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Effect of donor age on the developmental capacity of bovine cumulus oocyte complexes obtained by repeated OPU from nonstimulated and FSH-superstimulated German Simmental heifers and cows at different life cycle stages

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Meiner Familie und den Kühen

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List of abbreviations

Ac	Angus cross
ART	Assisted reproductive technologies
BB	Belgian Blue
Bc	Beef cross
B x F	Beef x Friesian
BSA	Bovine serum albumin
CH	Chinese Holstein
CIDR	Controlled intravaginal progesterone-releasing device
CL	*Corpus luteum*
COC	Cumulus oocyte complex
DF	Dominant follicle
DFR	Dominant follicle removal
E2	Estradiol
eCG	Equine chorionic gonadotropin
ECS	Estrus cow serum
ET	Embryo transfer
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GS	German Simmental
h	hour
HF	Holstein Friesian
ICSI	Intracytoplasmic sperm injection
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
LH	Luteinizing hormone
LUV	Luteinized unruptured follicles
M	Murrah
mg	milligram
min	minute
µL	microliter
mL	millilitre
Mo	Montbeliard
M x B	Murrah x Brahmann
n.p.	Not presented
NR	Nili Ravi
n.s.	Not significant
OHS	Ovarian hyperstimulation syndrome
p.w.	Per week
RW	Red and White breed
SB	Swamp Buffalo
SD	Standard deviation

1. Introduction

In humans, assisted reproduction technologies (ART) are usually applied in couples having fertility problems whereas in farm animals of high genetic value or endangered species they have become frequent to produce larger numbers of offspring than it would be possible with normal reproduction. These techniques applied to specific large animal research models, associated or not, with *in vitro* biomedical models potentially represent valuable tools in experimental studies of the physiology and pathology of human reproduction.

Since women that desire to have children are becoming older nowadays, such fertility problems may often reflect inevitable age-related changes. In Germany and other countries of the Western world, the age of first-bearing mothers is increasing, especially in social stratums with higher educational levels (Bundesamt, 2007). Highest fertility appears from 18 to 31 years of age followed by a slow and then more rapid period of decrease until the advent of menopause at an age of ~51. Nevertheless, the duration of the reproductive phase of the life cycle and the production of developmentally competent oocytes vary greatly among individual women. Evaluation of data from fertility clinics all over Europe and the USA revealed a strongly increasing use of ART (U.S. Department of Health and Human Services, 2007; Andersen *et al.*, 2009). To distinguish between pathological processes and the natural decline in fertility, basic research is needed to reveal physiological changes and the etiology of abnormal deviations.

An increasing need for suitable animal models for the study of reproductive aging in women has led to the introduction of several laboratory species, including frog, rabbit, mice, hamster and other rodents, as well as non-human primates. Years of research revealed that the bovine also represents many aspects of a good animal model for the study of reproductive aging and (in)fertility in women.

Therefore, the objective of the present study was to examine the potential of the bovine species as a large-animal model using donor heifers and cows at different life cycle stages. For a systematic evaluation, age-dependent effects of *in vitro* versus *in vivo* maturation of oocytes obtained by repeated transvaginal ultrasound-guided ovum pick-up (OPU) on the developmental capacity of embryos produced after *in vitro* fertilization (IVF) and culture (IVC) were studied in the same animals. In order to obtain a large number of *in vivo* matured oocytes by OPU for these studies a superstimulation protocol was established and the results of oocyte

retrieval and their development *in vitro* were compared to those of non-superstimulated donor animals. Further, the capacity of *in vitro* matured oocytes to reprogram nuclei of bovine fetal fibroblasts was evaluated in the different age classes.

2. Review of the literature

2.1. The cow as a model for reproductive aging in women

There are several legal, ethical and practical constraints that limit investigations involving human subjects, especially in basic research. Animal models have been used for centuries to overcome such obstacles. The development of contraceptive technologies has allowed women to control their fertility and thus to decide if or when they desire to have children in their life changing traditional conceptions of women's role in the society (te Velde *et al.*, 2002). In most countries of the Western world, this has led to women delaying child bearing until close to the end of their reproductive life span. In parallel, ART, such as artificial insemination, ovarian superstimulation, follicle aspiration and oocyte donation, *in vitro* maturation (IVM) of oocytes, IVF including intracytoplasmic sperm injection (ICSI), IVC and embryo transfer, have become accepted methods over the last decades and thus essential tools for women's socio-cultural perspectives (U.S. Department of Health and Human Services *et al.*, 2007; Andersen *et al.*, 2008; Deutsches IVF Register, 2008).

Several similarities in reproductive physiology have been found between woman and cow, such as aspects of oogenesis, folliculogenesis, follicular wave emergence and selection of the dominant follicle (DF), and - in the phase of reproductive aging - in hormonal patterns and in the age-dependent decline in ovarian reserve (Erickson, 1966; Wallace *et al.*, 2010). Both are monovulatory and polycyclic species and the morphology and the size of their ovaries are also similar (Adams, 1995). Furthermore, pathological conditions, such as the appearance of ovarian follicular cysts or the clinical picture of luteinized unruptured follicles (LUV) are supposed to be similar to the cystic ovarian degeneration in bovine (Adams, 1995). For over 15 years, the cow has been proposed as a valuable model for investigating reproductive aging in women, and these studies have led to the discovery of follicular waves in women (Baerwald *et al.*, 2003). In addition to these similarities, the bovine has several characteristics of a good animal model (easily available, easy to handle and work with, highly adaptable to changing conditions) (Adams, 1995).

In humans, IVM has become a very promising technique potentially offering treatment opportunities for women with fertility problems, especially for those

suffering from ovarian hyperstimulation syndrome (OHS). However, at present IVM of human oocytes results in rather low pregnancy rates and high miscarriage rates when compared to the use of *in vivo* matured oocytes (Andersen *et al.*, 2008; Buckett *et al.*, 2008).

Early signs of reproductive aging in women are increasing levels of circulating follicle stimulating hormone (FSH) due to decreasing levels of inhibin A and B, variations in menstrual cycle length, phases of amenorrhea, and finally menopause (Practice Committee of the American Society for Reproductive Medicine, 2008). There is no report of a menopause-like event in bovine (Grunert, 1999).

2.2. Age-related changes and effects on *in vitro* embryo production (IVP)

Age-related changes in fertility have been studied intensively in the bovine. Developmental competence of oocytes from prepubertal heifers improves with age with a remarkable increase of the potential to cleave between 7 and 9 months of age (Yang *et al.*, 1998). Malhi *et al.* (2005; 2006; 2007; 2008) investigated age-related changes in mother-daughter pairs regarding their recruitment and the growth of follicles during the estrous cycle and during hormonal stimulation, the superovulatory response and the developmental competence of their oocytes as well as the treatment-associated and age-related hormonal changes. They found fewer 4-5 mm follicles recruited into waves resulting in lower peak numbers of 6-8 mm follicles and a smaller diameter of the ovulatory follicle in older cows (Malhi *et al.*, 2005).

During superstimulation older cows had significantly fewer numbers of follicles smaller than 6 mm, between 9-11 mm and larger than 12 mm in diameter in comparison to their younger daughters (Malhi *et al.*, 2008) whereas these differences were not observed in a previous experiment (Malhi *et al.*, 2006). Plasma hormone measurements during non superstimulated cycles revealed significantly higher FSH concentrations during follicular waves in older cows. Plasma luteinizing hormone (LH) concentrations and the amplitude of the LH-surge did not differ between younger and older cows while its occurrence was delayed in the group of older animals. However, the time of ovulation did not differ significantly between the age groups (Malhi *et al.*, 2006). Older cows displayed significantly greater profiles of plasma estradiol (E2) in the 7 days

preceding ovulation (Malhi *et al.*, 2005).

When mother-daughter pairs were submitted to a superovulation-embryo recovery program, a higher proportion of unfertilized ova were obtained in older cows but no differences in the total number of ova/embryos or in the quality of the collected embryos were observed (Malhi *et al.*, 2007). These findings corroborate those by Faasch *et al*. (2009). Occytes from younger animals (1.5-3 years old) showed higher fertilisation rates after superstimulation and embryo recovery than 4-16-year-old animals. Lerner *et al.* (1986) observed decreased numbers of ova/embryos accompanied by lowered fertilization rates and lower embryo quality with advanced age of the donors.

Su *et al.* (2009) compared young (12 months), middle aged (7-8 years) and older cows (at least 15 years old) with respect to the *in vitro* developmental capacity of oocytes obtained by OPU. The authors observed the highest cleavage and blastocysts rates in young cows followed by middle aged and older cows. Young cows showed lower plasma progesterone (P4) levels than older animals during a whole estrous cycle without OPU as well as during the OPU period. E2 plasma concentrations differed between age groups. Roth *et al.* (2008) did not find differences in the numbers of COCs obtained or in the cleavage and blastocyst rates between Holstein-Friesian (HF) heifers and early- or mid-lactating multiparous cows after twice weekly OPU. An early study reported that 55.0% of 13-year-old cows were infertile when the selection criterion was “failure to deliver a calf during two successive years” (Erickson *et al.*, 1976).

In spite of more follicles aspirated and COCs collected from HF heifers compared to early lactating HF cows after twice weekly OPU, Rizos *et al.* (2005) found no differences in the developmental competence of oocytes *in vitro*. In this study, higher blastocyst rates *in vitro* were obtained in cows compared to heifers using COCs originating from slaughterhouse-+derived ovaries. No differences in the blastocyst rates obtained from *in vitro* matured oocytes from different age groups of crossbred beef heifers were observed. Another investigation that dealt with abattoir-derived ovaries from individual animals of different breeds found no differences in the number of COCs obtained and in the *in vitro* blastocyst rates between cows aged 1-3 years and older (Mermillod *et al.*, 1992). While the developmental capacity of oocytes seems to decrease with advancing age, their quality according to morphological criteria seems to remain relatively stable in aging cows (Katska and Smorag., 1984).

2.3. Oocyte developmental competence

Developmental competence of oocytes is commonly defined as the ability to resume meiosis, the ability to be fertilized, to cleave, and develop to blastocysts, establish pregnancies and result in healthy and fertile offspring (Armstrong, 2001). Mammalian oocytes acquire their developmental capacity over different periods of time (human: ~85 days, mouse: 2 weeks) involving a multitude of processes, from oocyte growth and folliculogenesis to meiotic resumption and cytoplasmic maturation (reviewed by Hunt *et al.*, 2008). During oocyte growth and maturation the cell organelles are formed and arranged, the produced proteins and mRNA are stored until final maturation, fertilization and early embryonic development (Ferreira *et al.*, 2009). These reserves facilitate the *in vitro* developing embryo to bypass the embryonic developmental block which occurs at the 8-16 cell stage in bovine (Duranthon *et al.*, 2001).

Until resumption of the meiosis, the oocytes surrounded by granulosa cells are arrested in the prophase of the first meiotic division. Granulosa cells have been considered to inhibit meiotic resumption, shown by the occurrence of spontaneous final maturation *in vitro* when cumulus-oocyte-complexes were removed from their follicles (Pincus, 1935) or after co-culture of oocytes with granulosa cells *in vitro* (Leibfried *et al.*, 1980). Not until exposure to the LH-surge, the oocyte undergoes final maturation to the metaphase II stage. Expansion of the cumulus occurs around 9-12 h after the LH-peak. At this same time the germinal vesicle breakdown occurs and around 19 h after the exposure to the LH-peak the first polar body is formed (Hyttel *et al.*, 1986). The expansion of the cumulus plays an important role for the ovulation, the proper transport into the oviduct and the fertilization (reviewed by Canipari, 2000). Correct completion of meiosis is supposed to be one of the most critical issues. Age-related degeneration of components of the cohesin complex might promote aneuploidy (Hunt *et al.*, 2008). Investigations in mice found an age-related decrease in transcript levels of oxidative stress genes and other genes that presumably affect the developmental competence of oocytes (Ottolenghi *et al.*, 2004).

2.4. Ovum pick-up (OPU)

Bovine IVP is highly standardized in laboratories worldwide and has been shown to be important for both the cattle breeding industry and basic research (Gordon, 2003).

Since Pieterse *et al.* (1988) developed transvaginal ultrasound-guided OPU as a minimal invasive method, it has become possible to collect COCs many times from the same donor animal. OPU can be performed for a long period without negative effects on the animal`s health, well-being and the chance to become pregnant afterwards (Santl *et al.*, 1998; Petyim *et al.*, 2007). Petyim *et al.* (2007) evaluated heart rates, stress hormones, physiological and estrous behaviour for short time and long term effects in donors submitted to OPU and showed that the technique does not have substantial negative consequences on animal well-being. Another substantial advantage of this approach consists on the possibility to obtain COCs from donors during the first trimester of their pregnancy (Meintjes *et al.*, 1995; Takuma *et al.*, 2010).

Possible negative consequences of OPU for the donors may be irregular ovarian cyclicity and a thickening of the tunica albuginea accompanied with a hardening of the ovaries after very prolonged use of the same animal. Further, aberrances of hormonal patterns due to the formation of progesterone secreting *Corpus luteum* (CL)-like structures after puncture of large follicles have been reported (Boni *et al.*, 1997; Petyim *et al.*, 2000, 2001).

It is also possible to obtain embryos from genetically valuable animals that cannot be dealt with by conventional ET technology (De Roover *et al.*, 2005a).

2.4.1. Factors influencing OPU and IVP results

2.4.1.1. Exogenous factors

There are several exogenous factors influencing the efficiency of OPU and IVP.

The OPU-technique and the team performance carried out have great impact on the results. The recovery rate expressed as the number of COCs retrieved per follicle aspirated is highly influenced by the team`s performance (Scott *et al.*, 1994).

Vacuum pressure of the OPU aspiration system has been shown to have an effect on the recovery rate and the oocyte quality and thus on their developmental competence *in vitro*. Increasing negative pressure higher than -50 mmHg

negatively influenced the recovery rate and COC quality with regard to the number of cumulus cell layers, cleavage and blastocyst rates *in vitro* (Ward *et al.*, 2000). In contrast, Sasamoto *et al.* (2003) achieved higher recovery rates when vacuum pressure was elevated from -50 mmHg to -50-100 mmHg with best results at -75 mmHg. No effects of puncture needle type were observed when single or double lumen needles were used in OPU, and twisting of the needle positively influenced the recovery rate.

Season influences the number of follicles aspirated and COCs recovered with higher numbers in autumn compared to summer but does not seem to influence the quality of COCs (Pieterse *et al.*, 1991; Takuma *et al.*, 2010). Silva *et al.* (2006) observed highest cleavage and blastocyst rates *in vitro* during the autumn months September to November from slaughterhouse-derived ovaries of heifers.

It has been demonstrated that nutrition is a major factor influencing follicle recruitment and the quality and the *in vitro* developmental competence of COCs. Over- or underfeeding as well as a wrong composition of the daily intake of proteins, lipids, carbohydrates and crude fibre have been shown to negatively influence the superstimulatory response of lactating dairy cattle in conventional ET programs (Santos *et al.*, 2008).

Merton *et al.* (2003) compared different treatment protocols and time intervals between two consecutive OPU sessions in order to optimize the number and the quality of COCs and transferable embryos. Twice weekly OPU often is performed in 3- or 4-day intervals aiming to yield high numbers of developmentally competent COCs. OPU has also been performed in 2- or 5-day intervals without negative influence on COC collection results. However, higher oocyte developmental rates were observed after 2 days compared to a 5-day OPU interval.

Twice weekly OPU led to higher yields of COCs (Bruggerhoff *et al.*, 2002), higher numbers of good quality COCs (Lopes *et al.*, 2006) and corresponding higher *in vitro* developmental rates (Goodhand *et al.*, 1999) when compared to once weekly OPU.

Similarly, Ding *et al.* (2008) yielded highest numbers of good quality COCs after twice weekly OPU compared to recoveries performed at 5 or 10 day intervals, once weekly or every two weeks. Chaubal *et al.* (2006) did not find differences between once and twice weekly OPU with regards to follicle and COC numbers, COC quality or IVP results per session. A lower proportion of small follicles and

higher blastocyst rates were observed when the DF was removed 72 h prior to once weekly OPU. However, on a per week basis, follicle and COC numbers were higher and blastocyst rates were similar to the OPU-Dominant Follicle Removal (DFR)-protocol in twice weekly OPU. Estrous cycle stage did not influence follicle and COC numbers, the quality of COCs and the cleavage and blastocyst rates *in vitro* in twice weekly OPU (Petyim *et al.*, 2003).

In numerous studies IVP conditions have been shown to affect embryo development results (Lonergan *et al.*, 1994) and to alter gene expression on both the maternal and embryonic side (Duranthon *et al.*, 2001; Lonergan *et al.*, 2003). The IVP conditions may lead to abnormal embryonal and foetal development (van Wagtendonk-de Leeuw *et al.*, 1998; Young *et al.*, 1998). In addition, semen quality and sire health status critically influences the fertilization results (Ward *et al.*, 2001). Table 1 shows results of twice weekly OPU and IVP in non-superstimulated donors.

