Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2022:22

In vitro embryo production in Norwegian Duroc and Landrace pigs

In vitro embryoproduksjon i norske duroc og landsvin-griser

Reina Jochems

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Hamar, March 2022

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Abbreviations and definitions

AI	Artificial insemination
AIL	Acrosome intact live
AN	Androstenedione
ART	Assisted reproductive technologies
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CA	Corpus albicans
CASA	Computer assisted sperm analysis
CG	Cortical granules
CH	Corpus hemorrhagicum
CL	Corpus luteum
COC	Cumulus-oocyte complex
DFI	DNA fragmentation index
DHEA	Dehydroepiandrosterone
E1	Estrone
E2	Oestradiol
Em	Emission
Ex	Excitation
FBS	Foetal bovine serum
FF	Follicular fluid
FITC	Fluorescence isothiocyanate
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GSH	Glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
ICSI	Intracytoplasmic sperm injection
IVC	*In vitro* culture
IVD	*In vivo* derived
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* produced
LH	Luteinizing hormone
ND	Norwegian Duroc
NL	Norwegian Landrace
OPU	Ovum pick up
OSF	Oocyte-secreted factor
P4	Progesterone
PGF2-α	Prostaglandin F2 alpha
PGM	Porcine gamete medium
PNA	Peanut agglutinin
POM	Porcine oocyte medium
PREG	Pregnenolone
PROG	Progressive motility
PXM	Porcine X washing medium
PZM	Porcine zygote medium
ROS	Reactive oxygen species
SAS	Statistical analysis system
SCSA	Sperm chromatin structure assay
TS	Testosterone
TNB	Total number piglets born
ZP	Zona pellucida

List of papers

Paper I Jochems, R., Gaustad A.H., Zak L.J., Grindflek E., Zeremichael T.T., Oskam I.C., Myromslien F.D., Kommisrud E., Krogenæs A.K. 2021. Ovarian characteristics, and *in vitro* nuclear and cytoplasmic oocyte maturation in Duroc and Landrace pigs. *Veterinary medicine and Science 7(5):1845-1853.*

Paper II Jochems, R., Gaustad A.H., Zak L.J., Grindflek E., Zeremichael T.T., Oskam I.C., Myromslien F.D., Kommisrud E., Krogenæs A.K. 2022. Effect of two 'progressively motile sperm-oocyte' ratios on porcine *in vitro* fertilization and embryo development. *Accepted in Zygote.*

Paper III Jochems, R., Gaustad A.H., Bjarne Styrishave, Zak L.J., Oskam I.C., Grindflek E., Myromslien F.D, Kommisrud E., Krogenæs A.K. 2022. Follicular fluid steroid analysis and *in vitro* embryo development in Duroc and Landrace pigs. *Submitted to Theriogenology.*

Summary

The use of embryo technology is of interest in pig breeding to increase genetic gain in the breeding program and disseminate genetics of superior animals worldwide with less transportation of live animals and a lower risk of disease transmission. In Norway, breeding programs exists for two breeds with different reproductive characteristics. The Norwegian Duroc sire line has on average 9.7 total number piglets born per litter compared to 14.3 piglets in the Norwegian Landrace dam line. Breed differences within this trait could be related to ovarian characteristics, which may also affect *in vitro* embryo production. This could require different optimizations to successfully obtain *in vitro* blastocysts in both breeds. Therefore, the overall aim of this thesis was to establish protocols for porcine *in vitro* embryo production and staining of oocytes and embryos to acquire knowledge about *in vitro* maturation, fertilization and embryo culture in Norwegian Duroc and Norwegian Landrace.

Firstly, ovarian characteristics and *in vitro* nuclear and cytoplasmic oocyte maturation were studied for both breeds one day after weaning in paper I. It was observed that Landrace ovaries contained significantly more 3-8 mm surface follicles compared to Duroc, and thus more oocytes can be collected for IVEP. The individual Duroc cumulus-oocyte complexes (COCs) covered a smaller area at time of collection, but a broader cumulus expansion was observed from 0-20 h compared to Landrace. More Duroc oocytes exhibited advanced stages of nuclear maturation at 20 h of maturation, while more Landrace oocytes showed advanced stages of cortical granule (CG) distribution. Nuclear maturation to MII stage at 48 h did not differ between Duroc and Landrace and was consistently high in both breeds. In addition, no significant differences were observed for glutathione content or CG distribution at the end of maturation. It was therefore questioned if subsequent *in vitro* fertilization and embryo development would be different between the breeds.

In paper II, *in vitro* fertilization was optimized and the effect of two 'progressively motile sperm to oocyte ratios' on fertilization and embryo development was assessed. Random sow oocytes of unknown breed in both the luteal and follicular phase of the oestrus cycle were fertilized with frozen-thawed sperm from three Duroc and three Landrace boars at a 250:1 and 500:1 progressively motile sperm to oocyte ratio. Fertilization with the 500:1 ratio resulted in a higher fertilization and blastocyst yield on day 6 compared to the 250:1 ratio, while no effect of ratio was observed for polyspermy, cleavage rate or blastocyst cell number. Individual differences between

boars were observed for fertilization, cleavage and blastocyst rates although all IVEP rounds were adjusted for the same number of progressively motile sperm cells present per oocyte. Promising blastocyst yield and high total blastocyst cell numbers were obtained with cryopreserved sperm from both Duroc and Landrace boars.

Since differences were observed between the breeds regarding ovarian characteristics, and nuclear and cytoplasmic oocyte maturation parameters at 20 h of maturation, the aim of paper III was to subsequently assess *in vitro* embryo development. In addition, follicle diameter and follicular fluid (FF) steroid hormones were measured to study possible relation to oocyte developmental competence. In paper I it was observed at the end of the study that parity, and thus age, was higher in Duroc animals compared to Landrace and this may have affected the maturation results. Therefore, only sows with one or two litters were selected in both breeds for the experiments in paper III. On average a larger follicle diameter was observed on Landrace ovaries compared to Duroc. Individual COC area was larger for Landrace at the start of maturation, while cumulus expansion from 0 to 20 h of maturation was greater for Duroc oocytes and this confirmed the results from paper I. After fertilization, cleavage rate was higher for Duroc oocytes and the highest blastocyst yield was obtained for Duroc oocytes fertilized with the Landrace boar. No significant differences were observed between the breeds for the individual steroid hormones, but differences in patterns were observed in the steroid pathways. Both the total level of oestrogens, and aromatase products/substrates ratio were higher in Landrace FF than in Duroc, indicating a higher degree of feminization in Landrace. In conclusion, breed differences were found at the early follicular phase regarding *in vitro* oocyte developmental competence and steroidogenesis.

In summary, differences between the breeds were observed during *in vitro* maturation, fertilization and embryo culture. Results suggest that Duroc oocyte have a better developmental competence during *in vitro* embryo production. Further studies are recommended to optimize IVEP protocols per breed and to find out how differences between breeds are related to oocyte developmental competence. The work in this thesis has direct practical relevance for Norsvin, as the established *in vitro* embryo production and staining protocols and acquired knowledge can be further used in follow-up studies related to *in vitro* embryo production, embryo quality and freezing and storage of embryos. Moreover, the acquired knowledge on embryo handling and quality is valuable for future commercialisation of embryo transfers.

Sammendrag (Norsk)

Bruk av embryoteknologi er av interesse i svineavl for å øke genetisk framgang i svinepopulasjonene og for å distribuere genetikk fra verdifulle dyr over hele verden. Produksjon og transport av embryo fører til mindre transport av levende dyr og redusert risiko for overføring av smittsomsykdom. I Norge finnes det avlsprogram for to raser med ulike reproduksjonsegenskaper. Den norske farrasen duroc har i gjennomsnitt 9,7 fødte grisunger totalt per kull sammenlignet med 14,3 i norsk landsvin, som er en morrase. Raseforskjellen i antall fødte grisunger kan skyldes forhold knyttet til eggstokkene, noe som også vil kunne påvirke embryoproduksjonen *in vitro* (IVEP). Dette kan kreve ulike optimaliseringer for å kunne produsere *in vitro* blastocyster i begge raser. Målet med denne avhandlingen var å etablere protokoller for *in vitro* embryoproduksjon hos gris samt protokoller for farging av eggceller og embryoer for å skaffe kunnskap om *in vitro* modning, befruktning og embryo kultur hos duroc og landsvin.

I artikkel 1 ble eggstokker fra begge raser samlet én dag etter avvenning. Eggstokkene ble karakterisert og nukleær og cytoplasmatisk modning av oocyttene ble studert. Eggstokkene hos landsvin hadde signifikant flere 3-8 mm follikler sammenlignet med duroc, og dermed flere eggceller tilgjengelige for IVEP. I gjennomsnitt var cumulus-oocytt-kompleksene (COCs) hos duroc mindre i størrelse ved starten av kultur (0 timer), men det ble observert en større grad av cumulusekspansjon fra 0-20 timers modning sammenlignet med landsvin. Flere eggceller fra duroc var kommet lenger i nukleær modning etter 20 timers modning, mens flere eggceller fra landsvin viste avanserte stadier av fordeling av de kortikale granula (CG). Prosent fullført nukleær modning til MII-stadiet etter 48 timer var høy og lik i begge raser. Det ble heller ikke observert signifikante forskjeller i innholdet av glutathion eller for CG-fordeling ved slutten av modningen. Det ble derfor interessant å undersøke om påfølgende *in vitro* befruktning og embryoutvikling ville være forskjellig mellom rasene.

I artikkel II ble *in vitro* fertilisering optimalisert, og effekten av to forskjellige forholdstall for "progressivt bevegelige spermier per eggcelle" ble undersøkt med hensyn på befruktning og embryoutvikling. Til forsøket ble det samlet eggceller fra follikler i både follikel – og lutealfase fra ukjent donor. Til befruktningen ble det benyttet kryokonserverte spermier fra 3 duroc og 3 landsvin råner i et forhold på 250:1 eller 500:1 «progressivt bevegelige spermier per eggcelle». Befruktning med forholdstallet 500:1 resulterte i høyere befruktningsprosent og høyere

blastocystutbytte på dag 6 sammenlignet med forholdstallet 250:1, men ingen effekt av forholdstallet ble observert for polyspermi (egg befruktet av 2 eller flere sædceller), celledeling på dag 2 (som angir % befruktning) eller celleantall i blastocystene. Individuelle forskjeller mellom råner ble observert for befruktning, celledeling og blastocystprosent, selv om alle IVEP-runder ble justert for det samme antallet progressivt bevegelige sædceller per eggcelle. Lovende blastocystutbytte og høye totale blastocystcelletall ble oppnådd med kryokonservert sæd fra begge raser.

Siden det ble observert forskjeller mellom rasene med hensyn til eggstokkenes karakteristika og parametre for nukleær og cytoplasmatisk modning etter 20 timer, var målet med artikkel III å studere *in vitro* embryoutvikling i de to rasene. I tillegg ble follikkeldiameter og steroidhormoner i follikkelvæske (FF) målt for å undersøke om disse var relatert til eggcellenes utviklingsevne. I artikkel I ble det observert at kullnummer, og dermed alder, var høyere hos duroc- enn landsvinpurkene, dette kan ha påvirket modningsresultatene. Derfor ble det kun selektert purker som hadde hatt ett eller to kull til forsøkene i artikkel III. I gjennomsnitt ble det observert en større follikkeldiameter på eggstokkene fra landsvin sammenlignet med duroc. Individuelt COC-areal var større for landsvin ved starten av modningen, mens cumulusekspansjonen fra 0 til 20 timers modning var større for duroc og dette bekreftet resultatene fra artikkel I. Etter befruktning var celledeling høyere for duroc, og det høyeste blastocystutbyttet ble oppnådd for duroc-eggceller befruktet med landsvinsæd. Det ble ikke observert signifikante forskjeller mellom rasene for de enkelte steroidhormonene, men forskjeller i mønstre ble observert for steroidveiene. Både det totale nivået av østrogener og forholdet mellom aromataseprodukter og substrater var høyere i landsvin FF enn i duroc, noe som indikerer en høyere grad av feminisering i landsvin.

Oppsummert ble det observert forskjeller mellom rasene under *in vitro* modning, befruktning og embryokultur. Resultatene tyder på at eggceller fra duroc har en bedre utviklingsevne under IVEP. Videre forskning anbefales for å optimalisere IVEP protokoller per rase og for å finne ut hvordan forskjeller mellom rasene er relatert til eggcellenes utviklingsevne. Arbeidet i denne oppgaven har direkte praktisk relevans for Norsvin, da de etablerte protokollene og tilegnet kunnskap kan brukes videre i oppfølgingsstudier knyttet til IVEP, embryokvalitet og embryo frysing og lagring. Dessuten er den tilegnete kunnskapen om embryohåndtering og kvalitet verdifull for fremtidig kommersialisering av embryooverføringer.

Samenvatting (Nederlands)

Het gebruik van embryotechnologie is van belang in de varkensfokkerij om de genetische vooruitgang in het fokprogramma te versnellen en om de genetica van waardevolle dieren wereldwijd te verspreiden. Wanneer dit kan door middel van embryos, is er daarnaast ook minder transport van levende dieren nodig en is er een verlaagd risico op overdracht van ziekten. In Noorwegen zijn er fokprogramma's voor twee rassen met verschillende reproductieve kenmerken. De Noorse Duroc berenlijn heeft gemiddeld 9.7 totaal aantal geboren biggen per worp vergeleken met 14.3 biggen in de Noorse Landras zeugenlijn. Deze rasverschillen kunnen gerelateerd zijn aan kenmerken van de ovaria, die ook van invloed kunnen zijn op *in vitro* embryo productie. Het is mogelijk dat verschillende aanpassingen in protocollen vereist zijn om succesvol *in vitro* blastocysten te kunnen produceren in beide rassen. Het hoofddoel van dit proefschrift was om protocollen op te zetten voor *in vitro* embryo productie (IVEP) in varkens en voor het kleuren van eicellen en embryos om kennis op te doen over *in vitro* rijping, bevruchting en embryo ontwikkeling in de Noorse Duroc en Landras rassen.

In artikel I werden de ovaria en *in vitro* nucleaire en cytoplasmatische eicelrijping bestudeerd in beide rassen één dag na het spenen. Landras ovaria hadden significant meer 3-8 mm follikels in vergelijking tot Duroc ovaria en hierdoor kunnen dus meer eicellen verzameld worden voor *in vitro* embryo productie. Gemiddeld waren de Duroc cumulus-eicelcomplexen (COCs) kleiner van grootte op 0 uur, maar een grotere mate van cumulusexpansie werd waargenomen van 0-20 uur rijping in vergelijking met Landras. Meer Duroc eicellen waren na 20 uur rijping in verder gevorderde stadia van nucleaire rijping, terwijl meer Landras eicellen vergevorderde stadia van corticale granule (CG) distributie vertoonden. Het percentage voltooide nucleaire rijping tot MII-stadium na 48 uur was hoog en gelijk in beide rassen. Er werden bovendien geen significante verschillen waargenomen tussen de rassen in het glutathiongehalte of de CG-verdeling aan het einde van de rijping. Het was daarom interessant om te onderzoeken of de daaropvolgende *in vitro* bevruchting en embryo ontwikkeling verschillend zou zijn in de rassen.

In artikel II werd de *in vitro* bevruchting geoptimaliseerd en werd het effect van twee 'progressief beweeglijke sperma-tot-eicelverhoudingen' op de bevruchting en embryo ontwikkeling bestudeerd. Voor het experiment werden willekeurige eicellen verzamelend in zowel de luteale- als folliculaire fase van de oestruscyclus van

onbekende zeugen. Voor de bevruchting werd ingevroren sperma van drie Duroc en drie Landras beren gebruikt in een verhouding van 250:1 en 500:1 progressief beweeglijke sperma per eicel. Bevruchting met de 500:1 verhouding resulteerde in een hoger bevruchtingspercentage en hogere blastocystopbrengst op dag 6 vergeleken met de 250:1 verhouding, maar er werd geen verschil waargenomen voor polyspermie (eicel bevrucht door 2 of meer zaadcellen), celdeling op dag 2 (wat aangeeft hoeveel eicellen bevrucht waren waarna celdeling begon) of het totale blastocyst celaantal. Individuele verschillen tussen de beren werden waargenomen voor bevruchting, celdeling en blastocystopbrengt, hoewel alle IVEP-rondes werden aangepast voor hetzelfde aantal progressief beweeglijke spermacellen die per eicel aanwezig waren. Veelbelovende blastocystopbrengst en hoge blastocyst celaantal werden verkregen met ingevroren sperma van beide rassen.

Aangezien er verschillen tussen de rassen werden waargenomen met betrekking tot het aantal follikels op de eierstokken en parameters voor nucleaire en cytoplasmatische rijping na 20 uur, was het doel van artikel III om vervolgens de *in vitro* embryo opkweek en ontwikkeling te bestuderen. Verder werden de follikeldiameter en steroïde hormonen in folliculaire vloeistof (FF) gemeten om te onderzoeken of deze verband houden met de ontwikkelingscompetentie van de eicel. In artikel I werd aan het einde van het onderzoek waargenomen dat pariteit, en dus leeftijd, hoger was bij Duroc dieren in vergelijking met Landras en dit kan de rijpings resultaten hebben beïnvloed. Daarom werden voor de experimenten in artikel III alleen zeugen met één of twee worpen geselecteerd. Gemiddeld werd een grotere follikel diameter waargenomen op de eierstokken van Landras zeugen in vergelijking met Duroc zeugen. Het individuele COC oppervlak was weer groter voor Landras op 0 uur, terwijl cumulusexpansie van 0 tot 20 uur rijping groter was voor Duroc eicellen en dit bevestigde de resultaten van artikel I. Na de bevruchting was de celdeling op dag 2 hoger voor Duroc eicellen en de hoogste blastocystopbrengst werd verkregen voor Duroc eicellen die waren bevrucht met de Landras beer. Er werden geen verschillen waargenomen tussen de rassen voor de individuele steroïde hormonen, maar er werden verschillen in patronen waargenomen in de steroïde routes. Zowel het totale gehalte aan oestrogenen als de verhouding aromataseproducten/substraten waren hoger in Landras FF dan in Duroc, wat wijst op een hogere mate van feminisering in Landras. Er werden dus verschillen gevonden in de vroege folliculaire fase met betrekking tot ontwikkelingscompetentie van de eicellen en steroïdogenese.

Samenvattend, in dit proefschrift werden er verschillen waargenomen tussen de rassen tijdens *in vitro* rijping, bevruchting en embryo ontwikkelng. De resultaten suggereren dat Duroc eicellen een betere ontwikkelingscompetentie hebben tijdens *in vitro* embryo productie. Verder onderzoek wordt aanbevolen om de IVEP protocollen te optimaliseren per ras en om uit te zoeken hoe verschillen tussen de rassen verband houden met de ontwikkelingscompetentie van eicellen. Het werk in dit proefschrift heeft direkte praktische relevantie voor Norsvin, aangezien de opgezette *in vitro* embryo productie en analyse protocollen en verworven kennis verder kunnen worden gebruikt in vervolgonderzoeken met betrekking tot *in vitro* embryo productie, embryokwaliteit en het invriezen en bewaren van embryos. Bovendien is de verkregen kennis over het hanteren en de kwaliteit van embryo's waardevol voor toekomstige commercialisering van embryo transplantaties.

1. Introduction

1.1 General Background

Assisted reproductive technologies (ART) are widely used to treat infertility and achieve pregnancy in both human and domestic animals by performing techniques such as artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and embryo transfer. The use of ART in human has increased tremendously in the past decades (de Geyter et al., 2020) and since 1978 over 9 million IVF babies have been born (ESHRE, 2020). In domestic animal species, the first successful IVF calf born was reported in 1982 (Brackett et al., 1982) followed by the first IVF piglets born in 1983 (Cheng et al., 1986). Embryo technology is nowadays commercially used in cattle breeding to select and breed superior animals (Lonergan, 2007; Sanches et al., 2019) and in combination with genomic selection, the rate of genetic gain in the breeding programs has significantly increased (Ponsart et al., 2014). It is also of great interest to establish commercial application of embryo technology and transfer in the pig breeding industry (Martinez et al., 2016). Similarly, this can increase genetic gain in the pig breeding program by increasing selection intensity and reducing generation interval when fewer sows can be selected if embryos can be collected repeatedly from younger animals. Furthermore, less transportation of live animals is needed when embryos can be transported across the world to distribute genetics, which enhances animal welfare, is better for the environment and decreases transportation and quarantine costs. Another important advantage is biosecurity, as risk of pathogen introduction and disease transmission on farms is minimized when embryos instead of live animals or semen can be imported (Stringfellow & Givens, 2009). It is in this way possible to introduce 100% new genetics on specific pathogen free farms and it might open new markets in other countries.

Non-surgical *in vivo* embryo collection is still a challenge in pigs due to the complex anatomy of the female reproductive tract with tight cervical folds and the coiled shape and long length of the uterine horns, which can be up to 1.5 to 2 meters (Geisert et al., 2019; Jochems et al., unpublished data). *In vivo* derived (IVD) embryos can be collected and transferred surgically (Hazeleger & Kemp, 2001), but this is an unpractical method and undesirable regarding animal welfare. Therefore, studies have focussed on establishing non-surgical methods (Ducro-Steverink et al., 2004; Li et al., 1996; Martinez et al., 2004). A non-surgical deep uterine embryo transfer

catheter has recently been developed that allows embryos to be deposited into the uterine horns within 5 minutes. With this technique an average farrowing rate of 80% and a litter size of 9.5 piglets are reported when transferring 25 to 30 fresh morulae and blastocysts (Martinez et al., 2013). This is the start of the potential commercial application of embryo transfer in porcine, but non-surgical collection of embryos is still a challenge.

Instead of using IVD embryos for transfer, it is also possible to use *in vitro* produced (IVP) embryos. In cattle breeding, ultrasound-guided ovum pick up (OPU) followed by *in vitro* embryo production (IVEP) has commercially been used since the early 1990s (Van Wagtendonk-De Leeuw, 2006). In 2017, the total production and number of bovine embryos transferred worldwide was for the first time greater for IVP embryos compared to IVD embryos (IETS, 2019). In horses, OPU-IVEP has only become commercially established in the last decade as oocyte recovery, fertilization and successful culture were too low to make it economically interesting for breeding. This changed by increasing recovery of multiple immature oocytes and performing fertilization by ICSI (Morris, 2018; Stout, 2020). In pigs, live born piglets have successfully been born using IVP blastocysts from abattoir oocytes using both surgical (París-Oller et al., 2021; Suzuki et al., 2006; Yoshioka et al., 2003) and non-surgical embryo transfer (Suzuki et al., 2004). However, porcine IVEP is still challenging due to a lower efficiency and variable outcomes compared to other livestock species. In cattle, blastocyst rates are higher when using *in vivo* matured OPU collected oocytes compared to *in vitro* matured abattoir oocytes (Rizos et al., 2002) and the use of OPU is therefore also of interest in pigs. Moreover, repeated collection or production of gilt embryos with high genetic value is highly desirable and is not possible with abattoir oocytes. Recently, the first piglets have been born after OPU-IVEP (Yoshioka et al., 2020) and commercial application may be possible in the future as instruments and techniques are further optimized. Another challenge in pigs is the relatively small litter size obtained after both surgical and non-surgical embryo transfer, often ranging from 4-12 piglets, while larger numbers of embryos are transferred (approximately 25-30 IVD morulae or blastocysts with non-surgical transfer and 25-50 IVP embryos with surgical transfer). This makes the use of embryo transfer less efficient compared to cattle and horse where only one embryo is transferred. Therefore, optimization to obtain more piglets after transfer is desirable before implementation.

Recent developments in new reproductive techniques have thus created the need for improved knowledge about embryo technology in pig breeding. Ovaries from prepubertal gilts without known background are usually used in IVEP studies, as prepubertal finisher pigs are slaughtered in the highest numbers at commercial abattoirs and are easily available. However, using oocytes from primi- and multiparous sows rather than from prepubertal gilts lead to better IVEP outcomes (Bagg et al., 2007; Grupen et al., 2003; Pawlak et al., 2015) and in cattle, outcomes vary between breeds with different reproductive performance (Abraham et al., 2012; Fischer et al., 2000). In Norway, breeding programs exist for two pig breeds that are bred for different traits. The Norwegian Landrace dam line has a larger litter size compared to the Norwegian Duroc sire line. An increase in litter size has been shown to be genetically correlated to a higher ovulation rate and a lower corpus luteum (CL) weight (Da Silva et al., 2018) and it has been suggested that CL*s* with a smaller diameter and lower weight originate from follicles having a smaller size at ovulation (Wientjes et al., 2012). This suggests that a higher TNB in Landrace might be related to smaller follicles on the ovary. Oocytes from larger follicles have an increased developmental competence (Bagg et al., 2007; Qian et al., 2001) and differences between breeds may require different optimizations in protocols to successfully obtain *in vitro* blastocysts.

The overall aim of this thesis was to establish porcine *in vitro* embryo production and staining protocols to acquire knowledge about *in vitro* maturation, fertilization and embryo culture in the Norwegian Duroc sire and Norwegian Landrace dam line. Ovaries and sperm from selected Norsvin sows and AI boars were used to investigate if breed differences could provide new insights in *in vitro* embryo production, as the use of embryo technology for dissemination and improvement of genetics is of interest for Norsvin.

1.2 Pig breeding in Norway

1.2.1 Breeding pyramid and three-way cross

Pig breeding in Norway is organized since 1958 by the farmer owned cooperative Norsvin SA. Norsvin merged in 2014 with the Dutch pig breeding company Topigs, and Topigs Norsvin has become the second largest pig breeding company in the world. Pig breeding is organized in a pyramid structure with a three-way cross (Fig. 1). The nucleus herds produce purebred animals from the sire and dam lines. The young boars are sent to the boar testing station and the gilts will be used for self-recruitment within the nucleus herd to produce the next generation, or they will be sold to multiplier herds. In the multiplier herds, sows are mated with a purebred boar of a different dam line, in this way a crossbred sow is produced. This F1 hybrid sow is then mated to a sire line to produce finisher pigs, i.e. slaughter pigs, that will be raised in the finisher herds. With crossbreeding the desirable traits of different lines are combined and a heterosis effect is included, which results in animals performing better than the average of their parents (Cassady et al., 2002; Dickerson, 1973). Embryo technology can be used in different parts of the breeding pyramid. First, using embryos can increase genetic gain at nucleus level since the sows with high genetic breeding values can be used much more extensively. Furthermore, genetics can be disseminated through embryos to a variety of nucleus herds to understand and account for environmental effect, and it is possible to disseminate genetics to other herds further down in the pyramid by the use of embryos.

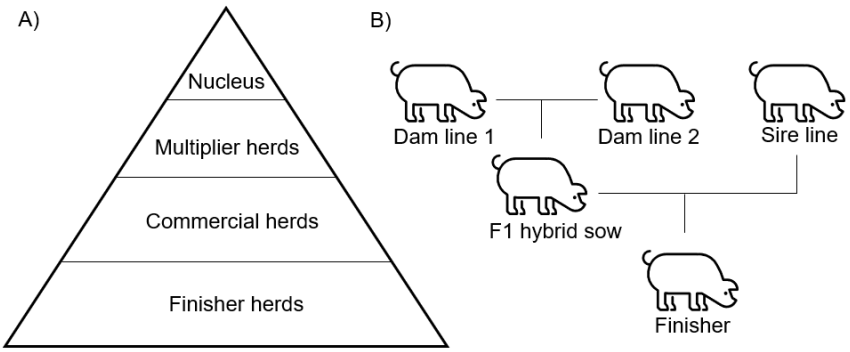


Figure 1. Pig breeding structure with A) the breeding pyramid and B) the three-way cross.

