, OPU-IVF and *in vivo*-derived embryos. Detailed whole genome overview of chromosomal anomalies corroborated by haplarithmisis. All embryos involved in the study are shown per pedigree. Each circos plot represents one embryo and each circle within the plot represents single blastomere derived from the embryo. The numbers above each line of a circos plot indicate chromosomes. Each chromosome is color coded according to the chromosomal status, indicating presence or absence of chromosomal anomalies (e.g. black for diploid, green for chromosome gain, red for chromosome loss). Androgenetic and gynogenetic cell lines are all depicted in either blue or purple color, respectively. Grey lines denote blastomere that did not pass the quality control, therefore the chromosomal status of these cells remained undetermined. Three embryos from Cross8301 that passed quality control had inconclusive data due to distort haplarithm profiles (depicted in yellow) and these embryos were also discarded from further investigation (E01, E09 and E10).



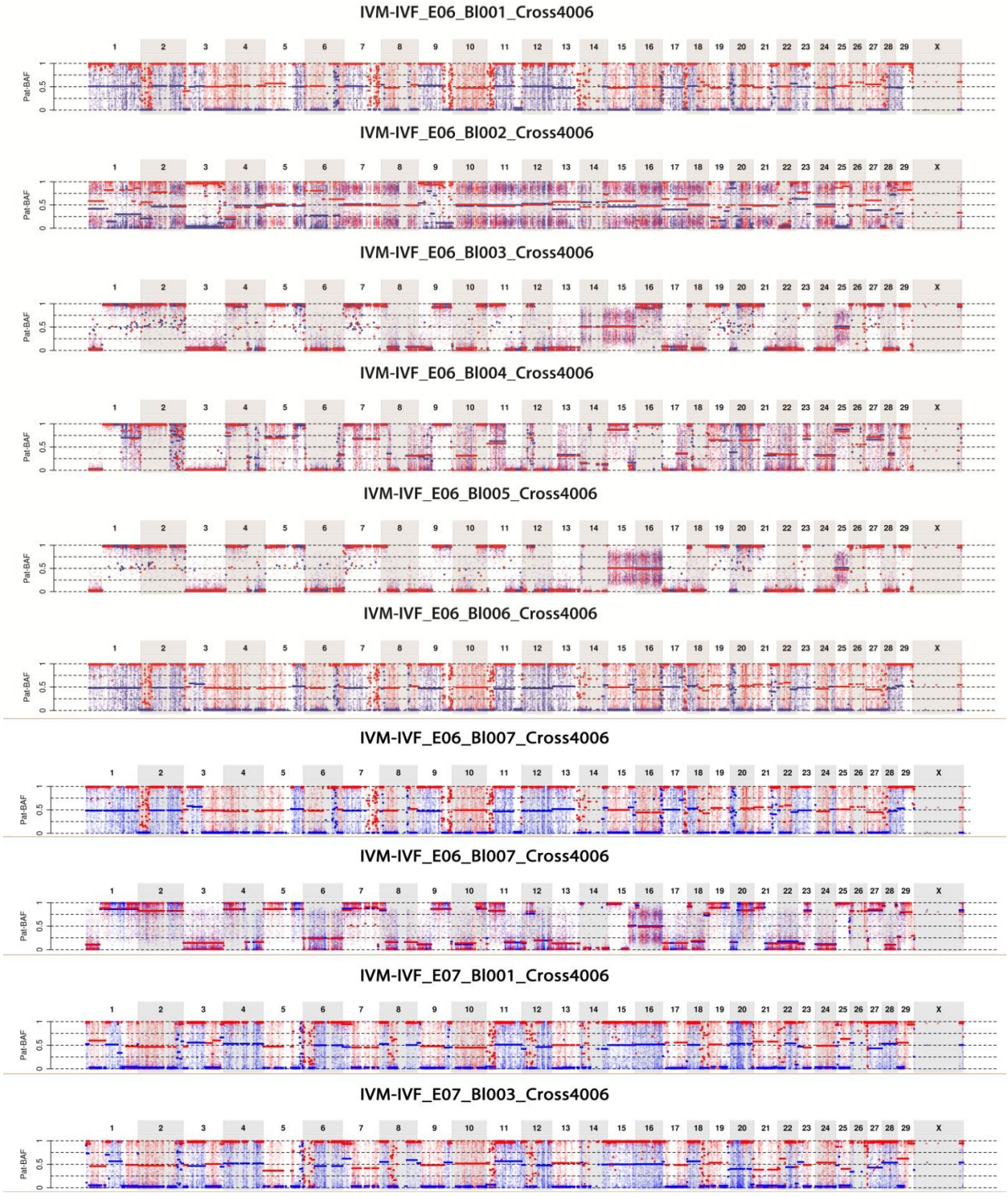
Supplementary Figure S2. The rate of CIN in monospermic *in vitro* and *in vivo*-derived embryos.

The numbers above the columns represent the total numbers of embryos (A) and blastomeres (B) included in the analysis. (A) Comparison of normally fertilized *in vitro* and *in vivo*-derived embryos. Although *in vivo*-derived embryos can present with genomic aberrations, *in vitro* maturation, fertilization and culture influences CIN rates; **P* = 0.029, two-tailed Fisher's exact test. (B) The total proportion of blastomeres with aneuploidies, segmental imbalances, and/or aberrant ploidy in IVF and *in vivo*-derived embryos. IVF embryos contain more blastomeres with aberrant genomes than their *in vivo* counterparts; *****P* < 0.0001, two-tailed Fisher's exact test.