Table 1: OPU and IVP results after twice weekly OPU

Reference	Breed	No. of donors	Age class	No. of sessions or period	No. of COC (mean)	Blastocyst rate (mean %)
(Roth *et al.*, 2008)	HF	7	Heifers	4	13.2	32.5
		5	Cows	4	17.2	39.5
		5	Cows,	4	16.0	22.8
(Rizos *et al.*, 2005)	HF	8	Heifers	10	4.7	12.4
		8	Cows	10	2.8	8.1
(De Roover *et al.*, 2008)	BB	81	1,5-15 years	Various	4.1	17.0
(Ding *et al.*, 2008)	CH	6	2	2 weeks	5.0	n.p.
(Chaubal *et al.*, 2006)	Ac	15	~4 years	10 weeks	3.9	21.0
(Liang *et al.*, 2008)	M/ NR	20	cows	n.p.	4.6	19.1
(Petyim *et al.*, 2003)	RW	8	Heifers	4 months	2.8	n.p.
(Petyim *et al.*, 2000)	RW	3	Heifers	4 and 5 weeks	1.5-5.9	n.p.
(Lopes *et al.*, 2006)	HF/ RW	5	Cows	9 sessions	6.0	~28.0
		8		8 sessions	4.3	~25.0
(Bruggerhoff *et al.*, 2002)	GS	13	Heifers, cows	6 weeks	6.1	15% (SCNT)
(Goodhand *et al.*, 1999)	GS	8	Heifers	n.p.	8.9/week	2.4/week
(Machado *et al.*, 2006)	GS	18	2-5 years	11 sessions	8.7	18.9
(Su *et al.*, 2009)	M x B	12	12 months	10 sessions	7.3	45.9
		15	7-8 years	10 sessions	6.1	30.2
		10	≥15 years	10 sessions	4.7	13.5
(Argov *et al.*, 2004)	HF	10	Cows	5 weeks	2.4	n.p.

n.p.: not presented; HF: Holstein Friesian, BB: Belgian Blue, CH: Chinese Holstein, Ac: Angus cross, M: Murrah, NR: Nili Ravi, RW: Red and white breed, GS: German Simmental, M x B: Murrah x Brahmann, SCNT: Somatic cell nuclear transfer

2.4.1.2. Ovarian status

In bovine two or three follicular waves emerge during the estrous cycle. In each wave a cohort of small follicles grows from which one is selected to become dominant. Products from the DF suppress FSH-release and the growth of the subordinate follicles. Under the influence of a progesterone producing CL the DF and the subordinates regress and the next wave emerges. After the induction of luteal regression the inhibiting influence of the CL is absent and the DF becomes the ovulatory follicle (reviewed by Adams *et al.*, 2008).

In OPU, a rise in FSH is detectable one day after each session accompanied with the emergence of a new follicular wave (Petyim *et al.*, 2001). FSH-peaks were also observed 16-24 h after DFR (Singh *et al.*, 2004).

Studies involving DFR aimed to remove inhibitory factors like inhibin and estradiol produced by dominant and antral follicles resulting in gonadotropin secretion by the pituitary like it occurs in regular cycles after physiological ovulations (Adams *et al.*, 2008).

Lower recovery rates have been observed for large (>10 mm in diameter) than for smaller follicles (Pieterse *et al.*, 1991). COCs originating from follicles smaller than 4 mm in diameter show lower developmental competence *in vitro* than those derived from follicles larger than 6 mm. Cumulus cell count revealed higher numbers in COCs from large follicles compared to those from smaller ones (Lequarre *et al.*, 2005). Oocytes removed from regressing subordinate follicles show similarities to those during the maturational phase. High developmental capacity for these oocytes have been demonstrated (Salamone *et al.*, 1999). The presence of a DF has been shown to have a negative influence on the developmental capacity of oocytes derived from the cohort of follicles that grows at the same time, especially on small follicles (Hagemann, 1999). Machatkova *et al.* (2000) suggested to perform the COC retrieval at the growing phase of the follicular wave, 3 days after ovulation, when no DF is yet selected, to optimize IVP results using ovaries of slaughtered animals. This is in agreement with Hagemann *et al.* (1999) who found higher blastocyst rates investing COCs collected during the follicular growth phase on days 2 and 10 than during the phase of dominance. Vassena *et al.* (2003) did not observe a negative influence of the presence of a DF or a CL on slaughterhouse-derived ovaries on the *in vitro* blastocyst rates which were higher on day 5 (regression phase) after follicular

wave emergence compared to days 2, 3 and 7 of the cycle. However, the cycle stage did not influence the quality of the COCs.

2.4.1.3. COC quality

COCs collected by OPU are normally classified into morphological categories regarding compactness and quantity of surrounding cumulus cells and homogeneity and transparency of the ooplasm. Merton *et al.* (2003) observed higher *in vitro* blastocyst rates using oocytes with a compact multilayered cumulus investment compared to those surrounded by a less compact cumulus. These authors reported a positive effect of the number of COC cultured together per well on blastocyst rates when using poorer quality COCs.

As mentioned above, vacuum pressure during follicle aspiration can negatively affect the quantity of surrounding cumulus cells and thereby COC quality. It was also observed that the number of COCs/embryos cultured together in one culture dish may influence the blastocyst rate suggesting positive interactions (Nagao *et al.*, 2008). Culture of single oocytes/embryos led to lower development to blastocysts compared to culture in groups of 5, 10 or 25. The amount of media used for IVC influenced the cell number of day 8 embryos (Ward *et al.*, 2000). To better evaluate the link between COC morphology and their developmental competence, other studies tried to overcome the low developmental capacity of individually cultured oocytes by altering ingredients of the culture medium (Carolan *et al.*, 1996; Hagemann *et al.*, 1998). It was demonstrated that culture of different qualities of COCs together would have a positive effect on their developmental capacity suggesting interactions between the different categories. While COCs graded as category 1 showed higher cleavage rates in culture than COCs of lower categories or of mixed categories, the blastocyst rates did not differ between category 1 and mixed categories but were lower in separately cultured COCs of lower grades (Kelly *et al.*, 2007).

2.4.1.4. Individual donor

The amount of germ cells and primordial follicles present in the ovaries at birth vary greatly between animals (Erickson, 1966). The number of follicles recruited into a wave later in life ranges between 8-41 (Adams, 1999), 9-45 (Ireland *et al.*,

2007) or 11-54 and 9-33 (Burns *et al.*, 2005) and can therefore vary greatly between different individuals. However, peak numbers of follicles during waves detected by ultrasound scanning of the ovaries are repeatable within individuals in successive estrous cycles (Ireland *et al.*, 2007). The number mostly remains constant or changes slowly over long periods in heifers and cows, even when the same animals as heifers were considered as cows and irrespective of lactation stage, season or the examining technician (Burns *et al.*, 2005).

Ovaries obtained at a defined cycle stage by ovariectomy from heifers with low numbers of antral follicles were smaller and lighter compared to those from animals with high numbers of antral follicles (Ireland *et al.*, 2008). Low antral follicle numbers were also associated with higher postovulatory serum FSH-peaks (Burns *et al.*, 2005; Ireland *et al.*, 2007), higher intrafollicular E2 production and alterations in the expression of several genes in the granulosa-, theca- and cumulus-cells. The influence of these factors on oocyte quality and their developmental competence are unknown (Ireland *et al.*, 2009).

Ireland *et al.* (2007) observed a higher proportion of transferable embryos after superovulation and embryo collection on day 7 when the donors showed low numbers of antral follicles at the beginning of the superovulatory treatment. However, low numbers of antral follicles on slaughterhouse-derived ovaries did not influence COC quality, cleavage and blastocyst rates in IVP (Ireland *et al.*, 2007). Burns *et al.* (2005) obtained repeatabilities between 0.86 and 0.96 for peak numbers of antral follicles during follicular waves. Boni *et al.* (1997) obtained a repeatability of 0.58 for follicle numbers recruited into waves during an OPU period in individual donors.

High inter-individual variations in collection results were observed between non-superstimulated (Petyim *et al.*, 2000) and FSH-superstimulated (van de Leemput *et al.*, 1999; Humblot *et al.*, 2005) OPU donors. Inter-individual variations in the number of recovered COCs and in blastocyst rates were also observed after IVP using slaughterhouse-derived ovaries (Mermillod *et al.*, 1992; Petyim *et al.*, 2000).

Investigations in mother-daughter pairs revealed that related animals develop equal numbers of follicular waves (two or three) during the estrous cycle (Malhi *et al.*, 2005). Brüggerhoff *et al.* (2002) observed an influence of maternal lineage on the quality of recovered COCs in OPU, and a study on monozygotic twin cows submitted to twice weekly OPU showed that COC collection and IVP results were

similar in and varied between pairs (Machado *et al.*, 2006).

2.4.2. OPU after exogenous hormonal stimulation

2.4.2.1. Physiology of hormones and the possibility of manipulation

During the estrous cycle the presence or absence of a progesterone producing CL is one of the most important factors regulating ovarian function. In the presence of a CL progesterone down-regulates the secretion of gonadotrophin releasing hormone (GnRH) by the hypothalamus so that the synthesis and release of the gonadotrophins FSH and LH by the pituitary is negatively influenced (negative feedback) leading to a decrease in their release frequency (Grunert, 1999). Plasma P4 levels have been described to increase with the formation of a CL from lower than 1 ng/mL at estrus (Grunert, 1999) and the following three days to 3 ng/mL on day 6 and peak at day 10 to 14 of the estrous cycle (>4 ng/mL) (Adams *et al.*, 2008). Between days 17 and 19 of the cycle, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is secreted by the uterus and induces regression of the CL. This leads to increasing secretion of GnRH and thereby more frequent FSH and LH pulses and the exhibition of the preovulatory LH-surge for a period of 6-10 h (Walters *et al.*, 1984). Ovulation is expected approximately 26 h (Grunert, 1999) or 29 h later (Saumande *et al.*, 2005).

Estradiol-17β is produced by aromatizing of androstendione in the granulosa cells. The enzyme is FSH-dependant which leads to increasing E2 levels when gonadotrophins are released more frequently. E2 peaks occur shortly before LH and the serum concentrations decrease thereafter (Grunert, 1999; Saumande *et al.*, 2005) probably because of the luteinization of the follicular wall (Dieleman *et al.*, 1983). It has been shown that LH leads to an inhibition of the aromatizing enzyme activity 14 h after its peak (Dieleman *et al.*, 1984).

If a functional CL is present on one of the ovaries, $PGF_{2\alpha}$ induces luteal regression accompanied by decreasing P4 levels within 24 h and increasing E2 levels. This leads to the preovulatory LH-surge and ovulation (Thatcher *et al.*, 1976).

The application of a progesterone intravaginal releasing device supports or mimics a functional CL and therefore prolongs the estrous cycle and these devices have been used in estrous synchronization programs (Gordon, 2003; Tauck *et al.*, 2007; Leitman *et al.*, 2008).

Administration of GnRH to induce a LH-surge in superstimulation protocols leads

to a detectable surge after 2 h (van de Leemput *et al.*, 2001) or 3 h (Bordignon *et al.*, 1997; van de Leemput *et al.*, 1999) in the peripheral blood. E2 concentrations during superstimulation were shown to rise steadily with exposure to FSH treatment (Kemper-Green *et al.*, 1996) and were positively correlated with follicle numbers and peaked shortly after the LH-peak with decreasing levels afterwards (van de Leemput *et al.*, 1999). Takagi *et al.* (2001) observed the peak of E2 shortly before the LH-peak during a superstimulation regimen. These authors also observed an asynchrony between follicular, nuclear and cytoplasmic maturation in stimulated preovulatory follicles and the occurrence of unovulated follicles seven days after estrus. In FSH-superstimulated animals ovulations occur between 60 h and 108 h after the induction of luteal regression (Bó *et al.*, 2006).

2.4.2.2. Ovarian superstimulation in OPU protocols

During the past decades, many protocols have been proposed for exogenous hormonal superstimulation in order to increase the number of developing ovarian follicles allowing the collection of larger numbers of developmentally competent oocytes. Sendag *et al.* (2008) obtained higher numbers of follicles and better quality of COCs after FSH treatment of OPU donors compared to superstimulation with equine chorionic gonadotropin (eCG). Superstimulation with either FSH or eCG in prepubertal swamp buffalo calves resulted in equal (Techakumphu *et al.*, 2000b) or higher ovarian responses and increased the COC yield after FSH (Techakumphu *et al.*, 2000a).

FSH treatment prior to OPU has been shown to shift the follicle size distribution from small (2-5 mm in diameter) to medium size (6-10 mm) compared to no stimulation (Goodhand *et al.*, 1999; Goodhand *et al.*, 2000; Durocher *et al.*, 2006). Once weekly OPU after three days of FSH-stimulation lead to the same number of follicles, COC yield and *in vitro* blastocyst rates per week in comparison to non-superstimulated twice weekly OPU. However, more good quality COCs were obtained from superstimulated donors (Goodhand *et al.*, 1999). Chaubal *et al.* (2006) compared non-superstimulated once or twice weekly OPU and once weekly OPU after DFR. In addition, this study included FSH-superstimulation in once or twice weekly OPU. The FSH-superstimulation resulted in higher numbers of follicles, COCs and good quality blastocysts. Similar results were obtained by Sirard *et al.* (1999) who observed increased total

numbers of follicles and of large ones and more category 1 COCs after three days of FSH treatment prior to OPU compared to non-superstimulated animals. As reviewed above, larger follicles (>6 mm) may contain COCs of higher morphological quality and thus improved developmental capacity *in vitro*. Similar findings were also reported after superstimulation of donors with eCG for a period of 60 h prior to OPU. COCs from follicles >8 mm in diameter showed higher developmental competence than those obtained from follicles 5-8 mm (Hendriksen *et al.*, 2000). Blondin *et al.* (2002) used either four or six doses of FSH with or without administration of LH 6 h prior to OPU performed 33 or 48 h after the last FSH injection, the so-called "coasting" period. It was observed that the administration of LH and a longer coasting period had positive effects on both follicle size and blastocyst rates after IVP.

In a study involving a large number of animals and including three different teams and the same FSH-superstimulation protocol, Humblot *et al.* (2005) demonstrated that OPU performed 12 h before and 12 h or 40 h after administration of $PGF_{2\alpha}$ does not influence the number of ovarian follicles available and COCs obtained as well as the *in vitro* blastocyst rates.

The FSH dosage also influences superstimulation results (Lerner *et al.*, 1986; Martens, 2004). Chaubal *et al.* (2007) examined the effect of single or multiple FSH administration when the same total dose was given to COC donors. Multiple FSH injections resulted in more aspirated follicles and recovered COCs. The highest *in vitro* developmental capacity of COC was found when LH was administered prior to OPU in protocols involving multiple FSH injections. In protocols without LH treatment, the administration of a controlled intravaginal progesterone-releasing device (CIDR®) had a negative influence on *in vitro* development of COCs/embryos but did not influence the ovarian response.

Ovarian response to FSH treatment differs individually between animals. Repeated superstimulation followed by OPU resulted in high, low and medium responders. While this categorization mostly remains relatively constant within individuals, the results varied in some cases between sessions (De Roover *et al.*, 2005a). In a recent study, FSH administration prior to OPU did not increase numbers of follicles but decreased the numbers of collected COCs compared to non-superstimulated donors in a design involving the same animals. There was no difference in the *in vitro* cleavage rates but higher blastocyst rates were achieved after FSH stimulation (Presicce *et al.*, 2011). In conventional ET programs, the

administration of eCG two days prior to FSH treatment increased the number of transferable embryos in poor responding cows (Bó *et al.*, 2008). This treatment protocol probably allowed the recruitment of more small follicles (1-3 mm) into a cohort (Jaiswal *et al.*, 2004).

De Roover *et al.* (2005b) tried to find an adequate dose of FSH for individual donors to increase their *in vitro* blastocyst production results. It was observed that higher doses of FSH resulted in enlarged follicles but did not increase the numbers of available ones. Contrarily to others, this study observed negative correlation between the number of large follicles and the COC quality. Table 2 shows OPU and IVP results in superstimulated donors.

Table 2: OPU and IVP results after superstimulation with FSH

Reference	Breed	No. of donors	Age class	Intervals (p.w.)	Period (weeks)	No. of COC (mean)	Blastocyst rate (mean %)
(Goodhand *et al.*, 1999)	GS	8	Heifers	1	6	6.1	39.0
(Goodhand *et al.*, 2000)	B x F	48	Cows	1	4	5.3	33.0
			Cows	1	5	5.9	37.0
(Blondin *et al.*, 2002)	HF	4	Heifers	4	16	7.3	20.0
						11.0	43.0
		4	Heifers	4	16	6.5	54.0
						8.5	41.0
		3	Heifers	4	8	n.p.	n.p.
						n.p.	52.0
		3	Heifers	4	8	n.p.	n.p.
						n.p.	63.0
(Techakumphu *et al.*, 2004)	SB	17	Prepubertal Heifers	2	10	5.4	n.p.
(De Roover *et al.*, 2005b)	HF	10	44-199 mo	2	12	5.6	42.0
(Humblot *et al.*, 2005)	BC/HF/ Mo	n.p.	Heifers/Cows	n.p.	n.p.	7.4-10.6	39.3-51.5
(Chaubal *et al.*, 2007)	AC	4	4 years	1	9	9.1	2.0
		4	4 years	1	9	9.5	1.8
		4	4 years	1	9	6.9	1.3
		4	4 years	1	9	7.2	1.3
		3		1	3	11.4	1.6
		3		1	3	8.3	1.6
		3		1	3	11.5	2.9
		3		1	3	6.9	1.3
(De Roover *et al.*, 2008)	BB	1,5-11	112	2	1999-2003	11.8	29.0

n.p.: not presented, p.w.: per week, GS: German Simmental, HF: Holstein Friesian, BB: Belgian Blue, AC: Angus Cross, SB: Swamp Buffalo, B x F: Beef x Frisian, Mo: Montbeliard, BC: Beef cross

2.4.2.3. Recovery of *in vivo* matured oocytes

Only few authors have dealt with protocols that aimed to collect *in vivo* matured oocytes at metaphase II directly before the expected time of ovulation.