1.2.2 Norwegian Landrace and Duroc

In Norway, breeding programs exist for the Landrace dam line and the Duroc sire line with different breeding goals. The breeding program for Norwegian Landrace was established in the late 1950s and has therefore a long selection history. The Landrace

line has consistently been selected for both production and reproduction traits, and since 1992 litter size has been included in the breeding goal with considerable weight. At the moment, the breeding goal includes traits for production, slaughter quality, meat quality, litter size, reproduction, mother ability and robustness and health. The average age of Landrace gilts at first insemination is 201 days and average gestation length reported is 118.8 days. Furthermore, the average total number of piglets born (TNB) per litter was 14.3 in 2020 and the weaning to oestrus interval was 5.7 days (Ingris, 2021).

The Norwegian Duroc has been bred since the 1990s and it has therefore a shorter selection history. Litter size has only recently been included in the breeding goal, as production and meat quality traits were most of interest in this line. The breeding goal now includes traits for production (feed intake and growth), slaughter quality, meat quality, litter size and robustness and health. The average age of Duroc gilts at first insemination is 224 days and average gestation length 116.9 days. Average total number of piglets born per litter was 9.7 in 2020 and the weaning to oestrus interval 5.7 days (Ingris, 2021). Differences in reproduction traits are thus observed between purebred pigs from these genetic nucleus lines. Over the years TNB has slightly increased for both breeds, but a difference of 4.6 piglets born can be observed between Landrace and Duroc (Fig. 2). The smaller litter size in Duroc is a challenge and using embryo technology in Duroc to obtain more animals and increase genetic gain would be beneficial for the breeding program and farmers.

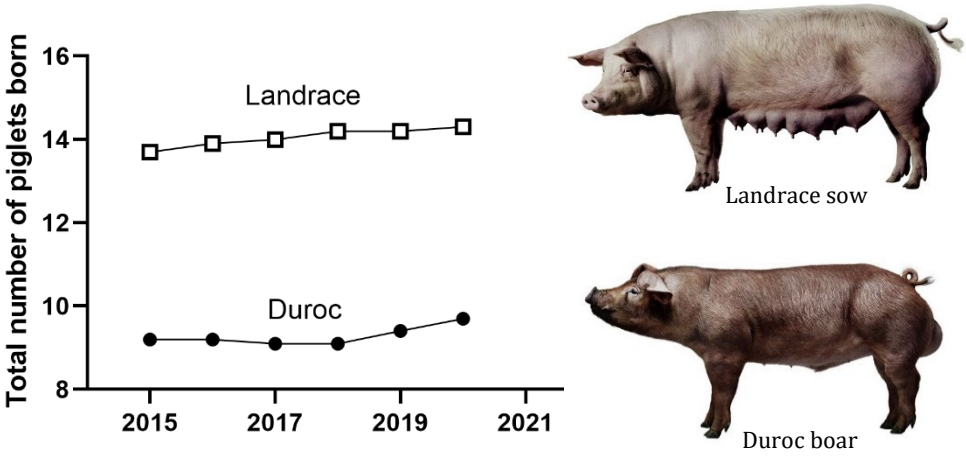


Figure 2. Total number of piglets born from 2015-2020 for the purebred Norwegian Landrace dam and Duroc sire line.

1.3 Pig reproduction

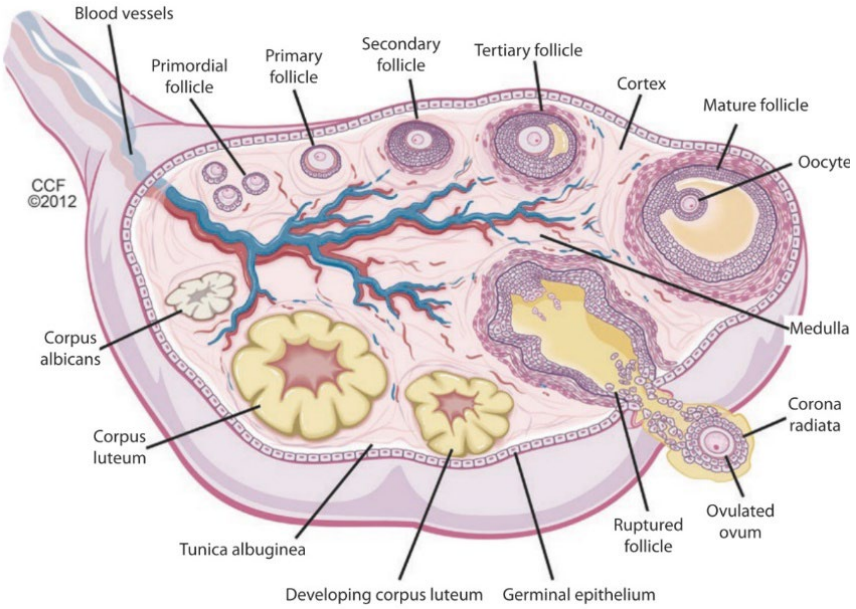
1.3.1 Oestrus cycle

The pig is a non-seasonal, polyoestrous species, with around 15-30 follicles ovulating in one oestrous cycle. The oestrous cycle lasts approximately 21 days (ranging from 18-24 days) and consists of a follicular phase of 5-7 days until ovulation and a following luteal phase of 13-15 days (Geisert et al., 2019; Soede et al., 2011). The oestrus is the period around ovulation, which is characterized by the sow showing standing behaviour in response to a boar. After successful fertilization, the pregnancy takes approximately 114 - 116 days and a weaning-to oestrous interval of 4-6 days is normally observed. The hypothalamic-pituitary-gonadal axis plays an important role in regulating reproductive activity and is activated at puberty (Desai et al., 2017). Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus which induces secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary gland. In the female, those gonadotropins in turn regulate the production of the sex steroid oestrogen and progesterone by the ovary (Noakes et al., 2018). The gonadotropins stimulate follicular growth and ovulation during the oestrous cycle, while the sex steroid progesterone prepares the reproductive tract for fertilization, implantation and pregnancy.

1.3.2 Follicular development

At birth, the ovaries contain primordial follicles with immature oocytes in the prophase of meiosis I that will serve a sow's entire reproductive life. Not alle primordial follicles are stimulated to grow at the same time, only a small proportion begin development while the rest remain quiescent in the ovary. After puberty, around 5-7 months of age in pigs (Senger, 2012), primordial follicles are continuously recruited to develop further. Primordial follicles will develop through primary, secondary, and antral (tertiary) follicle stages as shown in Figure 3A (Desai et al., 2017). The antral follicle is characterized by formation of a fluid filled cavity. This cavity enlarges further, and the follicles eventually reach the mature preovulatory follicle stage. In pigs, the estimated time to develop from an activated primordial follicle into an antral follicle of approximately 400 μm is around 84 days, which is followed by rapid growth and another 14 days are needed to reach a size of 3 mm. It was furthermore estimated that another 5 days are required to reach a size of 8 mm at the preovulatory follicle stage (Morbeck et al., 1992).

A)



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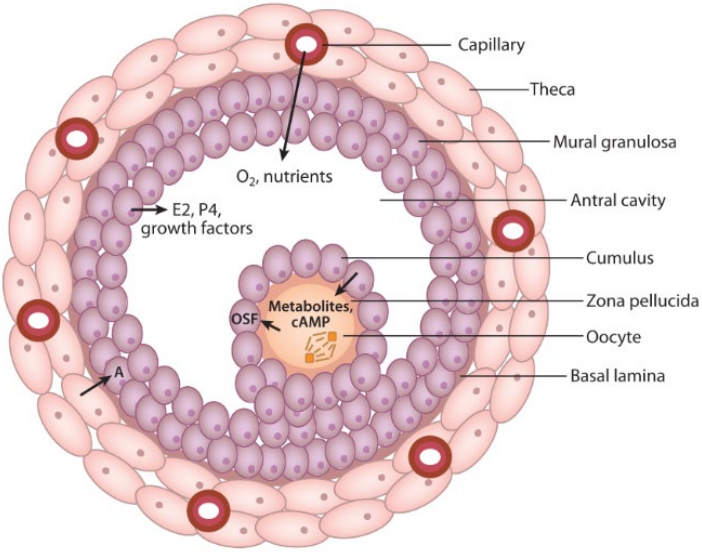


Figure 3. A) Schematic overview of the ovary representing follicle and oocyte development in the ovarian cortex (Desai et al., 2017) and **B)** Diagram of an ovarian preovulatory follicle showing the multiple cell types involved in maintaining the follicular environment and determining oocyte competence. Abbreviations: A, androgen; cAMP, cyclic AMP; E2, oestradiol; OSF, oocyte-secreted factors; P4, progesterone (Krisher, 2013).

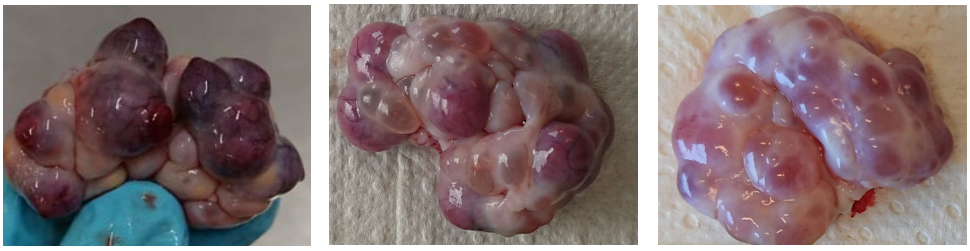
Individual preovulatory follicles contain an oocyte that is surrounded by the zona pellucida (ZP) and several layers of cumulus cells. The inside of the follicle is covered with an inner layer of mural granulosa cells and an outer layer of theca cells lining the antral cavity (Mcgee & Hsueh, 2000) (Fig. 3B). Oocytes develop and mature within the follicular environment throughout complex pathways and communication between granulosa cells, theca cells, cumulus cells and the oocyte itself (Krisher, 2013). The communication occurs via gap junctions and follicle development is regulated by paracrine signals produced by granulosa cells (Hunter, 2000) and endocrine signals from hormones transported through the bloodstream (Noakes et al., 2018).

Preantral follicle growth is gonadotropin independent, and mainly dependent on intrafollicular paracrine and autocrine signalling between the oocyte and somatic cells (Jones & Shikanov, 2019). Important factors related to this first stage of follicular growth are oocyte-secreted factors (OSF), growth differentiation factors and bone morphogenetic proteins (Hussein et al., 2006) and other factors like cyclic AMP (cAMP) which is involved in meiotic arrest and maturation, anti-Müllerian hormone (AMH), activin and inhibin (Desai et al., 2017). During the follicular phase, small antral follicles will develop into large pre-ovulatory follicles and this second part of continued growth of medium and large follicles is dependent upon the gonadotropins FSH and LH (Guthrie, 2005; Schwarz et al., 2008). Two processes are described in follicular growth at the antral follicle stage: recruitment and selection. Recruitment refers to the population of small and medium follicles that may be selected as ovulatory follicles, while selection refers to the follicles that escape atresia and eventually ovulate (Knox, 2005). Only the largest antral follicles from the ovarian follicular pool are recruited at the beginning of the follicular phase while the other follicles will undergo atresia and degenerate. It has been estimated that only 30-40% of the recruited follicles will finally mature and ovulate, whereas the others become atretic (Guthrie & Garrett, 2001). Antral follicles are recruited when the pulsatile GnRH/LH release shifts from a lesser frequency/greater amplitude pattern to a greater frequency/lesser amplitude pattern (Soede et al., 2011). Recruited antral follicles start to grow and begin to produce low levels of oestradiol and inhibin while the other antral follicles undergo atresia and degenerate. Inhibin level increases and gives negative feedback on FSH that will decline, and this will stop the recruitment process. Selection of follicles from the recruited pool is marked by a shift from FSH to LH as the selected follicles will start to synthesize LH receptors in the granulosa cells and will continue to grow into pre-ovulatory follicles (Schwarz et al., 2008). Pre-

ovulatory follicles produce high levels of 17β-oestradiol which causes positive feedback and increases the GnRH pulse, which induces oestrus behaviour and a LH surge that initiates ovulation of the pre-ovulatory follicles.

The follicular phase is followed by the luteal phase in which the ovulated and ruptured follicle forms a temporary structure called the corpus hemorrhagicum (CH) which develops further into a corpus luteum (CL) as shown in Figure 3A. The CLs primarily secrete progesterone, which is important for implantation and maintenance of pregnancy. When no pregnancy is established or if there are less than five embryos around day 12-14 in the cycle, the uterus starts to secrete prostaglandin F2 alpha (PGF2-α). This causes luteolysis, in which the CLs will start to regress into a corpus albicans (CA). Decreasing progesterone level after luteolysis causes an increase in FSH concentration that starts the recruitment of new follicles and it leads to pulsatile secretion of LH that enables follicular selection and maturation of new follicles (Schwarz et al., 2008).

Ovaries collected at the slaughterhouse are at different stages in the oestrous cycle and contain follicles at different stages of follicular development. Several sow ovaries corresponding to the follicular and luteal phase are shown in Figure 4.



Early luteal phase
CHs after ovulation

Late luteal phase
CLs and new follicles

Early follicular phase
One day after weaning

Figure 4. Sow ovaries related to the different phases of the oestrous cycle (R. Jochems).

1.3.3 Steroidogenesis

Follicular development is thus a complex process and growth of antral follicles is regulated by the gonadotropins FSH and LH which are transported to the ovaries via the blood (Senger, 2012). In early antral follicles, theca cells respond to LH and activate a cascade of events to convert cholesterol into androgens such as testosterone and androstenedione, while the granulosa cells are responsible for conversion of the androgens into oestrogens such as 17β-oestradiol and estrone by FSH induced aromatase (CYP19) as shown in Figure 5 (LaVoie, 2017). Briefly, steroidogenesis is initiated when steroidogenic acute regulatory protein (STAR) is synthesized which enables the import of cholesterol into the mitochondria. Cholesterol is then converted by CYP11A1 into pregnenolone (PREG) which moves to the smooth endoplasmic reticulum in the cytoplasm. Pregnenolone is converted to progesterone (P4) or dehydroepiandrosterone (DHEA) and can be converted further to androstenedione (AN) and testosterone (TS). Androstenedione and testosterone eventually move from the theca interna cells to the mural granulosa cells which are responsible for further conversion of the androgens into the oestrogens 17β-oestradiol (βE2) and estrone (E1) by FSH induced aromatase (CYP19).

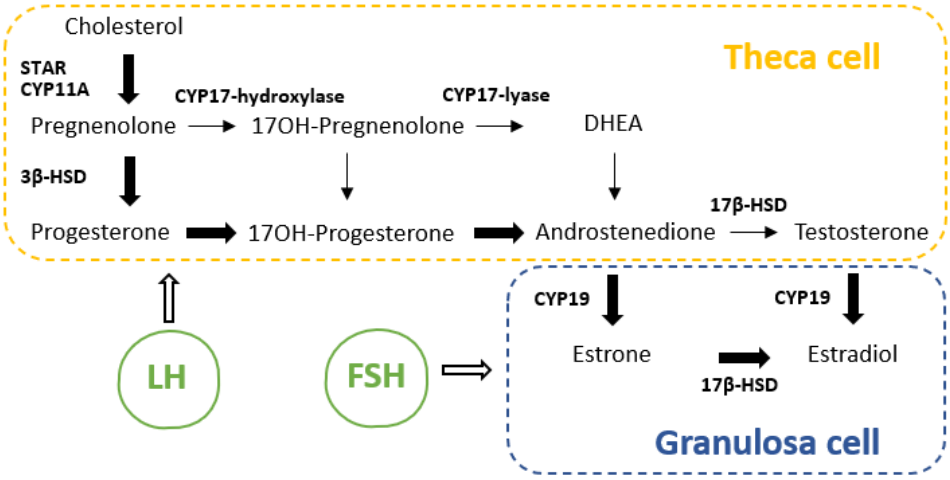


Figure 5. Steroidogenic pathways in the pig ovary. Bolded text next to arrows represents the major molecule(s) mediating the step or reaction. Thicker arrows signify a major pathway in pig follicles. DHEA, dehydroepiandrosterone (Adapted from LaVoie, 2017).

1.4 *In vivo* maturation and sperm-oocyte interaction

Primordial oocytes enter a prolonged resting stage at the end of prophase I of the first meiotic division just before birth. Further oocyte maturation consists of a period of growth and completion of both nuclear and cytoplasmic changes which is essential to acquire oocyte developmental competence (Eppig, 1996). Oocyte developmental competency is defined as the ability to resume and complete meiosis, successfully undergo fertilization and develop into a viable embryo (Conti & Franciosi, 2018). Resumption of meiosis is characterized by chromatin condensation, initiation of the germinal vesicle breakdown (GVBD), formation of the metaphase plate and spindle and extrusion of the first polar body. Oocytes then enter a second period of arrest at the metaphase II stage (MII) just before ovulation until sperm penetration. Sperm cells are deposited in the female reproductive tract and final maturation of the sperm cell is required which takes place while sperm transports through the female reproductive tract (Rodriguez-Martinez, 2007). When reaching the oviduct, sperm cells undergo a period of storage within the sperm reservoir in the oviduct. Upon fertilization, sperm cells must undergo capacitation which refers to biochemical and physiological changes at the plasma membrane so that they are able to bind to the zona pellucida and undergo the acrosome reaction (Fraser, 1998). The sperm cell will bind to the zona pellucida sperm binding protein 3 (ZP3) of the oocytes, which initiates the acrosome reaction and the released enzymatic acrosomal contents lyse the zona pellucida (Senger, 2012). Furthermore, capacitation changes sperm motility to a hyperactive motility which is characterized by a vigorous non-linear movement caused by an high amplitude and asymmetrical flagellar beating pattern (Ho & Suarez, 2001), which helps the sperm to penetrate the oocyte. Immediately after penetration, the cortical reaction is initiated in oocytes blocking other sperm cells from entering the oocyte by exocytosis of the cortical granules that hardens the zona pellucida. The oocyte is activated and will resume meiosis II and extrude the second polar body (Sun & Nagai, 2003). Both the oocyte chromatin and sperm nucleus will undergo decondensation and form a female and male pronucleus, after which the genetic material of the pronuclei will fuse to form the zygote (Kaser et al., 2019). Normal zygotes have two polar bodies and two pronuclei, but multiple pronuclei and decondensed sperm heads are observed when more sperm cells have fertilized the oocyte, which is called polyspermy (Oberlender et al., 2016).

1.5 *In vitro* embryo production

In vitro embryo production is divided into three main steps: *in vitro* maturation (IVM) which involves nuclear and cytoplasmic oocyte maturation, *in vitro* fertilization (IVF), in which matured oocytes are co-incubated with sperm cells, and *in vitro* culture (IVC), in which the fertilized zygotes develop until blastocyst stage. The maturation, fertilization and culture medium must mimic *in vivo* conditions to successfully produce embryos.

1.5.1 *In vitro* oocyte maturation

Nuclear maturation

Ovaries are normally collected regardless of the oestrous cycle stage and most oocytes can be found in the immature germinal vesicle (GV) or GVBD stage (Wang et al., 1997). Around 70 - 85% of the porcine oocytes that start maturation actually complete nuclear maturation and reach MII phase (Gil et al., 2017; Somfai et al., 2005; Yuan & Krisher, 2012). An increased oocyte developmental competence in relation to follicular size has been reported in cattle and pigs (Bagg et al., 2007; Carolan et al., 1996; Marchal et al., 2002; Qian et al., 2001). Throughout meiosis the oocyte will go through different phases. The prophase I is a long period in porcine and several GV stages can be distinguished which are related to differences in follicle size and growth of the follicles (Sun et al., 2001). The different GV stages are characterized and shown in Figure 6. Thereafter, chromosomes are fully compacted in the diakineses (pro-metaphase) and they start to align with the mitotic spindle, followed by the metaphase I (MI) stage with chromosomes clearly visible in the metaphase plate. In the anaphase I (A), chromosomes are migrating towards the spindle poles followed by the telophase I (T) in which chromosomes are separated at the poles. Finally, the oocytes will reach metaphase II (MII), exhibiting chromosomes in the metaphase plate and the first polar body that is extruded and that can be assessed examined under the microscope (Fig. 6).

In vitro, the germinal vesicle stage has been observed until 18-24 h after start of maturation in porcine after which the GVBD is initiated. Metaphase I is normally observed at 30-36 h and the anaphase and telophase are only observed for a short time around 36 h. Finally, the *in vitro* matured oocytes reach MII with extrusion of the polar body around 36-42 h (Yuan & Krisher, 2012). Therefore, cumulus-oocyte complexes (COCs) are cultured for around 40-42 h in pigs, and this is much longer than needed compared to some other species, for example 22-24 h in cattle.

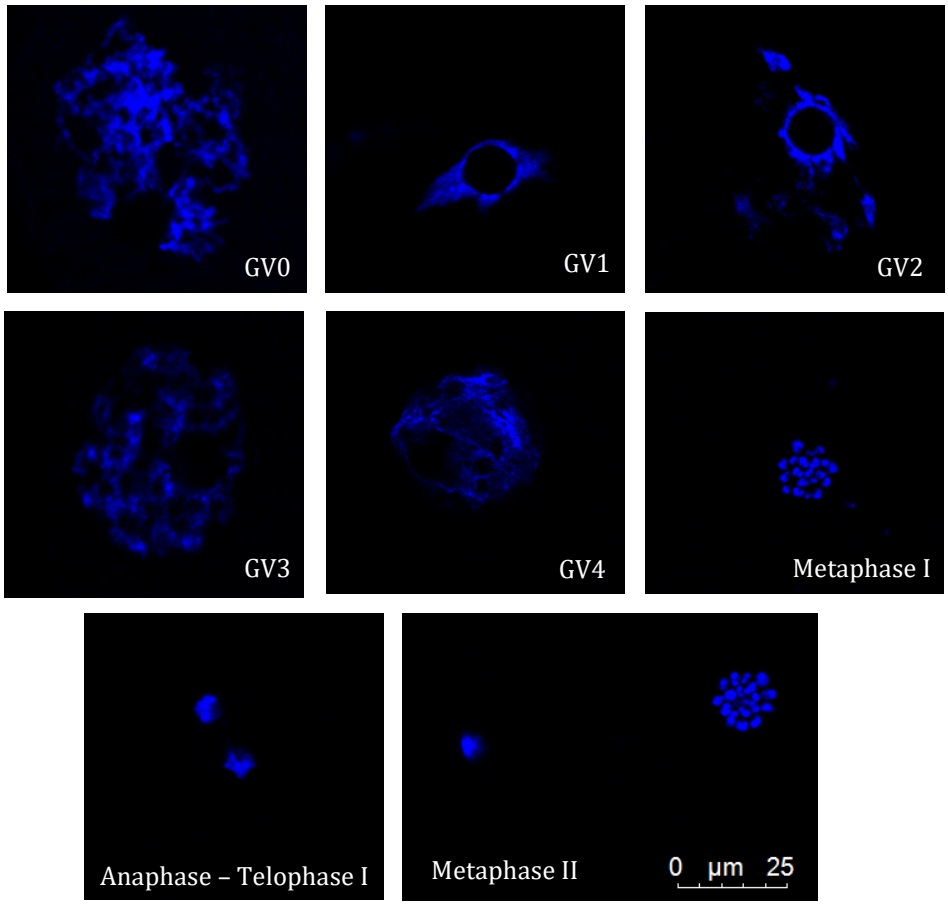


Figure 6. Nuclear morphology of porcine oocytes stained with Hoechst-33342 (R. Jochems). The germinal vesicle stages are defined as: GVO is characterized by a nucleolus, nuclear envelope and diffuse chromatin; GV1 is characterized by compact chromatin around the nucleolus which forms a halo in a horseshoe shape; GV2 is the first stage of the GVBD where condensed chromatin and 2-8 clumps are visible; in GV3 the nucleolus starts to disappear and many clumps are visible, and GV4; in which the nucleolus is not visible anymore, the nuclear envelope is less distinct and chromatin is compacted to form chromosomes (as described by Sun et al., 2004). Furthermore, the metaphase I, anaphase-telophase I and metaphase II are shown.

Cytoplasmic maturation

Oocytes that complete nuclear maturation and extrude the first polar body may still be incompetent to be fertilized, form pronuclei after sperm penetration and develop to an embryo. It has been indicated that this is related to incomplete cytoplasmic maturation (Abeydeera, 2002; Coy et al., 2002; Hirao et al., 1994). Cytoplasmic maturation is complex and includes many morphological ultrastructural changes in the cytoplasm. No method is available to assess complete cytoplasmic maturation, but different parameters have been correlated with cytoplasmic maturation and morphological changes in the oocyte. Reorganization and migration of different organelles such as cortical granules and mitochondria take place (Liu et al., 2010). Both cortical granules and mitochondria are distributed throughout the cytoplasm in immature oocytes, while they move towards the peripheral area near the oocyte membrane at the end of maturation (Cran & Cheng, 1985; Pawlak et al., 2012; Wang et al., 1997) as shown in Figure 7. Cortical granules are important for the cortical reaction that is induced after penetration of a sperm cell to block polyspermy (Coy & Avilés, 2010), while mitochondria are essential for energy production and migrate often to areas of high energy consumption, towards polar body extrusion (Babayev & Seli, 2015). Furthermore, glutathione (GSH) synthesis by cumulus cells throughout oocyte maturation has been suggested to be an important parameter. This antioxidant protects oocytes against oxidative stress. The intracellular GSH content of oocytes reaches a peak level at MII stage and higher GSH levels are positively associated with sperm nuclear decondensation and male pronucleus formation (Yoshida et al., 1993), blastocyst development and total blastocyst cell number (Abeydeera et al., 1998; Maedomari et al., 2007).

Incomplete cytoplasmic maturation has been associated with inadequate IVM conditions or due to poor quality of the collected oocytes. Supplementation of maturation media with cysteine, β-mercaptoethanol and follicular fluid affected cytoplasmic maturation and resulted in an increase in GSH content and better blastocyst rates in both pig and cattle (Abeydeera et al., 1998; de Matos & Furnus, 2000; Kobayashi et al., 2006; Yoshida et al., 1992).

