

## Improving oocyte quality in cows and heifers - What have we learned so far?

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### Abstract

The major challenge in assisted reproduction technologies remains producing oocytes with optimal developmental competence. Such developmental competence can be defined as the ability of the oocyte to fulfill maturation, undergo successful fertilization, reach the blastocyst stage and yield a viable and healthy progeny. The main follicular parameters linked to oocyte competence are presented in this review: follicle size, health/atresia status, effects of superstimulation and level of differentiation. In a commercial environment, exogenous FSH superstimulation combined with FSH starvation (referred to as the coasting period) and ovum-pick up followed by *in vitro* maturation and fertilization, leads to maximal blastocyst rates in *Bos taurus* heifers and cows. This coasting period increases the proportion of medium-to-large size follicles and may induce slight atresia in the follicular cells, which improves developmental competence. The optimal period for oocyte retrieval in this context has been recently characterized in cows and is related to follicular size and state of differentiation. Apoptosis related signals have been identified in various studies as implicated in antral folliculogenesis. Follicular somatic cell transcriptome studies are currently used to identify competence related markers. Finally, oocyte competence acquisition is a complex and dynamic process. The best oocytes are obtained from follicles of the optimal size and differentiation status, which can be manipulated with the right hormonal regimen.

**Keywords:** ART, *Bos taurus*, coasting period/FSH starvation, developmental competence/oocyte quality, follicle.

### Introduction

Over the course of the last decades, assisted reproductive technologies (ARTs) have been successfully used to enhance production in domestic animal species. Among these methodologies, *in vitro* fertilization (IVF) and *in vitro* embryo production have allowed both the quick propagation of embryos from parents with genetically desired characteristics for the animal breeding industry, and the supply of biological material for research in reproductive science. The major challenge for ARTs has been the acquisition of oocyte developmental competence. This review will summarize the advances made in determining the optimal

superovulation protocols for Holstein heifers/cows to produce a maximal number of developmentally competent oocytes for IVF use.

### Developmental competence

The quest in understanding acquisition of oocyte developmental competence is an ongoing process both in fundamental and applied research in several livestock species including the bovine. The literature has clearly shown that acquisition of this competence is a multi-factorial process that is difficult to assess using only morphological oocyte parameters currently reported worldwide (Sirard *et al.*, 2006). Oocyte competence is the ability of the oocyte to complete maturation, undergo successful fertilization, reach the blastocyst stage and yield a viable and healthy progeny following embryo transfer (Watson, 2007). This developmental ability is acquired by the oocyte through the biosynthesis and/or storage of many key molecules. These molecules are the result of key molecular processes that occur both in the nucleus and ooplasm during oocyte growth and maturation. The storage of a suitable stockpile of these molecules is referred to as molecular maturation (Sirard *et al.*, 2006). Molecular maturation is the process whereby a suitable supply (in a time-, space- and dose-dependent manner) of the required factors are acquired to render an oocyte developmental competent. The oocyte is almost constantly changing from the primordial stage to ovulation. It seems that even after the oocyte has reached its final size, the transformation continues. The final changes occur when the oocyte progressively decreases its transcriptional activities (Fair *et al.*, 1995). This transcriptional decline was shown by both uridine incorporation experiments and a progressive change in the nucleolar structures during the dominant preovulatory period (Lodde *et al.*, 2008; Tan *et al.*, 2009). For the mouse, rabbit, sheep, goat, cow, horse, and human, the progressive configuration from diffuse to more condensed chromatin and from non-surrounded nucleolus to surrounded nucleolus is associated with a progressive shutdown of the transcriptional machinery (Escrich *et al.*, 2009; Tan *et al.*, 2009). Therefore it is possible to conclude that when the oocyte finally has all the RNA required, it has acquired its optimal developmental competence.

Although the major molecular mechanisms involved in the acquisition of oocyte competence are not well known, many clues arise from the follicular

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environment (Barnes and First, 1991; Hyttel *et al.*, 1997; Sirard and Trounson, 2003). In many mammalian species, especially in large mono-ovulating ones, it has been established that the follicular environment has a clear impact on the oocyte's capacity to develop beyond fertilization. The main follicular parameters that can be assessed are size, health/atresia status, effects of superstimulation and level of differentiation.