Different superstimulation protocols to obtain *in vivo* matured oocytes from sexually mature animals have led to 60.0-84.0% of the collected oocytes surrounded by an expanded cumulus (Bordignon *et al.*, 1997; Merton *et al.*, 2003; Humblot *et al.*, 2005) and 25.0-26.0% (Techakumphu *et al.*, 2000a); and 0.0-4.4% (Techakumphu *et al.*, 2000b) in prepubertal swamp buffalo heifers. Humblot *et al*. (2005) observed increasing percentages of oocytes having an expanded cumulus from 5.8% at 40 h after $PGF_{2\alpha}$ to 76.8% at 60 h in superstimulated donors.

Since the final maturation is initiated by the preovulatory LH-surge, investigators tried to determine the adequate time interval between the LH-surge and OPU in order to obtain the highest number of developmentally competent COCs for IVP. Hendriksen *et al*. (2000) did not find an increase in blastocyst rates after 6 h of *in vivo* maturation and additional 16 h of IVM compared to IVM of standard IVP protocols.

Administration of GnRH 26 h before OPU in superstimulated donors increased both the number of animals exhibiting a LH-surge and the recovery rates compared to the same superstimulation protocol without exogenous GnRH. The treatment with GnRH led to more COCs with an expanded cumulus and oocytes at metaphase II. GnRH treatment in this superstimulation protocol positively influenced the synchronous activity of cumulus expansion and meiotic maturation. IVF directly after COC recovery resulted in higher blastocyst rates in the GnRH treated animals (Bordignon *et al.*, 1997). Rizos *et al.* (2002) investigated the effect of *in vivo* maturation on oocyte developmental capacity compared to *in vitro* matured oocytes obtained at different times before or after the LH-surge. *In vivo* matured oocytes, obtained 20 h after GnRH administration and immediate fertilization, led to higher blastocyst rates *in vitro* compared to those collected just before the induced LH-surge followed by IVM and those of standard IVP. Higher developmental rates were obtained when fertilization and culture were carried out *in vivo*. A similar study was conducted using COCs obtained 2 h before and 24 h after the LH-surge from ovariectomized donors. These studies did not detect differences in *in vitro* cleavage rates. Nevertheless, higher *in vitro* blastocyst rates were obtained (Dieleman *et al.*, 2002). Dielemann *et al.* (2002) observed higher

cell numbers in blastocysts derived from *in vivo* matured oocytes than after IVM. Humblot *et al*, (2005) obtained better results when COCs were retrieved by OPU 20 h after GnRH compared to collection performed earlier in donors submitted to a standardized FSH- superstimulation protocol.

Van de Leemput *et al.* (1999; 2001) attempted to increase the developmental capacity of *in vivo* matured oocytes by delaying the LH-surge using LH-suppressing Norgestomet ear implants and GnRH in order to induce a controlled LH-surge in eCG superstimulated animals. This treatment did not result in higher *in vitro* cleavage or blastocyst rates compared to animals that exhibited a spontaneous LH-surge but was more efficient than IVP using COCs from non-superstimulated animals. It was concluded by these researchers that *in vivo* maturation of oocytes generally results in higher developmental competence *in vitro*.

Faasch *et al.* (2009) successfully superovulated donor heifers and cows on a repeated basis at 5-week intervals using different protocols involving the use of CIDR®, FSH and GnRH. Table 3 shows OPU and IVP results from *in vivo* matured COCs obtained from FSH-superstimulated donors.

Table 3: OPU and IVP results after FSH-superstimulation and recovery of *in vivo* matured COCs

Reference	Breed	No. of donors	Age class	No. of COC (mean)	Blastocyst rate (mean %)
(Bordignon *et al.*, 1997)	HF	12	Heifers	18.4	60.0
	HF	11	Heifers	12.3	40.0
(Hendriksen *et al.*, 2000)	n.p.	n.p.	n.p.	n.p.	52.0
(Dieleman *et al.*, 2002)	n.p.	n.p.	Cows	n.p.	41.0
(Rizos *et al.*, 2002)	BC	n.p.	Heifers	n.p.	58.2
(Humblot *et al.*, 2005)	BC/HF/Mo	n.p.	Heifers/Cows	10.7	57.6

n.p.: not presented, HF: Holstein Friesian, BC: Beef cross, SB: Swamp Buffalo, Mo: Montbeliard

2.5. The effect of the donor in somatic cell nuclear transfer (SCNT)

Yang *et al.* (1998) suggested after a study of reciprocal nuclear transfer (NT) that both the maturity of the ooplasm and the nucleus of the oocyte plays an important role in developmental competence. Aston *et al.* (2006) observed higher developmental competence of oocytes derived from cows than from heifers in SCNT programs. Brüggerhoff *et al.* (2002) demonstrated that, irrespective of age class (heifer or cow), maternal lineage has a high influence on the fusion rates and the number of transferable embryos after SCNT, but not on cleavage and morula or blastocyst rates. The individual animal influenced both fusion and blastocyst rates after SCNT (Yang *et al.*, 2008). However, results after standard IVP from the same donor animals were in a different range than after SCNT, meaning blastocyst rates could be either higher or lower (Yang *et al.*, 2008).

3. Materials and Methods

3.1. Animals

For the selection of suitable and healthy donors, animals were clinically examined and the genital tract was controlled by transrectal palpation for normal ovarian cyclicity. There were no direct genetic relations between animals. 12 heifers (~14 months at the beginning of the experimental period), eight cows in their first lactation (2-4 years old) and eight old cows (10-15 years old), all of the German Simmental (GS) breed, were used in two experimental periods. Period 1: Five heifers, five young cows, and five old cows; Period 2: Seven heifers, three young cows, and three old cows. Animals are listed individually in Table 4. Cows were dried off four to six weeks prior to the experimental period. They were housed under the same conditions in an open free stall in groups of two to five and were fed grass silage and hay and received mineral supplementation. Animals had access to fresh water *ad libitum*. Before the start of the experimental period, animals of period 1 received two doses of $PGF_{2\alpha}$ (Estrumate®, Intervet, Germany, 2 mL, i.m.) 11 days apart to synchronize estrous cycles while animals of period 2 were not synchronized previously.

3.2. Experiment 1: COC retrieval from non-superstimulated donors

Experiments were conducted from November 2008 to May 2009 (experimental period 1) and during June and July 2010 (experimental period 2) (Table 4).

Table 4: Animals used in experiment 1

Age class	No. of donors	Animal identification	Experimental period	No. of OPU sessions
Heifers	5	No. 94 - No. 98	1	32
	7	No. 124 – No. 130	2	6
Young cows	5	No. 87 -No. 91	1	32
	3	Nr. 121 - Nr. 123	2	6
Old cows	5	No. 81, No. 82, No. 84, No. 92, No. 93	1	32
	3	No. 118 - No. 120	2	6

3.2.1. OPU

OPU was performed using an ultrasound unit (SSD 500; Aloka, Japan) equipped with a 5 MHz convex array transducer (UST-9111-5; Aloka) and a probe holder (Watanabe Tecnologia Aplicada , Brazil). For follicle aspiration, a 60 cm long single lumen 18 Gauge puncture needle connected to a 50 mL Falcon collection tube and to a vacuum aspiration unit (K-MAR-5000; Cook, Germany) regulated to -100 mmHg was used.

In each session animals from each age class were chosen in random order. OPU was carried out twice weekly in 3- and 4-day intervals (Mondays and Thursdays) 32 times for the fifteen animals of experimental period 1 and 6 times for the 13 animals of experimental period 2 (Table 4). Animals received an epidural anesthesia using 5 mL of procaine 5% solution (Isocain®; Selectavet, Germany) to avoid rectal straining. The rectum was emptied from manure manually and the vulva and perineum were cleaned wet and dry. The probe holder was tempered with water and placed into the vagina in front of the cervix. Ovaries were searched for by rectal palpation and placed in front of the ultrasound probe. Ovaries were imaged on the screen and prominent structures were recorded, such as CL, DF and follicular cysts. The puncture needle was guided through the vaginal wall and the

tip of the needle placed into the follicles under visual control on a monitor. A puncture line on the screen was used to facilitate the exact positioning of needle tip and follicles. When inside a follicle, the needle was twisted until all visible fluid was sucked out. The aspiration system was rinsed with TCM 199 supplemented with heparin and gentamycin before and during each OPU session. All visible follicles larger than 2 mm in diameter were successively punctured and aspirated. Before puncture, the size of each follicle was estimated and categorized as small (2-5 mm), medium (6-10 mm) or large (> 10 mm). Obtained follicular fluid was filtered and washed out using PBS (Euroflush, France). A small amount of fluid (~40 mL) was kept, transferred into a 92 x 17 mm dish (Nunc; Germany) and COCs were recovered under a stereomicroscope within 15 min, classified into four morphological categories based on number and density of cumulus cell layers and colour and structure of the ooplasm (Table 5). They were kept in TCM 199 on a warming plate (HT 300; Minitüb, Germany) regulated to 26°C until the end of the last aspiration session of each age class. COCs of classes 1, 2 and 3 of each age class were pooled and transferred into four-well dishes (Nunc; Germany) containing 400 µL of maturation medium.

OPU and laboratory work was carried out always by the same team.

Table 5: Morphological classification of COCs

Category	Cumulus investment	Ooplasm
Class 1	> 5 compact cell layers	Homogenous colour, not granulated
Class 2	3-5 compact cell layers	Homogenous colour
Class 3	Few cell layers, breaks	Granulated and inhomogenous
Class 4	Denuded	Small, granulated, inhomogenous

3.3. Experiment 2: COC retrieval after FSH-superstimulation

Three months after the last non-superstimulated OPU session of experimental period 1 the same animals were FSH-superstimulated and OPU was performed at 5-week intervals. OPU was carried out 9 times from August 2009 to July 2010.

The superstimulation protocol is shown in Fig. 1. For estrus synchronization animals received a controlled intravaginal progesterone-releasing device (CIDR®;

Pfizer, Germany) between days 7-10 of the estrous cycle (day 0 = estrus) for 9 days. Starting six days after the administration of the CIDR®, FSH (Pluset®; Pharmanovo, Spain) was administered twice a day at 12-h intervals in decreasing doses for 4 days. The total dose was 500 I.U. for heifers and 750 I.U. for cows (Table 6).

Table 6: FSH dosage for the superstimulation of heifers and cows

FSH dosage in mL	1	2	3	4	5	6	7	8	Total
Heifers	2.0	1.5	1.5	1.5	1.0	1.0	1.0	0.5	10.0
Cows	3.0	2.5	2.0	2.0	2.0	1.5	1.0	1.0	15.0

$PGF_{2\alpha}$ was administered twice at the time of the 6th and 7th FSH injections (61 and 49 h prior to OPU) 2 h apart for each age class. The CIDR® was removed age-class-wise at the second $PGF_{2\alpha}$ administration. 18 to 20 h before the particular OPU, 5 mL of a GnRH analogue (Receptal®; Intervet, Germany) were administered. The OPU procedure was the same as described for experiment 1. Animals of each age class were chosen in random order. Before aspiration, follicles were measured and categorized into five groups (2-5 mm, 6-10 mm, 11-15 mm, 16-20 mm, and > 20 mm in diameter). All animals of one age class were submitted to OPU within 2 h to make sure that the interval between $PGF_{2\alpha}$ administration, CIDR®-removal and GnRH application was equal.

COCs were retrieved within 20 min and classified and separated according to the presence of cumulus cells into expanded and non-expanded COCs. COCs were transferred to Fert-TALP medium and fertilized within 1 h after the last OPU session of each age class.

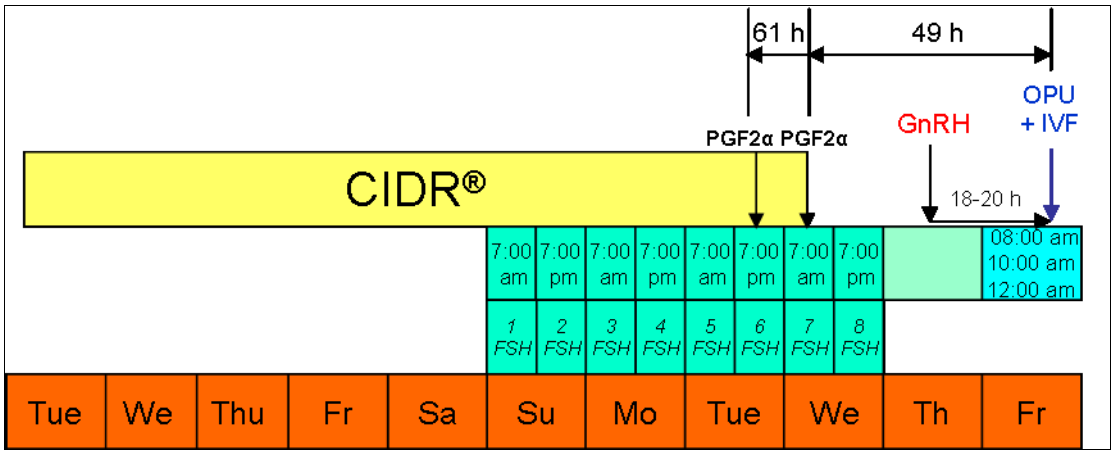


Figure 1: Superstimulation protocol for the collection of *in vivo* matured oocytes (experiment 2)

3.4. *In vitro* procedures

Media for *in vitro* procedures were prepared and stored for equilibration in the particular atmosphere 2 h prior to their usage.

3.4.1. *In vitro* maturation (IVM)

COCs obtained from non-superstimulated animals (experiment 1) were washed three times and matured *in vitro* in four-well dishes (Nunc, Germany) in Modified Parker Medium (MPM) supplemented with 0.025 U/mL FSH, 0.0125 U/mL LH and 5% (v/v) estrous cow serum (ECS) for 22 h in an atmosphere of 5% CO_2 in air at 39°C and maximum humidity.

3.4.2. *In vitro* fertilization (IVF)

For *in vitro* fertilization (IVF), *in vitro* (experiment 1) and *in vivo* (experiment 2) matured COCs were washed three times and transferred into four-well dishes containing 400 µL of Fert-TALP medium. Semen of an IVF-tested GS bull was used for IVF. A swim-up procedure was performed for sperm capacitation. Therefore, 10 mL plastic tubes were prepared with 1 mL Sperm-TALP medium. Frozen semen stored in liquid nitrogen at -196°C, was thawed in 37°C warm

water for 10 seconds. 100µL of semen per tube was immediately layered under the medium and stored in the incubator for 1 h. Meanwhile, healthy and motile sperms were supposed to swim into the medium phase. Afterwards, the medium above the sperm phase was removed from all tubes and collected in a single one and centrifuged at 612 g for 10 min in order to form a pellet of sperm on the bottom of the tube. The supernatant was discarded, leaving about 40 µL inside the tube. 10 µL of semen-medium suspension (1 x 10^6 spermatozoa/mL) was given to the COCs per well. COCs and semen were co-cultured for 20-22 h in an atmosphere of 5% CO_2 in air at 39°C and maximum humidity.

3.4.3. *In vitro* culture (IVC)

Presumptive zygotes were denuded mechanically by pipetting and cultured in four-well dishes in Synthetic Oviduct Fluid (SOF) supplemented with 5% (v/v) ECS, 40 µL/mL BME (Amino Acids Solution 50×, Sigma-Aldrich, Germany) and 10 µL/1 mL MEM (Non-essential Amino Acid Solution 100×, Sigma-Aldrich) covered with mineral oil at 39°C in maximum humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Cleavage and blastocyst rates were recorded on day 3 (IVF=day 0) and day 7, respectively.

In experiment 1 the cleavage rates are based on oocytes recovered in 14 OPU sessions (8 and 6 sessions for experimental period 1 and 2, respectively). Blastocyst rates are based on oocytes from 9 (3 and 6) sessions. This is due to the fact that a proportion of oocytes and developing embryos were submitted to other parts of this project where structural and proteomic analyses were performed. The results of these studies will be reported separately. For this reason, the cleavage and blastocyst rates in experiment 2 are based on 4 IVF experiments. The total numbers of COCs per age class and experiment that were used for the evaluation of cleavage and blastocyst rates are shown in Table 7.

Table 7: COC-input for evaluation of *in vitro* cleavage and blastocyst rates in experiment 1 and 2

COC-Input								
	Cleavage rates					Blastocyst rates		
	OPU sessions	Heifers	Young cows	Old cows		OPU sessions	Heifers	Young cows	Old cows
Experiment 1:								
Period 1	8	137	179	262		3	36	61	103
Period 2	6	274	135	182		6	274	135	182
Experiment 2:								
	4	140	130	185		4	140	130	185

3.4.4. Somatic cell nuclear transfer (SCNT)

The SCNT procedure was performed 6 times by Valeri Zakhartchenko (Chair for Molecular Animal Breeding and Biotechnology) using a standard protocol (Zakhartchenko et al., 1999). Embryos were cultured 3 times for 96 h and 3 times for 120 h. Results of cleavage and morula rate are based on 54, 81, 162 and 30, 44, 77 oocytes from heifers, young cows, old cows, respectively.