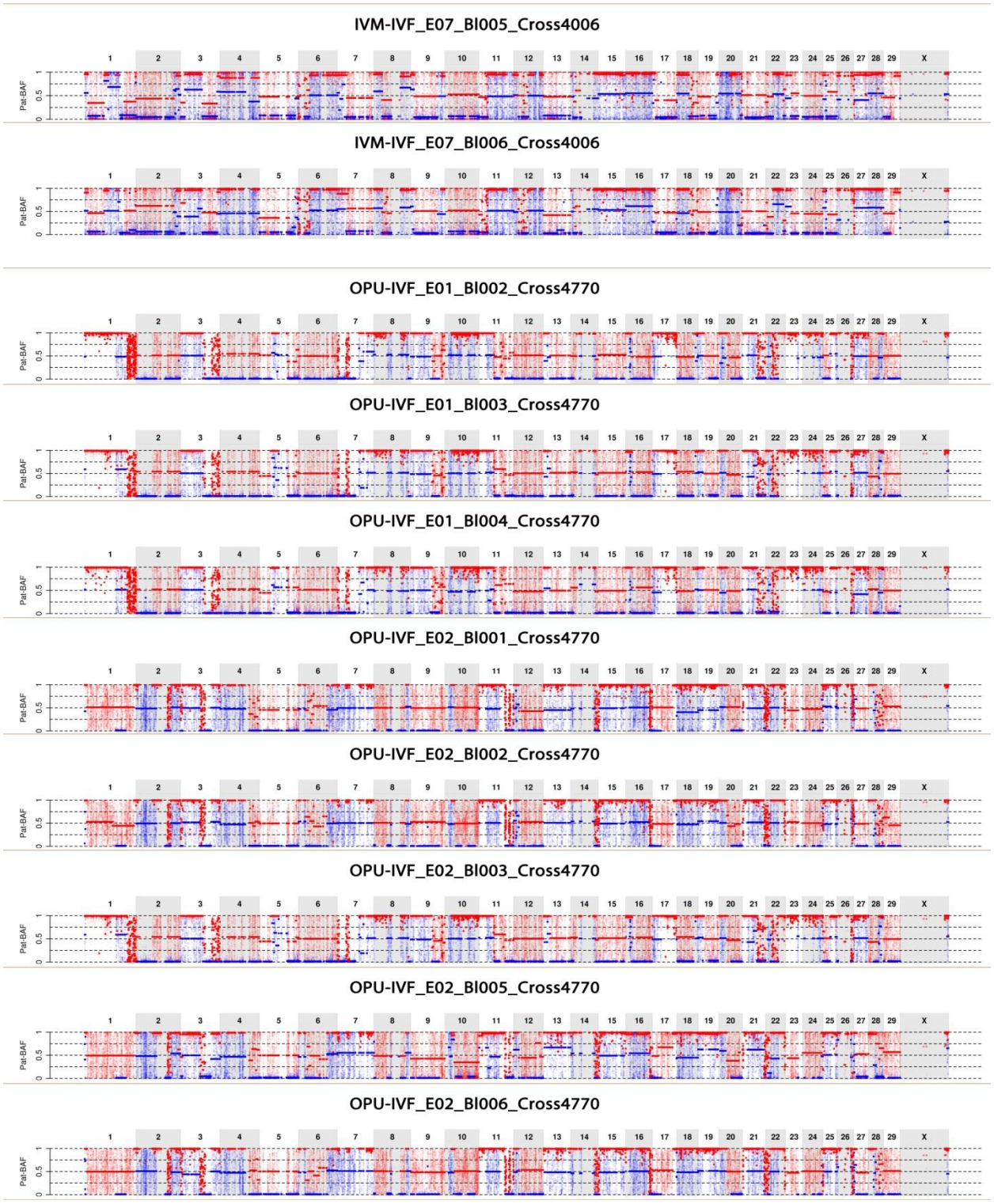
Supplementary Figure S3



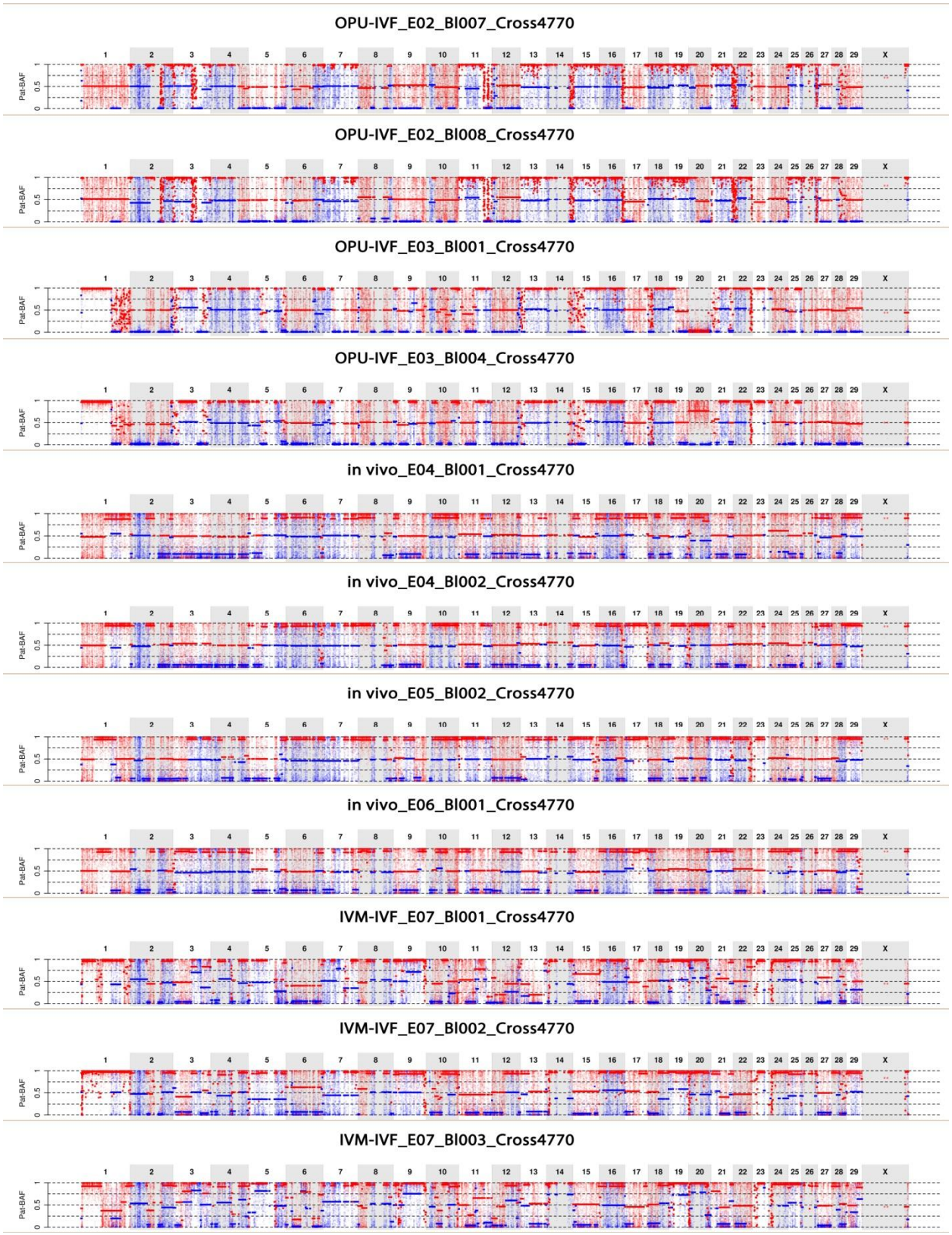
Supplementary Figure S3 (continued)



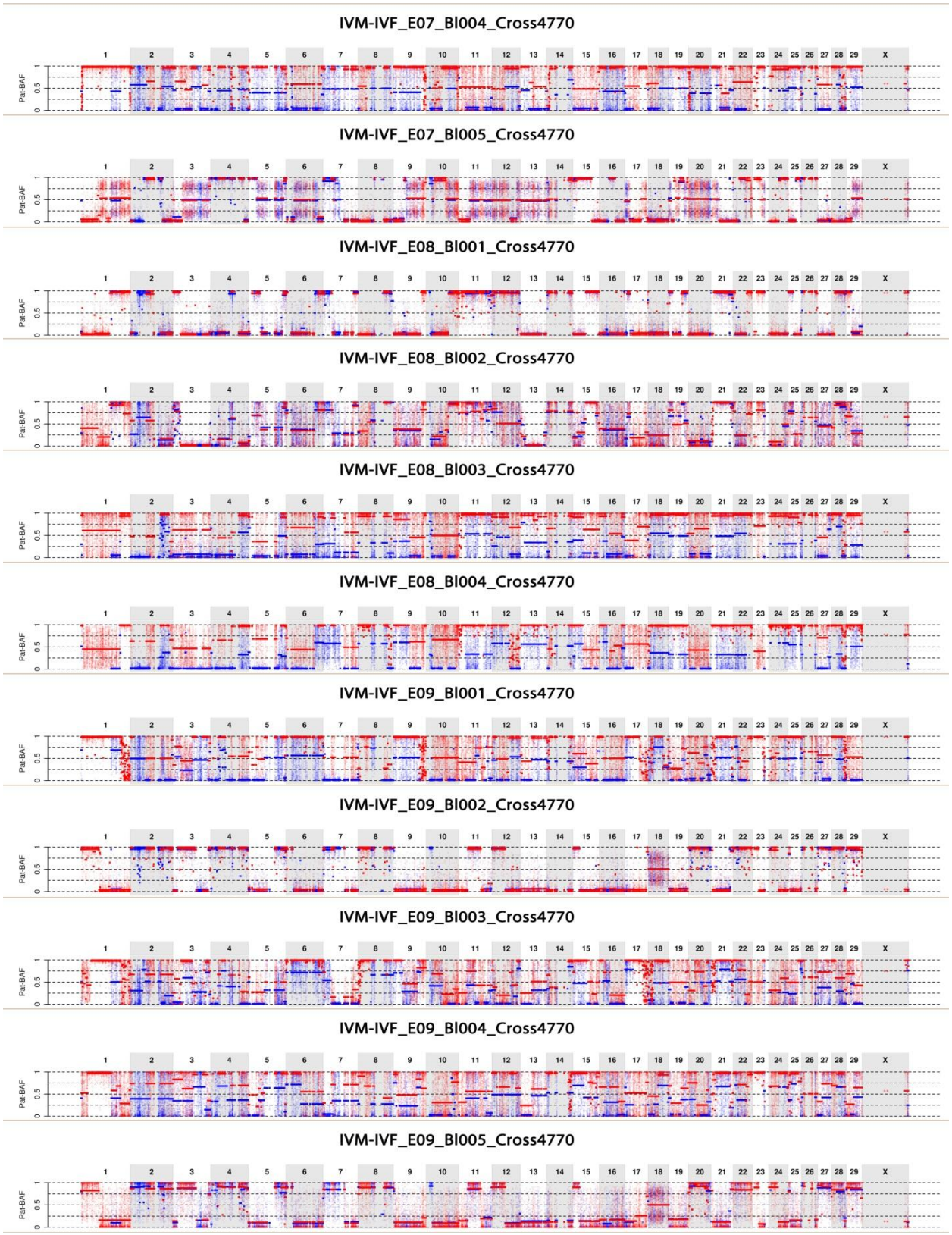
Supplementary Figure S3 (continued)



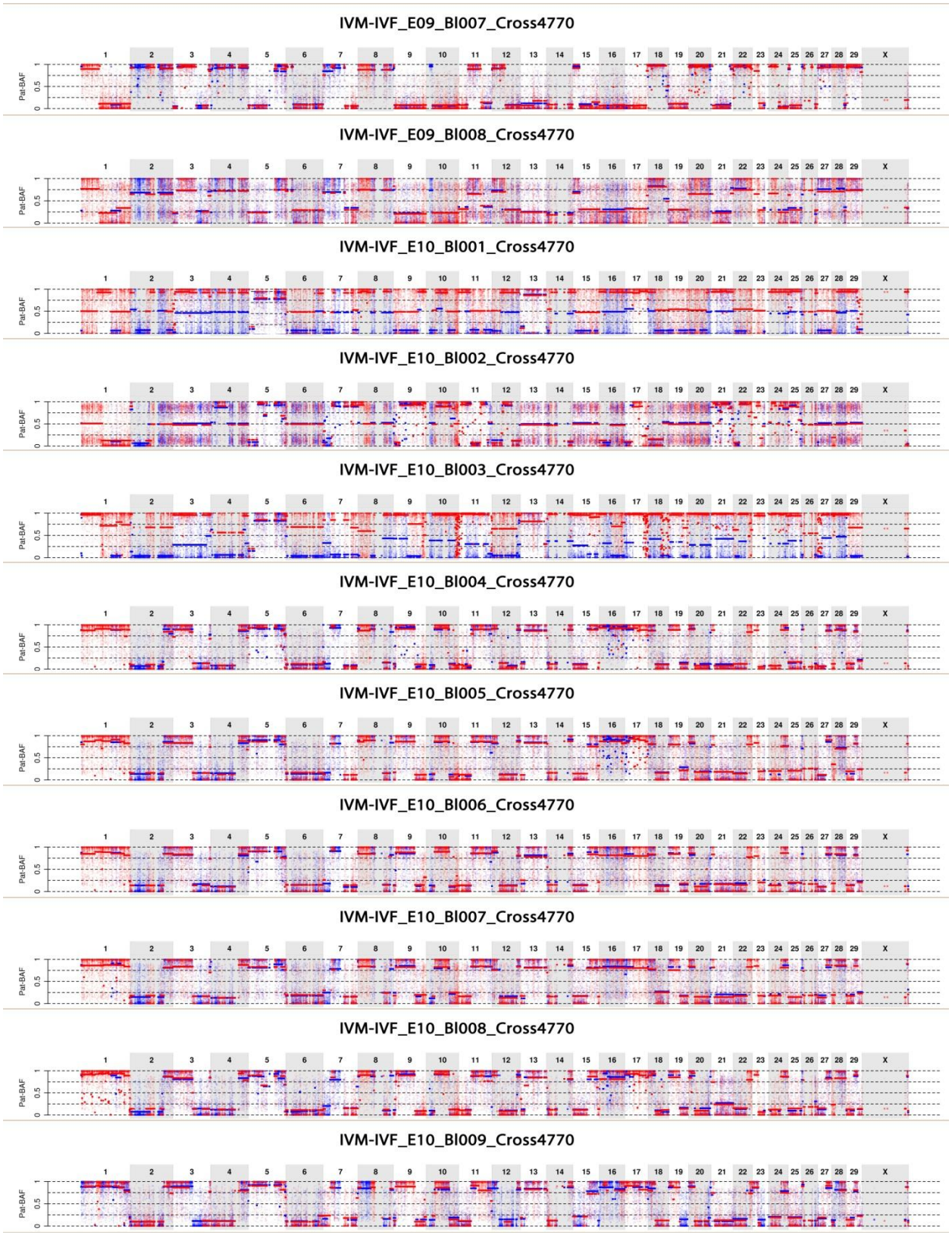
Supplementary Figure S3 (continued)