### Follicle size

Follicle size is an important parameter that influences oocyte competence. Although successfully fertilized after IVF, oocytes from smaller bovine and

porcine follicles (<3 mm) have reduced to no developmental competence (Lonergan *et al.*, 1994; Blondin and Sirard, 1995; Marchal *et al.*, 2002). These oocytes appear to have been recovered too early and therefore probably lacked some additional follicular factors that would have signalled the oocytes to acquire their full competence. Data collected from our commercial facility confirm this trend where larger follicles following superstimulation contain oocytes with higher developmental competence (Fig. 1). Indeed, the percentage of transferable embryos produced from the recovered oocytes increases with the diameter of the follicles at the moment of oocyte recovery after superstimulation and coothing.

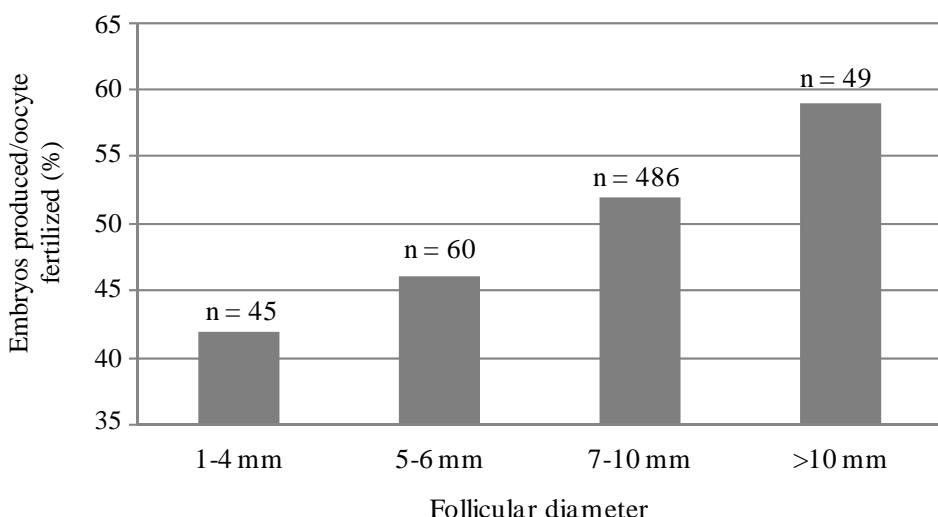


Figure 1. Relation between the percentage of transferable embryos (morula or blastocysts of quality 1 or 2) produced after 7 or 8 days of *in vitro* culture vs. follicle diameter of the majority of the follicles (>2-3 mm) at the time of OPU. (n = number of OPU sessions).

Previous studies have evaluated the effects of follicular characteristics (such as diameter and percentage of atretic granulosa and theca cells) on the oocyte's ability to acquire developmental competence (Pavlok *et al.*, 1992; Lonergan *et al.*, 1994). Only a few studies have evaluated this on an individual basis confirming an increased competence with follicle size (Blondin and Sirard, 1995; Hagemann *et al.*, 1999). Even cloning experiments support the concept of increased developmental competence of oocytes from large follicles (Barnes *et al.*, 1993). Although size has a significant effect, some oocytes originating from large follicles still fail to produce embryos, whereas some oocytes from medium-sized follicles (5-19 mm) already have this capacity. Recently, we demonstrated for the first time that, in extreme FSH starvation conditions (92 h), larger but still growing follicles contained oocytes associated with decreased competence (Fig. 2;

Nivet *et al.*, 2012). Those larger follicles were exposed to a very long starvation period, and thus it is difficult to exclude the possibility that this competence reduction is not related to some level of atresia caused by this longer FSH starvation in the absence of an increased LH pulsatility more than by the size of the follicles. This work has also demonstrated that the highest embryo production rate was obtained when the follicles were >7 mm following FSH superstimulation combined with shorter coothing durations (44 to 68 h; Fig. 2). This is consistent with the results obtained in our commercial setting (Fig. 1). It is however important to note that the ideal follicle diameter in a commercial context is 7 to 10 mm as follicles >10 mm are very challenging to recover using traditional aspiration techniques. Development of better ovum pick-up (OPU) procedures for large follicular diameters could be useful for veterinarian practitioners performing such procedures.