3.5. Blood sampling

For serum P4 determination blood samples were collected from the coccygeal vessels at the day of the second administration of $PGF_{2\alpha}$, 72 h later and right before the first OPU session in experiment 1.

For P4, E2 and LH determination blood samples were collected before the first OPU of experiment 2 from FSH-superstimulated animals in 2- to 3-h intervals beginning at the time of GnRH administration until the first particular OPU. Blood samples were collected in EDTA tubes (Monovette®, Sarstedt, Germany) and serum was separated by centrifugation at 1700 g and frozen at -20°C until analysis.

3.6. Enzyme immunoassays

The concentration of serum P4 (Prakash *et al.*, 1987), E2 (Meyer *et al.*, 1990) and LH (Mutayoba *et al.*, 1990) was determined by enzyme immunoassay. Enzymes and antibodies for the assays were donations from Univ.-Prof. Dr. H.H.D. Meyer, TU Munich, Freising-Weihenstephan, Germany.

3.7. Statistical analysis

The study was a completely randomized design with repeated measures (experiment 1: 32 non-superstimulated OPU sessions in experimental period 1, 6 non-superstimulated OPU sessions in experimental period 2; experiment 2: 32 non-superstimulated and 9 OPU sessions after FSH-superstimulation in experimental period 1). Data were tested for normality using PROC UNIVARIATE and were analyzed using PROC MIXED (SAS Version 8e; SAS Institute, Cary, NC, USA). For experiment 1, the model included age class (heifer, young cow, old cow), period (experimental period 1 and 2) and the interaction age class*period as fixed effects and animal as random effect. For experiment 2, the model included age class, treatment (non-superstimulated or FSH-superstimulated), and the interaction age class*treatment as fixed effects and animal as random effect. Data are shown as means and standard deviations (SD) together with the results of analysis of variance in Tables 9 and 10. In addition, least squares means were calculated for age class (experiment 1) and age class*treatment (experiment 2) and compared by using Student t-tests in order to facilitate direct comparisons of the experimental units. P values <0.05 were considered statistically significant.

Experiment 1:

$$Y_{ijklm} = \mu + animal_i + age_j + period_k + age*period_l + \varepsilon_{ijklm}$$

where:

Y_{ijklm}	=	observation of record ijklm
μ	=	expected mean of Y
$animal_i$	=	random effect of animal
age_j	=	fixed effect of age class j, j=1 to 3, (1:heifer, 2:first

lactating young cow, 3:old cow);

$period_k$ = fixed effect of period k, k=1, 2 (experimental period 1 and 2)

$animal*period_l$ = interaction of age class and period l, l= 1 to 6

ε_{ijklm} = random error term associated with record m on animal i at age class j, and period k and the interaction between age class and period l.

Experiment 2:

$$Y_{ijklm} = \mu + animal_i + age_j + trt_k + age*trt_l + \varepsilon_{ijklm}$$

where:

Y_{ijklm} = observation of record ijklm;

μ = expected mean of Y;

$animal_i$ = random effect of animal;

age_j = fixed effect of age class j, j=1 to 3, (1:heifer, 2:first lactating cow, 3:old cow);

trt_k = fixed effect of treatment k, k=1, 2 (FSH-superstimulated *vs.* non-superstimulated);

$animal*trt_l$ = interaction of age class and treatment l, l= 1 to 6;

ε_{ijklm} = random error term associated with record m on animal i at age j, and treatment k and the interaction between age class and treatment l.

3.7.1. Repeatability

Estimates of variance components and repeatability of OPU traits were obtained by using the MIXED procedure of SAS. The models used are described before.

Repeatability was calculated as:

$$r = \frac{\sigma^2_B}{\sigma^2_B + \sigma^2_W}$$

with:

σ^2_B = Variance between animals (B = *between*)

σ^2_W = Variance within animals (W = *within*)

3.7.2. Statistical significance of random effects - Likelihood ratio test

The likelihood ratio test evaluates the significance of a random parameter within a model, compared to the identical model excluding this parameter. The numerical values of the maximum of the likelihood function is estimated by restricted maximum likelihood (REML) for both models, and minus twice the difference in the two logL asymptotically has a χ^2 distribution with degrees of freedom equal to the number of parameters tested. The difference can than be compared to tabulated χ^2 values in order to decide whether the effect is statistically significant or not. For $p<0.05$; 1 degree of freedom (df) = 3.84 and for $p<0.01$, 1 df = 6.63).

3.7.3. Analysis of IVP, SCNT and hormone profil data

Due to the limited amount of data, and pooled COCs for IVP and SCNT, the model described before could not be used. To analyse IVP results, SCNT results and hormone profile analysis, the PROC GLM (SAS Version 8e; SAS Institute, Cary, NC, USA) was used. Fixed effects fitted in the model were in general age class and treatment, for SCNT data only age class and for hormone analysis, instead of treatment, the time point of blood sampling.

4. Results

4.1. Experiment 1: COC retrieval from non-superstimulated donors

All animals of both experimental periods showed good health and normal behaviour, with no contraindications to treatments and OPU throughout the experiment.

4.1.1. Estrus synchronization

Animals of the first collective were synchronized prior to the first OPU session, while animals of the second collective were not. Synchronization was successful in all animals of collective 1 as shown by a simultaneous drop in serum P4 concentrations 72 h after $PGF_{2\alpha}$ administration. Average serum P4 values were 2.2 ± 0.4, 2.0 ± 0.2 and 2.2 ± 0.4 [ng/mL] for heifers, young cows and old cows, respectively, at the time of the 2nd $PGF_{2\alpha}$ application, ≤ 0.2 ± 0.0 [ng/mL] for all age classes 72 h after the 2nd $PGF_{2\alpha}$ administration and 2.6 ± 0.9, 1.0 ± 0.3 and 1.2 ± 0.2 [ng/mL] at the time of the first OPU (Fig. 2). Ultrasonographic examination of the ovaries before puncture revealed CL in twelve of the fifteen animals. Eight of these twelve additionally presented a DF. One heifer, one young cow and one old cow did not develop a CL whereas the young cow showed a follicle theca cyst and a serum P4 value of 0.2 ng/mL. The old cow did not show any functional structure while P4 was 0.8 ng/mL, and the heifer developed a DF only while P4 value was 2.7 ng/mL.

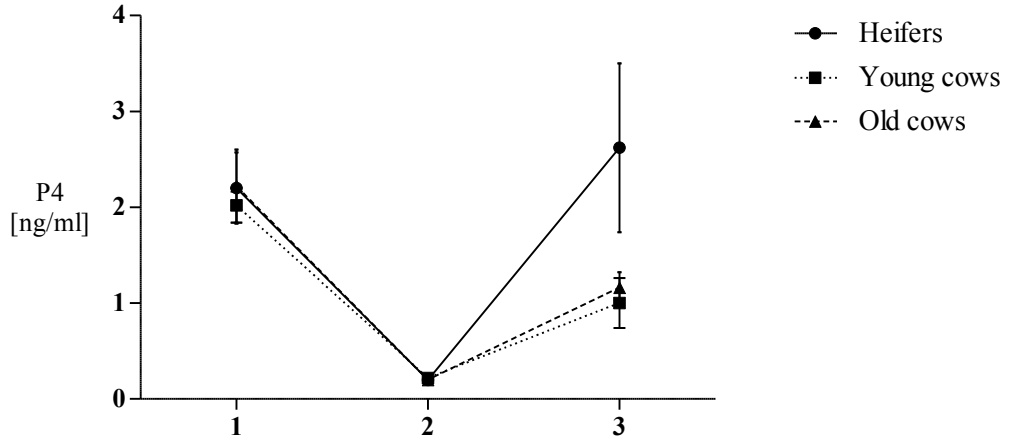


Figure 2: Serum P4 (ng/ml) concentration during estrous synchronization

4.1.2. Follicle numbers and COC yield

In 32 OPU sessions during experimental period 1 a total of 4388 follicles (1053 from heifers, 1499 from young cows, 1838 from old cows) were aspirated and 2238 COCs (heifers: 430; young cows: 698; old cows: 1110) were obtained. During the 6 OPU sessions of experimental period 2, a total of 775 COCs (heifers: 374; young cows: 169; old cows: 232) were collected from 1211 aspirated follicles (608 from heifers, 244 from young cows, 359 from old cows). The ranges of follicles aspirated and COCs obtained for experimental period 1 and 2 are shown in Table 8.

Table 8: Range of absolute and means for follicles aspirated and COCs obtained per period and age class

	Range					
	Heifers		Young cows		Old cows	
	absolute	means	absolute	means	absolute	means
Follicles aspirated						
Period 1	1 - 13	5.4 – 7.4	2 - 17	8.4 – 11.8	4 - 27	9.7 – 16.1
Period 2	7 - 24	11.3 – 16.8	1 - 27	6.2 – 20.5	13 – 32	18.8 – 21.2
COCs obtained						
Period 1	0 - 13	1.9 – 3.8	0 - 13	3.1 – 5.9	0 – 25	5.0 – 11.6
Period 2	1 - 22	5.8 – 14.0	1 - 22	2.5 – 14.3	6 - 19	10.7 – 14.3

Analysis of variance revealed that all parameters regarding follicle numbers and sizes as well as the recovery of COCs were significantly affected by the experimental period, with significantly higher total numbers of follicles aspirated and COCs recovered in period 2 than in period 1 (Table 9). In six sessions of period 2 more COCs were recovered than follicles aspirated.

Comparison of follicle size distribution revealed that higher percentages of small follicles were aspirated in all age classes of period 2 than 1. Conversely, higher percentages of medium and large follicles were aspirated in all age classes of period 1 compared to 2.

The total number of follicles increased with age ($P<0.001$). While heifers and young cows had similar numbers of small and medium size follicles, the proportion of small follicles was increased ($P<0.01$) while the proportion of medium size follicles was decreased in old cows ($P<0.05$). The proportion of

large follicles was not affected by donor age when data of the two experimental periods were combined. However, significantly more large follicles were aspirated in young cows of period 1 compared to heifers and old cows of the same period while the number of large follicles in young cows of period 2 was higher than in heifers but not in old cows.

Significantly higher percentages of class 1 COCs were obtained from heifers and young cows of period 1 compared to heifers and young cows of period 2. No difference was seen between old cows of period 1 and 2 in the percentage of class 1 COCs. Percentages of class 2 COCs were not different between experimental periods. Heifers and old cows of period 2 showed higher fractions of class 3 COCs than their counterparts of period 1, while fractions of young cows were not different. In experimental period 2, higher percentages of class 4 COCs were recovered from heifers and young cows compared to period 1. No difference was seen in the percentages of class 4 COCs between old cows of period 1 and 2.

In period 1, the total number of COCs recovered was also affected by donor age, with higher numbers in old cows than in young cows and heifers ($P<0.01$) (Table 9) while this parameter did not differ between age classes of period 2.

When periods 1 and 2 were combined, the recovery rates were not different between age classes. In period 1, the recovery rate in old cows was significantly higher compared to heifers ($P<0.01$) and young cows ($P<0.05$) (Table 9) while this difference was not present between age classes of period 2. The recovery rate in heifers of period 2 was significantly higher than in heifers of period 1.

4.1.3. Developmental competence after IVM

The developmental capacity of oocytes after IVM and IVF was not different between the two experimental periods.

Analysis of variance failed to detect a systematic effect of donor age on cleavage and blastocyst rates, however there was a clear trend of lower blastocysts rates for heifers as compared to young cows and old cows (Table 9). The least squares mean blastocyst rate was significantly smaller in heifers (5.1 %) than in young cows (17.4 %) when the two experimental periods were combined.

Table 9: Experiment 1: Effect of donor age on number and size of follicles, oocyte yield and developmental capacity after IVM

Parameter	Period	Donor age class			ANOVA		
		Heifers	Young Cows	Old Cows	Age	Period	Age*Period
Experimental period 1 (32 sessions per donor)		n = 5	n = 5	n = 5	F P	F P	F P
Experimental period 2 (6 sessions per donor)		n = 7	n = 3	n = 3			
Follicles							
n per donor and session	1	6.6 ± 2.3	9.4 ± 3.0	11.4 ± 4.4	9.2 ***	43.3 ***	1.6 n.s.
	2	14.5 ± 4.1	13.6 ± 7.1	20.0 ± 5.1			
Small (%) (2-5 mm)	1	33.9 ± 17.1	32.6 ± 12.0	36.8 ± 12.7	5.7 **	281.8 ***	2.0 n.s.
	2	70.4 ± 14.4	67.1 ± 17.9	82.4 ± 8.3			
Medium (%) (6-10 mm)	1	47.3 ± 16.2	43.4 ± 12.3	46.1 ± 12.9	4.4 *	143.2 ***	3.7 *
	2	26.2 ± 12.8	24.0 ± 14.6	13.5 ± 8.5			
Large (%) (>10 mm)	1	17.9 ± 18.9	24.9 ± 18.1	16.8 ± 16.0	2.7 n.s.	33.3 ***	0.1 n.s.
	2	3.4 ± 4.8	8.9 ± 11.1	4.1 ± 3.9			
Cumulus-oocyte-complexes							
n per donor and session	1	2.7 ± 2.1	4.4 ± 2.7	7.0 ± 4.1	4.7 **	27.8 ***	0.2 n.s.
	2	9.2 ± 4.9	9.4 ± 6.8	12.9 ± 3.5			
Class 1 (%)	1	32.5 ± 38.1	38.7 ± 34.6	25.8 ± 24.0	0.7 n.s.	9.6 **	0.2 n.s.
	2	15.7 ± 12.7	18.2 ± 16.9	14.7 ± 15.8			
Class 2 (%)	1	21.7 ± 31.2	22.2 ± 25.7	23.0 ± 21.8	0.1 n.s.	3.7 n.s.	0.2 n.s.
	2	16.6 ± 18.4	16.7 ± 18.5	13.0 ± 8.7			
Class 3 (%)	1	29.1 ± 35.9	24.0 ± 25.3	35.4 ± 26.8	2.1 n.s.	11.4 **	0.1 n.s.
	2	48.9 ± 24.8	38.0 ± 25.9	54.8 ± 22.8			
Class 4 (%)	1	7.3 ± 20.4	11.3 ± 21.8	14.6 ± 19.6	2.0 n.s.	13.8 **	1.6 n.s.
	2	18.8 ± 20.5	27.1 ± 30.2	17.5 ± 10.8			
Recovery rate (%)	1	39.9 ± 26.9	46.2 ± 25.0	60.8 ± 28.5	1.86 n.s.	9.3 **	1.1 n.s.
	2	64.8 ± 33.3	63.2 ± 28.6	67.5 ± 23.3			
In vitro development							
Cleavage rate (%)	1	49.5 ± 15.2	60.7 ± 22.3	52.7 ± 18.3	1.2 n.s.	0.9 n.s.	0.2 n.s.
	2	53.4 ± 15.8	62.5 ± 18.3	62.1 ± 13.7			
Blastocyst rate (%)	1	3.3 ± 5.8	21.9 ± 5.8	11.4 ± 3.5	2.6 n.s.	0.0 n.s.	0.5 n.s.
	2	5.9 ± 9.2	15.2 ± 17.5	15.8 ± 14.8			

Data are shown as means and standard deviations (SD). Cleavage and blastocyst rates are based on the number of presumptive zygotes cultured. The effects of Age of donors (heifer, young cow, old cow), Group (period 1 and 2), and the interaction Age*Group were estimated by analysis of variance. F values and levels of significance are shown: *P<0.05; **P<0.01; ***P<0.001; n.s. = not significant.

4.1.4. Cleavage and development *in vitro* after SCNT

COCs obtained from six OPU sessions were used for SCNT. Mean (± SEM) cleavage rates 96 h after activation were 28.9 ± 10.2 % for heifers, ranging from 0.0 to 60.0 %, 44.9 ± 6.7 % for young cows ranging from 22.2 to 64.2 % and 39.0 ± 6.3 % for old cows, ranging from 17.9 to 52.0 % and did not differ between age classes. The morula rate 120 h after activation ranged from 11.1 to 37.5 % in heifers, from 22.2 to 41.7 % in young cows, from 17.9 to 29.6 % in old cows and means (± SEM) were 23.9 ± 7.6, 33.2 ± 5.8 and 21.9 ± 3.9. There was no difference between age classes (Fig. 3). Results are based on numbers of cultivated reconstructed embryos.