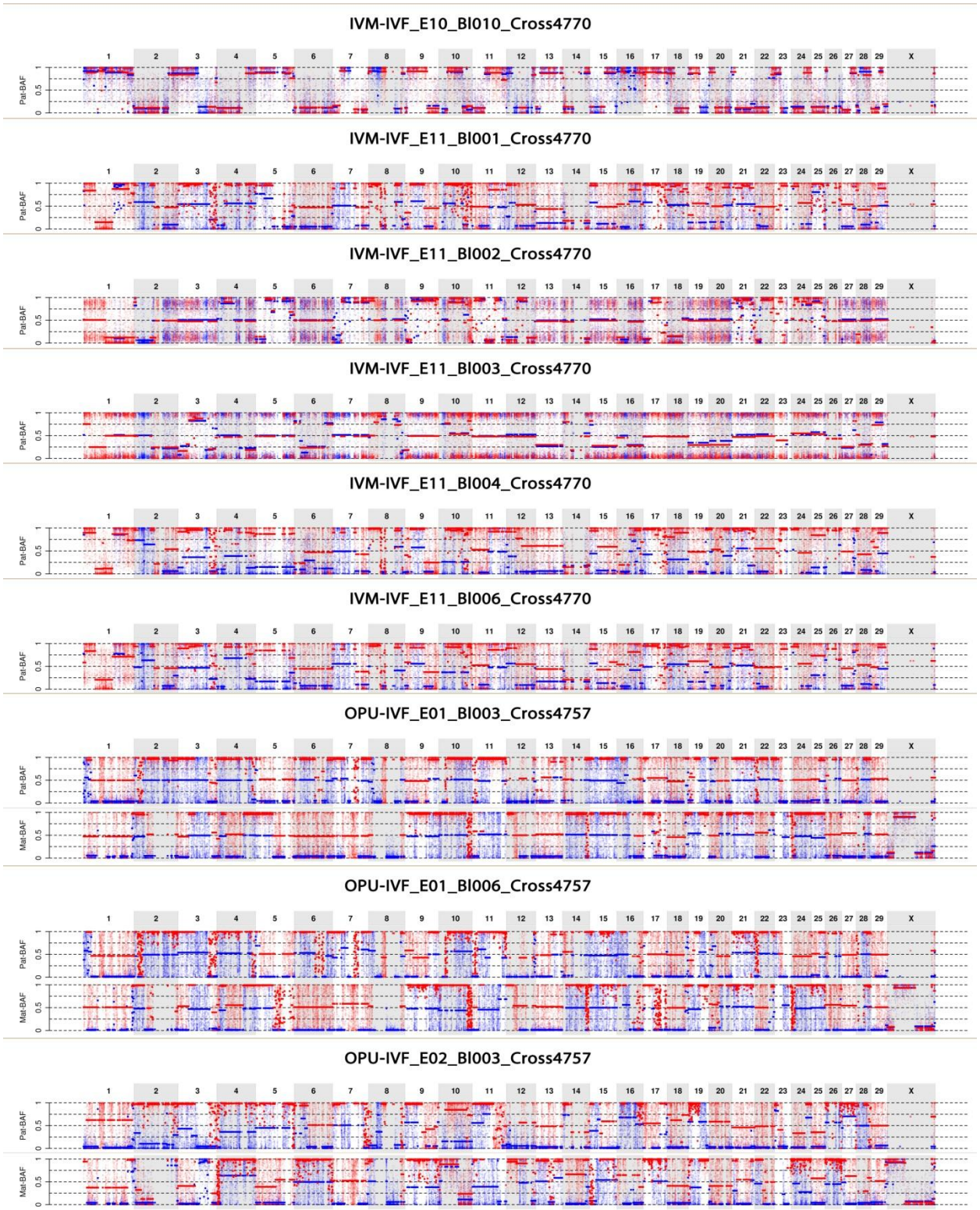
Supplementary Figure S3 (continued)



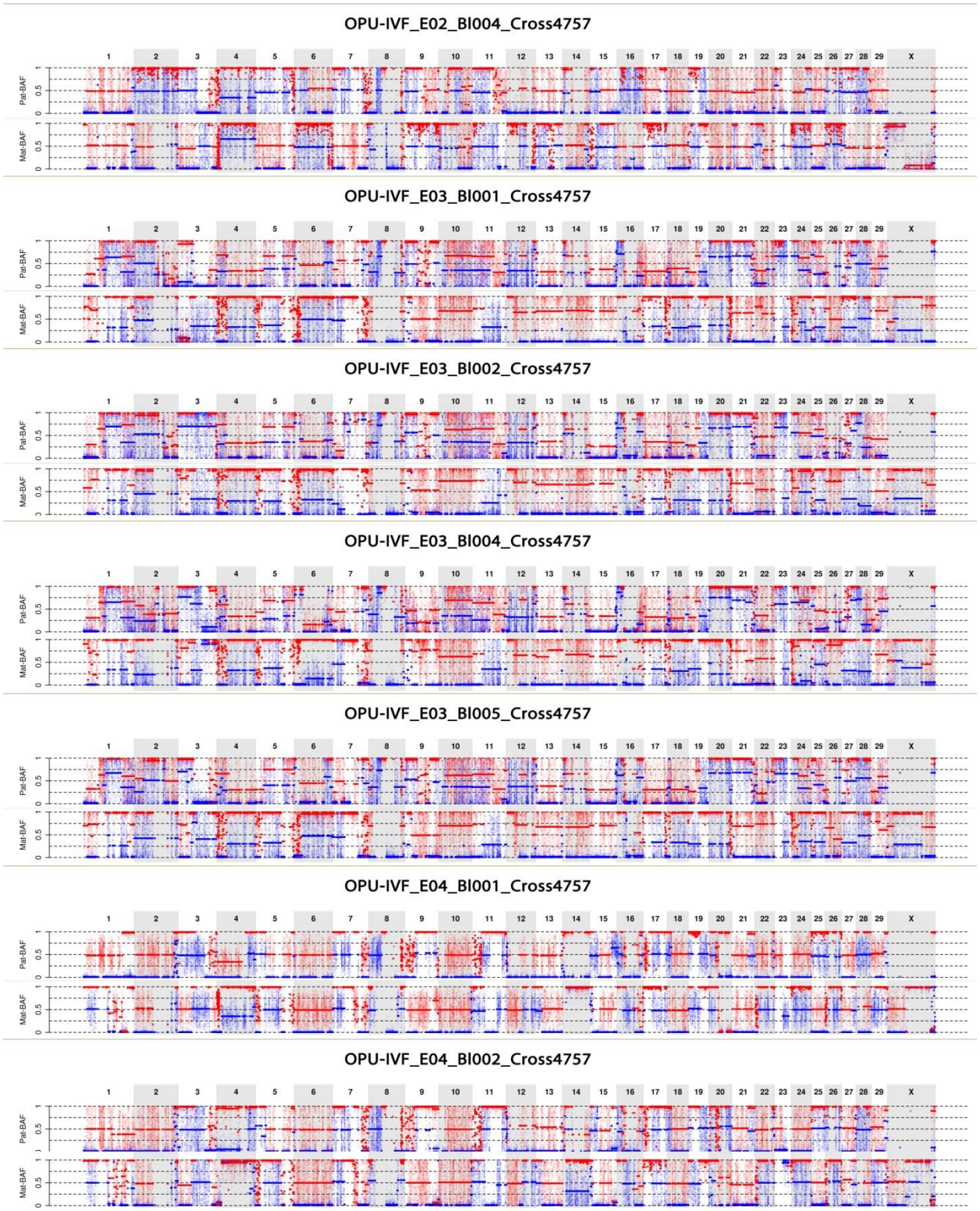
Supplementary Figure S3 (continued)