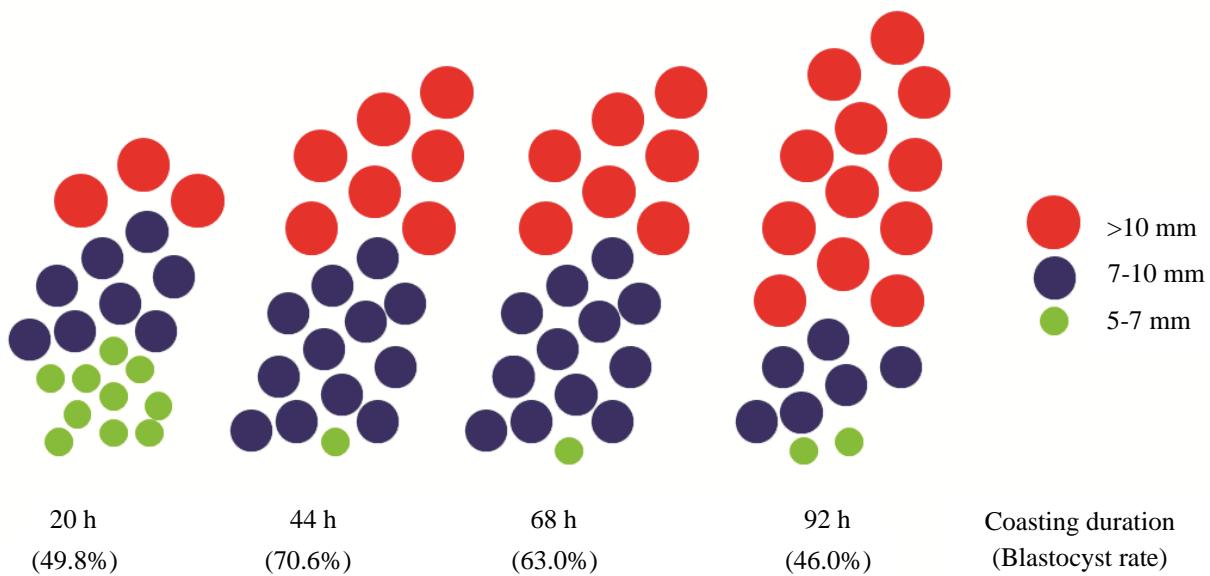


Figure 2. Relationship between the coasting time (FSH starvation) and follicle diameter and blastocyst rates. The follicular diameter is based on the frequency per class as determined by ultrasound.

#### Healthy/atretic status of the follicle

The dominant follicle, as well as large subordinate follicles, is generally healthy or very slightly atretic and their oocytes preserve good developmental competence (blastocyst rate; Vassena *et al.*, 2003). Follicular atresia begins with signs of follicular cell apoptosis in granulosa cells and then show signs in cumulus cells and finally the embedded oocyte (Zeuner *et al.*, 2003). Atretic follicles marked by disrupted cumulus layers demonstrated poor developmental competence. It was previously demonstrated that oocyte viability was not necessarily related to the degree of atresia of sheep follicles cultured *in vitro* to mature oocytes for fertilization and development to term (Moor and Trounson, 1977). Similar observations have been noted in rats (Tsafriri and Pomerantz, 1984) and other species including humans (Barnes *et al.*, 1993). In cattle, we were the first to demonstrate that higher developmental competence was achieved in bovine cumulus-oocyte complexes with slight signs of apoptosis in the outer layers of the cumulus (Blondin and Sirard, 1995; Zeuner *et al.*, 2003; Feng *et al.*, 2007). In fact, the group of oocytes with the highest competence possessed a less compact cumulus and originated from healthy or slightly atretic follicles, as measured by histology or flow cytometry (Blondin *et al.*, 1996). These early apoptotic events might be seen as a form of maturation-promoting/accelerating signals to the oocyte to improve its competence. Another study (Salamone *et al.*, 1999) revealed that during the growth of the first follicular wave, oocytes from the subordinate follicles remain viable throughout the static phase, but undergo degenerative changes in the regressing phase following the appearance of the dominant follicle. Yet another