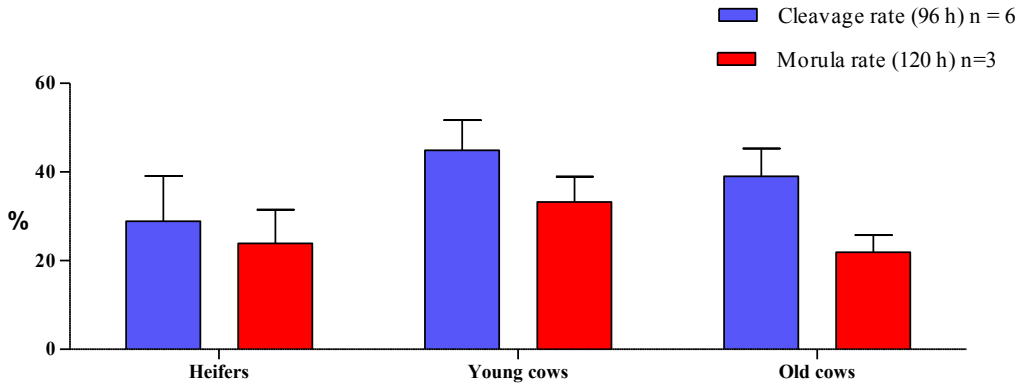


Figure 3: Cleavage and morula rates after SCNT, 96 h and 120 h after activation

4.2. Experiment 2: COC retrieval after FSH-superstimulation

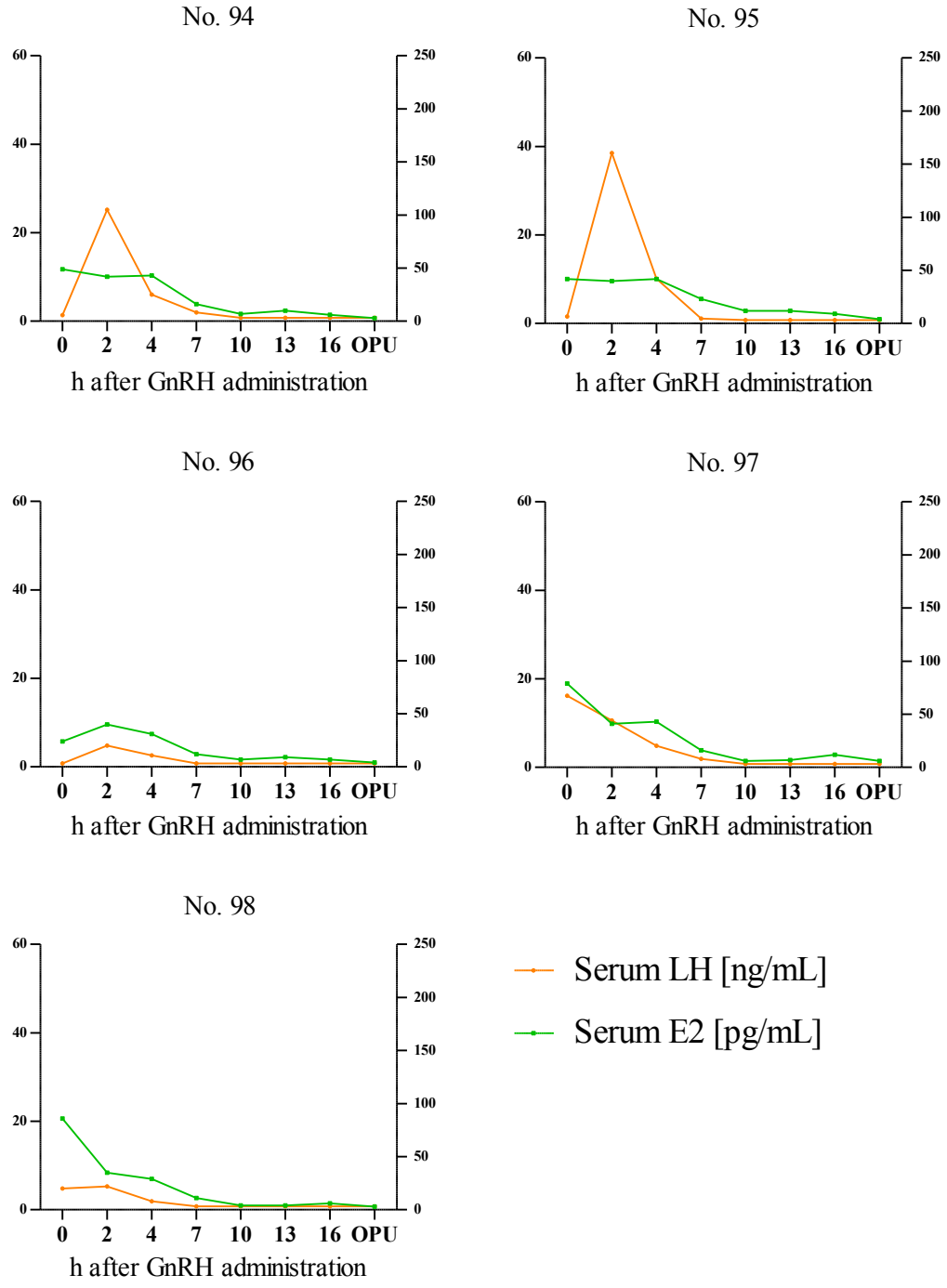
4.2.1. Hormone profiles

Prior to the first OPU session after FSH-superstimulation, blood samples were taken to detect whether animals exhibited an LH-surge after GnRH administration 18-20 h before OPU. Serum LH data revealed a detectable LH-surge in all age classes (Fig. 4) with higher ($P<0.01$) values 2 or 3 h after GnRH application than at any other time. The least square mean was significantly lower in heifers compared to young cows. However, values were rather variable between animals. In five animals (two heifers, one young cow and two old cows) plasma LH was already elevated at the time of GnRH administration. Serum E2 concentrations were elevated in all age classes, observing highest ($P<0.01$) concentrations at the time of GnRH administration (Fig. 4). Old cows showed higher E2 concentrations

than heifers at that time (97.4 ± 11.8 *vs.* 56.0 ± 11.8 pg/mL; P<0.05), while values of young cows were intermediate (72.4 ± 11.8 pg/mL) and did not differ significantly (P<0.14). Afterwards, E2 concentrations decreased steadily with time and did not differ between age classes. There was a correlation between E2 values at the beginning of the blood sampling period and number of follicles in the first OPU session with FSH-superstimulation.

Serum P4 stayed lower than 1 ng/mL during the sampling period in all animals and did not differ between age classes.

a) Heifers


No. 94
No. 95
No. 96
No. 97
No. 98
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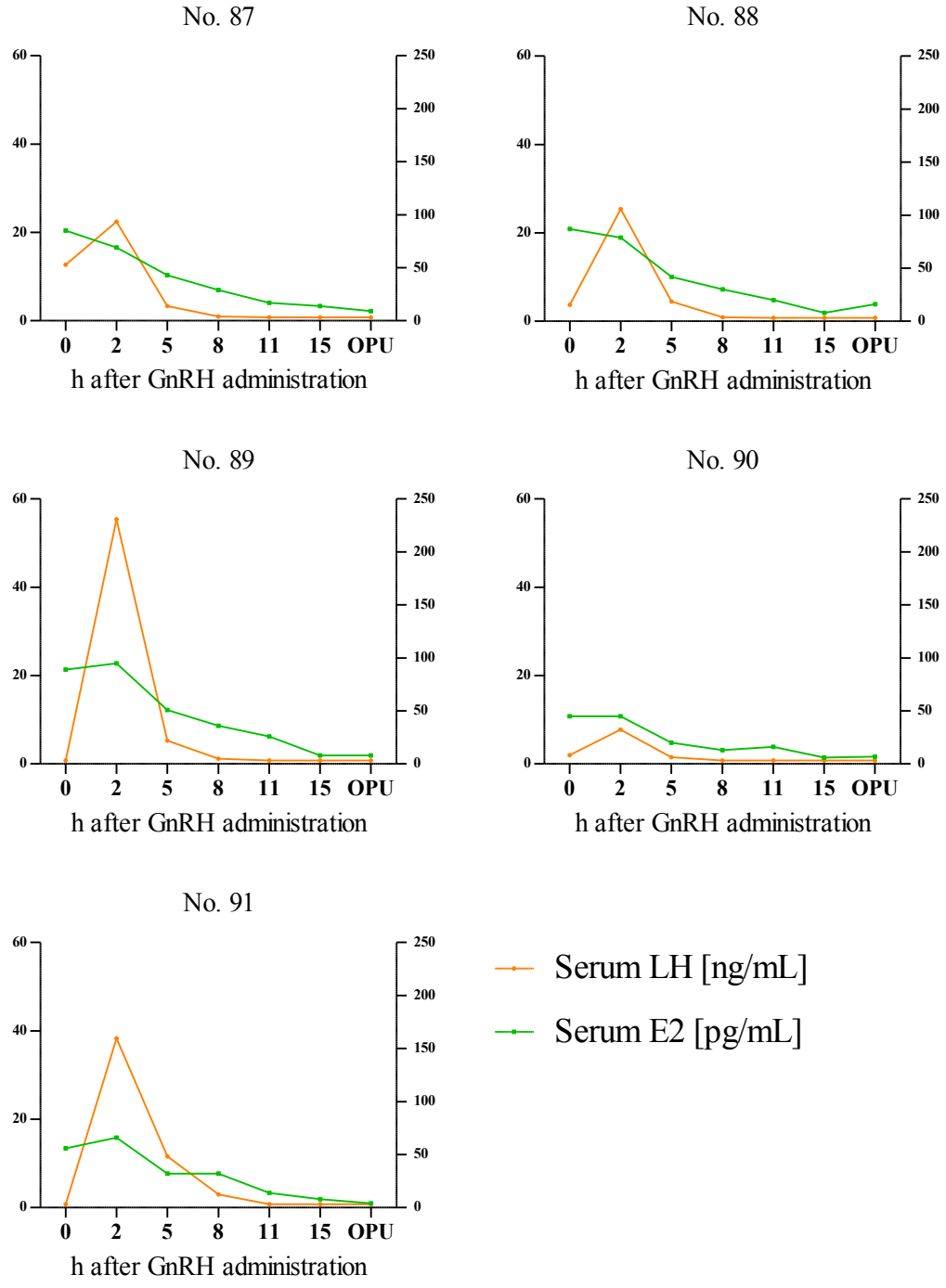
20
0
250
200
150
100
50
0
0 2 4 7 10 13 16 OPU
h after GnRH administration
Serum LH [ng/mL]
Serum E2 [pg/mL]

b) Young cows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h after GnRH administration
Serum LH [ng/mL]
Serum E2 [pg/mL]

c) Old cows

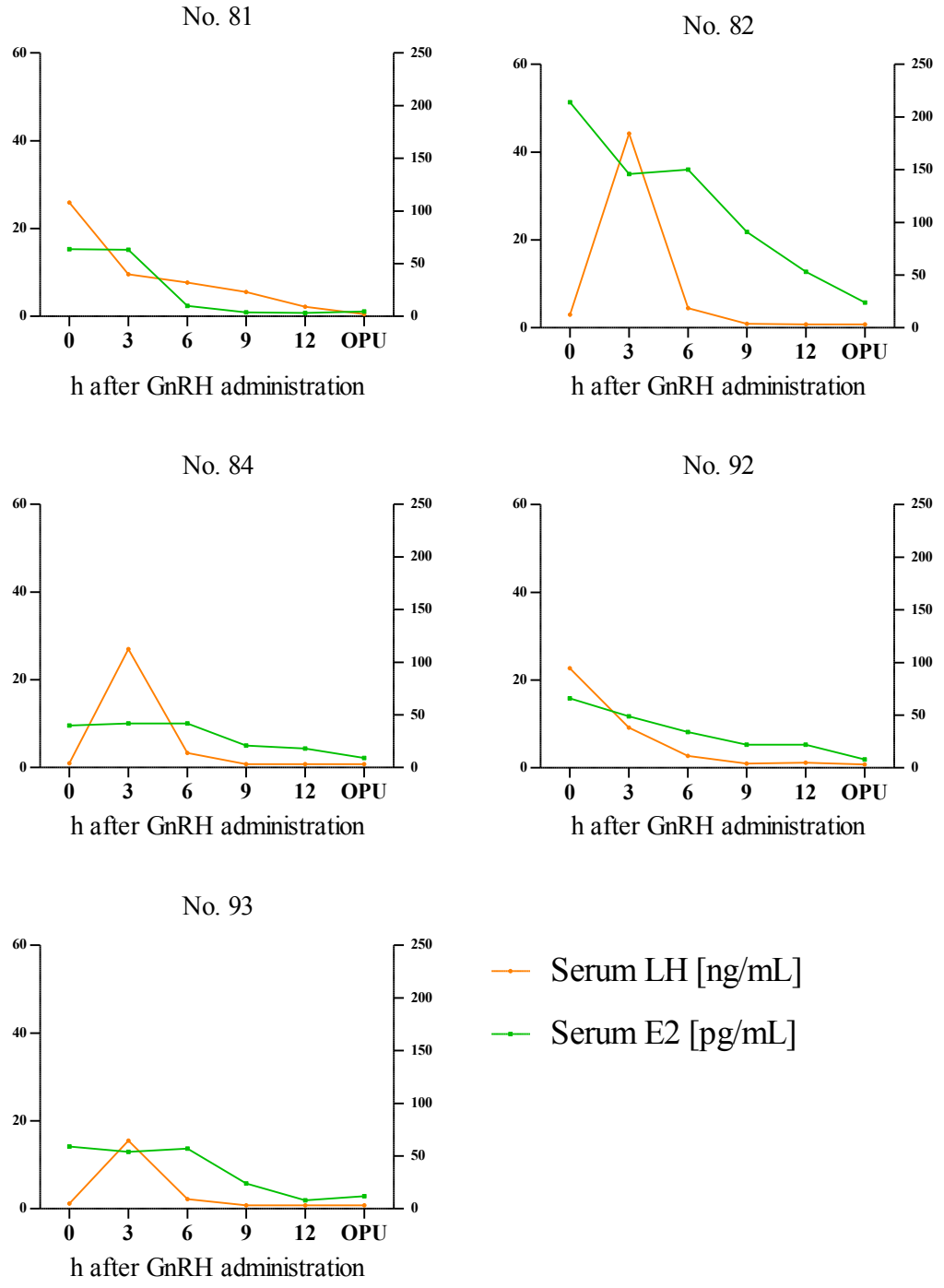

Figure 4: Individual serum LH (left Y-axis) and E2 (right Y-axis) concentrations prior to the first OPU session after FSH-superstimulation (a: heifers, b: young cows, c: old cows)

4.2.2. Follicle numbers and COC yield

During the nine OPU sessions after FSH-superstimulation 2494 follicles were aspirated (672 in heifers, 726 in young cows and 1096 in old cows) and 1405 COCs recovered (476 from heifers, 402 from young cows and 527 from old cows). Significantly more follicles were aspirated from old cows than from heifers and young cows. This age-dependent effect was more pronounced in FSH-treated than in non-treated animals as evidenced by the highly significant effect of the interaction age class*treatment. There was no difference in follicle size distribution between age classes. Further, there was an effect of age class on the total number of COCs obtained, with old cows yielding the highest numbers ($P<0.05$). The interaction age class*treatment was highly significant since the number of COCs was higher in young cows than in heifers in non-superstimulated animals while the opposite trend was observed in FSH-superstimulated animals.

The percentages of oocytes having an expanded cumulus were not significantly different between age classes (71.4% in heifers, 65.0% in young cows and 75.1% in old cows, respectively).

Analysis of variance revealed that the FSH-superstimulation treatment resulted in larger numbers of follicles aspirated ($P<0.001$) and COCs recovered ($P<0.001$). In addition, FSH-superstimulation affected the distribution of follicle sizes. FSH-treated donors of all age classes exhibited a smaller proportion of 2-5 mm follicles ($P<0.001$), and a larger proportion of 10-15 mm follicles ($P<0.001$; Table 10). As described below, follicle sizes even larger than listed in Table 9 were recorded. Percentages of 16-20 mm and >20 mm follicles were not different between treated age classes. Treatment had a positive effect ($P<0.01$) on the recovery rates. The recovery rates were significantly higher in experiment 1 than 2 for heifers and young cows, whereas it was the contrary for old cows. Fig. 5 shows the sonographic images of the ovaries of a non-superstimulated and a FSH-superstimulated donor at OPU.

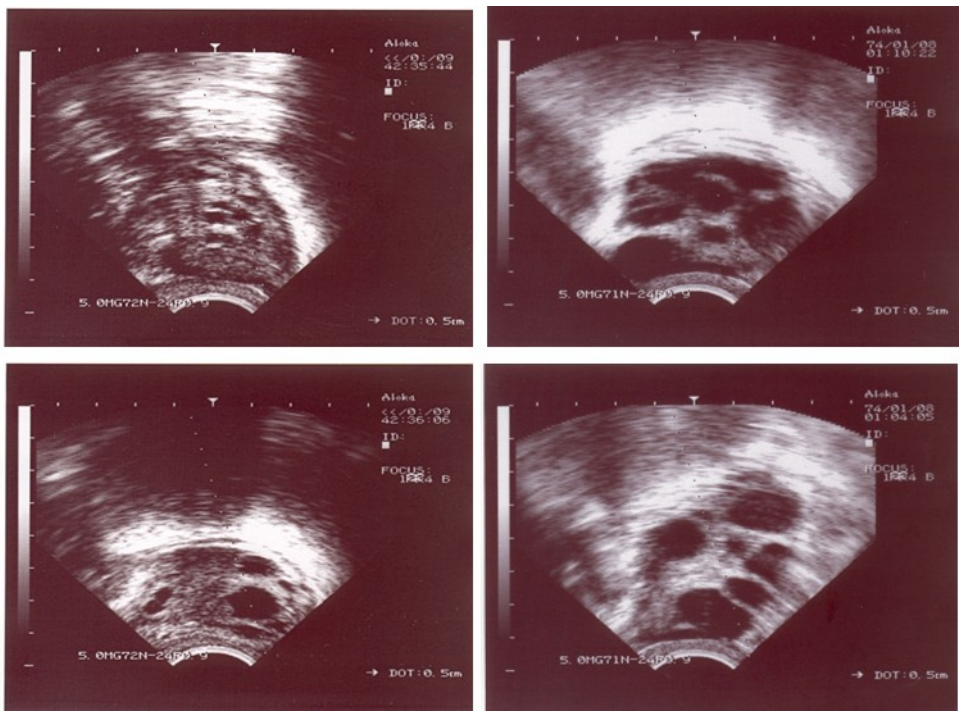

Figure 5: Left and right ovary of a non-superstimulated donor (left) (experiment1) and a FSH-superstimulated donor (right) (experiment 2)

4.2.3. Developmental competence of oocytes after *in vivo* maturation

There was a marked positive effect of FSH-superstimulation treatment on cleavage and blastocyst rates in all age classes ($P<0.001$), while these parameters were not affected by donor age (Table 10). Blastocyst rates were increased in a tenfold, twofold, and threefold manner for heifers, young cows and old cows, respectively. The culture of presumptive zygotes derived from immature COCs completely surrounded with unexpanded cumulus cells resulted in sporadic cleavage in all age classes and only one embryo developed to a single blastocyst in the age class of young cows.