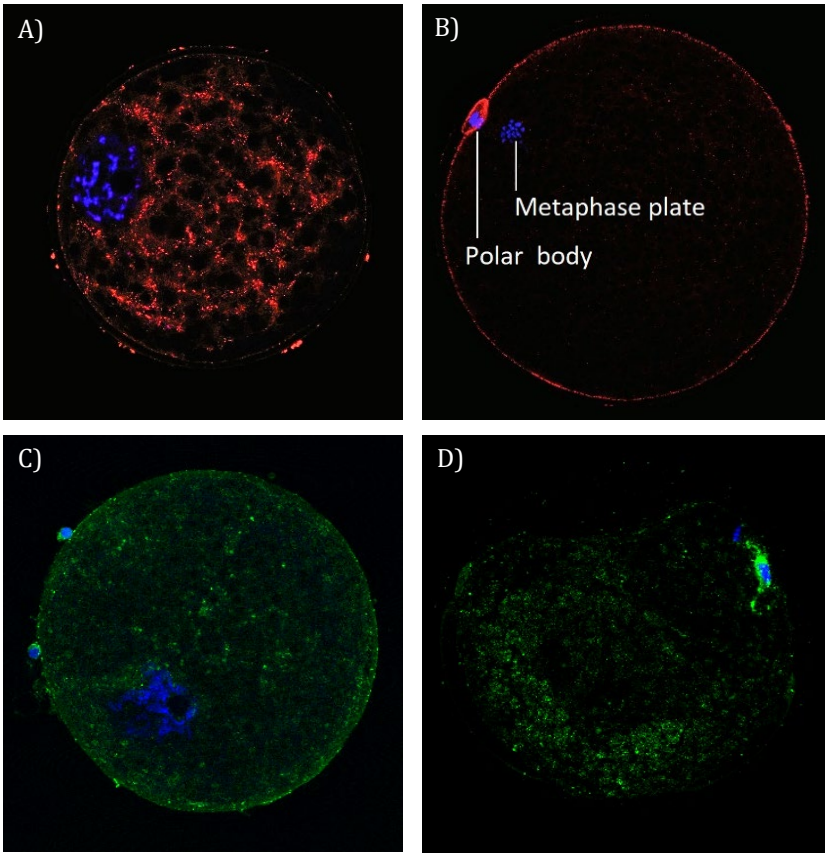


Figure 7. **A)** Immature porcine oocyte in the germinal vesicle stage (Blue; Hoechst-33342) with cortical granules (CGs in red; PNA Lectin Alexa Fluor 568) distributed throughout the cytoplasm, **B)** mature oocyte in the MII stage with a metaphase plate and extruded polar body and CGs in the outer cortex, **C)** immature oocyte in the germinal vesicle stage and mitochondria (Mitotracker Green) throughout the oocyte and **D)** mature oocyte in the MII stage and mitochondria around the polar body (R. Jochems).

Cumulus expansion

Cumulus cells surrounding the oocyte and cumulus expansion play an important role during oocyte maturation (Tanghe et al., 2002a). Intracellular communication take place via gap junctions and the cumulus cells supply the oocyte with nutrients and other regulatory molecules. In this way, the cumulus-oocyte complex supports nuclear maturation during both the meiotic arrest and resumption, as well as cytoplasmic maturation. A greater cumulus expansion has been correlated with a higher oocyte developmental potential and better development to blastocyst stage in different species (Han et al., 2006; Qian et al., 2003). *In vivo*, COCs start to produce hyaluronic acid in response to the LH surge and cumulus expansion is initiated. At the start of *in vitro* embryo production, the oocytes with a compact cumulus are selected and cumulus expansion throughout IVM is shown in Figure 8. In addition, cumulus cells have been suggested to play an important role during *in vitro* fertilization (Van Soom et al., 2002).

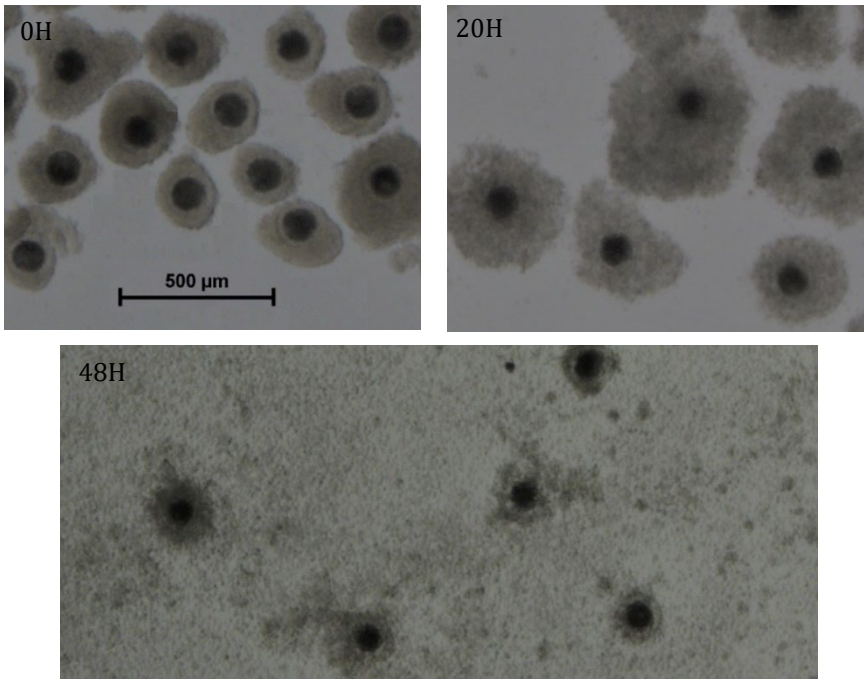


Figure 8. Cumulus-oocyte complexes at 0, 20 and 48 hours of IVM (R. Jochems).

1.5.2 *In vitro* fertilization

Successful *in vitro* fertilization of IVM oocytes has been realized with the use of fresh and frozen-thawed ejaculated sperm, and even fresh and frozen-thawed epididymal sperm (Rath & Niemann, 1997; Romar et al., 2005; Suzuki et al., 2005; Wang et al., 1991). Sperm survival and motility after freezing-thawing are often lower compared to fresh semen (Yeste, 2017). Nevertheless, using cryopreserved sperm is useful to increase repeatability as sperm from the same ejaculate can be used over a long period and throughout the same experiment.

For successful *in vitro* fertilization, the fertilization medium has to contain chemicals that induce sperm capacitation such as caffeine or theophylline (Romar et al., 2016) so that the sperm cell is able to bind and penetrate the zona pellucida. Caffeine is still mostly used in IVF protocols, but stimulates both capacitation and the acrosome reaction, which has been suggested to lead to higher polyspermy rates (Funahashi & Nagai, 2001). Theophylline was demonstrated to stimulate sperm penetration of IVM oocytes without being accompanied by polyspermy (Yoshioka et al., 2003). A high incidence in polyspermy, when an egg cell is fertilized by more than one sperm cell, has been seen as one of the limiting factors in porcine IVF (Funahashi, 2003; Suzuki et al., 2003). Polyspermic zygotes can develop further to blastocysts with a good morphology and it has been reported that a few polyspermic blastocysts developed to foetuses and piglets after transfer (Han et al., 1999a; Han et al., 1999b) but most do not develop further into viable piglets due to chromosomal abnormalities. In some laboratories polyspermy has been described as a significant problem with up to 50% polyspermic zygotes (Gil et al., 2010; Martinez et al., 2017; Yoshioka et al., 2008), while in other labs it is lately seen as less of a problem as only 20-30% polyspermy has been reported (Chen et al., 2021). Different studies have looked in reducing porcine polyspermy by decreasing the number of sperm cells present per oocyte (Gil et al., 2007; Xu et al., 1996), reducing co-incubation time (Almiñana et al., 2005; Gil et al., 2004) or using different IVF methods to increase sperm selection and reduce the number of sperm cells by a swim up method (Park et al., 2009) or straw IVF (Li et al., 2003). It has been shown that sperm-oocyte ratio affects fertilization and polyspermy rates, and that the optimal IVF ratio varies between boars (Gil et al., 2004, 2007; Wang et al., 1991; Xu et al., 1996). In addition to individual boar differences, differences between breeds have been reported for penetration and polyspermy rates (Suzuki et al., 2003). *In vitro* fertilization results are thus depended on several factors.

1.5.3 *In vitro* embryo culture

After fertilization, the embryos are cultured until blastocyst stage which is the longest step in the IVEP procedure. Cell cleavage is often assessed on day 2 of culture (fertilization is day 0) and pig embryos are normally cultured until day 5 or 6 in which they reach blastocyst stage. Further embryo culture behind this stage is possible to assess hatching, but hatching is not always observed in porcine IVEP. Embryos develop from 2-4 cells to 6-8 cells, morula, blastocyst, expanded blastocyst and eventually the hatched stage. Some of the pig embryos stages are shown in Figure 9.

Previously, a four-cell block was often observed during culture but medium is available now that overcomes this blockage and supports development up to blastocyst stage (Abeydeera, 2001). This developmental block has been observed in several species at different embryo stages and is suggested to be related to the maternal to embryo transition in which embryonic genome activation (EGA) is initiated, which is also known as zygotic gene activation (Meirelles et al., 2004). In pigs the EGA occurs at the 4-cell stage (Cao et al., 2014). Not all oocytes that start maturation are fertilized and not all fertilized zygotes will develop until blastocyst stage. Around 15-30% of pig oocytes starting culture develop to blastocyst stage (Grupen et al., 2003; Kidson et al., 2004; Martinez et al., 2017). Different non-invasive and invasive parameters can be used to assess blastocyst quality such as scoring morphology, kinetics of development by timelapse, counting total blastocyst cell number or the trophectoderm cells and inner cell mass, or study chromosomal abnormalities, blastocyst metabolism and apoptosis (Soom & Boerjan, 2002). The quality of IVP blastocyst is known to be less as compared to IVD embryos. In pigs, a decreased inner cell mass and trophectodermal cell numbers (Bauer et al., 2010), lower rates of glycolysis and metabolism of pyruvate, glutamine and glycose (Swain et al., 2002) and differences in lipid content dependent on developmental stage (Romek et al., 2009) have been shown in IVP blastocyst compared to IVD blastocysts. This indicates that IVEP is still suboptimal and can be improved to obtain blastocysts with better quality.

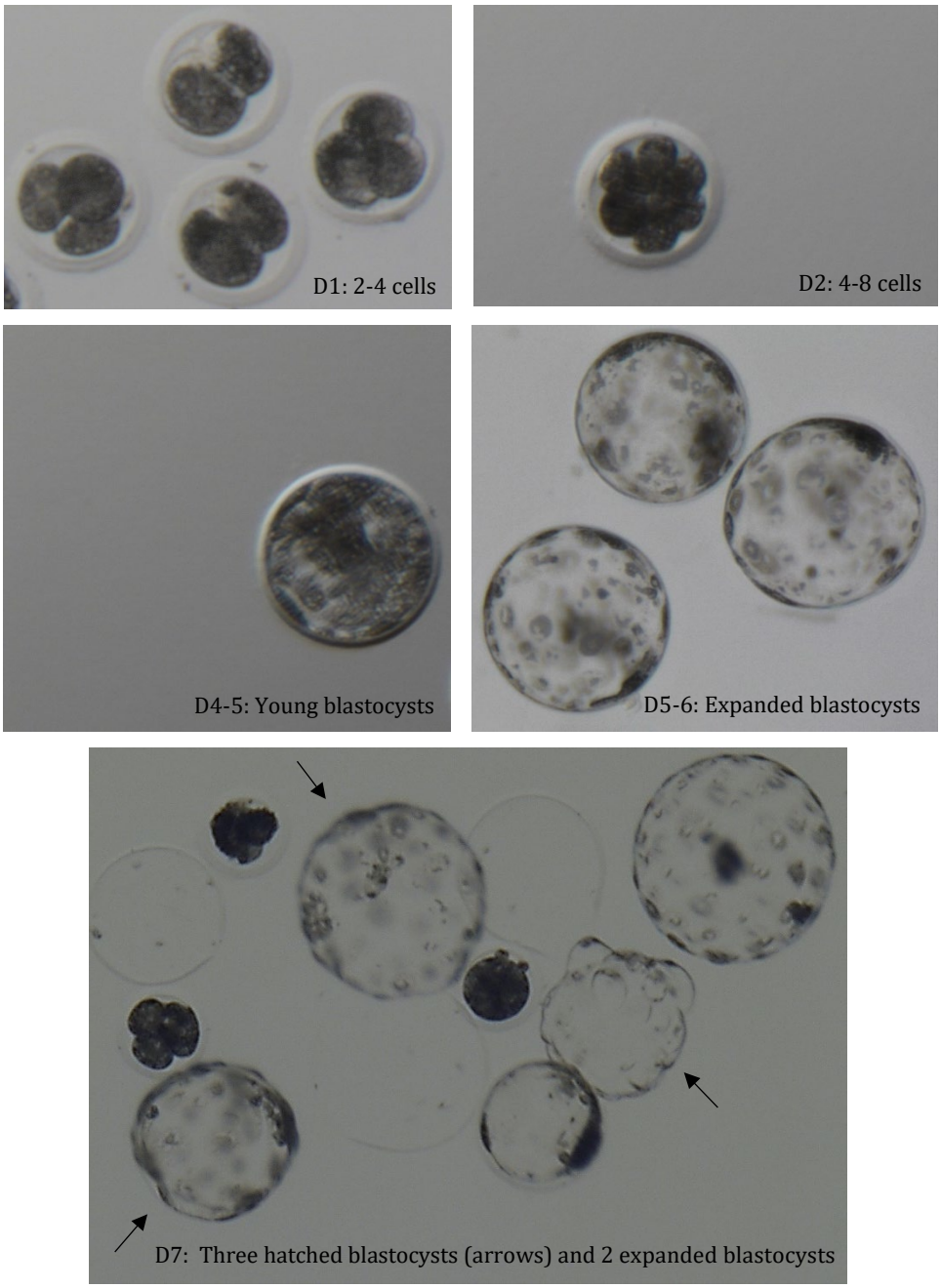


Figure 9. *In vitro* embryo culture with different pig embryo stages (R. Jochems).

2. Aims and outline of the thesis

The use of embryo technology is of interest in pig breeding to increase genetic gain in the breeding program and disseminate genetics of superior animals worldwide with less transportation of live animals and a lower risk of disease transmission. In Norway, breeding programs exist for two breeds with different reproductive characteristics. The Norwegian Duroc sire line has on average 9.7 total number piglets born (TNB) per litter compared to 14.3 TNB in the Norwegian Landrace dam line. Breed differences in this trait could be due to ovarian characteristics, which may also affect *in vitro* embryo production outcomes and could require different optimizations to successfully obtain *in vitro* blastocysts.

Therefore, the overall aim of this thesis was to establish porcine *in vitro* embryo production and staining protocols to acquire knowledge about *in vitro* maturation, fertilization and culture and differences between the Norwegian Duroc sire and Norwegian Landrace dam line.

The objectives of this thesis were to

- Characterize ovarian characteristics, and *in vitro* nuclear and cytoplasmic oocyte maturation in the Norwegian Duroc sire and Norwegian Landrace dam line one day after weaning (Paper I).

- Assess *in vitro* fertilization, polyspermy and embryo development after fertilization with a 500:1 and 250:1 'progressively motile sperm' to oocyte ratio and using cryopreserved semen from three Duroc and three Landrace boars (Paper II).

- Determine differences in *in vitro* embryo development of Duroc and Landrace oocytes fertilized with cryopreserved sperm from both a Duroc and Landrace boar (Paper III).

- Measure follicle size and follicular fluid steroid hormone levels from Duroc and Landrace sows in the early follicular phase to study possible relation to oocyte developmental competence (Paper III).

The outline of this thesis is illustrated in Figure 10. First, *in vitro* oocyte maturation protocols were established. Ovarian characteristics from Norwegian Duroc and Landrace sows and *in vitro* oocyte maturation were studied in paper I one day after weaning. After obtaining high maturation rates to MII stage, sperm preparation and *in vitro* fertilization protocols were optimized. Paper II studied differences in *in vitro* fertilization using two progressively motile sperm to oocyte ratios and using cryopreserved sperm from three Duroc and three Landrace boars to fertilize oocytes from random collected ovaries. Two boars from this study with similar fertilization, polyspermy, cleavage and blastocyst rates were selected for Paper III in which *in vitro* embryo development with both Duroc and Landrace oocytes and sperm were assessed. In addition, follicle size and follicular fluid steroid hormone levels were measured in paper III to study possible relation to oocyte developmental competence.

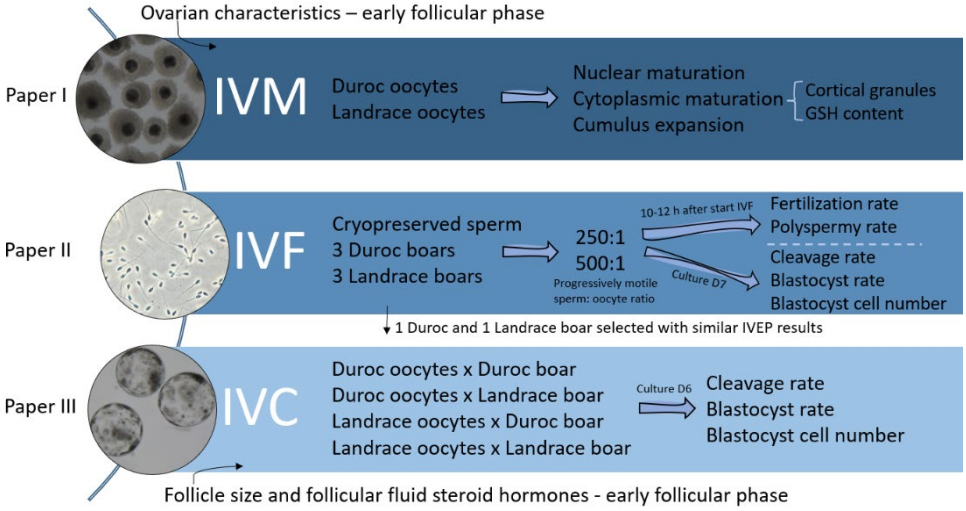


Figure 10. Schematic illustration of the outline of this thesis.

3. Results – Summary of individual papers

Paper I: Ovarian characteristics, and *in vitro* nuclear and cytoplasmic maturation in Duroc and Landrace pigs

Differences in reproduction traits are observed between pig breeds; on average 9.2 total number piglets born (TNB) per litter are reported in the Norwegian Duroc sire line compared to 13.8 TNB in the Norwegian Landrace dam line (Ingris, 2019). Breed differences in this trait could be due to ovarian characteristics, which might also affect *in vitro* embryo production outcomes. Therefore, the aim of this study was to assess ovarian characteristics and *in vitro* nuclear and cytoplasmic oocyte maturation in the Norwegian Duroc sire and Norwegian Landrace dam line. One day after weaning, follicular phase ovaries were collected from 37 Duroc and 20 Landrace sows. Ovary length and weight were measured, and the number of follicles (<3 mm and 3-8 mm) were counted. Cumulus-oocyte complexes (COCs) were collected and matured for 48 h. To assess individual COC area and cumulus expansion, images were taken at 0, 20 and 48 h by stereomicroscopy. Oocytes were stained at 20 and 48 h with 8 µg/ml Hoechst-33342 and 100 µg/ml Lectin PNA-Alexa Fluor 568 to evaluate nuclear maturation and cortical granule (CG) distribution, respectively. In addition, total glutathione (GSH) was measured at 48 h by a GSH/GSSG-Glo assay to further elucidate cytoplasmic maturation. In first parity sows (n = 11 Duroc and 10 Landrace sows) a smaller ovary length (3.0 ± 0.3 cm vs. 3.2 ± 0.3 cm, P = 0.01) and fewer 3 to 8 mm follicles (13.6 ± 5.4 vs. 21.6 ± 7.9, P<0.001) were observed for Duroc compared to Landrace. For all sows, Duroc COCs covered a significantly smaller area at 0 h, but a broader cumulus expansion was observed from 0-20 h compared to NL (364 ± 46% vs. 278 ± 27%, P < 0.001). At 20 h, more Duroc oocytes exhibited advanced stages of nuclear maturation, while more Landrace oocytes showed advanced stages of CG distribution. Nuclear maturation to MII stage at 48 h did not differ between Duroc and Landrace oocytes (90.1% and 87.7%, respectively). Moreover, no significant differences were observed for GSH content or CG distribution after maturation. In conclusion, differences with regard to ovarian characteristic as well as to cumulus expansion, and nuclear and cytoplasmic oocyte maturation at 20 h were observed between the breeds. Further studies are required to determine if this subsequently affects *in vitro* fertilization and embryo development.

Paper II: Effect of two 'progressive motile sperm to oocyte ratios' on porcine *in vitro* fertilization and embryo development

Using cryopreserved sperm during *in vitro* embryo production (IVEP) allow for increased repeatability. However, sperm motility and viability of cryopreserved sperm vary more between boars and straws and are often lower compared to fresh sperm. Fertilization is usually performed with a concentration based on total sperm cells per ml, whereas the percentage total motile or progressively motile sperm cells are often not considered. The aim of this study was to assess fertilization with a 500:1 and 250:1 'progressively motile sperm to oocyte' ratio on IVEP outcomes using cryopreserved sperm from three Duroc and three Landrace boars. Random sow ovaries were collected and follicles with a diameter of 3-8 mm were aspirated. Frozen-thawed sperm was centrifugated through a 45/90% Percoll® density gradient and sperm motility was analysed by computer-assisted sperm analysis (CASA) to determine sperm concentration and progressive motility after centrifugation. *In vitro* matured oocytes were fertilized with 250 or 500 progressively motile sperm cells per oocyte and co-cultured for 4 h, after which the presumptive zygotes were denuded by vortexing and cultured under mineral oil. To analyse fertilization and polyspermy rates, a portion of the presumptive zygotes were assessed 10 - 12 hours after start of fertilization, while the remaining zygotes were cultured up to day 7 to assess cleavage rate, blastocyst rate and total blastocyst cell number. The 500:1 ratio resulted in a higher fertilization (38.5 vs. 31.2%) and blastocyst yield on day 6 (26.9 vs. 20.3%) compared to the 250:1 ratio, while no effect of ratio was observed for polyspermy, cleavage rate or blastocyst cell number. Individual differences between boars were observed for fertilization, cleavage and blastocyst rates, but not for the other IVEP outcomes. The lowest blastocyst rate of 4.3% was observed for a Duroc boar with the 250:1 ratio, while the highest blastocyst rate of 37.0% was observed for a Landrace boar with the 500:1 ratio. In conclusion, a higher fertilization and blastocyst yield was obtained with the 500:1 ratio compared to the 250:1 ratio, while polyspermy level was consistent across ratios. Differences in IVEP outcomes were still observed between the individual boars although adjusted for progressive motility. Promising blastocyst yields and high total blastocyst cell numbers were obtained with sperm from both Duroc and Landrace boars.

Paper III: Follicular fluid steroid analysis and *in vitro* embryo development in Duroc and Landrace pigs.

Differences were observed between the Duroc sire and Landrace dam line regarding ovary characteristics and nuclear and cytoplasmic oocyte maturation parameters at 20 h of maturation in paper I. Therefore, the aim of paper III was to assess if *in vitro* embryo production (IVEP) subsequently differed for the Duroc and Landrace line with focus on both the maternal and paternal breed. In addition, follicle diameter and follicular fluid (FF) steroid hormones were measured to study possible relation to *in vitro* oocyte developmental competence. Sow ovaries in the follicular phase were collected one day after weaning from 23 Duroc and 36 Landrace sows and the 10 largest follicles were measured per ovary before aspiration of the 3-8 mm follicles. Cumulus-oocyte complexes (COCs) were matured *in vitro* and images were taken at 0 h and 20 h using a stereomicroscope, to assess individual COC area and cumulus expansion from 0 to 20 h. Fertilization of Duroc and Landrace oocytes was performed with both a Duroc and Landrace boar, resulting in four different groups to compare (ND x ND, ND x NL, NL x NL and NL x ND). These two boars were selected based on results from Paper II where they had similar fertilization, polyspermy, cleavage and blastocyst formation rates when fertilizing random oocytes. Cleavage rate at day 2 and blastocyst rate at day 6 were assessed, after which blastocysts were stained with 8 µg/ml Hoechst-33342 to evaluate total blastocyst cell number. Follicular fluid from all follicles aspirated was pooled per breed during each replicate and steroid hormones were measured using liquid chromatography tandem mass spectrometry. A larger follicle diameter was observed for Landrace animals (5.7 vs. 4.8 mm, $P < 0.0001$) and individual COC area was larger at 0 h after aspiration ($P < 0.0001$) compared to Duroc. Contrary, cumulus expansion from 0 to 20 h of maturation was greater for Duroc oocytes compared to Landrace (407 ± 67% vs. 319 ± 31%, $P < 0.0001$). After fertilization, cleavage rate was highest for Duroc oocytes. The highest blastocyst yield was obtained for Duroc oocytes fertilized with the Landrace boar, but no significant differences were found for total blastocyst cell number. No significant differences were observed between breeds for the individual steroid hormones but in Landrace FF, the oestradiol level tended to be higher ($P = 0.09$) compared to Duroc. Furthermore, differences in patterns were observed in the steroid pathways and both the total level of oestrogens ($P = 0.01$), and aromatase products/substrates ratio ($P < 0.01$) were higher in Landrace FF than in Duroc, indicating a higher degree of feminization in Landrace. In conclusion, breed differences were found at the early follicular phase with regard to *in vitro* oocyte developmental competence and steroidogenesis.

4. Discussion

The results in this thesis are based on three papers. The first section of this discussion provides methodological considerations, which is followed by a discussion focussing on the breed differences in relation to *in vitro* maturation, fertilization and embryo culture. Finally, progressive motility and steroid hormone levels in follicular fluid will be discussed.

4.1 Methodological considerations

The following section explains experimental designs and statistics used for the different papers. Furthermore, decisions for established protocols and methods used are discussed.

4.1.1 Experimental designs

Age of sows

In paper I of this thesis, ovaries were collected at a local abattoir from sows originating from two Norsvin nucleus herds. It was expected that most animals would only have had one or two litters due to the high replacement rate at nucleus level. However, after genotyping and identifying the sows it was discovered that the Duroc sows had a higher parity, and thus age, ranging from parity 1-6 compared to parity 1-2 in Landrace. This could have influenced the IVM results as maternal age is affecting oocyte developmental competence (Bagg et al., 2007; Grupen et al., 2003; Krisher, 2019). Therefore, only animals that had one or two litters were selected from the nucleus farms for further study in paper III.

Timing of ovary collection

The timing of ovary collection is important when comparing differences between two breeds, as follicular development and stages vary throughout the oestrous cycle. The weaning-to-service interval, which is defined as the days from weaning until the first insemination, is 5.7 ± 1.8 days in Duroc and 5.7 ± 1.3 days in Landrace. This suggests that follicular development and time of ovulation relative to oestrous length are comparable. In both paper I and III, sow ovaries were collected one day after weaning so that the stage of follicle development at a fixed time point after weaning would be comparable between the two lines. The ovaries and oocytes were thus in the early follicular phase. During antral follicle development, only 30-40% of the recruited follicles will finally mature and ovulate (Guthrie & Garrett, 2001), while the rest will undergo atresia. Collecting ovaries at day 4 or 5 post weaning would represent the

final selected ovulatory population and might provide better insights in relation to the difference in litter size. However, collection at that timepoint was challenging with the on-farm management system and planning at the slaughterhouse. It is furthermore known that there is great variation in oestrous length between animals (Soede et al., 2011). Collection at day 4 or 5 after weaning would thus additionally have increased variation in follicular development and the risk of collecting ovaries with ovulated follicles.

During the IVEP rounds for paper I and III, the number of Duroc and Landrace animals available was a limitation as a batch of approximately 8-15 sows were only slaughtered from the nucleus herds every 7th or 8th week. It was therefore decided to study fertilization in paper II with oocytes collected from random sows in both the follicular and luteal phase. In this way, many random ovaries and oocytes could be collected in a short time for this large study as most ovaries collected at the abattoir are in the luteal phase since this is the longest phase in the oestrous cycle.