study evaluated the number of blastocysts obtained when aspiration was done on days 3, 4 or 5 of the first follicular wave; three times more viable embryos were produced at day 5 when dominance had occurred, compared with day 3 where all follicles were growing (Machatkova *et al.*, 2000). A recent paper described the progressive chromatin condensation of the nucleus in bovine oocytes as they were harvested from follicles of different sizes and health statuses (Lodde *et al.*, 2008). Our interpretation of their results is that transcription is progressively shut down in follicles approaching ovulation, or in subordinate follicles beginning atresia, which lead to the same consequence: better ability to develop when the transcription machinery is off (increased developmental competence).

#### Superovulation and competence

Recent developments in our understanding of reproductive physiology and hormonal regulation of the follicular waves have improved superovulation strategies in order to mimic the physiological/normal hormonal patterns. This hormonal induction is normally used prior to the onset of dominance at 8.5 mm of follicular diameter in cows (Ginther *et al.*, 1997), therefore allowing the recovery of a homogenous population of follicles (Ginther *et al.*, 2000; Merton *et al.*, 2003).

Stimulation with gonadotropins is normally used for regular embryo transfer where cows are stimulated, inseminated and flushed on day 7. The use of stimulation to recover mature oocytes (post LH surge) prior to fertilization has been mainly developed for human IVF. Very few studies have been done in cows with IVF where mature oocytes are recovered as in humans (Sirard and Lambert, 1985) as IVM became

possible as early as 1988 (Sirard *et al.*, 1988).

However, despite hundreds of technical papers on the improvement of both oocyte and embryo *in vitro* culture conditions, the average developmental rate to blastocyst after IVM has not changed significantly before 2002 (Sirard *et al.*, 2006). This situation has changed with the introduction of FSH coasting i.e., FSH withdrawal before OPU (Blondin *et al.*, 2002).

In cows, coasting refers to the arrest of gonadotropin support in the presence of endogenous LH (Ginther *et al.*, 1998) to stimulate final follicular differentiation and oocyte competence (Sher *et al.*, 1995; Blondin *et al.*, 2002). This gonadotropin starvation (or coasting period) exerts a selective pressure that eliminates the smaller follicles and increases the proportion of medium-to-large follicles (Fluker *et al.*, 1999; Blondin *et al.*, 2002). Moreover, this coasting may induce slight atresia in the cumulus outer layers, which will result in higher numbers of grade 3 cumulus oocyte complexes and therefore improved developmental competence (Blondin and Sirard, 1995). This early atresia is consistent with the pro-survival role of FSH on antral follicles (Chun *et al.*, 1996). The coasting strategy is therefore a useful tool to improve oocyte competence and producing a higher number of a homogenous pool of competent oocytes.

The initial coasting time was chosen based on previous studies indicating that following one bolus injection of FSH, the optimal blastocyst rate was obtained when oocytes were recovered 48 h later instead of 24 or 72 h (Blondin *et al.*, 1997). Therefore, the initial clinical trial was set for a coasting of 33 vs. 48 h following the last FSH injection and both periods resulted in a significant increase in competence compared to slaughterhouse controls. These findings are confirmed by the high rates of cleavage (90%) and blastocyst (80%) following 48 h of coasting in the Holstein cow (Blondin *et al.*, 2002).

Assuming that a coasting period between the last FSH injection and the LH surge is favorable for acquisition of oocyte developmental competence, we hypothesize that competence is initially lower because of the growing status of the follicle where differentiation has not begun and on the other hand, if FSH withdrawal is done for too long, differentiation is too far advanced resulting in atresia because of the non-ovulatory context of the corpus luteum (Nivet *et al.*, 2012). Identifying the ideal 'window' of acquisition of developmental competence is the true challenge in FSH superstimulation protocols.