Table 10: Experiment 2: Effect of donor age and treatment on number and size of follicles, COC yield and developmental capacity

Parameter	FSH	Donor age class			ANOVA					
		Heifers	Young Cows	Old Cows	Age		Treatment		Age x Treatment	
		n = 5	n = 5	n = 5	F	P	F	P	F	P
Follicles										
n per donor and session	-	6.6 ± 2.3	9.4 ± 3.0	11.4 ± 4.4	11.7	***	313.7	***	12.1	***
	+	14.9 ± 9.5	16.1 ± 8.3	24.4 ± 13.3						
2-5 mm (%)	-	33.9 ± 17.1	32.6 ± 12.0	36.8 ± 12.7	0.7	n.s	723.4	***	1.1	n.s.
	+	1.7 ± 3.3	1.4 ± 2.8	1.4 ± 4.4						
6-10 mm (%)	-	47.3 ± 16.2	43.4 ± 12.4	46.1 ± 12.9	1.8	n.s.	12.5	**	0.2	n.s.
	+	42.3 ± 28.3	37.5 ± 29.3	39.0 ± 29.5						
>10 mm (%) 11-15 mm (%)	-	17.9 ± 18.9	24.9 ± 18.1	16.8 ± 16.1	1.6	n.s.	69.1	***	1.9	n.s.
	+	33.4 ± 20.6	36.8 ± 29.5	38.1 ± 29.5						
16-20 mm (%)	-	-	-	-	-	-	-	-	-	-
	+	9.0 ± 12.9	12.4 ± 16.4	9.0 ± 13.2						
>20 mm (%)	-	-	-	-	-	-	-	-	-	-
	+	2.0 ± 4.4	4.4 ± 6.2	2.9 ± 5.9						
Cumulus-Oocyte-Complexes										
n per donor and session	-	2.7 ± 2.1	4.4 ± 2.7	7.0 ± 4.1	4.3	*	309.1	***	11.9	***
	+	10.6 ± 5.6	8.9 ± 4.5	11.7 ± 5.0						
Expanded cumulus (%)	-	-	-	-	-	-	-	-	-	-
	+	71.0 ± 27.3	65.0 ± 29.0	75.1 ± 19.1						
Non-expanded cumulus (%)	-	-	-	-	-	-	-	-	-	-
	+	29.0 ± 27.3	35.0 ± 29.0	24.9 ± 19.1						
Recovery rate (%)	-	39.9 ± 26.9	46.2 ± 25.0	60.8 ± 28.5	0.1	n.s.	9.3	**	26.7	***
	+	73.5 ± 35.2	55.1 ± 30.9	47.2 ± 21.8						
In vitro development										
Cleavage rate (%)	-	49.5 ± 15.2	60.7 ± 22.3	52.7 ± 18.3	0.9	n.s.	32.2	***	0.1	n.s.
	+	82.8 ± 1.8	89.8 ± 8.1	86.3 ± 2.4						
Blastocyst rate (%)	-	3.3 ± 5.7	21.9 ± 5.8	11.4 ± 3.5	2.7	n.s.	26.4	***	0.2	n.s.
	+	34.4 ± 11.1	44.6 ± 21.3	36.7 ± 6.9						

Data are from 32 non-superstimulated OPU sessions and from 9 OPU sessions after FSH-superstimulation per donor. Cleavage and blastocyst rates are based on the number of presumptive zygotes cultured. Data are shown as means and standard deviations (SD). The effects of Age of donors (heifer, young cow, old cow), Treatment (non-superstimulated vs. FSH-superstimulated), and the interaction Age*Treatment were estimated by analysis of variance. F values and levels of statistical significance are shown: *P<0.05; **P<0.01; ***P<0.001; n.s. = not significant.

4.3. Repeatabilities of OPU results

The repeatability for numbers of follicles aspirated was 0.62 and 0.87 in experiment 1 and 2, respectively, and 0.57 and 0.83 for collected COCs. Particular repeatabilities for the three age classes in experiment 1 are presented in Table 11. Individual profiles for numbers of follicles aspirated and numbers of COCs collected in experiment 1 are shown in Fig. 6-11. The overall repeatability was higher than 0.9 for all follicle sizes in experiment 1 and 2, and for the COC quality in experiment 1. In experiment 2, the repeatability for the percentage of COCs having an expanded cumulus was 0.58.

Table 11: Repeatabilities of OPU results in experiment 1 (period 1 and 2)

Parameter	Repeatability
Follicles *(n per donor and session)*	
Heifers	0.88
Young cows	0.34
Old cows	0.73
Cumulus oocyte complexes *(n per donor and session)*	
Heifers	0.51
Young cows	0.41
Old cows	0.67
Recovery rate *(%)*	
Heifers	0.83
Young cows	0.99
Old cows	0.84

Period 1, heifers

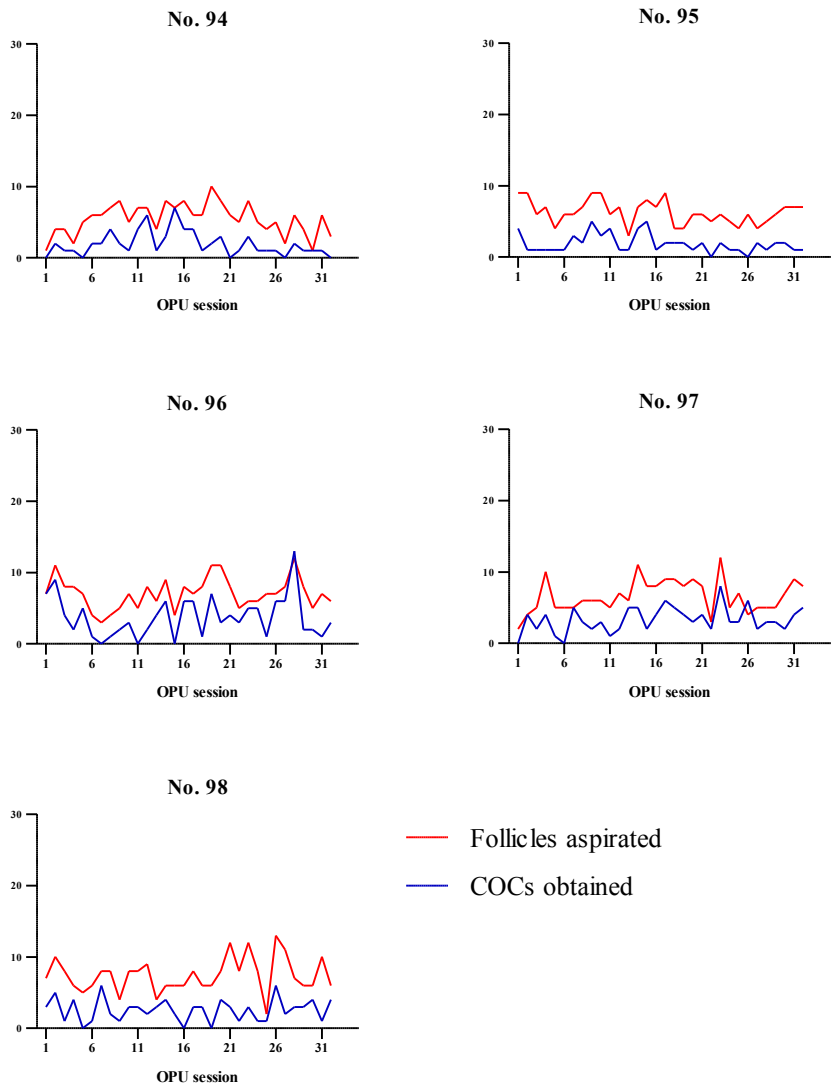

Figure 6: Individual profiles for numbers of follicles aspirated and numbers of COCs obtained during period 1 of experiment 1 in heifers

Period 1, young cows

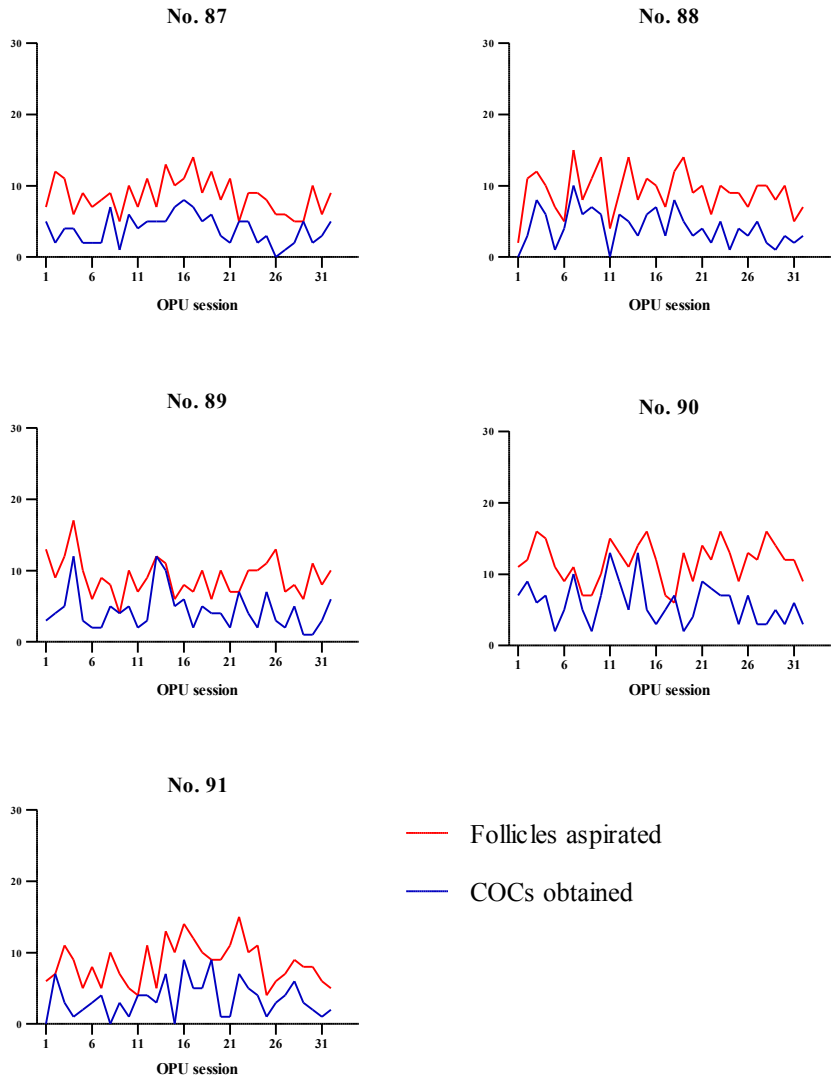

Figure 7: Individual profiles for numbers of follicles aspirated and numbers of COCs obtained during period 1 of experiment 1 in young cows

Period 1, old cows

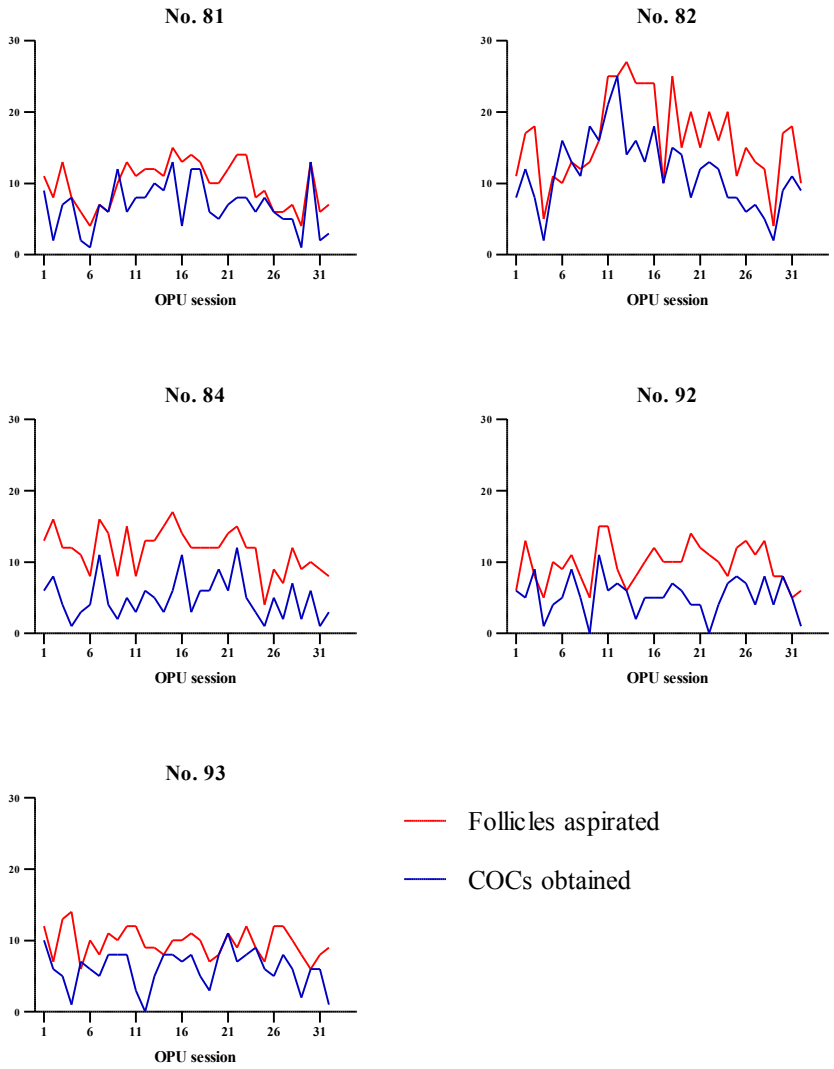

Figure 8: Individual profiles for numbers of follicles aspirated and numbers of COCs obtained during period 1 of experiment 1 in old cows

Period 2, heifers

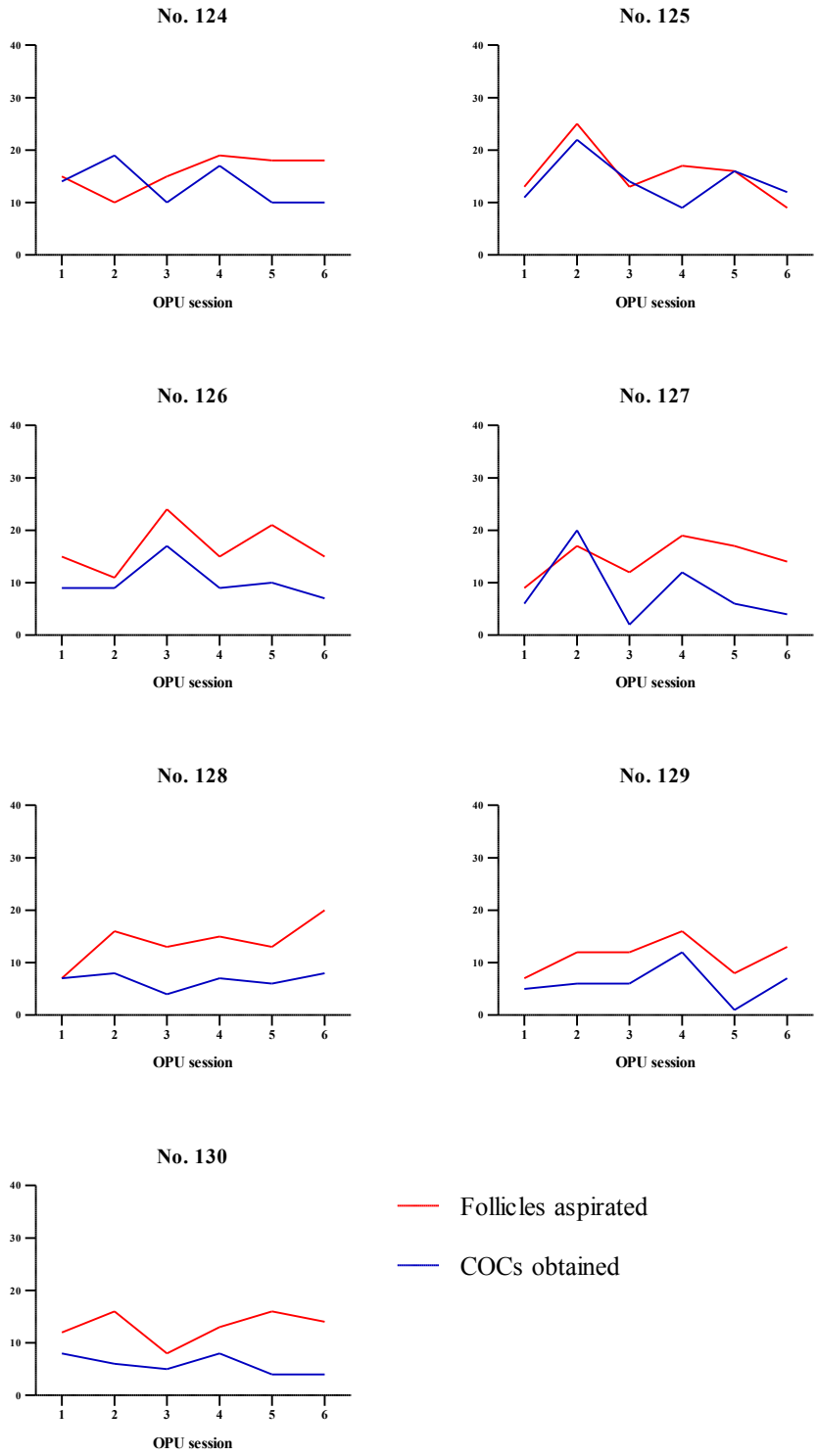

Figure 9: Individual profiles for numbers of follicles aspirated and numbers of COCs obtained during period 2 of experiment 1 in heifers