Paper I and paper III

It would have made the study in this thesis stronger if papers I and III could have been combined. If enough oocytes had been available, some of the oocytes could be stained during and after maturation, while the rest could be fertilized and stained to study fertilization and polyspermy rates and cultured to the blastocyst stage to assess embryo development. The results of *in vitro* maturation could then be related to the *in vitro* embryo production results when all parameters were analysed in the same groups of oocytes during the same IVEP weeks. However, this was not possible due to the small number of oocytes available per week. Especially the number of Duroc oocytes was a challenge and it was therefore not possible to assess fertilization and polyspermy rates for the four groups in paper III. Nevertheless, a smaller COC area at collection but a larger cumulus expansion from 0-20 h of maturation for Duroc oocytes compared to Landrace were observed in both paper I and paper III and suggest that the results from the two separate studies may complement each other.

Statistics

During analysis of *in vitro* embryo production, the number of cleaved oocytes and embryos were assessed per well to statistically analyse cleavage and blastocyst percentages. The number of observations was in this way restricted as 25-30 oocytes were cultured per well. Variation between weeks was observed while performing the experiments for paper II and III and this was confirmed when analysing the data.

Therefore, as many replicates as possible were carried out for the different experiments and variation between the different weeks was taken into account by including IVEP week as a random factor in the linear mixed models. This also covered the possible difference in relation to season that has been shown to affect oocyte developmental competence and quality (Bertoldo et al., 2010). Within week we also observed some variation between wells when oocytes were fertilized with the same boar and concentration. That is why as many oocytes as possible were used per week.

4.1.2 Protocol establishments

At the start of this PhD project, protocols had to be established for porcine *in vitro* embryo production. No commercially produced medium is available and the media in this thesis was therefore prepared daily. Decisions made during optimization of the maturation, fertilization and culture protocols are discussed below.

4.1.2.1 *In vitro* maturation

Numerous studies have been conducted to improve porcine *in vitro* embryo production and supplementation of maturation media with various components has improved oocyte maturation to MII stage, cleavage rate and blastocyst development (Abeydeera, 2002; Hunter, 2000). At the start of this PhD project, protocols had to be established and it was decided to start with a TCM-199 based IVM medium. In the first maturation rounds, the maturation medium consisted of TCM-199 medium supplemented with 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 50 ng/ml epidermal growth factor, 50 µg/ml gentamicin and 0.1% BSA. During the first 24 h of IVM the medium was supplemented with 0.05 IU/ml hFSH and 5 µl/ml ITS (mixture of recombinant human insulin, transferrin and sodium selenite) (Sjunnesson et al., 2013). During a valuable research stay at the University of Kent in Canterbury, their porcine IVEP protocol and knowledge was shared. The maturation medium was based on Porcine Oocyte Medium (POM) from Yoshioka et al. (2008) and higher nuclear maturation rates were obtained with this medium compared to the TMC-199 based medium. For this reason, and as it would be beneficial to compare results from different IVF labs when using the same media, it was decided to use the same medium in this thesis.

The POM medium was supplemented with 0.1 mM cAMP (Funahashi et al., 1997) during the first 20 h of maturation and with 10 ng/ml epidermal growth factor (EGF) and 40 ng/ml FGF2, 20 ng/ml LIF and 20 ng/ml IGF1 throughout the entire

maturation period (Yuan et al., 2017). In paper I, nuclear maturation percentages to MII phase were 90% and this was high compared to other articles in which around 70 - 85% of the oocytes that start maturation completed nuclear maturation and reached MII phase (Gil et al., 2017; Somfai et al., 2005; Yuan & Krisher, 2012). However, our IVM results were in line with the 89% nuclear maturation reported by Yuan et al. (2017), who supplemented IVM medium with FLI. As high nuclear maturation rates were obtained for both breeds in paper I, the sperm preparation and *in vitro* fertilization protocol was studied and optimized in paper II.

4.1.2.2 *In vitro* fertilization

Fresh vs. frozen-thawed sperm

Fresh sperm is easily available from the Norsvin AI station and has a high sperm motility. However, cryopreserved sperm from different boars was used during the studies to reduce boar and sample variation. Frozen straws from each individual boar were originating from the same ejaculate. Not all boars are good freezers and variation in semen freezability has been related to individual boar differences (Holt et al., 2005; Waterhouse et al., 2006), but variability has also been reported between breeds. When classified into good, medium and poor semen freezability, Thurston et al. (2002) showed that Landrace boars had significantly more good quality spermatozoa while Duroc boars were often classified in the poor quality group. In general, freezing spermatozoa from Norwegian Duroc boars is more difficult than from Landrace boars, but variation between individuals has indeed been observed (Norsvin, unpublished data). Semen from the boars used in this thesis were cryopreserved by Norsvin and they were selected based on their breeding values and cryotolerance. Straws of one of the Duroc boars were eventually not used for breeding as his freezing-thawing results were unsatisfactory. During analysis of his samples by CASA and flow cytometry in our IVF lab, sperm viability and motility were sufficient, and it was decided to include this boar in the IVF study in paper II.

Percoll density gradient centrifugation

Percoll® density gradient centrifugation has successfully been used in pig IVF to select acrosome intact live sperm cells with good motility after freezing and thawing. Centrifugation of sperm with Percoll® has been shown to result in faster male and female pronuclei formation (Matás et al., 2003) and better fertilization, cleavage and blastocyst rates (Jeong & Yang, 2001; Matás et al., 2003; Noguchi et al., 2013) compared to washing sperm in other solutions. For bovine, another sperm separation

and purification product is available which is known as Bovipure®. Higher cleavage and blastocyst rates have been reported in bovine IVEP when Bovipure® gradient was used compared to Percoll® gradient to wash frozen-thawed sperm (Samardzija et al., 2006). Therefore, we were interested whether this gradient also could work for the selection of frozen-thawed porcine sperm for IVF. The Bovipure® gradient worked well when centrifuging fresh sperm to select motile sperm and remove sperm extender and dead sperm cells, but a low sperm motility and viability were obtained using frozen-thawed porcine sperm. Therefore, it was decided to continue with Percoll® density gradient centrifugation. A 45/90% Percoll® gradient was used as it was shown that this concentration selected the highest percentage spermatozoa with normal morphology and less alteration in chromatin structure and DNA damage in fresh semen, which resulted in higher penetration rates compared to the 45/60% and 60/75% gradient (Matás et al., 2011). During IVF, Porcine Zygote Medium (PZM) with theophylline as capacitation factor was used (Yoshioka et al., 2008) and the Percoll® gradient was diluted with PZM to prepare the correct concentrations. Lately, a study with fresh sperm showed that the high 45/90% concentration resulted in a lower blastocyst rate and that hatched blastocyst showed a reduced oestrogen activity, which is essential for development from compact morula to blastocyst stage, compared to the 40/80% and 35/70% Percoll® density concentrations (Ohlweiler et al., 2020). It should thus be considered if it would be beneficial to use a lower Percoll concentration within our IVF protocol.

Co-incubation of oocytes and sperm

A sperm-oocyte ratio of 1000:1 is often used in porcine IVF (Gil et al., 2007; Kidson et al., 2004; Martinez et al., 2017). Progressive sperm motility is approximately 50% after freezing and thawing in our laboratory, but motility differs between boars and straws. Therefore, a 500:1 and 250:1 progressively motile sperm to oocyte ratio were studied in paper II. Different periods of co-incubation of pig sperm and oocytes have been reported, ranging from 24 hours (Kidson et al., 2004; Sjunnesson et al., 2013), to 8-10 hours (Suzuki et al., 2004; Yoshioka et al., 2008, 2020) and 4-5 hours (Chen et al., 2020; Martinez et al., 2017). A long co-incubation may expose zygotes to suboptimal culture conditions with increased reactive oxygen species (ROS) produced by dying and/or damaged sperm and shortening the co-incubation has been shown to reduce the incidence of polyspermy. It has been observed that sperm cells already attach to the zona pellucida within the first 10 minutes of co-incubation during IVF (Gil et al., 2007; Grupen & Nottle, 2000) and that sperm need another 2 hours to penetrate the oocytes (Gil et al., 2007). Also *in vivo,* boar sperm can reach

and penetrate the zona pellucida within 2 hours of insemination and this causes resumption of oocyte meiosis within 3 hours of insemination (Hunter & Dziuk, 1968). While some research groups denude oocytes from their cumulus cells before fertilization (Chen et al., 2020; Martinez et al., 2017; Yuan et al., 2017), others leave the COCs intact before fertilization and remove them before the zygotes go into culture (Suzuki et al., 2004; Yoshioka et al., 2020). Both methods have shown to result in blastocyst formation, but it has been suggested that the presence of cumulus cells reduces polyspermy and enhance embryo development (Li et al., 2018; Van Soom et al., 2002).

Within our IVF protocol, oocytes were co-incubated with sperm cells for 2 hours, after which the oocytes were moved to a new well with fresh PGM medium to remove the excess sperm cells that did not attach to the zona pellucida. After another 2 hours, the oocytes were removed from their cumulus cells by vortexing before they were cultured. Penetration rates of 50-70% and polyspermy rates of 20-50% are reported by others (Chen et al., 2021; Martinez et al., 2017; Yoshioka et al., 2008). An average fertilization rate of 39.6% was obtained for all boars with the 500:1 ratio in paper II and this was thus lower than expected. However, a difference was observed between individual boars and fertilization rates of 52.7% and 59.7% were found for the two best boars with the highest blastocyst rates, which are in line with the aforementioned results. In addition, average polyspermy for all boars with the 500:1 ratio in paper II was 25.3% (ranging from 11.9 - 43.0%). The incidence of polyspermy was relatively low in paper II, but a further reduction is always desirable. Therefore, it would be relevant to further study sperm selection and preparation methods and optimize IVF procedures within our protocol to increase fertilization rate, without increasing polyspermy.

4.1.2.3 *In vitro* embryo culture

Embryo culture is the longest period during *in vitro* embryo production. It has been stated that porcine IVEP is still suboptimal, as only about 15-30% of the oocytes that start maturation develop to blastocyst stage. Blastocyst rates of 20-40% have been reported in cattle (Ferré et al., 2020) and initially do not appear to be much different from IVEP in pigs. However, in pig IVEP results are often not constant over several weeks and very low blastocyst rates are sometimes suddenly observed during some weeks (Different laboratories, personal communications). In cattle, IVEP is more

constant and a blastocyst rate towards 40% is most of time obtained when using commercially available media.

In paper II, satisfying and promising blastocyst yields were obtained. This was the first study with successful culture of blastocysts and constant results were obtained over several months. The best boar and 500:1 ratio had an average blastocyst yield of 37.0% on day 6 which is in line with Yuan et al. (2017) who reported an average blastocyst yield of 40%. Average blastocyst yield for all boar at the 500:1 concentration was 27% in our study and lower due to individual boar differences. One of the boars had a higher blastocyst rate at the 250:1 ratio, and this is confirming the findings that variation in the optimal sperm-oocyte ratio during fertilization is shown between boars (Gil et al., 2007; Gil et al., 2004; Wang et al., 1991; Xu et al., 1996). Total blastocyst cell number in paper II was high for all boars (on average 59.8 ± 22.6 cells), as 30 - 45 cells are usually reported for good quality *in vitro* produced pig blastocysts (Gil et al., 2013; Yoshioka et al., 2020; Yuan et al., 2017). Bagg et al. (2007) reported a higher blastocyst cell numbers for adult sows (around 52 cells) compared to prepubertal pigs (around 20-40 cells). Only adult sow ovaries were used in this study and that may have resulted in a high blastocyst cell number. In paper III, a lower blastocyst yield (average 15.2% ranging from 7.8-24.2%) and lower blastocyst cell numbers (average 38.2 ± 17.8 cells) were reported compared to paper II. During experiments for paper III, challenges in the laboratory were faced with variable results over several weeks. To date, we have not found the reason for the problem and are still investigating the cause so that consistent blastocyst yield can be obtained again. We sometimes have weeks with good results, but it is a common and known problem with pig IVF as mentioned before. The bovine IVF performed simultaneously in the same incubators in our lab showed consistently good results, and variation is therefore not due to culture conditions in the lab, but probably due to medium.

Porcine zygote medium-5 (PZM-5) (Yoshioka et al., 2008) was used in this study and embryo development was assessed on day 5, 6 and 7 of culture in paper II. Hatching of oocytes was only observed a few times and some blastocysts started to collapse from day 6 to 7. Therefore, it was decided to only culture blastocysts until day 6 in paper III. Blastocyst hatching is not often observed during pig embryo culture. However, it has been suggested that the optimized porcine blastocyst medium (PBM) improves embryo quality, development to blastocyst stage and hatching (Mito & Hoshi, 2019). Additionally, supplementation of IVC medium with foetal bovine serum

(FBS) at the morula or blastocyst stage improves *in vitro* hatching (Ohlweiler et al., 2020; Yoshioka et al., 2005) but a defined or semi-defined medium is preferable as media supplemented with serum components can carry a higher risk of disease transmission when embryos are produced for transfers.

4.1.3 Confocal microscopy

Confocal microscopy is a useful tool for quantitative measurements within cells and tissues, when used appropriately (Jonkman et al., 2020). Confocal microscopy makes it possible to take sharp images of the plane of focus while blocking blurry features from outside of the focus as is the case with widefield microscopy. In addition, a confocal microscope can take optical sections throughout the sample which is useful to localize different cells in thick specimens. A single image may not represent the entire sample, and with stacking several images from the different planes, i.e Z-stack, the 3D structure can be reconstructed and analysed.

Oocyte maturation parameters

In paper I of this thesis, *in vitro* oocyte maturation was investigated by confocal microscopy. It was decided to stain oocytes with three different dyes; Hoechst-33342 to stain DNA and assess nuclear maturation, PNA lectin Alexa Fluor 568 to stain cortical granules and assess their distribution and MitoTracker™ Green FM (M7514, Invitrogen, Thermos Fisher Scientific, Waltham, MA, USA) to stain mitochondria. Overlapping excitation (Ex) and emission (Em) wavelength were avoided with a combination of those three dyes and the dyes had been used before in different combinations with other dyes (Lodde et al., 2021; Sun et al., 2001). The fixation, permeabilization and staining was optimized in relation to concentrations during different pilot studies. Negative controls of unstained oocytes were included during optimization of the concentrations. Especially the MitoTracker Green dye was challenging because on the one hand photobleaching, when the fluorophore loses its fluorescence due to exposure to light, is a known problem with this dye, while on the other hand the whole cytoplasm can be stained at too high concentrations. A successful protocol was established, and the experiment was started. However, in the last two IVEP rounds of paper I, MitoTracker Green was not clearly visible in the oocytes. It was decided to leave out the mitochondria staining from the results since new Duroc and Landrace oocytes were required for the follow-up study for paper III and Hoechst and PNA lectin Alexa Fluor 568 staining was successful in all oocytes. MitoTracker Green was chosen as it accumulates in the mitochondrial matrix

regardless of the mitochondrial membrane potential and it stains the total mitochondrial mass (Invitrogen, 2008), but it was found out later that MitoTracker Green is not retained well after aldehyde fixation of cells and therefore better used in live cells. In a further study it is recommended to use another Mitotracker dye (Red or Orange) that is retained after fixation. It is then recommended to change the staining of cortical granules with PNA lectin Alexa Fluor 568 to PNA-FITC to avoid overlapping Ex and Em wavelengths.

Plane of focus

Fixed oocytes and embryos can be mounted in fluorescence mounting media under a cover slip for analysis. They are not flat so during analysis of distributions of for example cortical granules or mitochondria it should be realised that the image should be taken at the right plane. An image captured at the bottom, or the top of the oocyte might represent the wrong distribution of organelles. In paper I, the images for maturation parameters were taken in the middle, the equatorial plane, of the oocytes. In future studies it would be of interest to look into 3D whole mount fixation and analysis (Yokomizo et al., 2012) or even assessing the changes in organelle distributions during IVM by timelapse microscopy (Lane et al., 2017). In paper II and III, polyspermy and total blastocyst cell number were assessed by going through the different planes of the microscope by hand while counting the sperm cells, pronuclei and blastocyst cells as it was not possible to take clear images for later assessments.

Image capturing and analysis

In paper I, images were taken and analysis were carried out with the same microscope wavelength settings. Images were taken the same day after staining, as we were worried about changes in the dyes during storage. Patterns of cortical granule distribution from images at the equatorial plane were classified simultaneously by two persons into one of the six categories to ensure correct classification. Zygotes and embryos for paper II and III were stored at -20 °C after Hoechst staining and polyspermy and total blastocyst cell number were analysed later in the slides as we have experienced before that this is not a problem with the Hoechst dye. The same person assessed the samples to avoid variation. Total blastocyst cell number was counted twice by the same person and if the numbers did not match, the blastocyst was recounted again until the person was certain about the cell number.

4.1.4 Computer assisted sperm analysis

Sperm motility is related to fertility in pigs (Gadea, 2005) and cattle (Nagy et al., 2015). Traditionally, sperm motility was assessed manually and subjectively using phase contract microscopy. An objective computer assisted sperm analysis system (CASA) is nowadays available that reduces bias compared to visual and subjective evaluation, but it has been shown that CASA results are still affected by several factors. Outcomes are among other things affected by sperm cell concentration, semen extender, instrument settings, chamber used and the technician handling the sperm samples (Amann & Waberski, 2014; Brito, 2010; Meara et al., 2022). Nevertheless, the system is commercially used to measure sperm concentration and the percentage of motile sperm to produce semen doses at cattle and pig breeding stations. To avoid variation and inaccurate results in this thesis, the same person prepared the samples and operated the instrument to assess the sperm samples. In paper II and III sperm parameters were assessed 30 minutes after thawing of the straws to check if the sperm cells were viable and motile, and after Percoll® density centrifugation to calculate how much sperm should be added to the oocyte to reach the correct sperm to oocyte ratio. During pilot studies and in experiments for paper II, we observed that Landrace boars have on average a better total motility after thawing compared to Duroc boars, but that Duroc boars often showed a higher hyperactivity.

4.1.5 Flow cytometry

Flow cytometry is a technology that can rapidly analyse single cells in populations with high precision and sensitivity within a short time. To ensure reliable output, appropriate calibration of the instrument is required before running sperm samples. Optical alignment using uniformly fluorescent beads (Flow-check TM Fluorospheres, Beckman Coulter Ltd) was run every time prior to analysis of sperm samples in paper II. Well-established protocols for assessing sperm DNA fragmentation with the patented SCSA® protocol (Evenson & Jost, 2000) and sperm viability and acrosome integrity were used. Optimization and adjustments of protocols were therefore not necessary. Sperm samples were prepared and analysed, and results were assessed by the same person for all the runs to avoid variation and inaccuracy in the studies.

4.2 Breed differences

4.2.1 In relation to *in vitro* oocyte maturation

Completion of nuclear and cytoplasmic maturation is important for the oocyte to acquire developmental competency so that it can be successfully fertilized and develop into a viable embryo. Follicle size, age of the animal, nutrition and season have been shown to affect oocyte developmental competence (Bertoldo et al., 2010; Bertoldo et al., 2019; Grupen et al., 2003; Zak et al., 1997), but also differences between breeds have been observed (Hunter, 2000). Ovarian characteristics and the *in vitro* maturation potential of Norwegian Duroc and Landrace oocytes were for the first time studied.

Ovarian characteristics and cumulus expansion

In paper I of this thesis, more 3-8 mm surface follicles were observed on Landrace ovaries, with an average of 8 more follicles per ovary compared to Duroc. In paper III, average follicle size of the 10 largest follicles was larger in Landrace animals (5.7 vs. 4.8 mm) compared to Duroc. The number of 3-8 mm follicles was not significantly different between the breeds in the last-mentioned study, but the mean number was still higher for Landrace compared to Duroc (23.2 vs. 21.4 follicles). In both studies, Duroc COCs had a smaller area at time of collection, but a broader cumulus expansion was observed from 0 – 20 h of maturation. These contradicts results from Procházka et al. (2000), who observed a larger cumulus expansion for oocytes originating from larger follicles. Another study that compared the Hungarian Mangalica breed, that has a low reproductive performance and a small litter size, to Landrace gilts found a significantly lower number of preovulatory follicles in Mangalica (6.8 vs. 19.6) with a smaller follicle size (5-7 mm vs. 6-9 mm) compared to Landrace. This is in line with results for the Duroc breed in this thesis work. Contrary, 34 h after the initiated LH surge a lower degree of *in vivo* cumulus expansion was observed for the Mangalica breed (Rátky et al., 2005), while we observed a better *in vitro* cumulus expansion for Duroc. A study comparing the Meishan breed, that has a high reproductive performance and a large litter size, with the Large White breed observed no difference in ovulation rate while Meishan pigs have more piglets. Preovulatory follicle diameter was in this study smaller for Meishan animals, but more oocytes were in more advanced stages of meiosis compared to Large White (Faillace & Hunter, 1994; Hunter et al., 1994). The aforementioned studies investigated preovulatory follicles and *in vivo* matured oocytes while ovaries in the present thesis were collected one day after weaning in the early follicular phase and matured *in vitro*. During

follicular development, the oocyte and follicle undergo growth and maturation and most of the antral follicles undergo atresia which makes it difficult to properly compare studies.

Nuclear and cytoplasmic maturation

In the present thesis, nuclear and cytoplasmic maturation were assessed at 20 and 48 h of maturation to study if there were differences between the breeds. More Duroc oocytes were found in the GV2 and MI phase at 20 h of maturation, while most Landrace oocytes were at the GV1 stage, which indicated a faster nuclear maturation in Duroc halfway maturation. For cytoplasmic maturation, cortical granule distributions were assessed at 20 h and 48 h of maturation and glutathione content of the oocytes was additionally measured at 48 h. Contrary to nuclear maturation, cortical granule distribution was more advanced for Landrace at 20 h. However, no differences were observed for nuclear maturation, cortical granule distribution or GSH content after maturation. It was not possible to assess cumulus expansion at the end of maturation, as cumulus cells from the individual oocytes were overlapping. Cumulus expansion is essential for meiotic progression (Suzuki & Saito, 2006) and it has been suggested that nuclear maturation is preceded by cumulus expansion (Torner et al., 2004). Although not significant, the average GSH content at the end of maturation was higher in Duroc oocytes than in Landrace (4.45 vs 4.11 pmol), which may be related to a higher cumulus expansion at the same timepoint. Cumulus cells play an important role in GSH synthesis (Maedomari et al., 2007) and greater cumulus expansion is positively correlated with a higher intracellular GSH content in bovine oocytes (Furnus et al., 1998).

Shorter maturation period for Duroc

There is a possibility that Duroc oocytes reached MII phase earlier and have been arrested for a longer time until fertilization, as they were further ahead at 20 h of IVM. Porcine oocytes normally reach MII stage around 36 h of maturation and it has been suggested that maturation percentages do not change any more towards the end of IVM (Kikuchi et al., 1999; Somfai et al., 2005). An increased incidence of chromosomal abnormalities has been reported when pig oocytes were matured for 40 hours or longer (Sosnowski et al., 2003) and it is thus worth investigating if shortening the IVM period for Duroc oocytes can increase blastocyst development and quality.

4.2.2 In relation to *in vitro* fertilization

Individual boar differences

Large variations have been reported between boars for *in vitro* fertilization rates with cryopreserved epididymal (Ikeda et al., 2002) and ejaculated sperm (Noguchi et al., 2013; Wang et al., 1991). In paper II, fertilization was studied using cryopreserved semen from three Duroc and Landrace AI boars. Individual differences between boars were observed for fertilization, cleavage and blastocyst rates although all IVEP rounds were adjusted for the same number of progressively motile sperm cells present per oocyte. In this study a Landrace boar had the highest cleavage and blastocyst rates, but also high blastocyst rates were observed for the two Duroc boars. The Duroc boar with the lowest cleavage and blastocyst rates was the boar that was ultimately not selected by Norsvin for breeding as his freezing-thawing results were unsatisfactory. During analysis of his samples by CASA and flow cytometry in our IVF lab, sperm viability and motility were sufficient after Percoll® density gradient centrifugation. Therefore, it was decided to include this boar in the IVF study in paper II. However, IVEP results show that sperm cells from this boar had a lower fertilization and blastocyst rate compared to the other boars, although sperm motility, AIL and DFI were sufficient after Percoll® density gradient centrifugation.

Breed differences

In addition to individual boar differences, differences between breeds have been observed during IVEP. Suzuki et al., (2003) reported higher penetration rates for Large White boars compared to Landrace and Duroc, which also lead to a higher polyspermy. We were originally interested in sperm motility patterns of cryopreserved Norwegian Duroc and Landrace semen, as differences in fresh semen motility were observed between the breeds by Tremoen et al. (2018). Fresh semen from Norwegian Duroc boars had a higher percentage of hyperactive sperm cells with a more circular pattern at the day of sperm collection compared to Norwegian Landrace, which showed a more linear pattern. However, hyperactivity in Landrace semen increased over four days' storage while it stayed constant for Duroc (Tremoen et al., 2018). In the same study it was suggested that sperm variables associated with hyperactivity affected the total number of piglets born. Moreover, Landrace boars have a higher semen volume at collection with a lower sperm cell concentration and a higher percentage of progressively motile sperm cells compared to Duroc (Smital, 2009; not Norwegian Duroc and Landrace). This has also been observed by Norsvin (Unpublished data). If breed differences in sperm motility patterns before and after

Percoll density centrifugation also exist in cryopreserved semen, this could affect the IVEP results. After Percoll® density centrifugation, hyperactivity was significantly higher for Duroc sperm while other motility parameters like VAP, VSL, LIN and WOB were higher for Landrace (Unpublished results). However, the number of boars included in this study was too small to conclude something about differences in sperm quality parameters between the breeds.

It was clear from the results that Percoll® density gradient centrifugation selected the motile and live sperm cells as it decreased DFI and increased the percentage of AIL sperm cells and increased motility in sperm samples from all boars. Individual boar differences have been observed in the response to sperm preparation by Percoll® (Martecikova et al., 2010; Suzuki & Nagai, 2003) and it has been indicated that preliminary screening for each individual boar is recommended to select their optimal IVF conditions (Almiñana et al., 2005; Gil et al., 2008). A large study including a sufficient number of Duroc and Landrace boars may give insight into the optimal sperm preparation and IVF conditions based on breed or individuals.

4.2.3 In relation to *in vitro* embryo culture

In paper III, Duroc oocytes showed a higher cleavage rate, and the highest blastocyst rate was obtained for the Duroc oocytes fertilized with semen from the Landrace boar. No significant differences in total blastocyst cell number were observed between the four groups. Overall, results suggest that Duroc oocytes have a better oocyte developmental competence during IVEP as a broader cumulus expansion and faster nuclear maturation at 20 h of IVM were observed in paper I, and higher cleavage and blastocyst rates were obtained in paper III compared to Landrace. It would be relevant to study fertilization and polyspermy rates for the different groups, as broader cumulus expansion has been related to a lower polyspermy rate (Costermans et al., 2019). Interestingly, the purebred Duroc and Landrace groups both resulted in a blastocyst rate of 14.4%, while the Landrace oocytes fertilized with semen from the Duroc boar only yielded a blastocyst rate of 7.8% and the Duroc oocytes fertilized with semen from the Landrace boar yielded the highest blastocyst rate of 24.2%. In breeding, the effect of heterosis is used to get offspring that perform better than their parents. In cattle, it has been investigated if heterosis also occurs in IVEP, but results suggested that crossbreeding was not always leading to better blastocyst yield (Fischer et al., 2000; Slade Oliveira et al., 2019).