#### Low responder vs. low potential cows

In 2005, we published a paper that described the difference between low responders and low potential cows. Low responders (less than 10% of the animals in that study) were defined as animals with a lower than average follicular response following superstimulation (Durocher *et al.*, 2006). Low potential animals were

defined as donors producing a limited number of embryos because of the limited population of small antral follicles present in the ovaries at initiation of FSH treatment. The paper concluded by indicating that embryo transfer practitioners must distinguish between low responders and low potential animals as modifications to the stimulation protocol for the latter group is unlikely to result in a higher number of transferable embryos. In fact, recent studies have demonstrated that heifers and cows with ovaries with inherent low ovarian reserves (low antral follicle count) will have this phenotype for their entire reproductive life (Abdullah *et al.*, 2008; Ireland *et al.*, 2011; Mossa *et al.*, 2012). These studies even suggest that low antral follicle count may be the result of inadequate maternal environment during fetal development. Therefore veterinarian practitioners must re-think their superstimulation strategies when it comes to low potential animals.

#### Follicular differentiation

Several morphological, ultrastructural and metabolic criteria have been used to predict oocyte competence. Despite improvements in pregnancy outcomes, these morphological criteria remain subjective, in some cases invasive and/or poorly correlated with oocyte competence. Considering the crucial contribution of the somatic follicular compartment to oocyte quality through bidirectional and continuous communication (Armstrong and Webb, 1997; Beg and Ginther, 2006) genomic studies have focused on the follicular status correlation with gene expression. Evans *et al.* (2004) reported an increase in differentially expressed genes associated with apoptosis in subordinate versus dominant follicles. These gene markers were suggested as possible markers of the bovine dominant follicle. Similar work has suggested key genes associated with follicular status progression and dominance acquisition (Bedard *et al.*, 2003; Fayad *et al.*, 2004; Mihm *et al.*, 2006, 2008; Forde *et al.*, 2008; Mihm and Evans, 2008; Wells and Patrizio, 2008). Overall, these studies demonstrated that dominant follicles were positively associated with enhanced expression of mRNAs in granulosa cells with genes involved in cellular survival, regulation of proliferation, prevention of apoptosis or DNA damage, and RNA synthesis. On the other hand, subordinate follicles were positively associated with enhanced expression of mRNAs in granulosa cells for genes that were associated with cell death and/or apoptosis (see references cited above for review).

Other studies explored the impact of superovulation treatment on follicular transcriptome. For example, LH was shown to induce an early effect (6 h after the LH surge) marked by the transcription of ovulation-related genes in bovine granulosa cells *in vivo*. In the case of the late LH effect (22 h after LH surge), the granulosa transcriptome was characterized by large sets of luteinization-related genes (Gilbert *et*

al., 2011). Moreover, we recently revealed a transcriptomic signature from granulosa cells associated with competent oocytes following superstimulation (Gilbert et al., 2012). Taken together, these findings confirm expectations regarding follicular cell behavior prior to ovulation and offer useful target genes to improve our understanding of molecular physiology and regulation (see references cited above for review).

Other reports have also analyzed the gene expression patterns associated with ovarian stimulation (Grondahl et al., 2009; Gilbert et al., 2011) or *in vitro* culture (Jones et al., 2008; Tesfaye et al., 2009) using the *in vivo* context as reference. The goal was to identify quantitative and non-invasive gene markers that accurately predict oocyte competence and reinforce the morphological criteria already used. These studies demonstrated that granulosa cells from follicles collected before the LH surge were associated with gene expression involved in typical tasks such as cell division, development, and proliferation, whereas granulosa cells from follicles subjected to the LH surge included features such as response to stimulus, vascularization, and lipid synthesis, which are indicative of cells preparing for ovulation. Furthermore, blastocysts that implanted and resulted in pregnancies expressed transcripts involved in cell adhesion and cell communication. Finally, cumulus cells derived from cumulus–oocyte complexes matured *in vivo* expressed transcripts of genes associated with cumulus expansion and regulation of oocyte maturation while cumulus cells derived from oocytes matured *in vitro* were enriched with genes involved in response to stress (see references cited above for review). Several studies performed in many mammalian species including cattle (Burns et al., 2003; Fayad et al., 2004; Bettegowda et al., 2008; Gilbert et al., 2011) have revealed key gene markers that could increase the efficiency of ARTs.