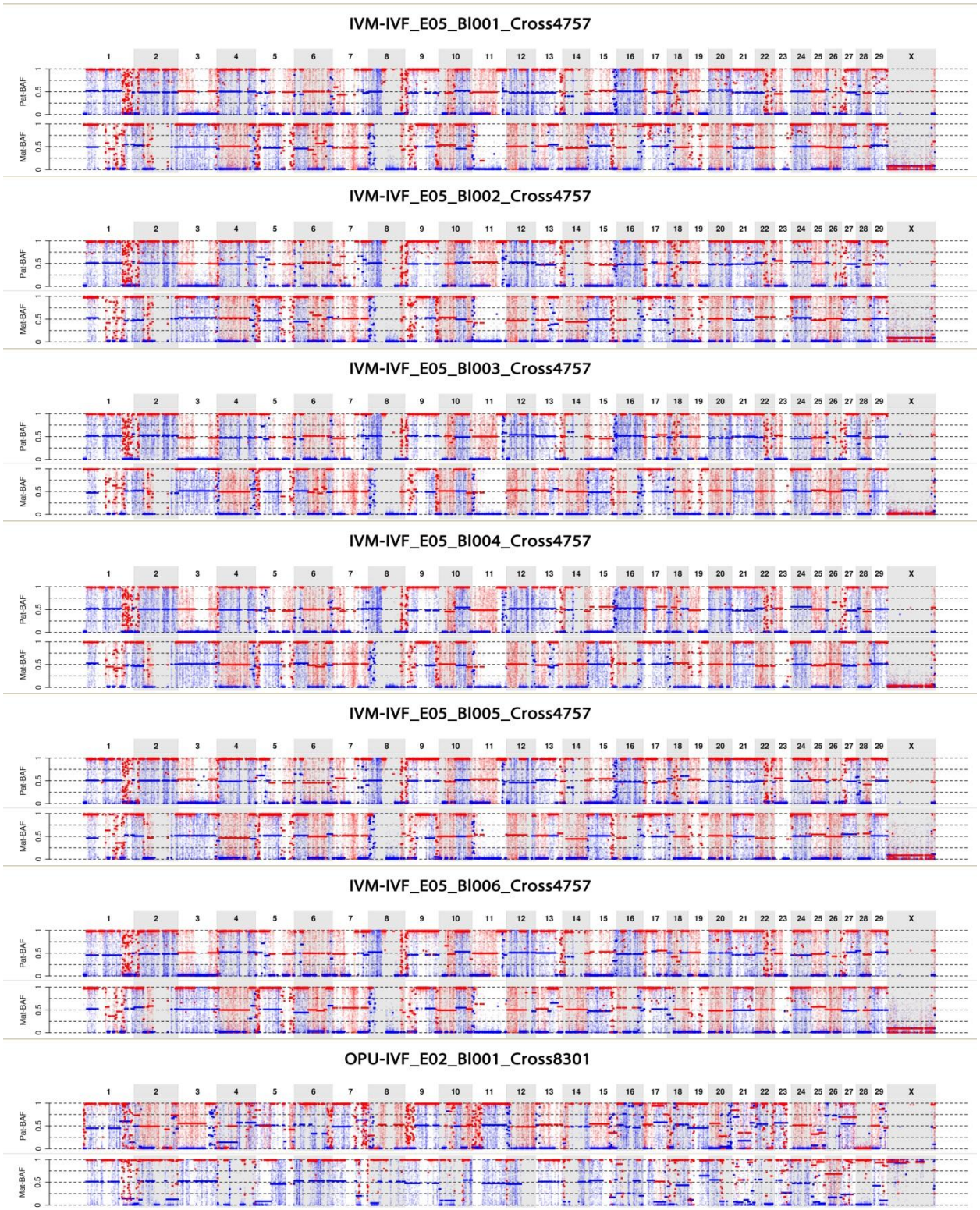
Supplementary Figure S3 (continued)



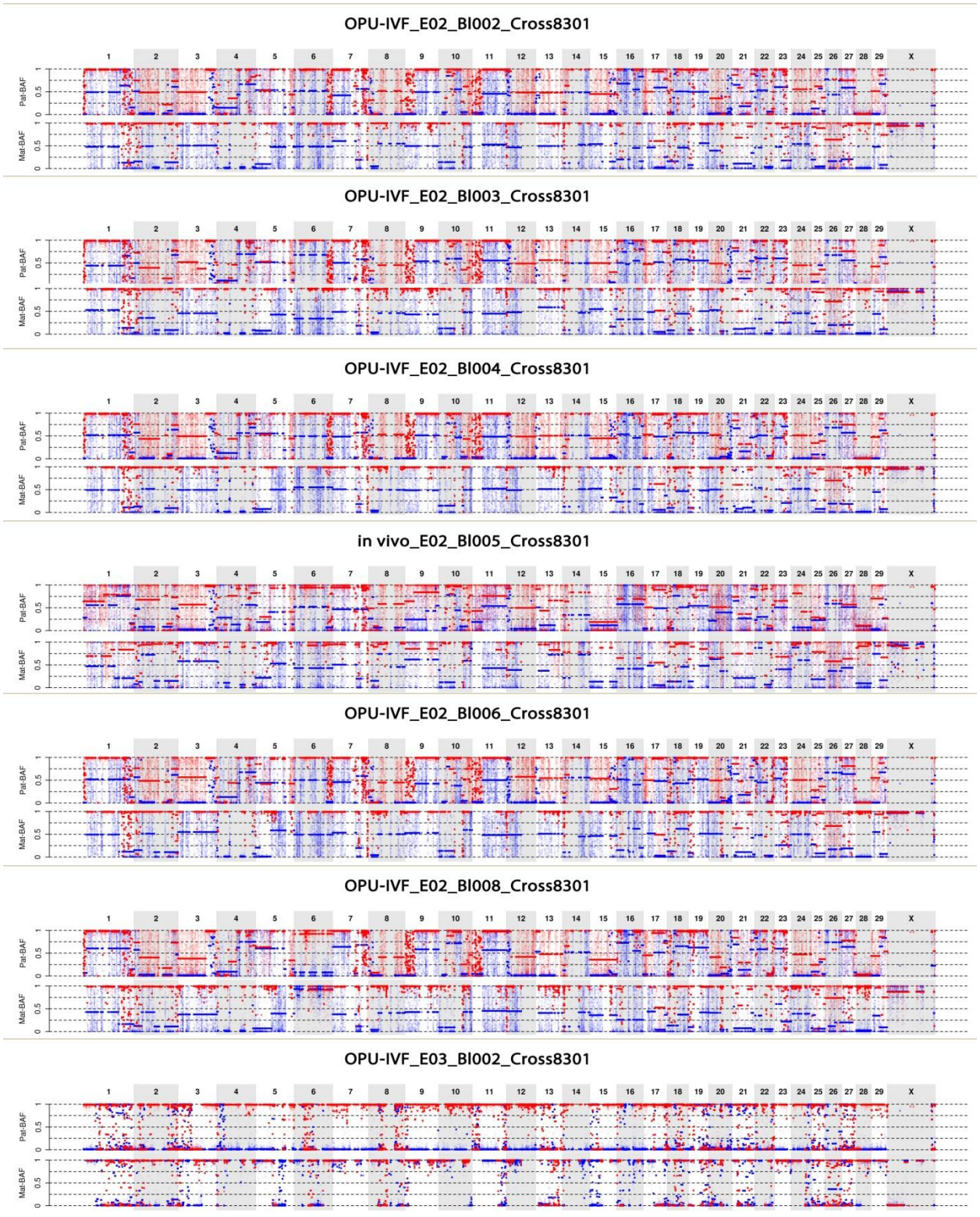
Supplementary Figure S3 (continued)



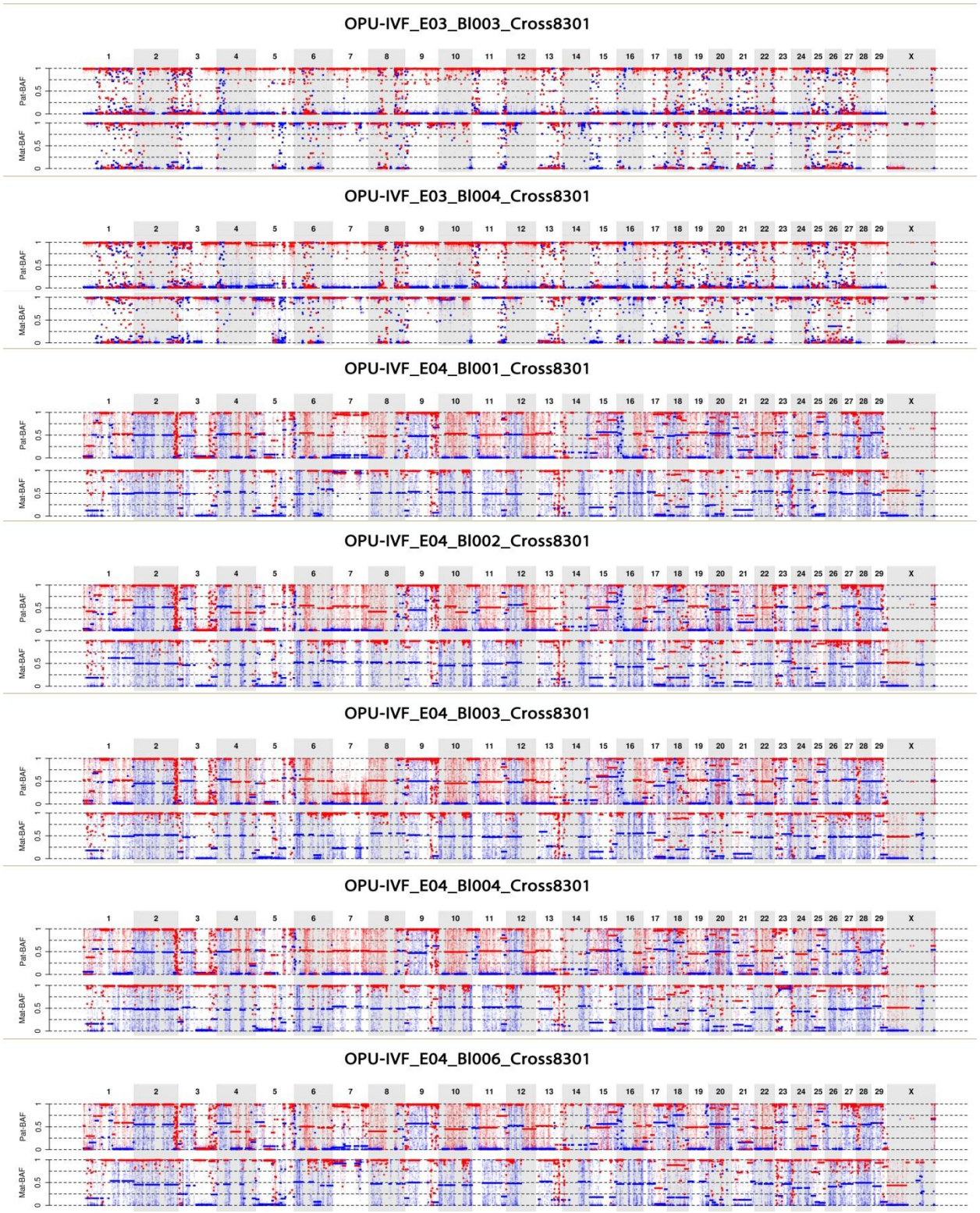
Supplementary Figure S3 (continued)