Although more follicles are present on Landrace ovaries one day after weaning and thus more oocytes can be collected for *in vitro* embryo production, the blastocyst yield was lower in paper III compared to Duroc. This suggests that more Landrace oocytes are required to obtain the same number of blastocysts as in Duroc for embryo transfers.

4.3 Progressive motility

During pig IVF, the sperm count per ml is normally reported, while the total motility or progressive motility of a sperm sample is often not considered. Progressive motility has been shown to be of importance during bovine IVF. Progressive motility of frozen-thawed sperm showed a good correlation with *in vitro* pronucleus formation (Tanghe et al., 2002b) and higher cleavage and blastocyst rates were obtained with high progressively motile sperm compared to low progressively motile sperm (Li et al., 2016). Valencia et al. (2018) showed furthermore in a pig IVEP study that progressive motility, total motility and normal sperm morphology were significantly higher in fresh boar semen for the group that had a high cell cleavage (defined as cleavage >23%) compared to the group with low cleavage (defined as cleavage <23%).

In paper II, differences in IVEP outcomes were observed between individual boars although adjusted for progressive motility. This suggests that there are factors other than just the number of progressively motile sperm that influence IVEP results. More spermatozoa were added for the IVF rounds when samples had a lower progressive motility to obtain the correct sperm-oocyte ratio. This increased the total number of sperm as well as the number of immotile or dead sperm cells present during fertilization for different replicates and boars. It has been indicated that a higher percentage of dead spermatozoa prior to freezing negatively affects *in vitro* fertilization, cleavage and blastocyst rates (Roca et al., 2013). Dead sperm cells produce reactive oxygen species and this creates suboptimal culture conditions that affect viable spermatozoa, as too high levels lead to sperm DNA damage, as shown in different species (Agarwal & Said, 2003; Simões et al., 2013; Takahashi et al., 2000). An *in vitro* study in bovine showed that DNA damage induced by sperm oxidative stress affected cleavage rate but not blastocyst formation rate or quality (Simões et al., 2013). The effect of the number of dead and/or immotile sperm cells would be a topic of interest for further research to understand differences between individuals and to optimize IVF protocols. Furthermore, in paper II we only assessed fertilization and embryo development after adjusting for the same progressivity, but we did not

analyse IVEP results without adjusting by using the same number of sperm cells /ml. Although it is well known that differences are observed between boars, the study in paper II may have provided more insight when comparing adjustment of progressive motility for all boars to not adjusting for the different boars.

4.4 Follicular fluid steroid hormones

The follicular fluid provides a microenvironment for the oocytes and contains the produced steroid hormones required for follicular development, reproductive function, and fertility (Drummond, 2006). No significant differences were observed between breeds for the individual FF steroid hormone levels, but differences in the pathways were observed with higher levels of progestagens and oestrogens in Landrace FF compared to Duroc. In addition, the total level of oestrogens was significantly higher in Landrace FF. A clear difference in follicular steroid hormones between the breeds was thus already observed at the early follicular phase, while oocytes in this phase are still growing and their granulosa cells only produce low levels of oestradiol. Differences between other breeds have also been observed in relation to steroid hormones. In Meishan, with a larger litter size but smaller preovulatory follicle diameter and more oocytes at the MII stage (Faillace & Hunter, 1994; Hunter et al., 1994), a higher oestradiol concentration and an increased aromatase activity in both granulosa and theca cells have been reported during the follicular phase compared to FF from Large White pigs (Biggs et al., 1993; Miller et al., 1998). This is in line with the Landrace line which has a larger litter size compared to Duroc. However, Duroc oocytes performed better during IVEP and no correlations between the individual steroid hormones and IVEP outcomes were observed in paper III. Besides steroid hormones, follicular fluid consist out of several other components such as growth factors, interleukins, anti-apoptotic factors, proteins, peptides and amino-acids that have been found to affect oocyte developmental competence during maturation (reviewed by Revelli et al. (2009). Further studies in follicular fluid components for the Duroc and Landrace lines may thus provide insight into the difference in oocyte developmental competence and how to improve media for *in vitro* embryo production.

5. General conclusions

In the current thesis, *in vitro* embryo production and staining protocols were established, and differences were observed for the Norwegian Duroc sire and Norwegian Landrace dam line throughout oocyte maturation, fertilization and embryo culture. Further research is recommended to optimize the protocols per breed and to find out how differences between the breeds are related to oocyte developmental competence. The work in this thesis has direct practical relevance to Norsvin as the established protocols can be further used in follow-up studies regarding *in vitro* embryo production, embryo quality and embryo freezing and storage. Furthermore, knowledge on reproductive traits in the Duroc breed is highly relevant as this breed has a smaller litter size and the use of embryo technology can be of genetic and economic importance within the breeding program. Moreover, the acquired knowledge on embryo handling and quality is valuable for future commercialisation of embryo transfers.

From the comparison of *in vitro* embryo production in Norwegian Duroc and Landrace the following specific conclusions can be drawn:

Ovarian characteristics and follicular fluid

- More follicles were present on Landrace ovaries one day after weaning and more oocytes can thus be collected for *in vitro* embryo production.
- Average follicle size was larger on Landrace ovaries one day after weaning.
- Individual cumulus-oocyte complex area was on average larger for Landrace oocytes at time of aspiration.
- No differences were observed in individual steroid hormones between the breeds, but differences in patterns were observed in the steroid pathways. Higher levels of total oestrogen and a higher ratio of aromatase products/substrates were found in Landrace, indicating a higher degree of feminization.

IVM

- Cumulus expansion from 0-20 h of maturation was broader for Duroc.
- Nuclear maturation was more advanced for Duroc oocytes at 20 h, while cortical granule distribution was more advanced for Landrace oocytes at the same timepoint.
- Nuclear maturation to MII stage at the end of maturation was consistently high in both breeds and no difference was observed between the breeds.

- No differences between the breeds were observed at the end of maturation for cortical granule distribution or glutathione content, which were assessed as parameters for cytoplasmic maturation.

IVF

- Cryopreserved sperm from Landrace boars normally had a better motility after thawing. However, sperm from both Duroc and Landrace boars led to successful fertilization and blastocyst development.
- Fertilization with the 500:1 ratio resulted in a higher fertilization rate compared to the 250:1 ratio, while polyspermy level was consistent across ratios.
- Differences between boars were observed for fertilization rate but not for polyspermy, although all IVEP rounds were adjusted to the same number of progressive motile sperm cells present per oocyte.

IVC

- Fertilization with the 500:1 ratio resulted in a higher blastocyst rate at day 6 compared to the 250:1 ratio, while no difference was observed for cleavage rate at day 2.
- Differences between boars were observed for cleavage and blastocyst rates, although all IVEP rounds were adjusted to the same number of progressive motile sperm cells present per oocyte.
- Duroc oocytes showed a higher cleavage rate at day 2 after fertilization than Landrace oocytes.
- The highest blastocyst rate was obtained for Duroc oocytes fertilized with the Landrace boar.
- High blastocyst cell numbers could be obtained with both Duroc and Landrace boars.
- Embryo culture up to day 6 is optimal with the current established protocol to obtain most blastocysts if embryo transfer is the goal.
- Hatching of blastocysts has been observed at day 7 of culture but is not common with the current established protocol.

6. Future perspectives

During this thesis, porcine IVEP and staining protocols have been established. In the future it is of importance to optimize the protocol regarding oocyte maturation, fertilization and embryo culture to obtain a high and consistent blastocyst yield. Decreasing the concentration of the Percoll® density gradient and using different methods of sperm selection could be relevant to study first. Furthermore, it is of importance to keep up with the latest developments to improve *in vitro* blastocyst yield in pig IVEP, as several research groups focus on optimization of media. Supplementation of IVF medium with haptoglobin, a protein produced by the liver but also present in the oviduct and the uterus, has recently shown to improve blastocyst rate (García-Vázquez et al., 2021). In addition, supplementation of culture media with GlutaMAX (Chen et al., 2021) or antioxidants such as lycopene which reduces oxidative stress (Kang et al., 2021) also have increased blastocyst rate. Optimization of the IVEP protocol will be beneficial for obtaining more blastocysts and a constant blastocyst yield. Both are important and necessary so that embryo technology can be applied in the pig industry.

Differences between breeds were the focus of this thesis and it is possible that we eventually end up with IVEP protocols that are optimal for the individual breeds. Investigating the effect of a shorter maturation period for Duroc oocytes is of interest to see if this improves fertilization and blastocyst development. Furthermore, a larger IVF study with a sufficient number of Duroc and Landrace boars could provide insight in fertilization and polyspermy rates and optimal sperm preparation and IVF conditions. It may also be of interest to culture random slaughterhouse oocytes with Duroc and Landrace FF to study if Duroc follicular fluid positively affects *in vitro* maturation and embryo production, as observed with Meishan conditioned medium (Xu et al., 1998). When differences are then observed, follicular fluid of the different breeds can be further analysed to try to find out what is causing this, which may provide insight in how to increase IVEP results.

A promising blastocyst yield was obtained during the experiments in these studies. If the quality of the produced blastocysts is sufficient, it is of interest for future studies to transfer them to a recipient sow with non-surgical embryo transfer to see if it is possible to establish pregnancy as previously reported by Suzuki et al. (2004). Therefore, we recently started to quantify chromosomal abnormalities and aneuploidy of *in vitro* produced blastocysts. Preliminary results demonstrate the

presence of chromosomal abnormalities and parthenogenesis in some IVP blastocysts. This could be related to the donor sow, or indicative of limitations in the current IVEP system. To address this aspect, *in vivo* derived embryos were in addition collected and comparisons will hopefully give valuable insight into aneuploidy at the different stages of embryo development *in vitro* and *in vivo.*

OPU-IVEP is of interest in the future for pig breeding. With this technique it will be possible to collect oocytes repeatedly from animals with a high genetic value to produce embryos. In addition, better fertilization and blastocyst rates are expected when *in vivo* matured oocytes can be used compared to *in vitro* maturated oocytes as observed in cattle (Rizos et al., 2002) and in the first pig OPU-IVEP study (Yoshioka et al., 2020). Furthermore, less aneuploidy is expected in those embryos as this has also been observed in sheep, cattle and horse (Coppola et al., 2007; Rambags et al., 2005; Tšuiko et al., 2017). Optimizations in ovum pick up equipment and techniques are promising and collecting of oocytes is possible (Aslak Oltedal, personal communications). Studying *in vitro* fertilization and embryo culture with OPU collected oocytes is thus of importance and one of the next steps in further research.

Finally, embryo transfer of blastocysts is the main goal to obtain live born piglets. At present, relatively small litter sizes are obtained after non-surgical embryo transfers. Litter size often ranges from 4-12 piglets, while around 25-30 IVD morulae and blastocysts (Martinez et al., 2013) and approximately 50 IVP blastocysts (Suzuki et al., 2004) are transferred for a successful pregnancy. Thus, improving the efficiency of embryo transfers is highly beneficial and necessary before embryo technology can be applied as standard practice in pig breeding.

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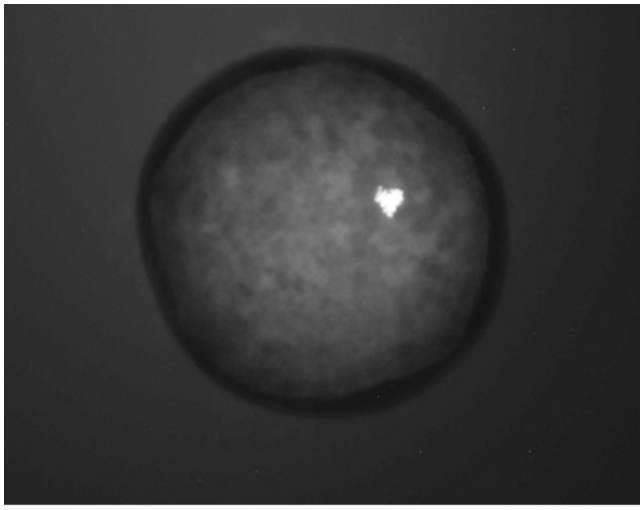
Paper I



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ORIGINAL ARTICLE

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Ovarian characteristics and in vitro nuclear and cytoplasmic oocyte maturation in Duroc and Landrace pigs

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Abstract

Differences in total number of piglets born per litter are observed between the Norwegian Duroc (ND) sire and Norwegian Landrace (NL) dam line. The aim of this study was to evaluate ovarian characteristics, and in vitro nuclear and cytoplasmic oocyte maturation in both breeds. One day after weaning, follicular phase ovaries were collected. Ovary length and weight were measured and the number of follicles (< 3 mm and 3–8 mm) was counted. Cumulus-oocyte complexes (COCs) were collected and matured for 48 hr. To assess cumulus expansion, COC area was analysed at 0 and 20 hr. Nuclear maturation and cortical granule (CG) distribution were analysed at 20 and 48 hr, and total glutathione (GSH) was measured at 48 hr to further elucidate cytoplasmic maturation. In first parity sows, a smaller ovary length and fewer 3 to 8 mm follicles were observed in ND compared to NL. For all sows, ND COCs covered a significantly smaller area at 0 hr, but a higher cumulus expansion ratio was observed at 20 hr compared to NL (364 ± 46% versus. 278 ± 27%, $p < 0.001$). At 20 hr, more ND oocytes exhibited advanced stages of nuclear maturation, while more NL oocytes showed advanced stages of CG distribution. Nuclear maturation to MII stage at 48 hr did not differ between ND and NL oocytes (90.1% and 87.7%, respectively). Moreover, no significant differences were observed for GSH content or CG distribution after maturation. In conclusion, differences with regard to ovarian characteristics as well as to cumulus expansion, and nuclear and cytoplasmic oocyte maturation at 20 hr were observed between the breeds. Further studies are required to determine if this subsequently affects in vitro fertilization and embryo development.

KEYWORDS

breed differences, Duroc, in vitro oocyte maturation, Landrace, ovary, porcine

1 | INTRODUCTION

In Norway, the farmer-owned cooperative Norsvin has been in charge for all pig breeding since 1958. Since the beginning, a breeding program was established for the Norwegian Landrace (NL) dam line and it has therefore a long selection history. The NL line has consistently been selected for both production and reproduction traits, and since 1992 litter size has been included in the breeding goal with considerable weight. The Norwegian Duroc (ND) sire line was established in 1986 and litter size has only recently been included in the breeding goal, as production and meat quality traits were most important in this line. Differences in reproduction traits are observed between purebred pigs from these genetic nucleus lines. The ND sire line has a smaller litter size with on average 9.2 total number of piglets born (TNB) compared to 13.8 TNB in the NL dam line (Ingris, 2019). An increase in TNB is genetically correlated to a higher ovulation rate and a lower corpus luteum (CL) weight (Da Silva et al., 2018). In addition, it has been shown that CLs with a smaller diameter and lower weight originate from follicles having a smaller size at ovulation (Wientjes et al., 2012). This suggests that a higher TNB in NL might be correlated to smaller follicles on the ovary. Possible differences in ovarian characteristics between the breeds could additionally lead to differences in in vitro oocyte maturation (IVM) as the use of oocytes derived from larger follicles results in better in vitro embryo production (IVP) outcomes (Bagg et al., 2007; Marchal et al., 2002; Qian et al., 2001).

In the past decades, numerous studies have been conducted to improve porcine IVP (Brüssow et al., 2000; Dang-Nguyen et al., 2011; Grupen, 2014). Supplementation of different components to maturation media has improved oocyte development to MII stage, and cleavage and blastocyst development after fertilization (Abeydeera, 2002; Hunter, 2000). Completion of both nuclear and cytoplasmic changes during oocyte maturation is essential to acquire oocyte developmental competence (Eppig, 1996) and while nuclear maturation can be analysed with nuclear staining, cytoplasmic maturation requires an indirect assessment. Among other parameters, migration of cortical granules (CGs) is often analysed to assess cytoplasmic maturation, whereby a distribution of CGs in the cortical cytoplasm is specific for immature oocytes while a distribution just beneath the plasma membrane is specific for mature oocytes (Cran & Cheng, 1985; Pawlak et al., 2012; Wang et al., 1997). CG exocytosis, after sperm penetration of the oocyte, is important to prevent polyspermy during fertilization, and migration of CGs during maturation is therefore required. In addition, intracellular glutathione (GSH) content of oocytes, which reaches a peak level at MII stage, has been used as an indicator for cytoplasmic maturation since a higher GSH level is positively associated with male pronucleus formation (Yoshida et al., 1993), blastocyst development and total blastocyst cell number (Abeydeera et al., 1998; Maedomari et al., 2007). Moreover, cumulus cells and cumulus expansion play an important role in oocyte maturation as reviewed by Tanghe et al. (2002) and a greater cumulus expansion has been correlated with a higher oocyte developmental potential in different species (Han et al., 2006; Qian et al., 2003).

A study in other pig breeds reported a significant lower number of preovulatory follicles in Hungarian Mangalica gilts, which have a relatively poor reproductive ability, compared to Landrace gilts (Rátky et al., 2005). Mangalica oocytes showed in addition a lower degree of cumulus expansion and fewer were matured, which suggested that reproductive ability was affected by a decreased follicular development and a prolonged intrafollicular oocyte maturation. To our knowledge, in vitro maturation potential of oocytes from the ND and NL breeds with different in vivo fertility have not been studied before. Therefore, the aim of this study was to compare 1) ovarian characteristics, 2) nuclear oocyte maturation and 3) cytoplasmic oocyte maturation by assessing CG distribution and intracellular GSH, between the ND sire and NL dam line.

2 | MATERIALS AND METHODS

2.1 | Chemicals and media

All chemicals and reagents were purchased from Sigma-Aldrich (Oslo, Norway) unless stated otherwise. Medium used for washing cumulus-oocyte complexes (COCs) was Porcine X Medium (PXM) supplemented with 4.0 mg/ml bovine serum albumin (BSA) and maturation was performed using Porcine Oocyte Medium (POM) as described by Yoshioka et al. (2008) with minor modifications. The POM medium was composed of 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 5.0 mM glucose, 0.91 mM Na-pyruvate, 2.0 mM Ca-$(lactate)_2 \cdot 5H_2O$, 2.0 mM L-glutamine, 5.0 mM hypotaurine, 20 ml/l BME amino acids, 10.0 ml/l MEM non-essential amino acid, 0.6 mM L-cysteine, 0.01 mg/ml gentamicin, 4.0 mg/ml BSA, serum substitute, 10 ng/ml epidermal growth factor (EGF) and 50 μM Mercaptoethanol (Gibco).

2.2 | Animals

Ovaries were collected at a commercial abattoir from 37 Duroc and 20 Landrace sows originating from two Norsvin nucleus herds. Since material was collected from animals that were routinely slaughtered, no ethical approval was required. Animals were cared for according to internationally recognized guidelines and regulations for keeping pigs in Norway (The Animal Welfare Act, 10 July 2009 and Regulations for keeping pigs in Norway, 18 February 2003). Lactating sows in both herds were liquid fed with a commercial diet with whey supplementation and feed was offered up to four times a day to ensure ad libitum access during lactation. Data were collected in three replicates from June to October 2019.

2.3 | Ovarian characteristics and genotyping

Follicular phase sow ovaries were collected 1 day after weaning and transported to the laboratory in 0.9% NaCl containing 2.5 μg/ml

kanamycin at 30–35°C within 2 hr of slaughter. Ovaries were dissected free of non-ovarian tissue before ovary weight and length were measured. The number of 3 to 8 mm follicles was counted per ovary during aspiration, and surface follicles smaller than 3 mm were subsequently counted. A tissue sample from each individual ovary was stored at −80°C for genotyping to identify the nucleus sows as it was not possible to track the animals along the slaughter line. In this way, ovaries could be matched to sow identity and ovarian characteristics could be analysed based on parity. DNA extraction from the ovaries was performed by BioBank, Hamar and genotyping was performed at CIGENE, Norwegian University of Life Sciences, Ås in Norway. Samples were genotyped using the Illumina GeneSeek custom 50K SNP chip (Lincoln, NE, United States).

2.4 | Oocyte collection and in vitro maturation

Follicles with a diameter of 3 to 8 mm were aspirated with an 18-gauge needle and 10 ml syringe. Oocytes with a compact cumulus and evenly granulated cytoplasm were selected and washed three times in PXM and twice in POM medium. Groups of 40 to 50 oocytes were transferred into each well of a Nunc four-well dish containing 500 μl of pre-equilibrated POM medium.

For the first 20 hr, COCs were matured in POM supplemented with 0.05 IU/ml porcine FSH and LH (Insight Biotechnology Ltd, Wembley, UK), and 0.1 mM dbcAMP. Subsequently, COCs were matured for another 28 hr in POM without hormones and dbcAMP. Oocytes were cultured for a total of 48 hr at 38.8°C under an atmosphere of 6% CO_2 in humid air.

2.5 | Assessment of cumulus expansion

At 0, 20 and 48 hr of maturation, images were taken from each well with a Nikon SMZ1500 stereomicroscope (Nikon, Tokyo, Japan) to analyse cumulus expansion. Individual COC area of each oocyte was analysed by ImageJ software (version 1.52a; NIH, Bethesda, USA) at 0 and 20 hr, and a cumulus expansion ratio per well was determined by dividing total COC area of each well at 20 hr by the total COC area of the well at 0 hr (Costermans et al., 2019). After 48 hr of maturation, it was not possible to assess cumulus expansion per individual oocyte as cumulus cells showed a high degree of expansion and were overlapping.

2.6 | Nuclear and cortical granule staining

Cortical granule staining was based on methods reported by Yoshida et al. (1993) and Wang et al. (1997) with a few modifications. At 20 and 48 hr, oocytes were stripped of cumulus cells by repeated pipetting, washed twice in PBS, fixed in 4% paraformaldehyde for 30 min at room temperature and washed three times for 5 min in 0.3% BSA in PBS. Oocytes were then permeabilized in 0.1% triton X-100 for 5 min, washed twice for 5 min in PBS and stained in 100 μg/ml Peanut agglutinin (PNA) lectin from *Arachis hypogaea* conjugated with Alexa Fluor 568 (L32458, Invitrogen, Thermos Fisher Scientific, Waltham, MA, USA) for 30 min at room temperature in the dark. After this staining step, oocytes were washed twice for 5 min in 0.3% BSA, 0.01% triton X-100 in PBS, moved to a droplet of PBS and stained with 8 μg/ml Hoechst (H-33342, B2261, Sigma). Oocytes were mounted in 6 μl fluorescence mounting medium (Dako, Glostrup, Denmark) on glass slides and a coverslip was placed on top. Slides were analysed using a Leica SP8 laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Hoechst staining was evaluated with a 405 nm excitation laser and a 410 to 480 nm emission filter and PNA lectin Alexa Fluor 568 with a 552 nm excitation laser and a 650 to 720 nm emission filter. Images of each oocyte were taken at the equatorial plane and at the top.

2.7 | Assessment of nuclear maturation

Nuclear maturation of oocytes was assessed at 20 and 48 hr by Hoechst staining and confocal microscopy. Samples were analysed according to morphological criteria for meiotic stages; GV0 -GV4, MI (metaphase I), AI/TI (anaphase I/telophase I), MII (metaphase II) and D (degenerated) oocytes (Appeltant et al., 2015; Sun et al., 2004). At 48 hr, maturation rate was expressed as the number of AI/TI and MII stage oocytes divided by the total number of oocytes in culture. Oocytes arrested at the GV stage or only progressed to MI were considered as immature.

2.8 | Assessment of cytoplasmic maturation

2.8.1 | Cortical granule distribution

Cortical granule distribution of oocytes was assessed at 0 and 20 hr by PNA lectin Alexa Fluor 568 staining and confocal microscopy. Classification is often based on only two or three different CG distribution patterns (Pawlak et al., 2012; Wang et al., 1997), but in this study, a distinguish was made between six different distribution categories. Patterns of CG distribution from images at the equatorial plane were classified simultaneously by two persons into one of the six categories described in Figure 1. Oocytes showing an abnormal CG distribution due to problems with mounting were not included in the CG results.

2.8.2 | Intracellular glutathione content

Total GSH content was determined from 28 Duroc and 26 Landrace oocytes at 48 hr of maturation. Oocytes were stripped of cumulus cells by repeated pipetting and the zona pellucida was removed using 0.05% Pronase in PBS. Oocytes were stored during every replicate in a ratio of one oocyte in 5 μl dH_2O and frozen at −20°C. Samples were frozen

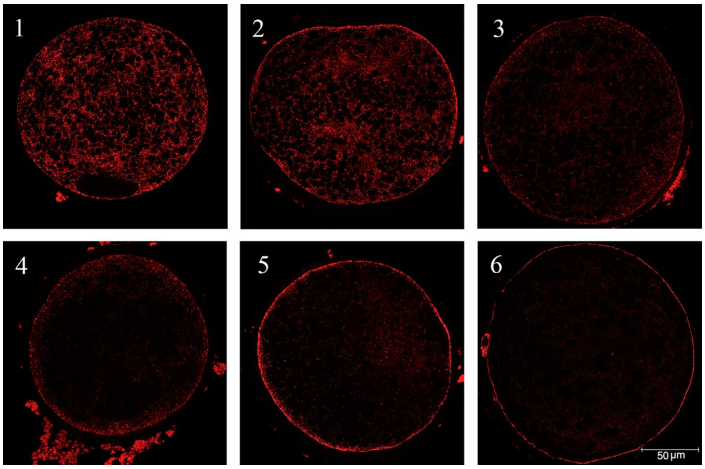

FIGURE 1 Classification of cortical granule distribution at the equatorial plane (PNA lectin). Oocytes were classified into one of the six categories; (1) Central, CGs distributed through the whole cortical cytoplasm, (2) Central distribution of CGs but already a layer under the plasma membrane in the periphery visible, (3) Outer layer in periphery and fewer CGs in the cytoplasm, (4) Clear outer layer and only a few CGs in the cytoplasm, (5) Partially complete, a thicker layer in the peripheral region, (6) Complete, a clear and thin layer under the plasma membrane in the peripheral region and around the polar body

and thawed three times for lysis of the oocytes to get a homogeneous mixture. Analysis was performed using the GSH/GSSG-Glo™ Assay (Promega, Madison, WI, USA) according to the manufacturer's manual. Luminescence was measured using a Fluostar Optima multiwell plate reader with Optima control software, version 2.20 (BMG LabTech, Ortenberg, Germany). The value for each sample, measured in relative luminescence units (RLU), was converted to the corresponding GSH value in pmol per oocyte using the standard curve values.

2.9 | Statistical analysis

Only data from first parity sows ($n = 11$ ND and $n = 10$ NL sows) were used for analysis of ovarian characteristics as different parities were not equally represented across breeds and it was therefore not possible to include parity in a statistical model. Data on IVM were based on oocytes derived from ovaries from all sows ($n = 37$ ND and $n = 20$ NL sows). All data were analysed using SAS 9.4 (SAS Inst. Inc., Cary, NC, United States). Distributions of means and residuals were examined to verify model assumptions of normality and homogeneity of variance. Data for individual COC area of each oocyte at both time points were log transformed to obtain normality before statistical analysis. Mean values for ovarian characteristics, individual COC area, COC expansion ratio and GSH content between the breeds were analysed using the Student's *t*-test for two independent samples. Proportion of oocytes in the different nuclear maturation stages and distribution classes of CGs were analysed between the breeds using Pearson's Chi-square test. Results are presented as mean ± *SD* and a probability of $p < 0.05$ was considered to indicate statistical significance.