### Benefits of superovulation with IVF

Table 1 demonstrates the advantage of IVF compared to *in vivo* embryo flushing for a 60-day period. It is clear that four IVF cycles using

superovulated donors can be done in the same time a single *in vivo* embryo flush is performed (Blondin et al., unpublished data). Therefore, when using conventional semen (non-sexed semen), dairy producers can expect to produce 5.2 times more female embryos (11.0 vs. 2.1, respectively) and 4.8 times more female day 60 gestations (5.7 vs. 1.2, respectively) using IVF compared to *in vivo* flushes. Additionally, if clients choose to use sexed semen, they can expect to produce 9.4 times more female embryos (19.8 vs. 2.1, respectively) and 7.9 times more female day 60 gestations (9.5 vs. 1.2, respectively) using IVF compared to *in vivo* flushes. It is important to note that every animal does not respond the same to superovulation protocols. As recently published, the optimal coasting window is quite large (20 to 92 h) due to variations between the different donors (Nivet et al., 2012). In fact, in this study, it was calculated that the ideal superstimulation protocol included 54 h of coasting. In our commercial context, we usually start with a standard protocol, which consists of 6 injections of FSH followed by 43 h of coasting before OPU. For the majority of the cases, this protocol gives a large proportion of follicles around 7 to 10 mm in diameter and produces a high proportion of competent oocytes. However, with some donors, modifications to the length of the coasting period and/or the number of FSH injections were applied to produce a high proportion of follicles around 7 to 10 mm. By modifying superstimulation parameters, it is possible to bring almost all donors in the optimal window of follicle size and therefore optimal window of acquisition of developmental competence. In our commercial production, 77% of the donors showed an optimal coasting duration of 43 h, whereas 10% showed better results with 30 h of coasting and 15% preferred 54 h of coasting. Usually, the standard superstimulation protocol is applied at least for the first two IVF sessions to confirm if the protocol needs to be modified and each animal is monitored carefully to determine its ideal protocol that will be applied for future superstimulation cycles. However, the application of that concept is not an exact science since variations occur also between different superstimulation cycles in the same animal and many other factors can influence the final outcome of IVF.

Table 1. Advantage of IVF over embryo flushing: comparison of a 60 day period.

Endpoints/semen	<i>In vitro</i> (IVF)		<i>In vivo</i> (flush)
	Non-sexed	Sexed	Non-sexed
OPU or flush	4	4	1
Oocytes	41.6	41.6	7.3
Viable embryos	23.3	20.4	4.3
Female embryos	11.0	19.8	2.1
Gestation (Day 30) - Female calf	6.3 (57%)	11.1 (56%)	1.3 (61.0%)
Gestation (Day 60) - Female calf	5.7 (52%)	9.5 (48%)	1.2 (59.4%)

Average rates based on 895 OPU sessions from 275 superstimulated Holstein heifers or cows.

There are commercial groups that perform IVF cycles using natural, non-superovulated cycles (Galli *et al.*, 2004; Merton *et al.*, 2012). It is clear that the developmental competence of oocytes from natural cycles will be lower than oocytes from superovulated cycles. In our commercial setting using superovulation and coasting, non-sexed semen results in average blastocyst rates of 56%. The commercial groups referenced above obtain 16 to 25% average blastocyst rates using non-superovulated donors. Using natural cycles, a reproductive specialist could perform OPUs once or twice a week subjecting the donors to many more invasive OPU sessions augmenting the risks to the donors. With the correct superovulation strategies, it is clear that there are more benefits to use such strategies

and perform OPUs every 2 weeks diminishing any risks and maximizing the embryo production rates from a donor. It is important to note that natural cycles remain an alternative to donors that do not respond well to superovulation (true low responders) or are allergic or insensitive to the hormones used in superovulation protocols. In our commercial setting in 2010 and 2011, 64 IVF sessions were done using oocytes from non-superstimulated animals with a blastocyst developmental rate of 23% (which is comparable to the other studies cited above) compared to the 56% obtained from superovulated animals with coasting. The lower blastocyst rates (and thus lower oocyte developmental competence) correlates with smaller follicle diameters at the time of OPU as shown in Fig. 3.