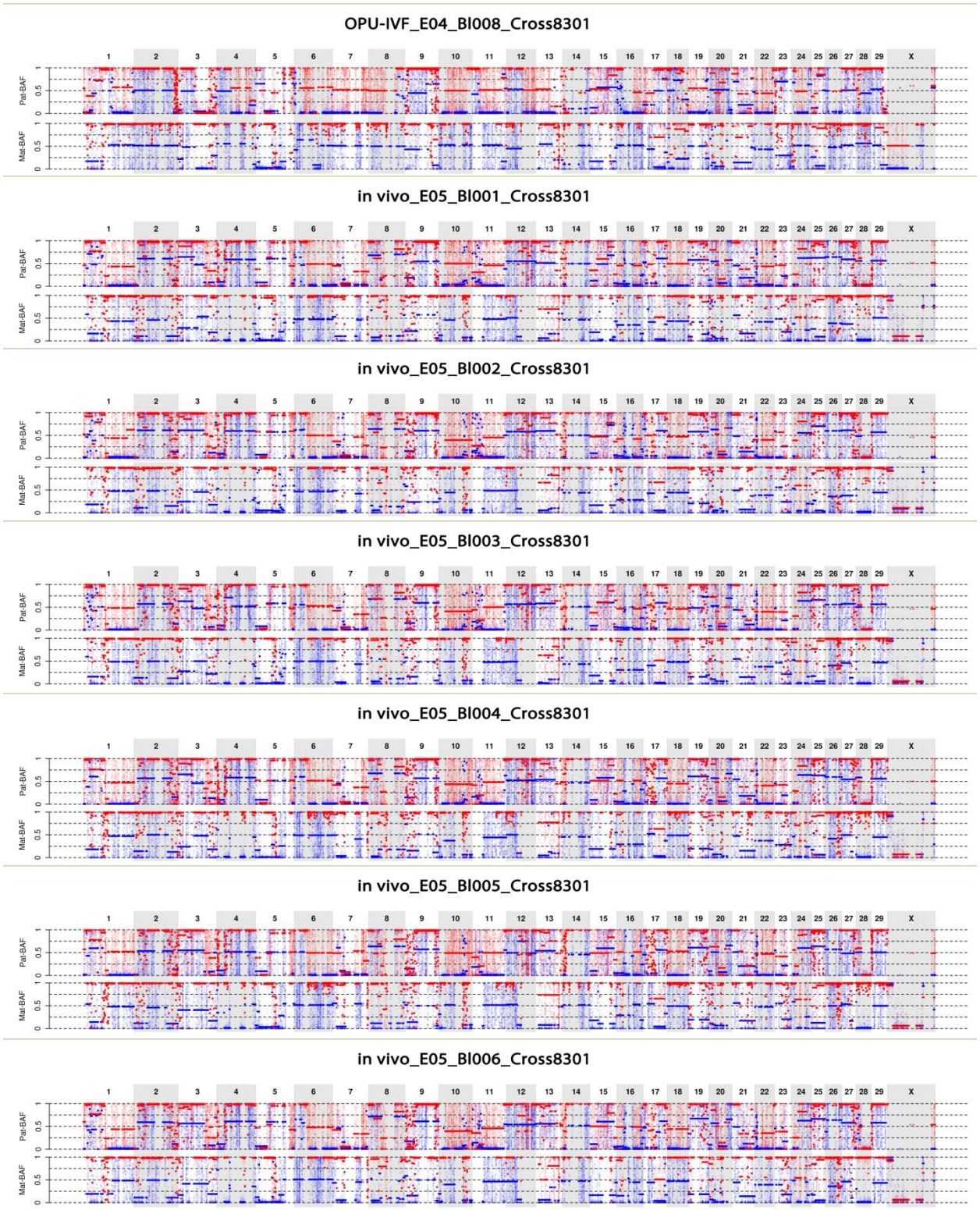
Supplementary Figure S3 (continued)



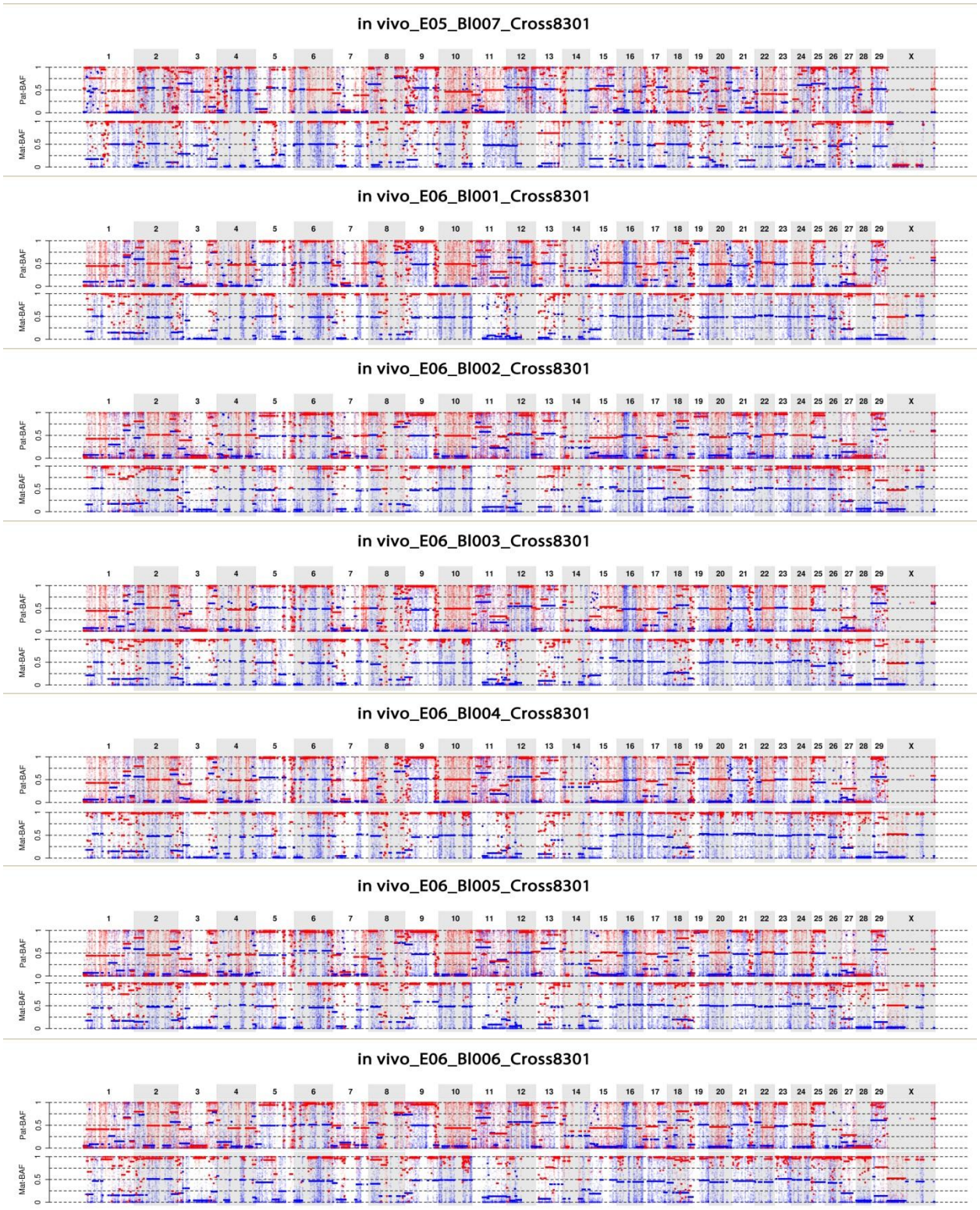
Supplementary Figure S3 (continued)



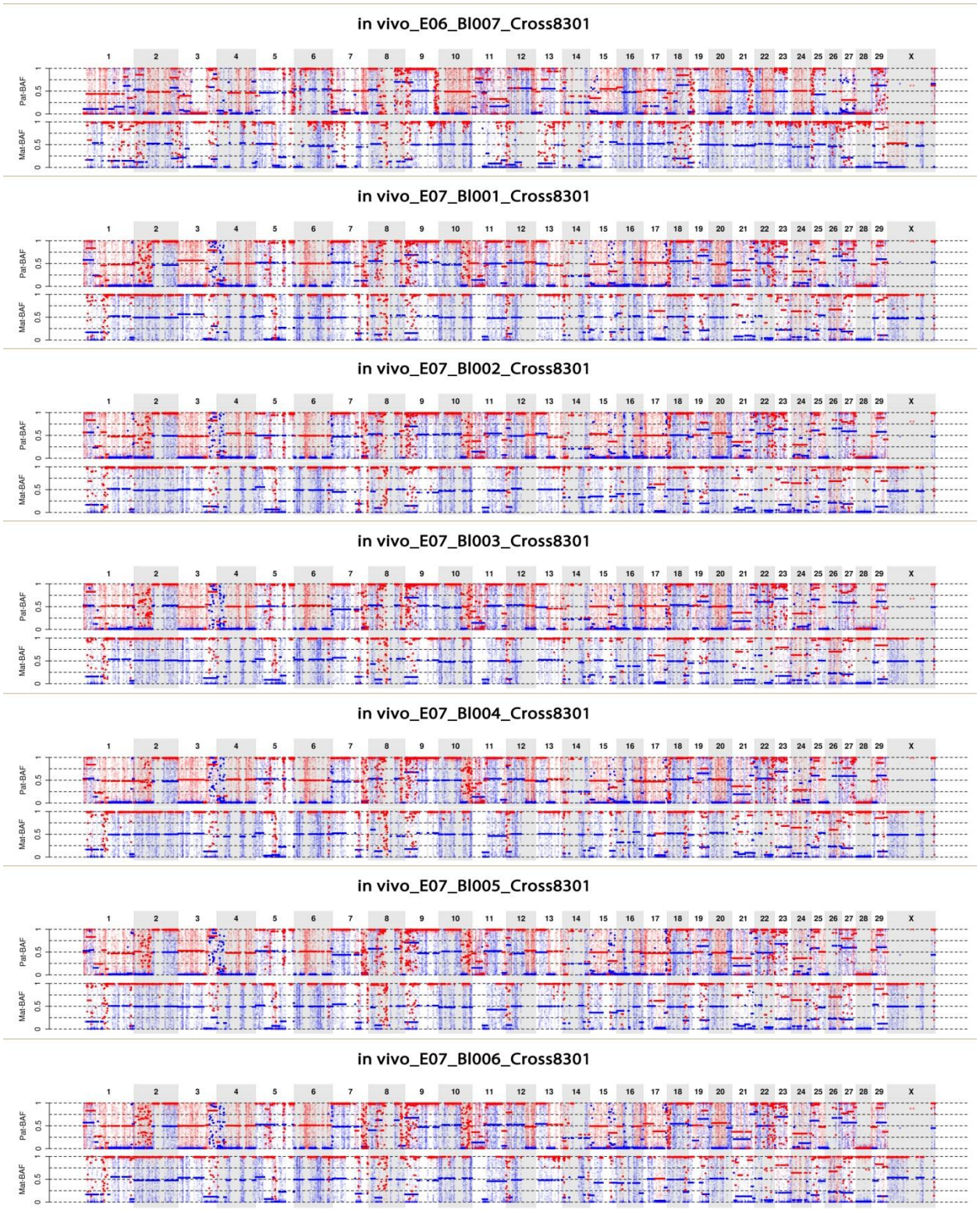
Supplementary Figure S3 (continued)