3 | RESULTS

3.1 | Ovarian characteristics

In total, 32 Duroc and 14 Landrace sows could be identified by genotyping with an average parity of 1.9 ± 1.3 (ranging from 1 to 6) and 1.1 ± 0.3 (ranging from 1 to 2), respectively. Data from first parity sows only were used for analysis of ovarian characteristics. A larger average ovary length was observed in first parity Landrace sows compared to Duroc ($p = 0.01$), whereas no significant difference was observed in ovary weight between the two breeds (Table 1).

TABLE 1 Ovarian characteristics from 11 Norwegian Duroc (ND) and 10 Norwegian Landrace (NL) first parity sows

Characteristics	Breed	Ovaries	Mean ± SD	Min	Max	*P*-value
Length (cm)	ND	22	3.0 ± 0.3	2.2	3.6	0.01
	NL	20	3.2 ± 0.3	2.8	3.6	
Weight (gr)	ND	22	4.5 ± 1.0	2.7	6.8	0.24
	NL	20	4.8 ± 1.0	3.3	6.6	
Number of < 3 mm follicles[1]	ND	22	12.3 ± 8.4	0.0	37.0	0.13
	NL	20	15.9 ± 5.9	8.0	28.0	
Number of 3 to 8 mm follicles[2]	ND	22	13.6 ± 5.4	5.0	24.0	<0.001
	NL	20	21.6 ± 7.9	9.0	38.0	

[1]Total of 271 ND and 317 NL follicles counted

[2]Total of 300 ND and 431 NL follicles counted

Furthermore, on average eight additional 3 to 8 mm sized follicles were found on the surface of Landrace ovaries compared to Duroc ovaries ($p < 0.001$).

3.2 | Cumulus expansion

Cumulus expansion was analysed for 375 ND and 304 NL oocytes. Average individual COC area per oocyte was significantly smaller for Duroc compared to Landrace at 0 hr ($p < 0.0001$). Contrary, average individual COC area tended to be larger for Duroc at 20 hr compared to Landrace COC area ($p = 0.06$). A larger variation was observed for Duroc COC area at this time point (Figure 2). A significantly higher cumulus expansion ratio was observed after 20 hr of maturation for Duroc COCs compared to Landrace COCs, 364 ± 46% and 278 ± 27%, respectively (Figure 3).

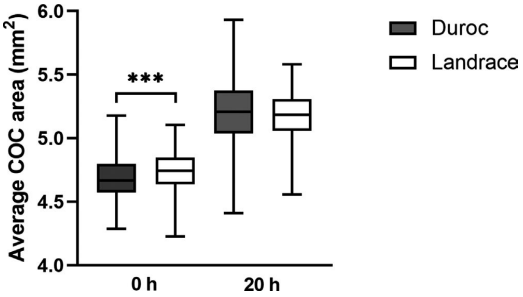

FIGURE 2 Average cumulus-oocyte complex (COC) area at 0 hr and 20 hr of maturation for 375 Norwegian Duroc and 304 Norwegian Landrace COCs. The data were log transformed before analysis. ***$p < 0.0001$

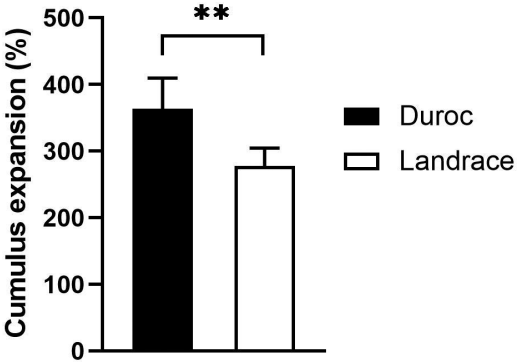

FIGURE 3 Cumulus expansion ratio (%) from 0 hr to 20 hr of maturation for Norwegian Duroc and Norwegian Landrace cumulus-oocyte complexes (COCs). The cumulus expansion ratio was defined as the total COC area per well at 20 hr divided by the total COC area per well at 0 hr. **$p < 0.001$

3.3 | Nuclear maturation

At 20 hr of maturation, Duroc oocytes exhibited advanced stages of maturation based on chromatin configuration compared to Landrace oocytes. A significantly higher percentage of Duroc oocytes was found in the GV2 and MI phases at 20 hr compared to Landrace oocytes, while more Landrace oocytes were present in the GV1 stage (Figure 4a). Maturation rate to MII stage, recorded at 48 hr, was not significantly different between the breeds. The MII rate for both Duroc and Landrace oocytes was consistently high, 90.1% (136/151) and 87.7% (142/162), respectively (Figure 4b).

3.4 | Cytoplasmic maturation

At 20 hr of maturation, significantly more Landrace oocytes were classified to the CG distribution group 4 compared to Duroc oocytes, while more Duroc oocytes were found in group 3 (Table 2). In addition, analysing the proportion of all distribution groups ≥4 showed more Landrace oocytes in the more advanced distribution groups compared to the Duroc oocytes ($p = 0.0016$; ND = 34% and NL = 56%), suggesting that Landrace oocytes showed advanced stages of CG distribution at 20 hr. No significant difference between the breeds for the distributions was observed at the end of maturation.

At the end of maturation, total GSH concentration was assessed to further elucidate cytoplasmic maturation. Total GSH at 48 hr was on average 4.45 ± 0.56 pmol per Duroc oocyte and 4.11 ± 1.25 pmol per Landrace oocyte (Figure 5). No significant difference was observed between the breeds.

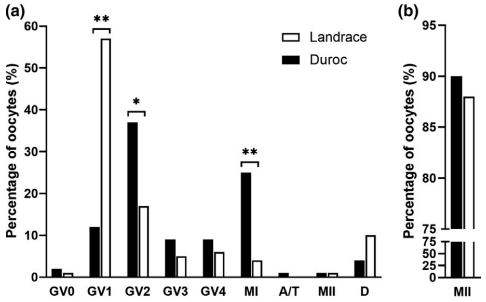

FIGURE 4 Nuclear morphology of Norwegian Duroc (ND) and Norwegian Landrace (NL) oocytes. Chromatin configuration of oocytes was classified in the categories; germinal vesicle stage 0 (GV0), 1 (GV1), 2 (GV2), 3 (GV3), 4 (GV4), metaphase I (MI), anaphase/telophase (A/T), metaphase II (MII) or degenerated (D). A) Oocytes at 20 hr of maturation (114 ND and 109 NL oocytes). B) Maturation rate to MII at 48 hr (151 ND and 162 NL oocytes). * $p < 0.01$; *** $p < 0.0001$

TABLE 2 Percentages of oocytes derived from 37 Norwegian Duroc (ND) and 20 Norwegian Landrace (NL) sows classified in six different cortical granule distributions at 20 hr and 48 hr of maturation

	20 hr[1] (%)		48 hr[2] (%)	
Distribution	ND	NL	ND	NL
1	10	11	0	0
2	25	24	5	6
3	31[a]	9[b]	0	0
4	11[a]	21[b]	1	3
5	23	35	1	2
6	0	0	93	89

Values per time point in the same row with different superscript letters represent a significant difference ($p < 0.05$).

[1]Total of 112 ND and 106 NL oocytes analysed

[2]Total of 147 ND and 161 NL oocytes analysed

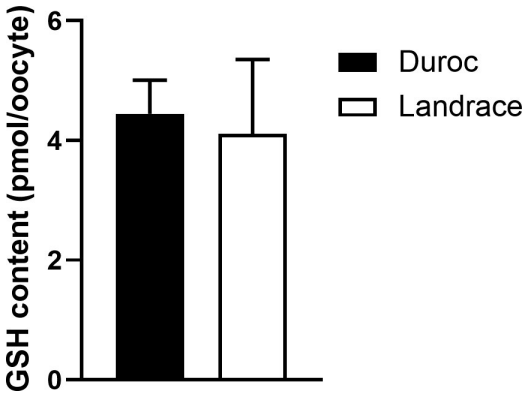

FIGURE 5 Average total glutathione content (GSH) for 28 Norwegian Duroc and 26 Norwegian Landrace oocytes at 48 hr of maturation

4 | DISCUSSION

This study evaluated differences in ovarian characteristics, and IVM between the ND sire and NL dam line. First parity Landrace sows had on average eight surface follicles (3–8 mm) more per ovary compared to Duroc. At a phenotypic level, this observation can explain the difference in total number of piglets born and is in agreement with selection in the breeding program, since litter size has been an important trait in the breeding goal of the dam line (NL). However, with the on-farm management system, oocytes were collected 1 day after weaning and are representative of the early follicular phase follicle population. Collecting ovaries at day 4 or 5 post weaning would therefore represent the final selected ovulatory population and provide better insights regarding litter size. Sows from both breeds were in the same phase of the oestrous cycle and the weaning-to-service interval, defined as the days from weaning until the first service, is similar across both breeds (5.7 ± 1.8 days and 5.7 ± 1.3 days for ND and NL, respectively; unpublished data). This suggests that follicular development and time of ovulation relative to oestrous length are similar in Duroc and Landrace animals and that the stage of follicle development at a fixed time point after weaning would be comparable between the breeds. However, results indicated furthermore that ovary length was larger for Landrace, while no difference was observed in ovary weight. Taking into account that more follicles were present on Landrace ovaries but that weight was similar, variation in follicle size within the 3–8 mm class and follicle development might therefore exist between the breeds.

Besides assessment of ovarian characteristics, IVM was studied and cumulus expansion was analysed. Results indicate that average COC area after aspiration at 0 hr was significantly smaller for Duroc oocytes compared to Landrace. However, when cultured for 20 hr and re-evaluated, the same COCs exhibited a larger cumulus expansion than Landrace. This greater cumulus expansion ratio from 0 to 20 hr for Duroc is of interest since a broader cumulus expansion has been associated with lower polyspermy rates (Costermans et al., 2019) and higher in vitro fertilization (IVF) and embryo development rates (Marchal et al., 2002; Qian et al., 2001).

Our results suggest furthermore that, similar to cumulus expansion, nuclear maturation at 20 hr was more advanced for Duroc oocytes compared to Landrace. At this time point, Landrace oocytes were found in earlier stages of nuclear maturation while having a smaller cumulus expansion, which might be related to a smaller follicle size at collection. A large variation in nuclear morphology at aspiration has been observed in porcine oocytes (Funahashi et al., 1997) and it would have been of interest to determine if variation in nuclear stage between the breeds already existed at the start of IVM. Nuclear maturation to MII stage at 48 hr was high in both breeds with 90.1% for Duroc and 87.7% for Landrace, and no difference was observed anymore between the breeds. This is not in line with observations from Rátky et al. (2005), who observed a significant lower percentage of matured oocytes with a lower degree of cumulus expansion in the breed with a smaller litter size. However, those oocytes were matured in vivo and collected by endoscopic ovum pick up which could differ from in vitro maturation. Cumulus expansion is essential for meiotic progression (Suzuki & Saito, 2006) and it has been suggested that nuclear maturation is preceded by cumulus expansion (Torner et al., 2004). It could be that Duroc oocytes reached MII stage earlier and that more Landrace oocytes completed nuclear maturation in the last part of maturation, as porcine oocytes normally reach MII stage around 36 hr and percentages do not change any more towards the end of in vitro maturation (Kikuchi et al., 1999; Somfai et al., 2005).

Furthermore, cytoplasmic maturation was analysed by assessing CG distributions and GSH content. In contrast to nuclear maturation, more Landrace oocytes showed advanced stages of CG distribution at 20 hr compared to Duroc. No differences in CG distribution were observed after maturation and almost all oocytes showed a clear and thin layer of CG under the plasma membrane,

which is important for CG exocytosis after sperm penetration to prevent polyspermy. In line with the other parameters studied, no difference in GSH content between breeds was observed after maturation. Due to a limited number of ovaries and oocytes available, GSH levels were only analysed at 48 hr. Since Duroc COCs had a larger cumulus expansion at 20 hr, a higher GSH level would be expected as cumulus cells play an important role in GSH synthesis (Maedomari et al., 2007) and as a greater expansion is positively correlated with a higher intracellular GSH content in oocytes (Furnus et al., 1998).

The results in this study suggest that it is more efficient to use Landrace ovaries for IVP as more oocytes can be collected per animal, but it is important to examine if differences at 20 hr of maturation lead to differences in in vitro fertilization (e.g. polyspermy and fertilization rates) and embryo development. Moreover, it is worth investigating if IVF should be carried out earlier for Duroc as an increased frequency of chromosomal abnormalities has been reported when oocytes were matured over a longer time period than necessary (Sosnowski et al., 2003). The larger variation observed for parity, and thus age, in Duroc sows after genotyping could have resulted in greater variation in oocyte maturation. The use of ovaries from primi- and multiparous sows instead of from prepubertal gilts lead to better IVP outcomes (Bagg et al., 2007; Grupen et al., 2003; Pawlak et al., 2015), and differences might also be observed with higher parity animals as they age (Krisher, 2019). Furthermore, it is relevant to assess distribution of follicle size in a further study to better understand differences between the breeds. It is of interest if a lower TNB in Duroc is related to less follicles found on the ovary surface, to further challenges during fertilization and embryo development or if there are other factors that must be considered. Brüssow et al. (2006) indicated that the presence of COCs in the oviduct influences sperm release from the oviductal sperm reservoir. Significantly more spermatozoa were found in the ampulla and isthmus when COCs were present in the oviduct compared to oviducts without COCs. Differences in the number of COCs at ovulation in Duroc compared to Landrace, as can be indicated from this study regarding the number of follicles on the ovaries, might thus affect sperm release and fertilization. In addition, studies on uterine horn and oviduct lengths could provide a better insight into the reproductive performance in both breeds.

In conclusion, differences with regard to ovarian characteristics as well as to cumulus expansion, and nuclear and cytoplasmic oocyte maturation at 20 hr were observed between the breeds. This could subsequently affect IVP outcomes even though the two breeds showed similar maturation results at 48 hr. Therefore, further experiments are required to study if there are differences in in vitro fertilization and embryo development between the ND sire and NL dam line.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION

Reina Jochems: Conceptualization; Formal analysis; Investigation; Visualization; Writing-original draft. **Ann Helen Gaustad:** Conceptualization; Data curation; Formal analysis; Writing-review & editing. **Louisa J Zak:** Conceptualization; Writing-review & editing. **Eli Grindflek:** Conceptualization; Writing-review & editing. **Teklu Tewoldebrhan Zeremichael:** Investigation; Writing-review & editing. **Irma C Oskam:** Conceptualization; Writing-review & editing. **Frøydis Deinboll Myromslien:** Conceptualization; Writing-review & editing. **Elisabeth Kommisrud:** Conceptualization; Writing-review & editing. **Anette Kristine Krogenæs:** Conceptualization; Writing-review & editing.

AUTHOR CONTRIBUTIONS

RJ and AHG took part in conception and design, acquisition of data and analysis and interpretation of data. LJZ, EG, ICO, FDM, EK and AKK took part in conception and design, and interpretation of data. TTZ took part in analysis and interpretation of data. RJ drafted the manuscript which was critically reviewed and approved by all authors.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the material was collected from animals that were routinely slaughtered.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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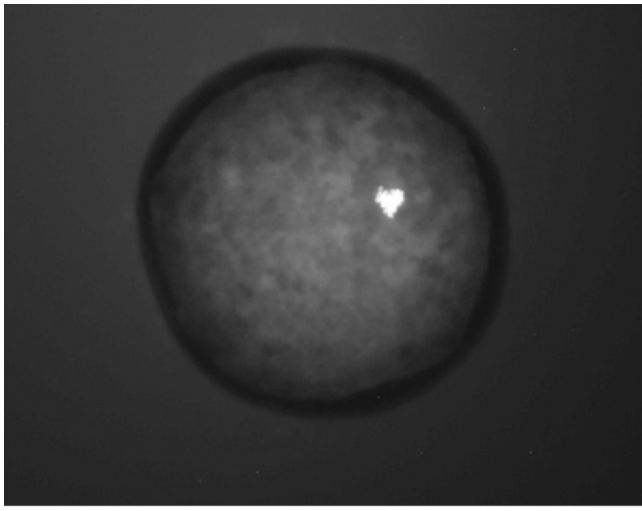
Paper II



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Effect of two 'progressively motile sperm–oocyte' ratios on porcine *in vitro* fertilization and embryo development

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Summary

Sperm motility and viability of cryopreserved semen vary between boars and straws, which influences the outcomes of *in vitro* embryo production (IVEP). However, progressive motility is usually not considered during IVEP. The aim of this study was to assess fertilization with a 500:1 and 250:1 'progressively motile sperm to oocyte' ratio on IVEP outcomes using semen from three Duroc and three Landrace boars. Frozen–thawed sperm was centrifugated through a 45/90% Percoll® density gradient and sperm quality parameters were assessed. *In vitro* matured oocytes were fertilized at the two ratios, a portion was stained 10–12 h after start of fertilization to analyze fertilization and polyspermy, while the remaining zygotes were cultured up to day 7. The 500:1 ratio resulted in a higher fertilization and blastocyst yield on day 6 compared with the 250:1 ratio, but no effect of ratio was observed for polyspermy, cleavage rate or blastocyst cell number. Individual differences between boars were observed for fertilization, cleavage and blastocyst rates, but not for the other IVEP outcomes. In conclusion, a higher fertilization and blastocyst yield was obtained with the 500:1 ratio compared with the 250:1 ratio, while polyspermy level was consistent across ratios. Differences in IVEP outcomes were still observed between the individual boars although adjusted for progressive motility. Promising blastocyst yields and high total blastocyst cell counts were obtained with sperm from both breeds.

Introduction

Using cryopreserved sperm in *in vitro* embryo production (IVEP) is useful to increase repeatability during an experiment and sperm from the same ejaculate can be used over a long period of time. However, sperm motility and viability after freezing–thawing vary between straws and individual boars (Holt *et al*., 2005; Waterhouse *et al*., 2006; Yeste, 2017) and are often lower compared with fresh semen. During *in vitro* fertilization (IVF) the final concentration of all sperm cells per ml during co-incubation with oocytes is usually reported, whereas the percentage total motile or progressively motile sperm cells are often not considered. *In vivo*, the facilitation of sperm transport through the female reproductive tract is not only due to uterine contractions but, among other things, also due to progressive motility of spermatozoa, which is defined as the straightforward movement in a clear direction. This is supported by the finding that progressive motility had a significant effect on *in vivo* farrowing rate (Broekhuijse *et al*., 2012). Moreover, progressive motility has been shown to be of importance during bovine IVF as progressive motility of frozen–thawed sperm showed a good correlation with *in vitro* pronucleus formation (Tanghe *et al*., 2002) and higher cleavage and blastocyst rates were obtained with high progressively motile sperm compared with low progressively motile sperm (Li *et al*., 2016).

It is well known that large variations are observed between individual boars when studying IVEP outcomes using both fresh and frozen–thawed sperm (Wang *et al*., 1991; Xu *et al*., 1996; Almiñana *et al*., 2005; Gil *et al*., 2008). It is further shown that the sperm–oocyte ratio affects fertilization and polyspermy rates, and that the optimal ratio even varies between boars (Wang *et al*., 1991; Xu *et al*., 1996; Gil *et al*., 2004, 2007). In addition to individual boar differences, differences between breeds have been reported for penetration and polyspermy rates (Suzuki *et al*., 2003). Therefore, it has been suggested that preliminary screening for each individual boar is required to select optimal IVF conditions (Almiñana *et al*., 2005; Gil *et al*., 2008). A sperm–oocyte ratio of 1000:1 is often used in porcine IVF (Kidson *et al*., 2004; Gil *et al*., 2007; Martinez *et al*., 2017. Progressive sperm motility is ~50% after freezing and thawing in our laboratory and,

as this differs between boars and straws, we were interested in performing fertilization with an adjusted sperm–oocyte ratios for all IVF rounds.

The aim of this study was to assess fertilization with a 250:1 and 500:1 'progressively motile sperm–oocyte' ratio using cryopreserved semen from three Duroc and three Landrace boars. It was hypothesized that: (1) a higher 500:1 sperm–oocyte ratio results in higher *in vitro* fertilization and blastocyst yield but also in an increase in polyspermy compared with a lower 250:1 ratio; and that (2) adjustment to the same number of progressively motile sperm cells per oocyte at fertilization will reduce variation in IVEP results between individual boars.

Materials and methods

Animals and experimental design

Oocytes were collected from random sow ovaries without specified breed in both the luteal and follicular phase of the oestrus cycle. Cryopreserved boar semen was available from six artificial insemination (AI) boars originating from two purebred breeds; three Duroc and three Landrace boars were used. All animals were cared for according to internationally recognized guidelines and regulations for keeping pigs in Norway (The Animal Welfare Act, 10 July 2009 and Regulations for keeping pigs in Norway, 18 February 2003). In total, 2456 oocytes were matured *in vitro* and fertilized with the two sperm–oocyte ratios and semen from the six boars during 14 IVEP rounds. Three to four replicates were carried out per boar and sperm–oocyte ratio. During each IVEP round, a random subset of presumptive zygotes was fixed and stained per boar and sperm–oocyte ratio to assess fertilization ($n = 1019$ oocytes). The remaining presumptive zygotes ($n = 1437$) were cultured to assess *in vitro* embryo development. Data were collected from January to August 2020.

Chemicals and media

All chemicals and reagents were purchased from Sigma-Aldrich (Oslo, Norway) unless stated otherwise. Washing of cumulus–oocyte complexes (COCs) was performed using porcine X medium (PXM), maturation using porcine oocyte medium (POM), fertilization using porcine gamete medium (PGM) and embryo culture using porcine zygote medium-5 (PZM-5) (Yoshioka *et al.*, 2008). Polyvinyl alcohol in original medium was replaced by 0.4% bovine serum albumin (BSA) in POM and PZM-5 medium and 0.6% BSA in PGM medium. Minor changes were made to the POM medium and the final composition was: 108 mM NaCl, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 5.0 mM glucose, 0.2 mM Na-pyruvate, 2.0 mM Ca-(lactate).$2.5H_2O$, 2.0 mM L-glutamine, 5.0 mM hypotaurine, 20 ml/l BME amino acids, 10.0 ml/l MEM non-essential amino acid, 0.6 mM L-cysteine, 0.01 mg/ml gentamicin, 4.0 mg/ml BSA, serum substitute, 10 ng/ml epidermal growth factor, and 50 µM β-mercaptoethanol (Gibco).

Oocyte collection and *in vitro* maturation (IVM)

Random sow ovaries in the luteal and follicular phase of the oestrus cycle were collected at a commercial slaughterhouse and transported to the laboratory in 0.9% NaCl at 35–38°C. Upon arrival, ovaries were washed with 0.9% NaCl containing 2.5 µg/ml kanamycin and placed in a beaker in a water bath at 30–35°C until follicle aspiration. Follicles with a diameter of 3–8 mm were aspirated 4 h after slaughter using an 18-gauge needle and 10 ml syringe. Oocytes with a compact cumulus and evenly granulated cytoplasm were selected under a Leica MS5 stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany), washed three times in PXM and once in POM medium, and transferred in groups of 30 oocytes into each well of a Nunc four-well multidish containing 500 µl of pre-equilibrated POM medium. For the first 20 h, COCs were matured in POM supplemented with 0.05 IU/ml porcine follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Insight Biotechnology Ltd, Wembley, UK), and 0.1 mM dbcAMP. Subsequently, COCs were matured for another 24 h in POM without hormones and dbcAMP. Oocytes were matured at 38.8°C in an humified atmosphere containing 6% CO_2 in air.

Sperm preparation and *in vitro* fertilization (IVF)

Fertilization was performed with cryopreserved sperm from three Duroc and three Landrace boars. Frozen straws from each individual boar originated from the same ejaculate. Each 2.5 ml straw was thawed at 50°C for 50 s (Waterhouse *et al.*, 2006) and diluted in 40 ml Tri-X-cell (IMV technologies, L'Aigle, France) at room temperature (RT). Sperm cells were washed and selected at RT using Percoll® density gradient centrifugation by layering 2 ml of 45% Percoll® on top of 2 ml 90% Percoll®. Finally, 1 ml of semen was carefully placed on top, and the sample was centrifugated at 700 *g* for 20 min. Supernatant was removed by aspiration, the pellet was resuspended in 4 ml PGM without BSA and centrifuged at 500 *g* for 5 min. The pellet was then resuspended in 150–200 µl PGM without BSA. Sperm concentration and progressive motility was measured using computer-assisted sperm analysis (CASA) and spermatozoa were diluted to 5×10^5 progressively motile sperm cells/ml in 300 µl PGM with BSA. The COCs were carefully washed once in PGM and groups of 30 oocytes were co-incubated with 15 µl sperm suspension (1 oocyte:250 progressively motile sperm cells, i.e. 1.5×10^4 progressively motile sperm cells/ml) or 30 µl (1 oocyte:500 progressively motile sperm cells, i.e. 3.0×10^4 progressively motile sperm cells/ml) in a final volume of 500 µl PGM per well. After 2 h of co-incubation, oocytes were transferred to a new well with 500 µl PGM medium to remove an excess of sperm cells.

In vitro culture (IVC)

After 4 h co-incubation, presumptive zygotes were denuded of cumulus cells by vortexing for 1 min in 2 ml PXM. The zygotes were washed twice in PXM medium and once in PZM-5 before culture in 500 µl PZM-5 under 400 µl mineral oil (IVF Biosciences, Falmouth, UK) at 38.8°C in an humified atmosphere containing 6% CO_2 and 7% O_2. At day 4 of culture (fertilization = day 0), PZM-5 medium was refreshed by taking 250 µl out and replacing it with 250 µl new equilibrated PZM medium.

Assessment of sperm motility by CASA

Sperm motility parameters were assessed after Percoll density gradient centrifugation using a Sperm Class Analyzer® version 6.1 (Microptic SL, Barcelona, Spain), equipped with a phase contrast Eclipse Ci-S/Ci-L microscope (Nikon, Japan) and Basler digital camera (Basler Vision Technologies, Ahrensburg, Germany). Per sample, 3 µl was loaded into a pre-warmed Leja-4 chamber slide (Leja Products, Nieuw-Vennep, The Netherlands) and analyzed with a frame rate of 45 frames per second and a minimum of eight microscope fields and 800 cells. Total motility (MOT) was

defined as sperm cells with curvilinear velocity (VCL) > 10 μm/s and progressive motility (PROG) with straightness (STR) > 45 %.

Sperm plasma membrane and acrosome integrity by flow cytometry

All analyses by flow cytometry were performed with a Cell Lab Quanta™ SC MPL flow cytometer (Beckman Coulter, Fullerton, USA). Sperm plasma membrane and acrosome integrity, were analyzed after Percoll centrifugation. Sperm samples were diluted to a concentration of 1×10^6 sperm cells/ml and stained with 0.05 mg/ml Lectin peanut agglutinin (PNA) conjugated with Alexa Fluor 488 (PNA-Alexa 488, L21409, Invitrogen) and 0.48 mM propidium iodide (PI) in PBS with 1% guaiacol glycerol ether (GGE) to identify live and dead sperm cells and acrosome-reacted spermatozoa, respectively. Sperm cells were incubated for 10 min at RT before analysis. Acrosome-intact and live spermatozoa (AIL) were recorded and analyzed using Cell Lab Quanta™ SC MPL software (Beckman Coulter, software version 1.0 A).