Period 2, young cows

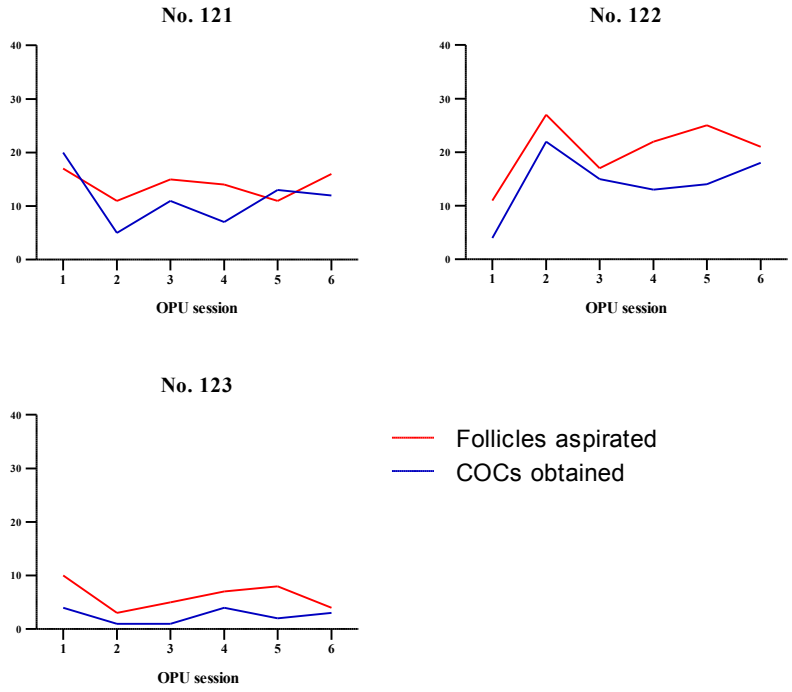

Figure 10: Individual profiles for numbers of follicles aspirated and numbers of COCs obtained during period 2 of experiment 1 in young cows

Period 2, old cows

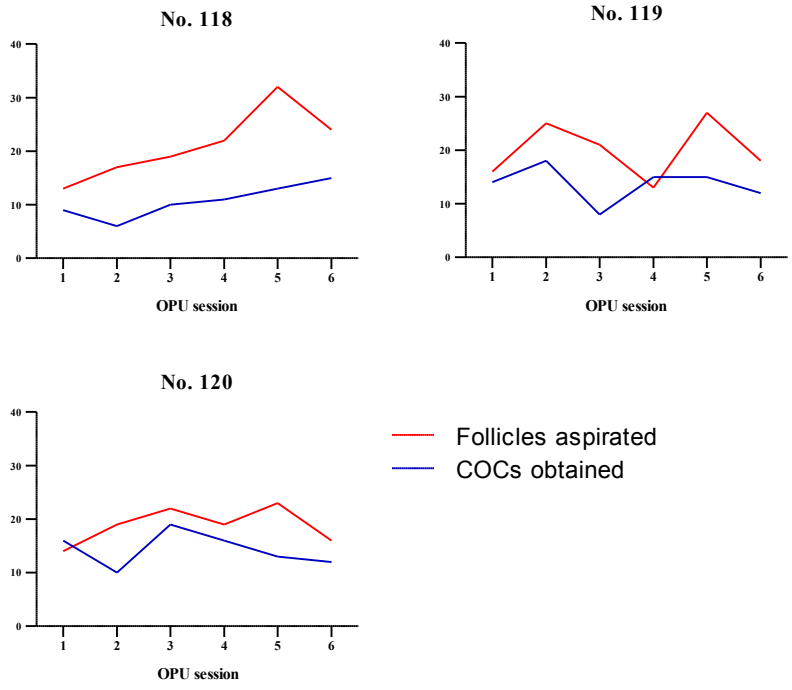

Figure 11: Individual profiles for numbers of follicles aspirated and numbers of COCs obtained during period 2 of experiment 1 in old cows

5. Discussion

The present study investigated for the first time in a two-factorial design the effects of donor age and FSH-superstimulation treatment on ovarian follicle number and size distribution, the yield and morphological quality of recovered COCs and the *in vitro* capacity of oocytes to cleave and to develop to the blastocyst stage after IVM/IVF or *in vivo* maturation/IVF.

As already described previously, the technique of OPU does not affect the health and the animal`s well-being (Petyim *et al.*, 2007). All animals used in the present study were healthy and over the course of the investigations no adverse effects of treatments on health and well-being were observed.

5.1. Hormone determinations in experiment 1 and 2

During estrous synchronization before the experimental period 1 of experiment 1, all animals showed estrous behavior and had low (≤0.2 ng/mL) serum P4 values 72 h after the second $PGF_{2\alpha}$ administration due to the absence of a progesterone producing CL during estrus as it has been described previously for estrous synchronization in heifers (Thatcher *et al.*, 1976). Our observations of differences in P4 levels between young and old cows agree with the results obtained by Malhi *et al.* (2006). As expected, serum P4 values were elevated seven days after induced estrus at the time of the first non-superstimulated OPU session in almost all animals. The ultrasonographic imaging of the ovaries revealed a CL in twelve of the 15 animals. One young cow developed a follicle theca cyst explaining the low serum P4 concentrations between days 0 (estrus) and 7 of the cycle. The two other animals showed P4 concentrations of 0.8 ng/mL and 2.7 ng/mL, suggesting the presence of a CL that was not clearly identifiable at the time of ultrasonographic examination.

In experiment 2 GnRH administration led to a LH-surge 2 to 3 h later in 10 animals considering the treatment as successful. Similarly, Bordignon *et al.* (1997) and Van de Leemput *et al.* (1999; 2001) observed LH-surges 2 to 3 h after treatment with GnRH. Five animals already showed elevated LH values at the time of the first blood sampling, indicating that they had a LH-surge. Therefore it can be concluded that the proposed time for GnRH administration in the superstimulation protocol was right determined. The above facts may justify the difference between young cows and heifers with regard to their least square mean

LH values at the time of the second blood sample. In addition, the interval between successive blood samples for GnRH determination was 2-3 h. The actual LH-peak might occasionally have occurred between samplings. In this case its identification and interpretation became doubtful. The maximal peak height of serum LH during the preovulatory LH-surge only remains detectable within a 6-10 h lasting period where the interval between highest LH pulses appears to be only around 23 min (Walters *et al.*, 1984).

It was reported for heifers that E2 concentrations rise with FSH-superstimulation (Kemper-Green *et al.*, 1996) and correlate positively with the number of developing follicles. Van de Leemput *et al.* (1999) observed the E2-peak shortly after the preovulatory LH-surge and declining concentrations afterwards. Contrarily in our study, serum E2 concentrations were highest at GnRH application and decreased steadily thereafter. Takagi *et al.* (2001) observed the E2-peak shortly before the LH-peak in superstimulated animals. Since blood sampling in our study started at GnRH administration the E2-peak in the superstimulated animals may already have occurred before the first sampling. However, there was a correlation between the height of serum E2 concentration at the time of GnRH administration and the number of follicles aspirated in the following OPU session in old cows, being higher compared to young cows and heifers. This correlation was also visible in heifers (lowest serum E2 concentration and follicle numbers) and young cows (intermediate serum E2 concentration and follicle numbers). However, a significance in differences in E2 concentrations at the time of the first blood sampling was only observed when the values were compared to those of heifers ($P<0.05$). We could not identify increasing E2 levels during FSH-superstimulation as described by Kemper-Green *et al.* (1996) since plasma hormone profiles were not determined during FSH-treatment in our study.

5.2. Experiment 1: OPU and IVP results - non-superstimulated

Experiment 1 investigated the effect of donor age in non-superstimulated animals. This was done in two independent experimental periods using two different sets of donor animals in order to evaluate independent experimental trials and individual animal effects. In fact, there was a marked effect of experimental period on all

parameters related to the number of follicles and the total number of COCs recovered. This may in part reflect seasonal influences (Takuma *et al.*, 2010) but to a large extent represent individual animal effects. This is in accordance with previous findings from other authors who reported high repeatabilities for follicle numbers recruited into waves during OPU periods (Boni *et al.*, 1997) or regular cycles, irrespective of season, age and lactation stage (Burns *et al.*, 2005). Given the restricted number of donor animals per group that can be investigated in such an experimental setting, individual animal effects are likely to be the major factor contributing to the variance between the first and the second experimental period. There was a high repeatability in all parameters related to OPU irrespective of age class (Table 11, Fig. 6-11).

Our study detected clear effects of donor age on the total number of follicles that increased with age ($P<0.001$), and on the follicular size distribution, with a larger proportion of small (2-5 mm; $P<0.01$) and a smaller proportion of medium-sized follicles (6-10 mm; $P<0.05$) in old cows as compared to heifers and young cows. In addition, more COCs per session were recovered from old cows than from young cows and heifers ($P<0.01$). Contrarily, Malhi *et al.* (2005) found fewer 4-5 mm follicles recruited into waves and lower peak numbers of 6-8 mm follicles in old cows compared to their younger daughters and Su *et al.* (2009) aspirated fewer follicles and obtained lower COC numbers in old cows than in middle-aged and young cows. Considering the fact that Malhi *et al.* (2005) used genetically related animals which has been shown to influence aspects of the estrous cycle and results of OPU and IVP (Bruggerhoff *et al.*, 2002; Machado *et al.*, 2006) our results, regarding follicle and COC numbers, may be more driven by individual effects. This seems likely, since initial recruitment of follicles and regulation of early follicle growth largely varies between animals and has been shown to be individually influenced (Adams, 1999; Burns *et al.*, 2005; Ireland *et al.*, 2007).

More follicles were aspirated in all age classes of period 2 than in period 1 resulting in higher numbers of collected COCs per animal and session in period 2. In six sessions of period 2 more COCs were recovered than follicles aspirated. Ireland *et al.* (2008) reported the existence of polyovulatory follicles, meaning that more than one oocyte was surrounded by one follicular wall. This fact might partially explain our findings but it is more likely that a number of follicles were concomitantly aspirated by passing the tip of the puncture needle through the ovarian stroma during a puncture procedure.

The recovery rates were higher in heifers of period 2, while this parameter did not differ between the other age classes. There is evidence showing a relationship between follicle size and recovery rates at OPU. According to Pieterse *et al.* (1991) aspiration of small follicles results in significantly higher COC yields than follicles above this size. The authors suggested that in larger follicles the follicular wall might fold around the aspiration needle and thereby avoid COC pick-up. This may eventually explain the lower recovery rates for heifers in period 1 since heifers in period 2 had significantly more small follicles aspirated. However, it remains unclear why there was no difference in the recovery rates between young cows and old cows of period 1 and 2 although the numbers of small sized follicles were lower in period 1 compared to 2.

Notably, there was a clear trend of lower *in vitro* blastocyst rates for heifers as compared to young cows and old cows. Statistically, we found a significantly smaller least squares mean blastocyst rate in heifers (5.1%) as compared to young cows (17.4%) when data of period 1 and 2 were combined.

After morphological classification, COC class distribution was different between the two periods. Although higher percentages of class 1 and lower percentages of class 3 COCs were retrieved in heifers and young cows of period 1, blastocyst rates were not different. Merton *et al.* (2003) found a positive effect of the numbers of COCs cultured together per droplet of culture medium on IVP results. Regarding the smaller number of COCs obtained per animal and session in period 1, especially in heifers, the greater numbers of COCs cultured together per well in the 4-well dishes in period 2 may have compensated the lower developmental capacity of categories 2 and 3 COCs in our study. Nevertheless, IVP employing COCs of different morphological categories was observed to yield similar blastocyst rates as IVP using only high class COCs (Kelly *et al.*, 2007). It can be concluded that the rate of *in vitro* development may be positively affected when COCs of considerable morphological variability are cultured together in larger numbers per group and unit volume of IVC medium, as previously reported by others.

Our main findings from experiment 1, a large effect of experimental trial and clear age-related differences, reflect the spectrum of findings described in the literature in relation to this topic. For instance, Rizos *et al.* (2005) did not find differences in the developmental competence *in vitro* of oocytes obtained from HF cows compared to heifers of the same breed after twice weekly OPU. The same study

on the other hand revealed higher blastocyst rates *in vitro* in cows compared to heifers when COCs originated from slaughterhouse ovaries. In contrast, Su *et al.* (2009) found the highest cleavage and blastocyst rates after *in vitro* fertilization of oocytes from 12 months old heifers and a decrease of the oocyte developmental competence in middle-aged and old cows. In contrast to Malhi *et al.* (2007) we did not detect a decreased fertilization rate of oocytes from old cows. However, as detailed above it is difficult to compare age-related effects that have been found in different studies since they may be markedly influenced by different breeds, differently defined age groups, environmental conditions, experimental settings and – probably most important – the individual donor animals used in the respective study. Previous investigations on age-related differences in bovine oocyte quality used either genetically related animals (Malhi *et al.*, 2005; Malhi *et al.*, 2006; Malhi *et al.*, 2007; Malhi *et al.,* 2008) or ovaries originating from slaughterhouses with unknown animal history (Rizos *et al.*, 2005); further complicating factors are different breeds (Su *et al.*, 2009) in parallel with different ways of utilization and levels of performance and nutrition (Roth *et al.*, 2008).

In our study, cleavage and morula rates after IVM and SCNT did not differ between age classes. Similarly, Brüggerhoff *et al.* (2002) did not find an influence of the age class of the donor animals (heifer or cow) on these parameters. In contrast, another study observed higher blastocyst rates after SCNT when used oocytes originated from slaughtered cows than heifers (Aston *et al.*, 2006). However, the numbers of COCs used in our SCNT experiment were relatively small and thus the results have to be interpreted carefully.

5.3. Experiment 2: OPU and IVP results after FSH-superstimulation

In order to at least rule out an inter-individual effect, experiment 2 of our study used the same donor animals to address age-related effects of FSH-superstimulation on the oocyte yield and the developmental potential of *in vivo* vs. *in vitro* matured oocytes recovered by OPU. Most studies dealing with *in vivo* matured oocytes used donor animals only once, often because animals were ovariectomized (Hendriksen *et al.*, 2000; Dieleman *et al.*, 2002; Humblot *et al.*, 2005). We found that repeated FSH-superstimulation prior to OPU is possible in at least 5-week intervals as it has been described for repeated superovulation and

embryo transfer programs by Faasch *et. al.* (2009). As well as in experiment 1, the repeatabilities of results following FSH-superstimulation were very high in individuals confirming findings by Malhi *et al*. (2008).

FSH-superstimulation significantly ($P<0.001$) increased the number of follicles aspirated and the number of COCs recovered in all three age classes. Recently, a positive effect of FSH-superstimulation prior to OPU on follicle number and yield of COCs per animal and session has been demonstrated in beef cattle (De Roover *et al.*, 2008). In our study, this effect was most marked in heifers ($P<0.001$ for the interaction age class*treatment).

Follicle size was shifted from mostly small and medium sized ones in experiment 1 to higher percentages of medium, large and even larger sized ones. This observation is in line with other studies where FSH-superstimulation was performed prior to OPU and similar size shifts were observed (Goodhand *et al.*, 1999; Goodhand *et al.*, 2000; Durocher *et al.*, 2006). Follicle size distribution was not different between age classes. Malhi *et al*. (2008) observed lower ovarian responses estimated by follicle size after FSH-superstimulation in old cows compared to their younger daughters in one experiment. In contrast to this but in agreement with our finding, in a similar study the response to FSH-superstimulation did not differ between the group of old and young cows (Malhi *et al.*, 2006).

Although Pieterse *et al.* (1991) found lower recovery rates for larger follicles in heifers and young cows, in our study the rates were significantly higher in experiment 2 where follicles were larger than in experiment 1. One could consider the fact that due to the expansion of the cumulus surrounding the oocyte the COC loses connection to the follicular wall and would be obtainable more easily. On the other hand, recovery rates decreased in old cows. It has been described that during superstimulation an asynchrony between follicular and oocyte maturation may occur (Takagi *et al.*, 2001) what possibly led to delayed cumulus expansion and still tight connections of the COC to the follicular wall. Further, the time span in which ovulations occur after superstimulation was described to be between 60 h to 108 h after $PGF_{2\alpha}$ administration (Bó *et al.*, 2006). It is likely that some follicles and the COC inside just would have needed more time to finish final maturation. Whatever the reasons for the lower recovery rate were in old cows, it may have altered the proportions of oocytes having an expanded cumulus but

these remain unknown. However, since the proportion of oocytes having an expanded cumulus is in line with other investigations that dealt with the recovery of *in vivo* matured oocytes (Bordignon *et al.*, 1997; Merton *et al.*, 2003; Humblot *et al.*, 2005), we consider the timing of our stimulation protocol as suitable.