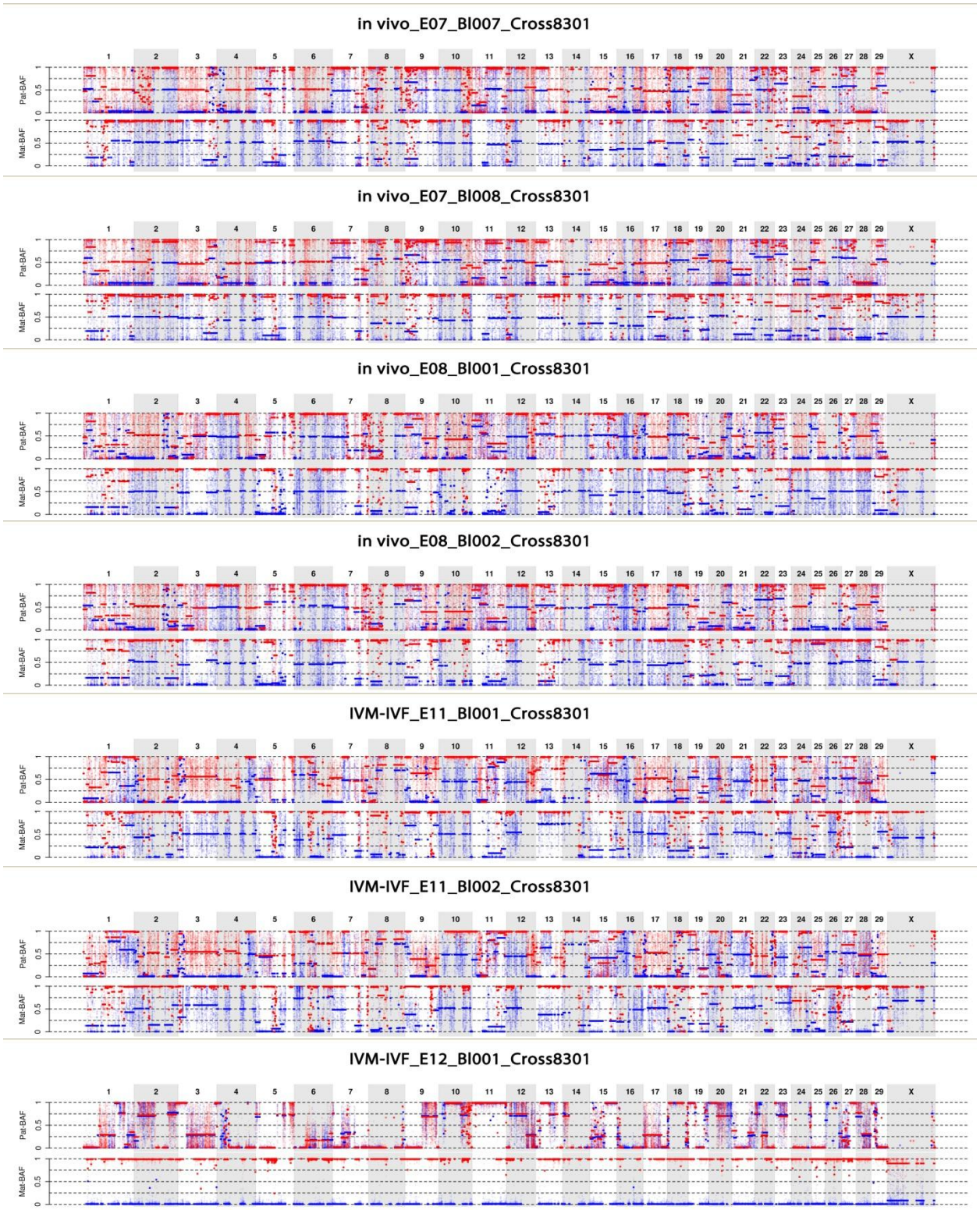
Supplementary Figure S3 (continued)



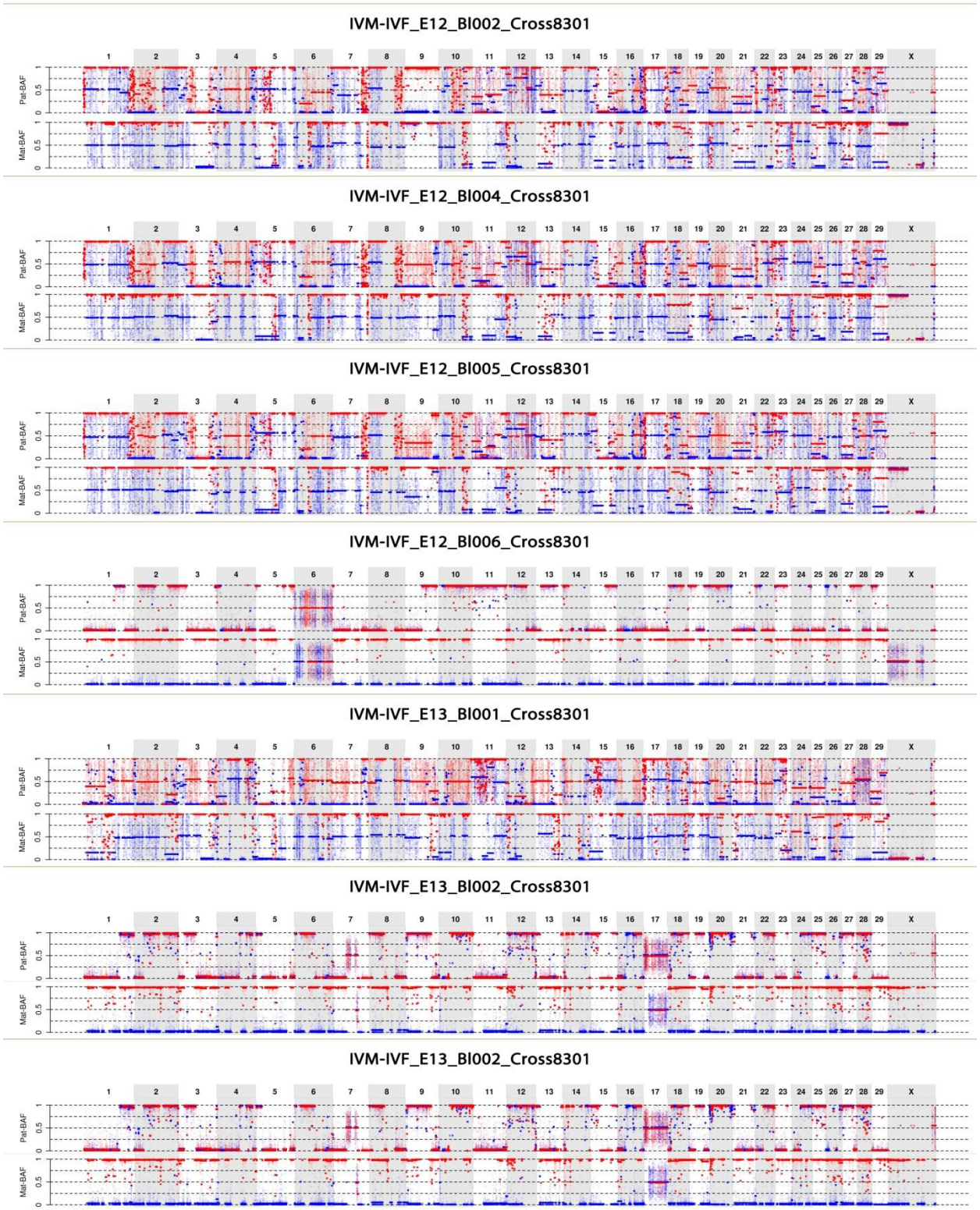
Supplementary Figure S3 (continued)