Sperm DNA fragmentation index (DFI) by flow cytometry

Sperm chromatin integrity was analyzed after Percoll centrifugation using the sperm chromatin structure assay (SCSA) (Evenson and Jost, 2000; Boe-Hansen *et al.*, 2005). Sperm samples were diluted to a concentration of 2×10^6 sperm cells/ml in TNE buffer (10 mM Tris–HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4) in a final volume of 200 μl. Immediately after dilution, 400 μl acid detergent solution [0.38 M NaCl, 80 mM HCL, 0.1% (w/v) Triton X-100, pH 1.2] was added to denature the samples. After 30 s of incubation at RT, denatured samples were stained with 1.2 ml of 6 μg/ml acridine orange staining solution (A3568, Invitrogen) in a buffer (37 mM citric acid, 0.126 M Na_2HPO_4, 1.1 μM EDTA and 0.15 M NaCl, pH 6). The samples were run in setup mode for 3 min, after which data acquisition started with 5000 events collected for each sample. The percentage of red (single-stranded DNA) and green (double-stranded DNA) fluorescence was determined using FCS Express 6 flow cytometry software (De Novo Software, USA). The percentage of DFI was calculated based on the fluorescence ratio red/(red + green).

Assessment of fertilization and polyspermy

To analyze fertilization and polyspermy rates, presumptive zygotes were assessed 10–12 h after start of fertilization. During each IVF replicate, one well with zygotes was fixed per sperm–oocyte ratio and boar. The presumptive zygotes were kept overnight in 4% PFA at 4°C, stained the next morning for 5 min in 8 μg/ml Hoechst stain (H-33342, B2261, Sigma) and mounted in 6 μl fluorescence mounting medium (Dako, Glostrup, Denmark) under a coverslip. Pronucleus formation was assessed using a fluorescence microscopy and a Leica SP8 laser scanning confocal microscope. Hoechst staining was evaluated with a 405 nm excitation laser and a 410 to 480 nm emission filter. Oocytes were classified as fertilized when they had one or more swollen sperm heads and pronuclei, and polyspermy was defined as the proportion of zygotes with more than two pronuclei or swollen sperm heads as described in del Olmo *et al.* (2013).

Assessment of embryo development and quality

Cleavage rate at day 2 and blastocyst rates at day 6 and 7 of culture were assessed per well using a Leica DM IL inverted microscope. The rates were defined as the number of cleaved oocytes or blastocysts divided by the total number of oocytes cultured. An embryo with a clear blastocoel was defined as a blastocyst. Day 7 blastocysts were fixed ($n = 260$) in 4% PFA at RT for 30 min and stained with 8 μg/ml Hoechst stain to assess total blastocyst cell number. Blastocysts that showed apoptotic nuclei with fragments after staining, and in which the total blastocyst cell number was difficult to count, were not included in the analysis.

Statistical analysis

Statistical analysis was performed using SAS v.9.4 (SAS Institute Inc., Cary, NC, USA). Distributions of the means and residuals were assessed to verify normality using Shapiro–Wilk's test and homogeneity of variance using Levene's test. Sperm DFI was log transformed to obtain normality before statistical analysis. Differences in fertilization, polyspermy, cleavage rate, blastocyst rate and total blastocyst cell number were studied using a mixed linear model (proc mixed). Sperm–oocyte ratio (250:1 or 500:1) and boar (1:6) were set as fixed effects and IVF week as a random effect as different oocyte materials over different seasons was used. Interactions between sperm–oocyte ratio and boar were not significant, and therefore not included in the models. When analysis of variance (ANOVA) revealed a significant effect, values were compared using the post hoc multiple pairwise-comparison Tukey test. Results are presented as least squares means ± standard error of the mean (SEM) and $P \leq 0.05$ was considered statistically significant. Figures were plotted using GraphPad Prism v.9.0 (GraphPad Software, San Diego, USA).

Results

Sperm motility, viability and DNA fragmentation

Sperm parameters after centrifugation are shown in Table 1 for the different boars. A variation in total and progressive motility between boars and straws was observed for the individual sperm samples. However, average total and progressive motility were not significantly different between the boars. Average total motility ranged from 57.1% to 74.5%, of which 38.1–61.0% of the sperm showed progressive motility. Therefore, the number of progressively motile sperm cells per oocyte was adjusted for each sperm sample to obtain either a ratio of 250:1 or 500:1 across all IVF rounds. Furthermore, no significant difference in DFI was observed, while the average percentage acrosome-intact live sperm cells was higher for Duroc boar 2 compared with Landrace boar 2 ($P < 0.05$).

IVEP outcomes

Averages and standard deviations for the IVEP outcomes are presented in Table 2 for each individual boar and sperm–oocyte ratio. Results indicated that Landrace boar 3 had a higher average blastocyst rate for the 250:1 ratio compared with the 500:1 ratio (29.5 % vs 17.1%, respectively), while the other boars had a higher blastocyst rate at the 500:1 ratio. It was observed that blastocysts sometimes started to collapse on day 7, independent from the ratio or boar. Average blastocyst yield on day 7 was 22.4 ± 12.7% and average total blastocyst cell number was 59.8 ± 22.6 cells.

Sperm–oocyte ratios

A higher fertilization rate and blastocyst formation rate at day 6 ($P < 0.05$) were observed for the 500:1 ratio compared with the

Table 1. Sperm parameters per boar after Percoll density gradient centrifugation and before adjustment (mean ± standard deviation (SD))

Boar	n	MOT (%)	PROG (%)	Range PROG (%)	AIL (%)	DFI (%)
Duroc 1	3	57.1 ± 3.5	38.1 ± 5.0	32.4–41.8	72.0 ± 2.1[a,b]	0.24 ± 0.09
Duroc 2	3	73.2 ± 12.2	56.1 ± 11.3	49.3–69.1	79.2 ± 4.5[a]	0.41 ± 0.36
Duroc 3	3	60.3 ± 6.5	46.3 ± 6.0	42.3–53.3	74.4 ± 3.7[a,b]	0.23 ± 0.04
Landrace 1	4	74.5 ± 17.4	61.0 ± 17.1	42.2–75.9	70.4 ± 8.8[a,b]	0.26 ± 0.04
Landrace 2	4	61.8 ± 9.6	47.2 ± 7.7	36.9–55.5	66.7 ± 1.8[b]	0.40 ± 0.20
Landrace 3	3	60.9 ± 18.5	45.2 ± 14.8	29.1–58.2	75.1 ± 2.6[a,b]	0.20 ± 0.07

n, number of straws analyzed and used for IVF.
AIL, Acrosome intact live; DFI, DNA fragmentation index; MOT, total motility; PROG, progressive motility.
Each sperm sample was evaluated for the percentage of total motile and immotile sperm. Total motility consists of a percentage of progressive motile and non-progressive motile sperm cells.
[a,b]Values with different superscript letters within a column are significantly different ($P < 0.05$).

250:1 ratio, while the polyspermy level was consistent across ratios (Table 3). No significant effect of sperm–oocyte ratio was observed for cleavage rate, blastocyst rate at day 7 or total blastocyst cell number.

Individual boar difference

Differences in IVEP outcomes between boars are shown in Figure 1. A significant effect of boar was observed for fertilization rate ($P < 0.05$), cleavage rate ($P < 0.001$) and blastocyst rates ($P < 0.001$). Fertilization rate (Fig. 1A) and blastocyst rates on day 6 and 7 (Fig. 1D) were significantly lower for Duroc boar 3 compared with Landrace boar 1. Cleavage rate at day 2 was lower for Duroc boar 3 compared with Landrace boars 1 and 3 (Fig. 1C). No significant differences in polyspermy (Fig. 1B) and total blastocyst cell number on day 7 of culture were observed between the boars.

Discussion

The aim of this study was to assess fertilization with a 250:1 and 500:1 'progressively motile sperm to oocyte' ratio using cryopreserved semen from three Duroc and three Landrace boars. To our knowledge this is the first study assessing IVEP outcomes after adjustment for the number of sperm cells based on progressive motility. The 500:1 ratio resulted in a higher fertilization rate and blastocyst percentage on day 6 of culture compared with the 250:1 ratio, but no effect of sperm–oocyte ratio on polyspermy, cleavage rate, blastocyst formation rate on day 7 or total blastocyst cell number was observed. A higher incidence of polyspermy was expected for the highest oocyte-sperm ratio as a decrease in penetration rate and polyspermy is normally observed when the sperm concentration is reduced in IVF as reviewed by Coy and Avilés (2010), but this was not the case in our study. One explanation, however, could be that the highest ratio in this study (500:1) was lower compared with other studies on sperm–oocyte ratios, ranging from 2000:1 to 8000:1 and 3000:1 to 50.000:1 (Xu *et al.*, 1996; Gil *et al.*, 2004). On day 7 of culture, no more difference was observed anymore in blastocyst formation between the two ratios. It was noticed that blastocysts sometimes started to collapse from day 6 to day 7 of culture, independent of the boar or ratio, and this could have affected the results. Furthermore, apoptotic nuclei were observed in some of the blastocysts and no hatching was observed. This can indicate suboptimal culture conditions and may even be related to degeneration of polyspermic zygotes. In further studies it will be of interest to use porcine blastocyst medium (PBM) during IVC, as this optimized culture medium is shown to improve embryo quality, development to blastocyst stage and hatching (Mito and Hoshi, 2019). Additionally, fetal bovine serum has improved hatching (Yoshioka *et al.*, 2005). However, a defined or semi-defined medium is preferable as medium supplemented with serum components carry a risk of disease transmission. Embryo transfers with *in vitro*-produced blastocysts on days 5 and 6 have successfully resulted in liveborn piglets (Mito *et al.*, 2015; París-Oller *et al.*, 2021; Suzuki *et al.*, 2006). When performing embryo transfers, it is beneficial to get as many blastocysts as possible, but a longer blastocyst culture than needed is unnecessary and might impair embryo quality and increase the risk of embryo hatching. Therefore, it was concluded that the 500:1 sperm–oocyte ratio and culture until day 6 is optimal within our IVF system for both breeds.

Variation in sperm motility between boars and between straws from the same boar was observed. Although adjusted to the same number of progressively motile sperm cells per oocyte for all boars and replicates in this study, differences in IVEP outcomes were still observed between some of the boars. Duroc boar 3 differed most and showed the lowest results, while the best blastocyst results were obtained for Landrace boar 1 with an average blastocyst formation rate of 37.0% on day 6 for the 500:1 ratio. No difference in blastocyst cell count was observed between the boars, but there was variation for all boars. Total blastocyst cell number was relatively high in this study for all boars (on average 59.8 ± 22.6 cells), as 30–45 cells are usually reported for good quality *in vitro*-produced blastocysts (Gil *et al.*, 2013; Yoshioka *et al.*, 2020; Yuan *et al.*, 2017). Average blastocyst yield in our study was lower (22.4 ± 12.7% for all boars) compared with the study that also supplemented IVM medium with serum substitute and reported a blastocyst yield of 40% (Yuan *et al.*, 2017). However, findings are similar for the best boar and 500:1 ratio which resulted in a blastocyst yield of 36.2 ± 6.4%. It should be considered that different media have been used that can affect culture success. Interestingly, results indicated that average blastocyst rate for Landrace boar 3 was higher for the 250:1 ratio compared with the 500:1 ratio. This is in line with others who reported differences in optimal sperm–oocyte ratio between boars (Xu *et al.*, 1996; Gil *et al.*, 2004, 2007) and the fact that preliminary screening for each individual boar is recommended before IVF.

Adjustment for the same number of progressively motile sperm cells present per oocyte did not result in similar and high blastocyst rates among the boars. This suggests that there are factors other

Table 2. Descriptive statistics for fertilization, polyspermy, cleavage and blastocyst formation rates per boar and 'progressively motile sperm–oocyte' ratio (mean ± standard deviation (SD))

Boar	Sperm–oocyte ratio	Zygotes analyzed[a]	Fertilization rate (%)	Polyspermy rate (%)[b]	Zygotes cultured[c]	Cleavage rate D2 (%)	Blastocyst rate D6 (%)	Blastocyst rate D7 (%)
Duroc 1	250	83	26.3 ± 13.4	30.0 ± 26.5	116	41.4 ± 2.4	18.1 ± 2.2	18.1 ± 2.2
	500	86	31.7 ± 20.9	23.0 ± 10.0	117	59.0 ± 7.5	33.2 ± 14.5	27.3 ± 12.4
Duroc 2	250	85	40.6 ± 16.9	15.3 ± 16.8	118	58.3 ± 20.9	22.0 ± 9.5	21.2 ± 11.3
	500	84	52.7 ± 18.9	43.0 ± 3.5	119	54.7 ± 10.6	28.7 ± 8.6	27.0 ± 12.3
Duroc 3	250	83	11.0 ± 3.9	36.1 ± 12.7	116	37.7 ± 8.8	4.3 ± 4.2	4.3 ± 4.2
	500	90	17.8 ± 10.7	19.4 ± 17.3	114	32.9 ± 13.4	13.6 ± 12.4	11.0 ± 11.3
Landrace 1	250	82	45.3 ± 27.4	23.3 ± 25.2	117	61.8 ± 11.6	24.9 ± 8.2	27.5 ± 8.2
	500	87	59.7 ± 9.5	34.6 ± 32.7	119	58.9 ± 6.9	37.0 ± 5.4	36.2 ± 6.4
Landrace 2	250	87	26.2 ± 12.0	27.2 ± 11.8	118	36.3 ± 21.3	21.2 ± 14.8	19.5 ± 12.3
	500	84	36.9 ± 20.6	20.2 ± 21.5	118	51.5 ± 12.1	28.7 ± 10.9	31.3 ± 8.5
Landrace 3	250	83	37.8 ± 22.6	29.8 ± 12.9	118	64.4 ± 7.0	31.2 ± 13.9	29.5 ± 18.0
	500	85	38.7 ± 8.5	11.9 ± 20.6	147	61.2 ± 15.1	21.8 ± 7.9	17.1 ± 10.4

[a]Number of presumptive zygotes analyzed for fertilization and polyspermy rates.
[b]Percentage of fertilized oocytes that were polyspermic.
[c]Number of zygotes cultured to assess cleavage rate at day 2 and blastocyst formation rate on days 6 and 7.
Cleavage and blastocyst rates were defined as the number of cleaved oocytes or blastocysts divided by the total number of presumptive zygotes cultured.

Table 3. Effect of 'progressively motile sperm cell to oocyte' ratio on fertilization, polyspermy, cleavage and blastocyst formation rates and total blastocyst cell number (least squares (LS) means ± standard error of the mean (SEM))

Sperm–oocyte ratio	Number of zygotes analyzed[1]	Fertilization rate (%)	Polyspermy[2]	Number of zygotes cultured[3]	Cleavage rate D2 (%)	Blastocyst rate D6 (%)	Blastocyst rate D7 (%)	Number of blastocysts analyzed	Total blastocyst cell number
250:1	503	31.2 ± 4.3[a]	27.2 ± 5.1	734	50.2 ± 2.9	20.3 ± 2.2[a]	20.0 ± 2.3	100	61.3 ± 3.6
500:1	516	38.5 ± 4.4[b]	24.8 ± 5.3	703	52.1 ± 2.9	26.9 ± 2.2[b]	24.7 ± 2.3	160	59.1 ± 3.1

[1]Number of presumptive zygotes analyzed for fertilization and polyspermy rates.
[2]Percentage of fertilized oocytes that were polyspermic.
[3]Number of presumptive zygotes cultured to assess cleavage rate at day 2 and blastocyst formation rate on day 6 and 7.
Cleavage and blastocyst formation rates were defined as the number of cleaved oocytes or blastocyst divided by the total number of presumptive zygotes cultured.
[a,b]Values with different superscript letters within a column are significantly different ($P < 0.05$).

than just the number of progressively motile sperm that influence IVEP results. Suzuki and Nagai (2003) observed that epididymal sperm samples with high total and progressive motility did not always result in high fertilization rates. In the present study, more spermatozoa were added for the samples with a lower progressive motility, which increased the total number of sperm and also the number of immotile or dead sperm cells present during fertilization for the different replicates and boars. Roca *et al.*, (2013) indicated that a higher proportion of dead spermatozoa but same number of viable sperm cells in raw semen before freezing negatively affected *in vitro* fertilization, cleavage and blastocyst formation. The dead sperm cells affect the intracellular reactive oxygen species (ROS) in the viable spermatozoa after freezing and thawing that, at too high levels, led to sperm DNA damage, as shown in different species (Takahashi *et al.*, 2000; Agarwal and Said, 2003; Simões *et al.*, 2013). Interestingly, an *in vitro* study in bovine showed that DNA damage induced by sperm oxidative stress affected cleavage rate but not blastocyst formation rate or quality (Simões *et al.*, 2013). In our study, Duroc boar 3 had significantly lower fertilization, cleavage and blastocyst rates compared with Landrace boar 1, but progressive motility was not lower. In contrast, Duroc boar 1 had generally a lower progressive motility after Percoll density centrifugation and therefore more sperm was added during the IVF rounds compared with the other boars, which have resulted in more immotile or dead sperm cells present during fertilization, but IVEP results were good for this boar. This suggests that a higher proportion of immotile or dead sperm cells did not affect IVEP outcomes in our study. In cattle it has been observed that after IVF, only the percentage of live spermatozoa was associated with fertilization outcomes (Tanghe *et al.*, 2002). Furthermore, several studies have observed positive correlations between progressive motility and penetration rate (Xu *et al.*, 1996; Gadea and Matás, 2000) while others did not (Suzuki *et al.*, 1996; Popwell and Flowers, 2004). The sperm parameters in the present study were assessed as means per sperm sample but, lately, evaluation has been based on individual sperm kinetics and subpopulations. In bovine it was shown that the population characterized with rapid and progressive motility had the greatest

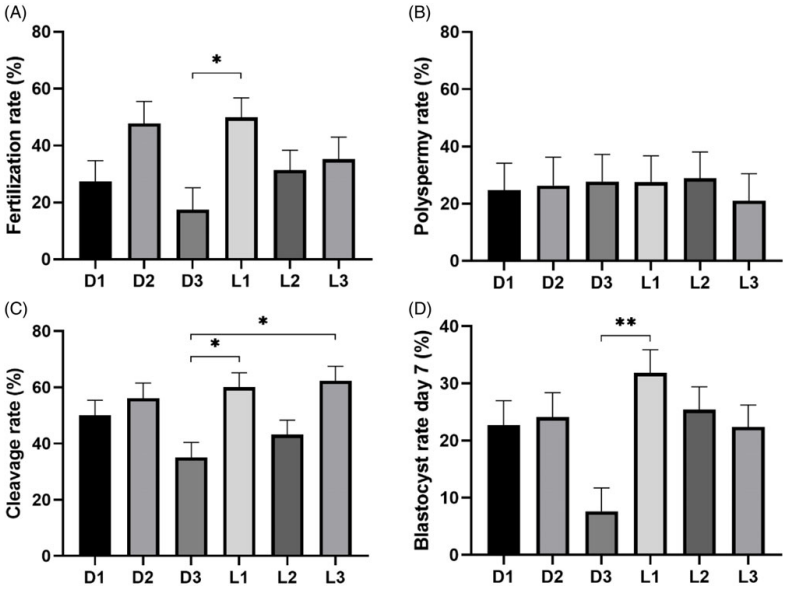

Figure 1. *In vitro* embryo production outcomes for the different boars for both ratios. (A) Fertilization rate, (B) polyspermy rate, (C) cleavage rate at day 2, and (D) blastocyst formation rate at day 7 per Duroc (D) and Landrace boar (L). *$P < 0.05$; **$P < 0.01$. Results are presented as least squares (LS) means ± standard error of the mean (SEM).

effect on IVEP outcomes (Peres Campanholi *et al*., 2021) and was more resistant to cryopreservation (Muiño *et al*., 2008). Therefore, it could be of interest to evaluate sperm subpopulations with specific movement patterns by a cluster analysis for individual boars and breeds in relation to IVEP outcomes.

In conclusion, fertilization with the 500:1 ratio resulted in a higher fertilization rate and blastocyst yield on day 6, while polyspermy did not increase with the higher sperm–oocyte ratio. Differences in IVEP outcomes were still observed between the individual boars although adjusted for progressive motility. Promising blastocyst yields and high blastocyst cell numbers were obtained with cryopreserved sperm from both Duroc and Landrace boars.

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Conflict of interest. The authors declare no conflict of interest.

Ethical standards. Oocytes were collected from routinely slaughtered animals, a procedure that did not require ethical approval.

Availability of data and materials. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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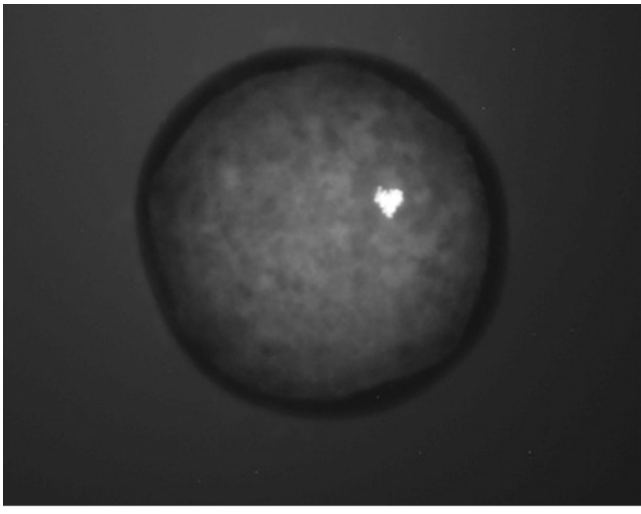
Paper III



Follicular fluid steroid hormones and *in vitro* embryo development in Duroc and Landrace pigs

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Keywords

Cumulus expansion, follicle diameter, follicular fluid, *in vitro* embryo production, porcine, steroid hormones

Abstract

The Duroc sire line has a smaller litter size and we have previously observed fewer surface follicles on the ovary one day after weaning compared to the Landrace dam line. In that same study, a broader cumulus expansion and faster nuclear maturation were observed for Duroc oocytes at 20 h of *in vitro* maturation (IVM), while Landrace oocytes showed more advanced stages of cortical granule distribution. However, no differences were observed after the final IVM period of 44 hours. The aim of this study was to assess subsequent *in vitro* embryo production (IVEP) in Duroc and Landrace. Furthermore, follicle diameter and steroid hormone levels in follicular fluid (FF) were measured to study possible relation to oocyte developmental competence. Follicular phase sow ovaries were collected one day after weaning and the 10 largest follicles were measured per ovary before aspiration. Cumulus-oocyte complexes (COCs) were matured *in vitro*, and cumulus expansion was analysed by assessing individual COC areas at 0 and 20 h. Fertilization of Duroc and Landrace oocytes was performed with both semen from a Duroc and a Landrace boar. A larger follicle diameter was observed for Landrace animals (5.7 vs. 4.8 mm, $P < 0.0001$) and individual COC area was additionally larger at 0 h after aspiration ($P < 0.0001$) compared to Duroc. Contrary, cumulus expansion from 0 to 20 h of maturation was greater for Duroc oocytes than for Landrace ($407 \pm 67\%$ vs. $319 \pm 31\%$, $P < 0.0001$). After fertilization, cleavage rate was higher for Duroc oocytes, and the highest blastocyst yield was obtained for Duroc oocytes fertilized with the Landrace boar. Steroid hormone analysis of the follicular fluid showed differences in the pathways with a higher total level of oestrogens ($P = 0.01$) and aromatase products/substrates ratio ($P < 0.01$) in Landrace FF compared to Duroc, which indicate a higher degree of feminization in Landrace. In conclusion, results suggest that Duroc oocytes have a better *in vitro* oocyte developmental competence and breed differences in steroidogenesis were found in the early follicular phase.

1. Introduction

Ultrasound-guided ovum pick-up (OPU) followed by *in vitro* embryo production (IVEP) is used worldwide in cattle breeding to select genetically superior animals and decrease the generation interval. In combination with genomic selection, the rate of genetic gain in breeding programs has significantly increased [1,2]. The use of embryo technology is also of interest in pig breeding to increase genetic gain and to disseminate genetics of superior animals worldwide with less transportation of live animals and a lower risk of disease transmission. However, there are still challenges to be overcome before embryo technology can be used commercially in porcine, mainly because of the complex female reproductive anatomy which makes it challenging to collect *in vivo* embryos or perform OPU. Furthermore, the efficiency of both IVEP [3] and embryo transfers [4] is low compared to other species. In cattle it has been shown that IVEP outcomes vary with breeds showing different reproductive performance [5,6]. Reproductive performance also differs among purebred pig breeds as they are bred for different traits. The Norwegian Duroc sire line has been bred for production and meat quality traits and has a smaller litter size with on average 9.2 total number of piglets born (TNB) compared to 13.8 TNB in the Norwegian Landrace dam line which has been selected for production and reproduction traits [7]. An increase in TNB is genetically correlated to a higher ovulation rate and a lower corpus luteum (CL) weight (Da Silva et al., 2018) and CLs with a smaller diameter and lower weight have been suggested to originate from smaller follicles at ovulation (Wientjes et al., 2012). As a larger follicle size has been related to an improved oocyte developmental competence *in vitro* [8–10], ovarian characteristics and *in vitro* oocyte maturation (IVM) in Norwegian Duroc and Landrace have previously been studied [11]. Significantly fewer follicles were observed on Duroc ovaries one day after weaning, which additionally contained smaller cumulus-oocyte complexes (COCs) at collection compared to Landrace. However, Duroc oocytes had a significantly broader cumulus expansion from 0 to 20 h of IVM. In addition, Duroc oocytes exhibited at this time point more advanced stages of nuclear maturation, while Landrace oocytes showed more advanced stages of cortical granule distribution. No differences were observed after the final maturation period and a similar percentage of oocyte reached MII stage with 90.1% for Duroc and 87.7% for Landrace [11]. Based on these results, it was questioned whether *in vitro* embryo

development subsequently differs between the two breeds and whether this could be related to follicle development and size on the ovaries.

Follicular development is a complex process regulated by the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus and induces secretion of both FSH and LH by the pituitary gland which are transported to the ovaries via the blood [12]. In antral follicles, theca cells respond to LH and convert cholesterol into androgens such as testosterone and androstenedione. The granulosa cells are responsible for conversion of the androgens into oestrogens such as 17β-oestradiol and estrone by FSH induced aromatase (CYP19) [13]. During follicular development the oocyte acquires developmental competency, which is defined as the ability to be fertilized and develop into a viable embryo [14]. The follicular fluid (FF) provides a microenvironment for the oocytes that, together with other factors, contain the produced steroid hormones that are required for follicular development, reproductive function and fertility [15]. In follicular fluid from the highly reproductive Meishan pig a higher oestradiol concentration and increased aromatase activity in both granulosa and theca cells have been reported during the follicular phase compared to FF from Large White pigs [16,17]. In later studies it was shown that a smaller preovulatory follicle diameter was observed for Meishan animals, but more oocytes were in advanced stages of meiosis and developed to MII stage compared to Large White [18,19]. On the contrary, others have shown higher levels of β-oestradiol in FF from sows with larger follicles compared to sows with smaller follicles (Costermans et al., 2020; Liu et al., 2002). In addition, sows in the early follicular phase with a high percentage high-quality COCs showed higher concentrations of β-oestradiol, progesterone, 19-norandrostenedione and α-testosterone in follicular fluid compared to low COC-quality sows, while cortisol concentration was lower [21]. Differences in FF steroid hormone concentrations are thus already present at the start of the follicular phase and suggested to be related to oocyte quality and developmental competence.