In our study the very low developmental capacity of *in vitro* matured oocytes recovered from non-superstimulated heifers was entirely rescued by FSH-superstimulation and *in vivo* maturation. We cannot completely exclude that the significantly higher developmental capacity of *in vivo* matured oocytes from FSH-superstimulated heifers is - at least in part - due to the fact that the heifers became inevitably older until the series of ovarian stimulation cycles started. However, we consider this potentially confounding factor less important than the large variance between individual animals. In addition, no differences in the blastocyst yield from *in vitro* matured oocytes from different age groups of crossbred beef heifers were observed (Rizos *et al.*, 2005) which supports the legitimation of our experimental design. Furthermore, cleavage and blastocyst rates also increased significantly in the age classes of young and old cows. Regarding the fact, that follicle size shifted to more large and even larger sizes and that this has been shown to increase developmental competence *in vitro* (Hendriksen *et al.*, 2000; Lequarre *et al.*, 2005), this finding seems logical.

In addition, Rizos *et al*. (2002) demonstrated the importance of *in vivo* maturation for the developmental competence of oocytes. In a study of Dieleman *et al*. (2002) *in vitro* produced blastocysts from *in vivo* matured oocytes showed a lower incidence of chromosome aberrations and mixoploidy and higher cell numbers than their counterparts from *in vitro* matured oocytes, while the cleavage rates were similar. Investigations on gene expression of the oocyte surrounding cumulus cells revealed higher expression of stress-related genes in *in vitro* matured COCs while those associated with oocyte maturation regulation and cumulus expansion were more abundant in cumulus cells from *in vivo* matured oocytes (Tesfaye *et al.*, 2009).

Moreover, multifarious protocols of FSH-superstimulation prior to OPU have been reported to have positive effects on the developmental competence of bovine oocytes *in vitro* (Goodhand *et al.*, 1999; Goodhand *et al.*, 2000; De Roover *et al.*, 2008).

As expected and described by Merton *et al*. (2003), fertilization and culture of

oocytes with a non expanded cumulus led to only sporadic cleavage. The one blastocyst found from young cows should be interpreted with care. It is likely that during the procedure of classification and separation one COC having an expanded cumulus was transferred to the wrong well. Another explanation for the occurrence of cleavage and further development might be the fact that COCs spontaneously resume meiosis and become competent when removed from antral follicles (Pincus, 1935).

Our study in the bovine model reveals - in all three donor age classes, but most pronounced in the heifer group - highly significant positive impacts of FSH-superstimulation and *in vivo* maturation on the oocyte developmental competence in comparison to *in vitro* maturation of oocytes derived without prior FSH-superstimulation.

Further, results of the present study underline earlier suggestions that the bovine provides a powerful animal model to demystify the complex relationship between female age, ovarian functions and the developmental competence of oocytes in large mammals, including humans.

In humans, hormonal ovarian stimulation and retrieval of mature oocytes are well established and frequently applied: pregnancy rates after IVF and ICSI are in the range of 30.0% (Andersen *et al.*, 2008). To a large extent, female reproductive aging is associated with a decrease in fertility due to a decrease of oocyte quality and, in particular, an increase in aneuploidies of oocytes and embryos resulting in early embryonic death and foetal loss at different stages of the pregnancy (te Velde *et al.*, 2002). However, in humans cleavage rates *in vitro* have been shown to stay relatively constant with increasing age (Ottolenghi *et al.*, 2004). Notably, this finding is in accordance with our study in the bovine model: irrespective of oocyte maturation *in vitro* or *in vivo*, there was no statistically significant difference in the *in vitro* cleavage rate between the three age classes.

It has been suggested that blastocyst formation *in vitro* as a sign of developmental competence should be interpreted carefully because culture conditions may affect the expression of genes and thereby lead to alterations in maternal, embryonic and even foetal metabolism (Duranthon *et al.*, 2001). This may result in effects on weight and size, known as “large offspring syndrome”, as well as physio-anatomical abnormalities (van Wagtendonk-de Leeuw *et al.*, 1998; Young *et al.*, 1998). Taking these facts into consideration, further investigation is needed to

prove if higher blastocyst rates from *in vivo* derived oocytes would improve pregnancy rates and proper embryonic and foetal development to term in an age-dependant manner.

6. Summary

Effect of donor age on the developmental capacity of bovine cumulus oocyte complexes obtained by repeated OPU from nonstimulated and FSH-superstimulated German Simmental heifers and cows at different life cycle stages

Similarities between the bovine female and women in terms of reproduction and fertility, such as oogenesis, folliculogenesis and reduced fertility with advanced age make the bovine a valuable model for the study of ovarian function and dysfunction as well as reproductive aging in women. The aim of the present work was to investigate the influence of donor age on follicle numbers, yield and quality of COCs obtained by repeated OPU and on the developmental competence *in vitro* after oocyte maturation *in vitro* versus *in vivo*. Further, the ability of oocytes from different age classes to reprogram nuclei of bovine fetal fibroblasts was studied. Since the individual is a major factor influencing parameters of fertility and results of ART in both humans and cattle, the present study used in parts the same animals to rule out inter-individual effects on the response to one or the other approach.

Experiment 1 investigated the effect of donor age in non-superstimulated German Simmental heifers (n = 12, 14 months at the beginning of the experiments), young cows in their first lactation (n = 8, 2-4 years) and old cows (n = 8, 10-15 years). A total of 38 OPU sessions were performed in two experimental periods on independent sets of animals from all age classes: 5/5/5 (32 sessions) and 7/3/3 (6 sessions). In spite of a marked influence of the experimental period, a number of parameters were also significantly affected by donor age. The total number of follicles increased with age ($P<0.001$) and similarly, the total number of cumulus-oocyte-complexes (COCs) recovered was higher in old cows than in young cows and heifers ($P<0.01$). Evaluation of the follicle size distribution revealed higher percentages of small and lower proportions of medium size follicles in old cows while the COC quality was not affected. Further, the least squares mean blastocyst rate obtained after IVM and IVF of COCs was significantly ($P<0.05$) higher in young cows (17.4%) than in heifers (5.1%). The culture of oocytes after SCNT resulted in similar cleavage and morula rates.

Experiment 2 investigated the interaction of donor age and FSH-superstimulation

using the set of animals from experimental period 1 (n = 5 per age class). During 9 OPU sessions performed in 5-week intervals proportions of *in vivo* matured oocytes between 65.0% and 75.1% on average were obtained. FSH-superstimulation significantly increased the numbers of follicles aspirated ($P<0.001$) and COCs recovered ($P<0.001$) as compared to non-superstimulated donors. Follicle size was shifted to less small and more medium and large follicles. Further, there was a marked positive effect of FSH-superstimulation on cleavage and blastocyst rates in all age classes ($P<0.001$). Importantly, the developmental deficit of heifer COCs after IVM was rescued by FSH-treatment and *in vivo* maturation.

7. Zusammenfassung

Einfluss des Spenderalters auf die Entwicklungskapazität boviner Kumulus-Oozyten-Komplexe nach wiederholten Follikelpunktionen an unstimulierten und FSH-superstimulierten Färsen und Kühen verschiedener Altersklassen der Rasse Deutsches Fleckvieh

Fruchtbarkeitsrelevante Ähnlichkeiten zwischen dem weiblichen Rind und der Frau hinsichtlich der Follikulogenese, Oogenese und der natürlichen altersbedingten Abnahme der Fertilität erlauben die Nutzung des bovinen Modells für Forschungsarbeiten im Bereich der Physiologie und Pathologie der Fruchtbarkeit. Das Ziel der vorliegenden Arbeit war es, den Einfluss des Alters auf die Follikelanzahl, den COC-Ertrag und die COC-Qualität sowie auf die Entwicklungskompetenz von *in vitro* vs. *in vivo* gereiften Oozyten nach Follikelpunktion von nicht-superstimulierten und FSH-superstimulierten Spendertieren zu untersuchen. In einem weiteren Versuch wurde die Entwicklungskompetenz von den gewonnenen Oozyten nach Kerntransfer mit bovinen fetalen Fibroblasten untersucht. Nachdem das Individuum selbst, sowohl beim Rind als auch beim Menschen, einen großen Einfluss auf die Ergebnisse von Reproduktionsbiotechniken ausübt, wurden die vorliegenden Untersuchungen an den selben Tiergruppen durchgeführt, um inter-individuelle Einflüsse möglichst gänzlich auszuschließen. Im Versuch 1 wurde der Einfluss des Alters von nicht-superstimulierten Oozytenspendern der Rasse Deutsches Fleckvieh auf die Anzahl und Qualität der COCs untersucht. Es wurden Färsen (n = 12, 14 Monate alt zu Beginn der Untersuchungen), junge Kühe in deren ersten Laktation (n = 8, 2-4 Jahre alt) und alte Kühe (n = 8, 10-15 Jahre alt) in zwei unabhängige Versuchsdurchgänge aufgeteilt (5/5/5 im ersten Durchgang und 7/3/3 im zweiten Durchgang) und jeweils 32 mal und 6 mal punktiert. Trotz unterschiedlicher Follikelpunktionsergebnisse zwischen den Durchgängen, wurden einheitlich einige Parameter durch das Alter der Spender beeinflusst. Die Follikelanzahl stieg mit dem Alter der Spender ($P<0,001$), genauso wie die Zahl der gewonnenen COCs ($P<0,01$). Bezüglich der Follikelgrößen wurden prozentual mehr kleine und weniger mittelgroße Follikel in der Gruppe der alten Kühe beobachtet, wobei die Qualität der COCs nicht unterschiedlich war. Die Least Squares-basierte Analyse der Mittelwerte zeigte, dass die Blastozystenraten nach IVM und IVF signifikant

höher (P<0,05) in der Gruppe der jungen Kühe (17,4%) als bei den Färsen (5,1%) waren. Die Ergebnisse des Kerntransfers zeigten keine Unterschiede zwischen den Altersgruppen hinsichtlich der Teilungs- und Morularaten.

Im Versuch 2 wurde die Interaktion zwischen Spenderalter und FSH-Superstimulation untersucht, wobei hierfür die selben Tiere des ersten Durchgangs des ersten Versuchs verwendet wurden (n = 5 pro Altersgruppe). Bei den im 5-Wochen-Rhythmus durchgeführten 9 Follikelpunktionssitzungen, wurden im Durchschnitt 65,0% - 75,1% *in vivo* maturierte Eizellen gewonnen. Die FSH-Superstimulation der Spender führte zu einem signifikanten Anstieg der Follikelzahlen (P<0,001) und der gewonnenen COCs (P<0,001) im Vergleich zu den Ergebnissen von nicht-superstimulierten Tieren. Die Verteilung der Follikel nach ihrer Größe veränderte sich nach FSH-Superstimulation der Spender von prozentual weniger kleinen zu mehr größeren Follikeln. Desweiteren wurden nach FSH-Superstimulation in allen Altersgruppen höhere Teilungs- und Blastozystenraten erzielt (P<0,001). Die Ergebnisse der vorliegenden Arbeit erlauben den Schluss, dass das schlechtere *in vitro* Entwicklungspotential von Eizellen aus Follikelpunktion bei Färsen im Vergleich zu dessen bei älteren Tieren durch eine FSH-Superstimulation der Spender und somit durch die *in vivo* Maturation der Eizellen verbessert werden konnte.

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9. Index of Figures

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11. Appendix

11.1. Apparatuses

11.1.1. OPU

Aloka, Tokyo, Japan:

SSD 500 with a UST-9111, 5 MHz convex array transducer

Sony, Japan:

Magnification screen Triniton KX 14 CP1

Tecnologia Aplicada (WTA), Cravinhos, SP, Brazil,

Probe holder (60 cm)

11.1.2. Follicle aspiration unit

William Cook Europe, Mönchengladbach, Germany:

V-OPAA-1860 puncture needle for follicle aspiration

V-OPAL-1800 flexible tube for follicle aspiration

V-MAR-CT-300 LL disposable KMAR vacuum hose with bacteria filter

K-MAR Vacuum pump

11.2. Laboratory equipment

Binder GmbH, Tuttlingen, Germany:

CO_2-Incubator

Medcenter Einrichtungen GmbH, Planegg/München, Germany:

CO_2-O_2-Incubator

Holten, Allerød, Denmark:

Flow, Lamin Air, HV 2448

Wild Heerbrugg, Switzerland:

M3B Stereomicroscope

Leica, Wetzlar, Germany:

MS 5 Stereomicroscope

Heraeus Sepatech GmbH, Osterode, Germany:

Megafuge 1.0 R, Centrifuge

11.3. Consumables

Nunc, Roskilde, Denmark:

Falcon tube (50 mL) for collection of follicular fluid

Sarstedt, Nümbrecht, Germany:

Serum-Monovetten, 9 mL for blood collection

Henry Schein® vet GmbH, Hamburg, Germany:

18 G hypodermic needles

20 G hypodermic needles

2 mL (3 mL) single use syringes, sterile

5 mL (6 mL) single use syringes, sterile

10 mL (12 mL) single use syringes, sterile

Nunc, Roskilde, Denmark

4-well dishes

92 mm × 17 mm dishes

Millipore, Bedford, MA, USA:

Millex®-GP sterile filters (pores: Ø = 0.22 µm)

BVN Lindenhof, Germany:

Sperm, Sire „Mindel“ 9957197, 400 µL/portion

11.4. Drugs

Intervet, Unterschleißheim, Germany:

Cloprostenol-Na, Estrumate® 20 mL

Buserelinacetat, Receptal® 10 mL

Pharmanovo, Barcelona, Spain

Follitropin (FSHp), Lutropin (LHp), Pluset® 20 mL

Pfizer, Zaventem, Belgium

Controlled intravaginal progesterone-releasing device, CIDR®

Selectavet, Weyarn-Holzolling

Procainhydrochlorid + Epinephrin, Isocain® 100 mL

11.5. Media and solutions for follicle aspiration and *in vitro* procedures

If not declared differently, all chemicals used were from Sigma, St. Louis, USA.

Medium for follicle aspiration

TCM:	500.0 mL	TCM 199 Hepes (Biochrome AG, Berlin, Germany)
	30.0 mg	heparin

Filter medium

PBS-basic mix	95.5 g	PBS powder
	10.0 L	H_20 bidest
	400.0 mg	streptomycin
	300.0 mg	penicilline
	1320.0 mg	$CaCl_2H2O$
	1210.0 mg	$MgCl_2H_2O$

Maturation medium

Modified Parker`s Medium (MPM)

100 mL solution 1:	600.0 mg	lactic acid
	100.0 mL	aqua bidest
1000 mL solution 2:	1000.0 mL	Tissue Culture Medium TCM 199 (Life Technologies, Karlsruhe,

		Germany)
	100.0 mg	L-gluthamine
	800.0 mg	$NaHCO_3$
	1400.0 mg	Hepes
	250.0 mg	pyruvic acid
	1100.0 µL	gentamycin stock solution
Supplementation (10 mL):	5.0%	Estrus cow serum (ECS)
	50.0 µL	(= 0.025 U/mL maturation medium) FSH
	50.0 µL	(= 0.0125 U/mL maturation medium) LH

Swim-up medium for sperm capacitation

Sperm TALP

500 mL solution:	2900.0 mg	NaCl
	1045.0 mg	$NaHCO_3$
	20.0 mg	$NaH_2PO_4H_2O$
	1190.0 mg	Hepes
	5.0 mg	phenol red
	1825.0 µL	Na lactate sirup (60%)
	155.0 mg	$MgCl_2H_2O$
	192.0 mg	$CaCl_2H_2O$
Supplementation (10 mL):	60.0 mg	bovine serum albumine (BSA)
	500.0 µL	pyruvate stock

Medium for *in vitro* fertilization

Fert TALP

500 mL solution:	3330.0 mg	NaCl
	117.5 mg	KCl
	1051.5 mg	$NaHCO_3$
	23.5 mg	$NaH_2PO_4H_2O$

	32.5 mg	penicilline
	5.0 mg	phenole red
	930.0 mg	Na lactate sirup (60%)
	50.0 mg	$MgCl_2H_2O$
	198.5 mg	$CaCl_2H_2O$
Supplementation (10 mL):	60 mg	BSA
	100 µL	pyruvate stock
	250 µL	heparin stock

Medium for *in vitro* culture

Synthetic oviduct fluid (SOF)

500 mL solution:	31460.0 mg	NaCl
	267.0 mg	KCl
	81.0 mg	KH_2PO_4
	123.9 mg	$CaCl_2H_2O$
	48.3 mg	$MgCl_2H_2O$
	1053.0 mg	$NaHCO_3$
	0.7 mg	phenol red
	181.5 mg	pyruvate
	2500.0 mg	L-gluthamine stock
	235.3 µL	Na lactate sirup (60%)
Supplementation (10 mL):	400 µL	amino acid solution B-6766 50x, BME
	100 µL	non-essential aminoacid solution M-7145 100x, MEM
	5.0 %	EC

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