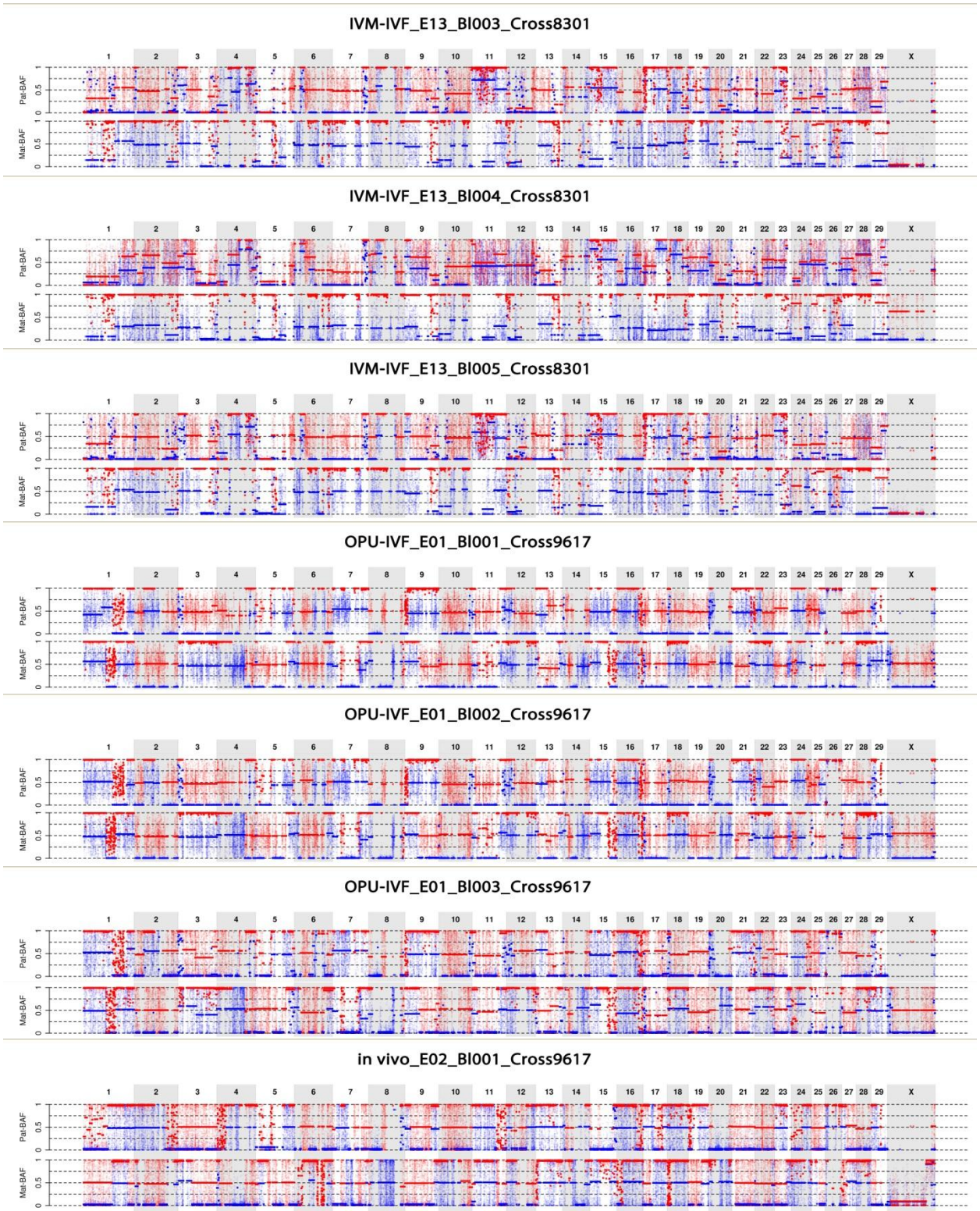
Supplementary Figure S3 (continued)



Supplementary Figure S3 (continued)

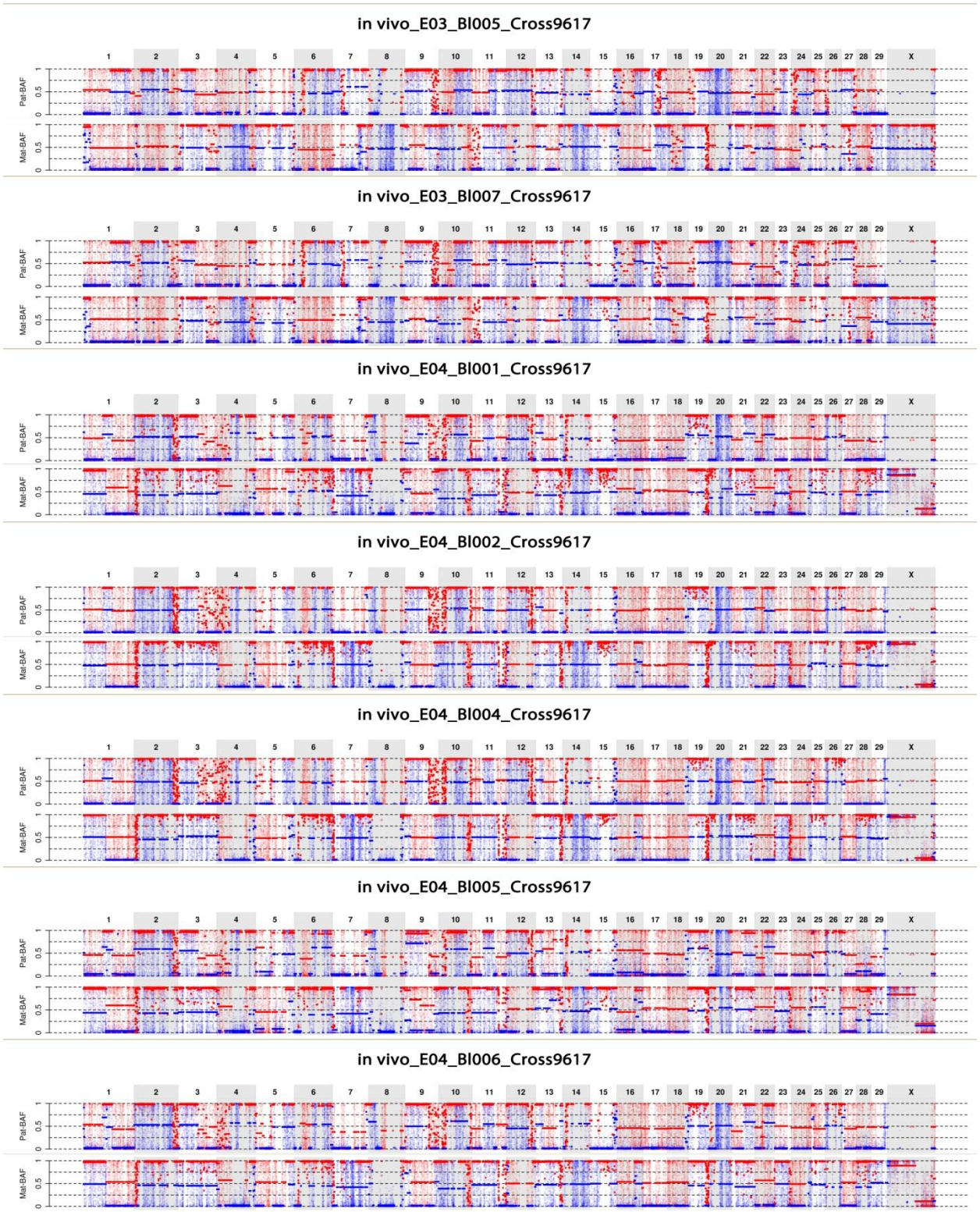


Supplementary Figure S3 (continued)

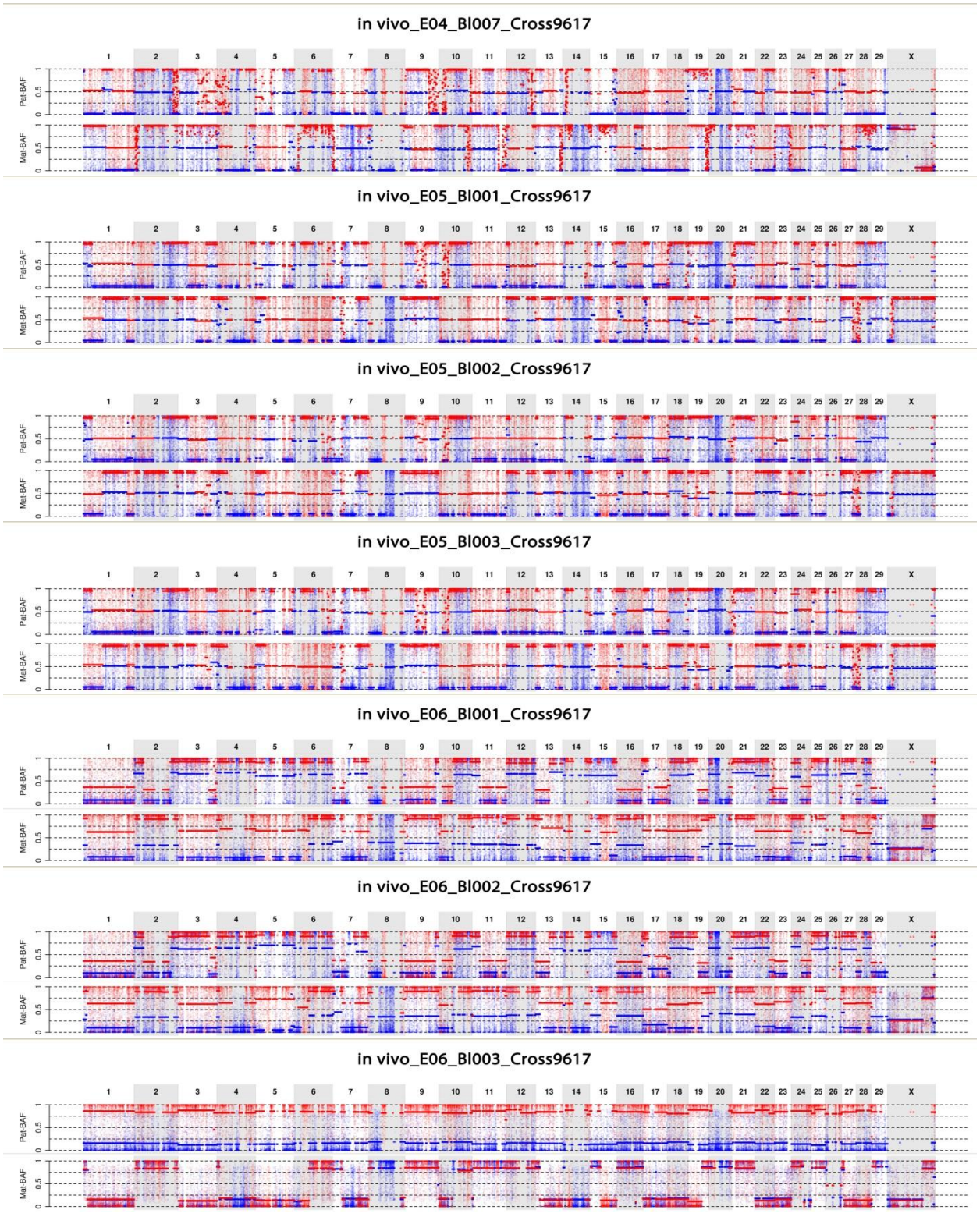


Supplementary Figure S3 (continued)