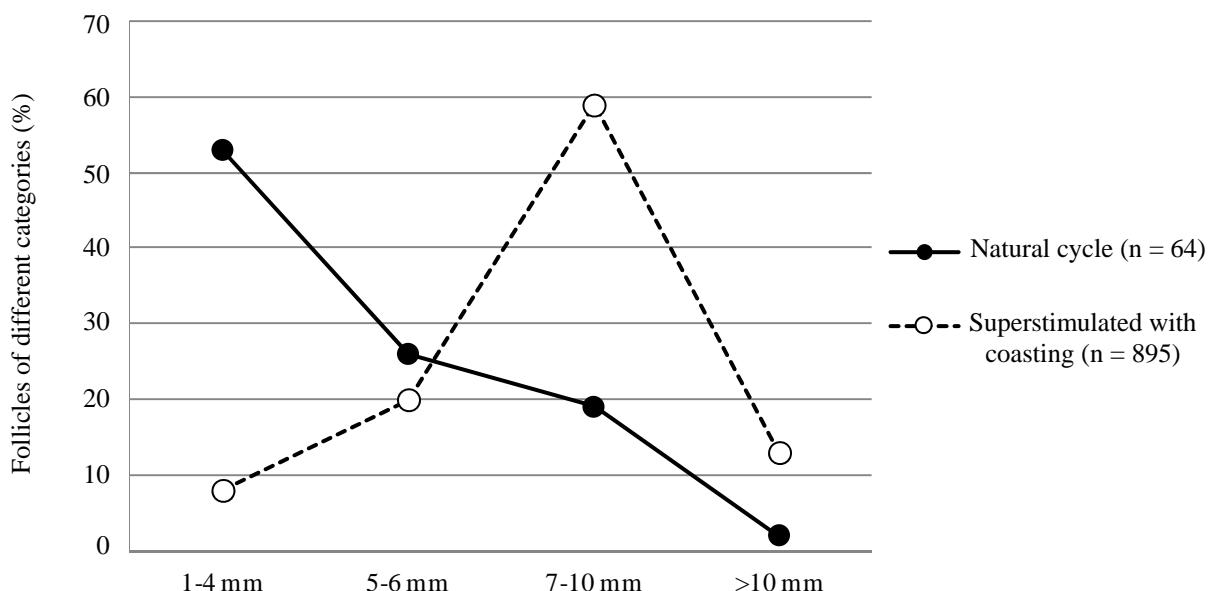


Figure 3. Mean representation of the follicle status at time of OPU in natural *vs.* superstimulated cycles followed by a FSH coasting period.

### Conclusions

Oocyte developmental competence is a complex process that translates to the ability of the oocyte to complete maturation, undergo successful fertilization, reach the blastocyst stage and yield a viable and healthy progeny following embryo transfer. The successful achievement of each one of the levels of competence discussed in this review requires the ideal and well-orchestrated effect of sequences of molecules (and molecular pathways) at specific space, time and magnitude levels. Although still not fully defined, scientists are getting a better idea of the conditions necessary to achieve complete developmental competence. These include decreased oocyte transcriptional activities and accumulation of all necessary RNA, increased diameter of the follicles at the moment of oocyte recovery after FSH superstimulation with shorter coasting durations (44 to 68 h for Holstein cows), oocytes

originating from healthy or slightly atretic follicles (early apoptotic events could be seen as a form of maturation-promoting/accelerating signals to the oocyte to improve its competence), and activation of specific genes associated with preovulatory follicles as described in this review. The ideal 'window' of acquisition of developmental competence during follicular growth is the true challenge in FSH superstimulation protocols and may even vary in different species of different animals.

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