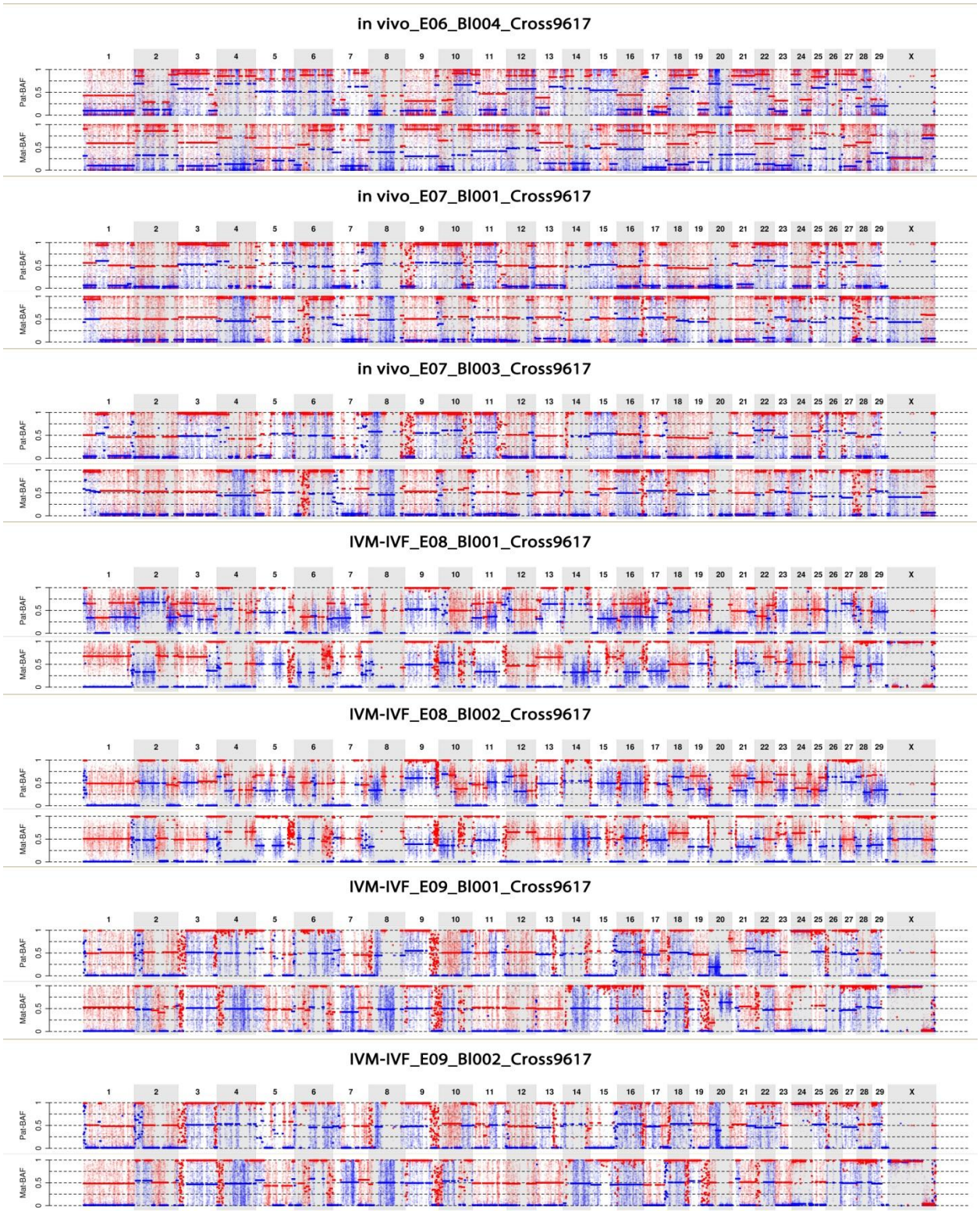
Supplementary Figure S3 (continued)



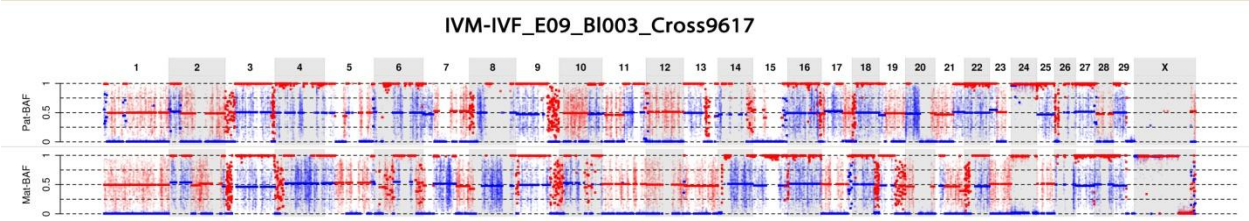
Supplementary Figure S3 (continued)



Supplementary Figure S3 (continued)



Supplementary Figure S3 (continued)



Supplementary Figure S3. Genome-wide haplarithm plots of single bovine blastomeres. For crosses 4006 and 4770 only paternal haplarithm is depicted. For crosses 4757, 8301 and 9617 both paternal and maternal haplarithm plots were obtained. Defined single cell BAF values of the segmented P1 or M1 (blue lines) and P2 or M2 (red lines), as well as the distance between the P1-P2 or M1-M2 denote the origin and nature of copy number (CN) alterations. In total, the distance in paternal haplarithm profile and distance in maternal haplarithm profile must equal 1. As example, the P1-P2 or M1-M2 distance in a diploid blastomere is 0.5 in both paternal and maternal haplarithm profiles, while any deviation from this value would indicate loss (distances 0 and 1) or gain (distances 0.67 and 0.33). For more information see (Zamani Esteki *et al*, 2015)

Supplementary Table S1. Description of individual donor response to hormonal treatment prior to ovum pick up

	Cross 4770		**Cross 4757**		**Cross 4006**		**Cross 8301**		**Cross 9617**	
OPU session	# oocytes	# cleaved embryos	# oocytes	# cleaved embryos	# oocytes	# cleaved embryos	# oocytes	# cleaved embryos	# oocytes	# cleaved embryos
1	7	3	1	0	5	5	4	3	1	1
2	2	2	15	4	2	1	5	4	1	0
3	4	4	2	1	-	-	-	-	-	-

Supplementary Table S2. Description of bovine cleavage stage embryo cohorts

Embryo ID	Origin	Time of isolation (day pi)	# of cells in the embryo	# of hybridized cells	# of QC passed cells (embryo representation %)
E01_Cross4006	OPU-IVF	2	6	6	2 (33%)
E02_Cross4006	OPU-IVF	2	4	4	3 (75%)
E03_Cross4006	*in vivo*	2	2	2	2 (100%)
E04_Cross4006	*in vivo*	2	4	3	2 (50%)
E05_Cross4006	*in vivo*	2	2	2	2 (100%)
E06_Cross4006	IVM-IVF	2	8	8	8 (100%)
E07_Cross4006	IVM-IVF	2	7	6	4 (57%)
E01_Cross4757	OPU-IVF	3	7	7	2 (28%)
E02_Cross4757	OPU-IVF	2	4	4	2 (50%)
E03_Cross4757	OPU-IVF	2	5	5	4 (80%)
E04_Cross4757	OPU-IVF	3	3	3	2 (66%)
E05_Cross4757	IVM-IVF	2	6	6	6 (100%)
E01_Cross4770	OPU-IVF	2	4	4	3 (75%)
E02_Cross4770	OPU-IVF	3	8	8	7 (87%)
E03_Cross4770	OPU-IVF	2	5	5	2 (40%)
E04_Cross4770	*in vivo*	2	3	3	2 (66%)
E05_Cross4770	*in vivo*	2	4	2	1 (25%)
E06_Cross4770	*in vivo*	2	2	1	1 (50%)
E07_Cross4770	IVM-IVF	2	6	6	5 (83%)
E08_Cross4770	IVM-IVF	2	4	4	4 (100%)
E09_Cross4770	IVM-IVF	2	8	8	7 (87%)
E10_Cross4770	IVM-IVF	2	10	10	10 (100%)
E11_Cross4770	IVM-IVF	2	6	6	4 (66%)
E01_Cross8301	OPU-IVF	2	8	8	8 (100%)*
E02_Cross8301	OPU-IVF	2	8	8	6 (75%)
E03_Cross8301	OPU-IVF	3	4	4	3 (75%)
E04_Cross8301	OPU-IVF	3	8	8	6 (75%)
E05_Cross8301	*in vivo*	2	8	7	7 (87%)
E06_Cross8301	*in vivo*	2	7	7	7 (100%)
E07_Cross8301	*in vivo*	2	8	8	8 (100%)
E08_Cross8301	*in vivo*	2	2	2	2 (100%)
E09_Cross8301	*in vivo*	2	4	4	4 (100%)*
E10_Cross8301	*in vivo*	2	3	3	2 (66%)
E11_Cross8301	IVM-IVF	2	2	2	2 (100%)
E12_Cross8301	IVM-IVF	2	6	6	6 (100%)
E13_Cross8301	IVM-IVF	2	5	5	5 (100%)
E01_Cross9617	OPU-IVF	2	3	3	3 (100%)

E02_Cross9617	*in vivo*	2	4	4	4 (100%)
E03_Cross9617	*in vivo*	2	7	7	6 (85%)
E04_Cross9617	*in vivo*	2	8	7	6 (75%)
E05_Cross9617	*in vivo*	2	4	4	3 (75%)
E06_Cross9617	*in vivo*	2	7	4	4 (57%)
E07_Cross9617	*in vivo*	2	3	3	2 (66%)
E08_Cross9617	IVM-IVF	2	2	2	2 (100%)
E09_Cross9617	IVM-IVF	2	3	3	3 (100%)

*The haplarithm profiles from these embryos were inconclusive for the majority of chromosomes, therefore they were removed from further study. The percentage indicates the overall representation of the embryo based on the initial number of blastomeres within an embryo and the final number of QC passed blastomeres that were analysed. IVP, *in vitro* produced; OPU, ovum pick up; pi, post-insemination.