The aim of this study was to assess *in vitro* embryo development in the Duroc sire and Landrace dam line by fertilizing both Duroc and Landrace oocytes with semen from a Duroc and Landrace boar, leading to four groups being compared: D x D, D x L, L x D, L x L. Furthermore, follicle diameter and FF steroid hormone levels were measured in both breeds to study possible relation to oocyte developmental competence.

2. Materials and methods

2.1 Animals and ethics

Ovaries from 23 Duroc and 36 Landrace sows (parity 1 and 2) were collected one day after weaning at a commercial abattoir. The sows originated from two Norsvin nucleus herds. The weaning-to-service interval in Duroc and Landrace is 5.7 ± 1.8 days and 5.7 ± 1.3 days, respectively. This indicates that ovarian dynamics and follicular development are comparable in the breeds. No ethical approval was required since material was collected from animals that were routinely slaughtered. All animals were cared for according to internationally recognized guidelines and regulations for keeping pigs in Norway (The Animal Welfare Act, 10 July 2009 and Regulations for keeping pigs in Norway, 18 February 2003). Lactating sows in both herds were liquid fed with a commercial diet with whey supplementation and feed was offered up to four times a day to ensure ad libitum access during lactation. Data were collected in three replicates from June 2020 to June 2021.

2.2 Chemicals and media

All chemicals and reagents were purchased from Sigma-Aldrich (Oslo, Norway) unless stated otherwise. Different media were used for the IVEP steps: porcine X medium (PXM) for washing cumulus-oocyte complexes, porcine oocyte medium (POM) for maturation, porcine gamete medium (PGM) for fertilization and porcine zygote medium-5 (PZM-5) for embryo culture [22]. Polyvinyl alcohol in original media was replaced by 0.4% bovine serum albumin (BSA) in POM and PZM-5 medium, and 0.6% BSA in PGM

medium. Minor changes were made to the POM medium and final composition was: 108 mM NaCl, 10 mM KCl, 0.35 mM KH2PO4, 0.4 mM MgSO4·7H2O, 25 mM NaHCO3, 5.0 mM glucose, 0.2 mM Na-pyruvate, 2.0 mM Ca-(lactate)2·5H2O, 2.0 mM L-glutamine, 5.0 mM hypotaurine, 20 mL/l BME amino acids, 10.0 mL/l MEM non-essential amino acid, 0.6 mM L-cysteine, 0.01 mg/mL gentamicin, 4.0 mg/mL BSA, serum substitute, 10 ng/mL epidermal growth factor, and 50 µM β-mercaptoethanol (Gibco).

2.3 Ovarian characteristics, oocyte collection and in vitro maturation (IVM)

Early follicular phase sow ovaries were collected one day after weaning and transported to the laboratory in 0.9% NaCl at 25 to 30 °C within an hour after slaughter. Upon arrival, ovaries were washed with 0.9% NaCl containing 2.5 µg/mL kanamycin and placed in a beaker in a water bath at 30 °C until follicle aspiration. Ovaries were dissected free of non-ovarian tissue before ovary weight and length were measured. The 10 largest follicles per ovary were measured with a calliper rule and all 3 to 8 mm follicles were aspirated with an 18-gauge needle and 10 mL syringe, after which surface follicles smaller than 3 mm were counted. Oocytes with a compact cumulus and evenly granulated cytoplasm were selected and washed three times in PXM, once in POM medium, and transferred in groups of 25 oocytes into each well of a Nunc four-well multidish containing 500 µL of pre-equilibrated POM medium. For the first 20 h, COCs were matured in POM supplemented with 0.05 IU/mL porcine FSH and LH (Insight Biotechnology Ltd), and 0.1 mM dbcAMP. Subsequently, COCs were matured for another 24 h in POM without hormones and dbcAMP. Oocytes were cultured for the first 44 h at 38.9 °C in an humified atmosphere containing 6% CO2 in air.

2.4 In vitro fertilization (IVF) and culture (IVC)

Fertilization was performed with cryopreserved sperm from one Duroc and one Landrace boar. The two boars were selected based on a previous IVEP study with random ovaries, where similar fertilization, polyspermy, cleavage and blastocyst formation rates were obtained (Jochems et al., unpublished data). Frozen straws from each individual boar originated from the same ejaculate. Each 2.5 mL straw was thawed at 50 °C for 50 seconds (Waterhouse et al., 2006) and the semen was diluted in 40 mL Tri-X-cell (IMV

technologies, L'Aigle, France) at room temperature (RT). Sperm cells were washed and selected at RT using Percoll® density gradient centrifugation by layering 2 mL 45% Percoll on top of 2 mL 90% Percoll. Finally, 1 mL of semen was carefully placed on top followed by centrifugation at 700g for 20 min. The supernatant was removed by aspiration, the pellet was resuspended in 4 mL PGM without BSA and centrifuged at 500g for 5 min. The pellet was then resuspended in 150-200 µL PGM without BSA. Sperm concentration was measured by computer assisted sperm analysis (CASA) using a Sperm Class Analyzer® version 6.1 (Microptic SL, Barcelona, Spain), equipped with a phase contrast Eclipse Ci-S/Ci-L microscope (Nikon, Japan) and Basler digital camera (Basler Vision Technologies, Ahrensburg, Germany). The sperm sample was diluted to 5 x 10^5 progressively motile sperm cells /mL in 300 µL pre-equilibrated PGM with BSA. The COCs were carefully washed once in PGM during centrifugation of the sperm and groups of 30 oocytes were co-incubated with 3.0 x 10^4 progressively motile sperm cells/mL (ratio of 1:500). After 2 h of co-incubation, oocytes were transferred to a new well with 500 µL PGM medium to remove an excess of sperm cells.

After a total of 4 h fertilization, presumptive zygotes were denuded of cumulus cells by vortexing for 1 min in 2 mL PXM in a 15 mL tube. The presumptive zygotes were washed twice in PXM medium and once in PZM-5 before culture in 500 µl PZM-5 under 400 µL mineral oil (IVF Biosciences, Falmouth, UK) at 38.9 °C in an humified atmosphere containing 6% CO2 and 7% O2. At day 4 of culture (fertilization day 0), PZM-5 medium was refreshed by taking 250 µl out and replacing it with 250 µL new equilibrated PZM media.

2.5 Assessment of cumulus expansion

To analyse cumulus expansion, images were taken from each well at 0 and 20 h of maturation with a Nikon SMZ1500 stereomicroscope (Nikon, Tokyo, Japan). Individual COC area of each oocyte was analysed by ImageJ software (version 1.52a; NIH, Bethesda, USA) at both timepoints, and cumulus expansion per well was determined by dividing total COC area of each well at 20 h by the total COC area of the well at 0 h (Costermans et al., 2019). After 44 h of maturation, it was not possible to assess cumulus expansion per individual oocyte as cumulus cells showed a high degree of expansion and were overlapping.

2.6 Assessment of embryo development and quality

Cleavage rates at day 2 and blastocyst rates at day 6 of culture were evaluated with a Leica DM IL Inverted microscope. Cleavage and blastocyst formation rate were defined as the number of cleaved oocytes, or blastocysts, divided by the total number of oocytes cultured. An embryo with a clear blastocoel was defined as a blastocyst. Blastocysts were fixated in 4% PFA at RT for 30 min, stained with 8 µg/mL Hoechst (H-33342, B2261, Sigma) and mounted in 6 µL fluorescence mounting medium (Dako, Glostrup, Denmark) under a coverslip to assess embryo quality. Total blastocyst cell number was assessed by fluorescence microscopy using a Leica SP8 laser scanning confocal microscope. Hoechst staining was evaluated with a 405 nm excitation laser and a 410 to 480 nm emission filter.

2.7 Follicular fluid collection and steroid hormone analysis

Follicular fluid (FF) was pooled per breed during each replicate from the 3-8 mm follicles aspirated and total FF volume collected was recorded. Average FF volume per follicle was calculated by dividing the total collected FF volume by all follicles aspirated per replicate. To remove cellular debris, FF was centrifugated at 1,900g for 30 minutes and supernatant was poured off and stored in 1.5 mL aliquots at -80 °C. Upon analysis, samples were shipped on dry ice to the Department of Pharmacy at the University of Copenhagen for steroid analysis. Follicular fluid steroids were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS) and steroids were extracted, purified and analysed as described by Weisser et

al. (2016). Online clean-up and chromatographic separation of steroids was performed using a binary 1290 Agilent Infinity Series system combined with a binary 1100 Agilent HPLC pump. Thereafter, an AB SCIEX 4500 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionization Turbo V source was used for detection. Multiple reaction monitoring was performed in positive mode during analysis with target scan time of 0.8 s. The LC and MS optimization was conducted using Analyst v. 1.6.2 software package and obtained data was processed in MultiQuant v. 3.0 software (AB SCIEX). Steroids analysed were androstenedione (AN), pregnenolone (PREG), progesterone (PROG), dehydroepiandrosterone (DHEA), testosterone (TS), estrone (E1), 17β-oestradiol (βE2), cortisol (COR), corticosterone (COS), 17α-hydroxyprogesterone (17-OHPROG), 17α-hydroxypregnenolone (17-OHPREG), 11-deoxycorticosterone (11-deoxyCOS), 11-deoxycortisol (11-deoxyCOR) and cortisone (CORNE).

2.8 Statistical analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Distributions of the means and residuals were assessed to verify normality using Shapiro-Wilk's test and homogeneity of variance using Levene's test. Individual COC area data at both timepoints and total blastocyst cell number were log transformed to obtain normality before statistical analysis. Mean values for ovarian characteristics, individual COC area, COC expansion and steroid concentrations between the two breeds were analysed using the Student's t-test for two independent samples. The effect of maternal and paternal breed on cleavage rate, blastocyst rate and total blastocyst cell number were studied using mixed linear model (proc mixed). The maternal and paternal breed (Duroc or Landrace) were defined as fixed effects and replicate as random effect. An interaction between maternal and paternal breed was only observed for blastocyst rate, and therefore not included in the other models. When ANOVA revealed a significant effect, values were compared using the post hoc multiple pairwise-comparison Tukey test. The results are presented as mean ± SD unless stated otherwise and $P \leq 0.05$ was considered statistically significant. Figures were plotted using GraphPad Prism version 9.0 (GraphPad Software, San Diego, USA).

3. Results

3.1 Ovarian characteristics

A higher average ovary weight (5.2 ± 1.2 g vs. 4.4 ± 0.9 g, $P < 0.001$) and ovary length (3.0 ± 0.5 cm vs. 2.7 ± 0.3, $P = 0.01$) were observed for Landrace ovaries compared to Duroc one day after weaning. The number of 3 to 8 mm follicles aspirated was not significantly different, but the average was higher for Landrace with 23.2 ± 7.0 follicles compared to 21.4 ± 6.5 follicles for Duroc. In addition, no difference was observed in the number of follicles with a diameter below 3 mm (12.6 ± 7.1 for Landrace and 11.0 ± 6.3 for Duroc).

3.2 Follicle diameter

The 10 largest follicles were measured on 43 Duroc and 67 Landrace ovaries. Follicle diameter of the 10 largest follicles per ovary ranged from 3 to 8 mm for both breeds (Fig. 1), but average follicle diameter was larger for Landrace compared to Duroc (5.7 ± 1.0 mm vs. 4.8 ± 1.0 mm, $P < 0.0001$). Average FF volume per follicle was 58.0 ± 10.6 µl and 42.1 ± 10.4 µl for Landrace and Duroc, respectively.

3.3 Cumulus-oocyte complex area and cumulus expansion

Individual COC area was analysed for 251 Duroc and 500 Landrace oocytes. After aspiration, average Duroc COC area was significantly smaller compared to Landrace ($P < 0.0001$). At 20 h of maturation, COC area was similar between the breeds. Therefore, a higher cumulus expansion per well (n = 10 Duroc and 20 landrace wells) was observed from 0 h to 20 h for the Duroc oocytes compared to Landrace with 407 ± 67% and 319 ± 31% expansion, respectively (Fig. 2).

3.4 In vitro embryo development

Cleavage and blastocyst rates and total blastocyst cell number are shown per group in Table 1. Cleavage rate was higher for Duroc oocytes ($P = 0.03$), and the highest blastocyst rate was obtained for Duroc oocytes

fertilized with the Landrace boar (P = 0.02). Total blastocysts cell number was not significantly different between the groups.

3.5 Steroid hormones in follicular fluid

The levels of steroid hormones in follicular fluid were investigated for both breeds and differences in the hormone pathways were observed with higher levels of progestagens and oestrogens for Landrace, and lower levels of androgens and corticosteroids compared to Duroc (Fig. 3). No significant differences for the individual steroids were observed between the breeds, but 17β-oestradiol tended to be higher for Landrace compared to Duroc (P = 0.09).

The aromatase products (E1 + βE2) were higher in Landrace follicular fluid compared to Duroc (P = 0.01), while no significant difference in aromatase substrates (AN + TS) was observed (Fig. 4A). The aromatase products/substrates ratio, i.e oestrogen:androgen ratio, was therefore 2 times higher in Landrace than in Duroc (P < 0.01, Fig. 4B).

A strong correlation between 17β-oestradiol concentration and the average follicle diameter of the 10 largest follicles was observed (Fig 5A.) Furthermore, correlations between average follicle diameter and progesterone and 17-OH Progesterone were found (Fig. 5B and C).

4. Discussion

In this study we investigated *in vitro* embryo development in Duroc and Landrace with focus on both the maternal and paternal breed. Furthermore, follicle diameter and follicular fluid hormone steroids were measured one day after weaning to study possible relation to oocyte developmental competence.

Follicle diameter of the 10 largest follicles per ovary ranged from 3 to 8 mm for both breeds, but average diameter was 0.9 mm larger on Landrace ovaries compared to Duroc. Individual COC area at time of

aspiration was larger for Landrace oocytes compared to Duroc and this is in line with Liu et al. (2002) and Yoon et al. (2000) who reported a relationship between follicle size and a larger oocyte diameter with more layers of cumulus cells in porcine. Additionally, cumulus expansion was analysed during *in vitro* maturation. In a previous study, cumulus expansion from 0 to 20 h of maturation was larger for Duroc, but Duroc animals had a higher variation in parity in that study (parity 1 to 6) compared to Landrace (parity 1 to 2) which could have affected the IVM outcomes [11]. Therefore, only parity 1 and 2 animals were selected in the present study but as previously observed, a smaller COC area at collection and a higher degree of cumulus expansion from 0 to 20 h of maturation was observed for Duroc oocytes compared to Landrace. In other studies assessing cumulus expansion at the end of maturation, larger cumulus expansion was observed for oocytes originating from larger preovulatory follicles [25] and a lower degree of cumulus expansion and percentage of matured oocytes was observed in Mangalica gilts having a low productivity and a smaller litter size compared to Landrace [26]. This is thus contrary to results in the present study. Broader cumulus expansion has furthermore been related to a lower polyspermy rate [27] and higher *in vitro* fertilization and embryo development [9]. In the present study, cleavage and blastocyst rates were higher for Duroc oocytes compared to Landrace after fertilization. This is in line with the findings mentioned above related to cumulus expansion but not with follicle diameter. No differences in total blastocyst cell number were observed between the groups. Overall, results suggest that Duroc oocytes have a better *in vitro* oocyte developmental competence as a broader cumulus expansion and faster nuclear maturation at 20 h of IVM have previously been observed [11] and higher cleavage and blastocyst rates were obtained in the present study compared to Landrace.

To better understand differences in oocyte developmental competence, levels of steroid hormones in follicular fluid were studied in both breeds. Clear differences in the steroid pathways were observed with higher levels of progestagens and oestrogens in Landrace FF compared to Duroc. Additionally, the total level of oestrogens was significantly higher in Landrace FF. Thus, a clear difference in follicular steroid

hormones between the breeds is already found at the early follicular phase, while oocytes in this phase are still growing and their granulosa cells only produce low levels of oestradiol. Biggs et al. (1993) also observed a higher oestradiol level in the higher reproductive pig breed in the late follicular phase. The androgens produced by theca cells are transferred to granulosa cells and converted by aromatase into oestrogens, i.e. oestradiol and estrone. Higher oestrogen concentrations in Landrace could be related to an increased androgen substrate that was available and could be converted. No significant difference between the breeds in total substrate level was found at the time of analysis but the mean availability of substrates was lower in Landrace. Furthermore, the higher oestrogen level could be due to an increased number of granulosa cells converting the substrates or due to increased aromatase activity. Higher aromatase protein abundance has been reported in follicular fluid from sows with high-COC-quality compared to sows with low-COC-quality [21] and a higher aromatase activity was additionally observed in granulosa cells from the highly reproductive Meishan pigs that had a higher oestradiol level compared to FF from Large White [19]. Therefore, aromatase activity in follicles from both breeds would be a topic of interest for further research. The steroid hormones and pathways indicate a higher degree of feminization in Landrace, which may be explained by the fact that the Landrace dam line has been selected for production and reproduction traits, while the Duroc sire has been bred for production and meat quality traits and androgens influence the growth. Interestingly, differences in steroid hormones between this sire and dam line have previously been demonstrated in boars, with higher plasma levels for both oestradiol and testosterone for Duroc compared to Landrace [28].

A strong positive correlation was observed between 17β-oestradiol and average follicle diameter of the 10 largest follicles, as also reported previously by others (Costermans et al., 2020; Liu et al., 2002). In addition, correlations between follicle diameter and the steroid hormones progesterone and 17-OH progesterone were found in the present study. A significantly higher progesterone level has previously been reported in large follicles (7-8 mm) compared to small follicles (1-2 mm), but not compared to medium follicles of 3-6 mm

(Liu et al., 2002). Better cleavage and blastocyst outcomes for Duroc were not significantly correlated to the individual follicular fluid steroid hormones levels in the current study. However, both Duroc and Landrace oocytes were removed from their follicular environment and matured *in vitro* in maturation media supplemented with the same concentrations of epidermal growth factor (EGF; 10 ng/mL), FSH and LH (0.05 IU/mL) and dbcAMP (0.1 mM) and it has been suggested that oocytes already acquire their developmental competence within the follicle before aspiration. Duroc oocytes, originating from smaller follicles and having a smaller COC area may have a better intrafollicular environment for maturation, as has also been hypothesized for the Meishan follicles [29]. Besides steroid hormones, follicular fluid consist of several other components such as growth factors, interleukins, anti-apoptotic factors, proteins, peptides and amino-acid also shown to affect oocyte developmental competence during maturation (reviewed by Revelli et al., 2009). A study in oocyte developmental competence in ewes with a higher number of preovulatory follicles found no differences in steroid hormones, but an earlier initiation of maturation and an increased responsiveness to gonadotropins were indicated compared to the breed with fewer follicles. Moreover, a lower metabolic activity was observed in those follicles which resulted in higher levels of amino acids and metabolic substrates that protect the oocytes from reactive oxygen species [31]. Although pigs have multiple ovulations during an oestrous cycle, further studies in follicular fluid components between Duroc and Landrace may provide insight into differences in oocyte developmental competence and how to improve IVEP. Furthermore, it could be of interest to culture random slaughterhouse oocytes with Duroc and Landrace FF to study if Duroc follicular fluid is positively affecting *in vitro* maturation and embryo production, as was observed with Meishan conditioned medium [32].

In conclusion, a larger follicle diameter and an individual larger COC area at aspiration was observed for Landrace animals one day after weaning compared to Duroc, whereas cumulus expansion from 0 to 20 h of maturation was larger for Duroc cumulus-oocyte complexes. Additionally, cleavage rate was higher for Duroc oocytes and the highest blastocyst yield was obtained for Duroc oocytes fertilized with semen from

the Landrace boar. The results suggest that Duroc oocytes have a better *in vitro* oocyte developmental competence than Landrace oocytes. Steroid hormone analysis of the follicular fluid showed differences in the pathways with a higher total level of oestrogens and aromatase products/substrates ratio for Landrace, indicating higher feminization in the Landrace dam line. Breed differences were thus found at the early follicular phase for *in vitro* oocyte developmental competence and follicular fluid steroid hormones.

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Conflict of interest Statement

The authors have no conflict of interest to declare.

Author contributions

RJ and AHG took part in conception and design, acquisition of data, and analysis and interpretation of data. LJZ, EG, ICO, FDM, EK and AKK took part in conception and design, and interpretation of data. BS took part in analysis and interpretation of data. RJ drafted the manuscript which was critically reviewed and approved by all authors.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Cleavage and blastocyst rates and total blastocyst cell number for the different groups

Oocytes	Sperm	Wells (zygotes) analysed	Cleavage rate D2 (%)	Blastocyst rate D6 (%)	Blastocysts analysed	Total blastocyst cell number
Duroc	Duroc	5 (125)	66.1 ± 19.7[a]	14.4 ± 8.3[ab]	15	34.7 ± 15.8
Duroc	Landrace	4 (99)	71.6 ± 10.2[a]	24.2 ± 2.9[a]	14	34.9 ± 16.9
Landrace	Duroc	8 (194)	54.4 ± 13.6[b]	7.8 ± 9.6[b]	15	44.3 ± 20.4
Landrace	Landrace	8 (194)	50.1 ± 20.9[b]	14.3 ± 6.2[ab]	26	38.1 ± 18.0

Cleavage and blastocyst formation rates were defined as the number of cleaved oocytes or blastocyst divided by the total number of oocytes cultured

[a-b] Values with different superscript letters within a column are significantly different ($P < 0.05$)

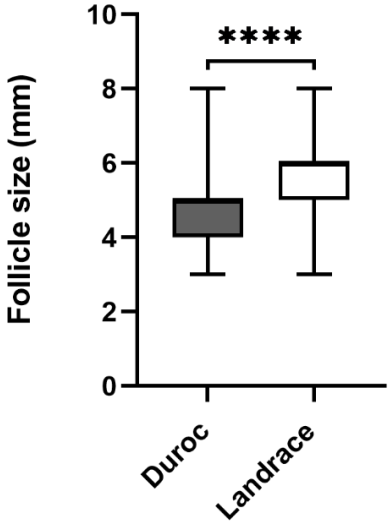

Figure 1. Boxplot of average follicle diameter (mm) for the 10 largest surface follicles on 43 Duroc and 67 Landrace ovaries one day after weaning. **** $P < 0.0001$

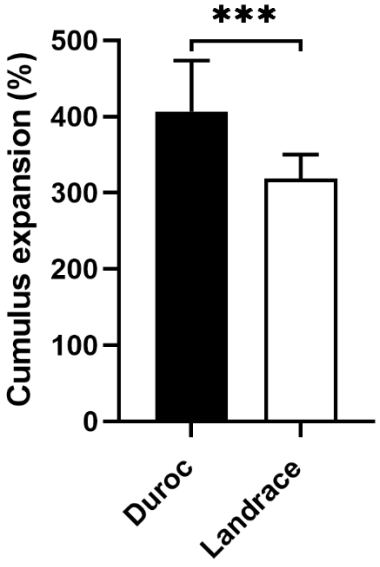

Figure 2. Cumulus expansion (%) from 0 to 20 hr of maturation for Duroc and Landrace cumulus-oocyte complexes (COCs). The cumulus expansion ratio was defined as the total COC area per well at 20 hr divided by the total COC area per well at 0 hr. *** $P < 0.001$

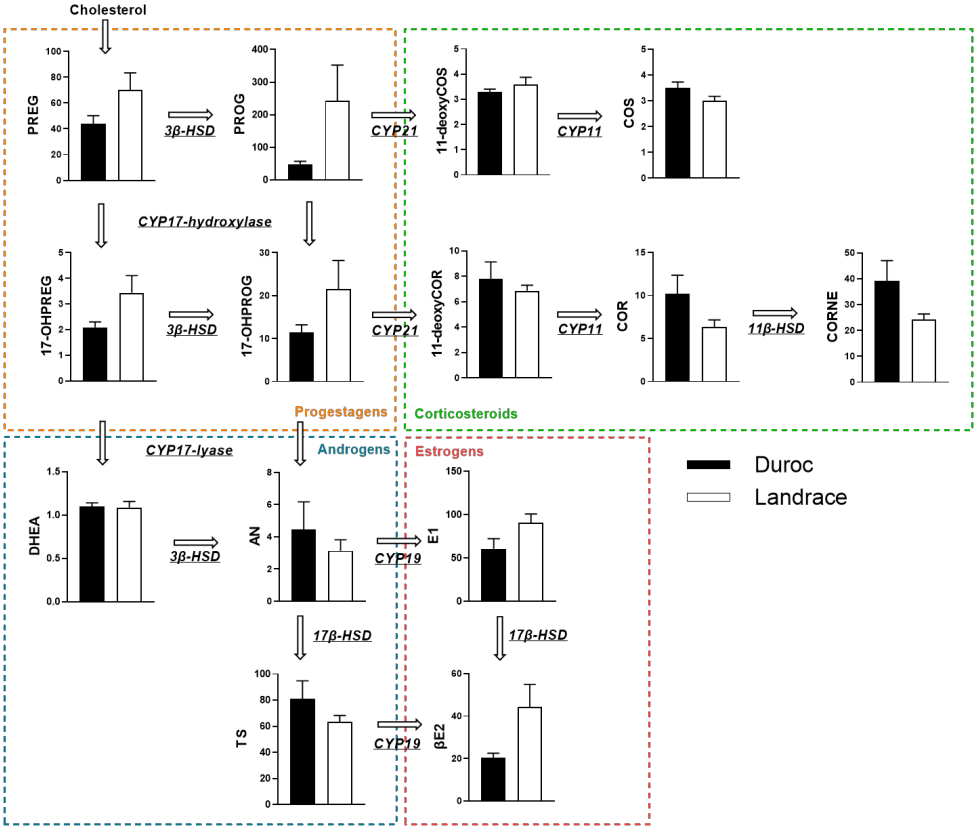

Figure 3. Steroidogenic overview of the Duroc and Landrace follicular fluid collected in the early follicular phase one day after weaning. Steroid levels were measured in ng/ml.

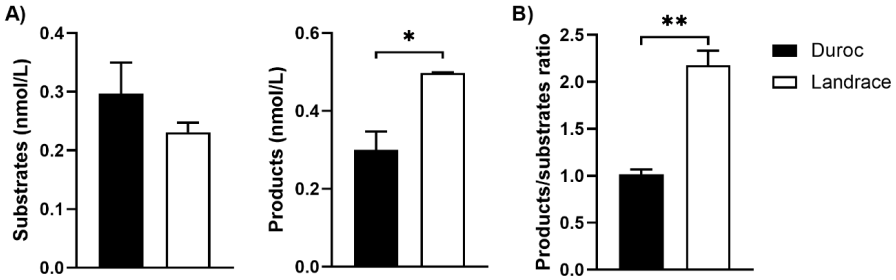

Figure 4. A) Aromatase substrates (AN + TS) and products (E1 + βE2) and B) Aromatase products/substrates ratio in Duroc and Landrace follicular fluid collected in the early follicular phase.

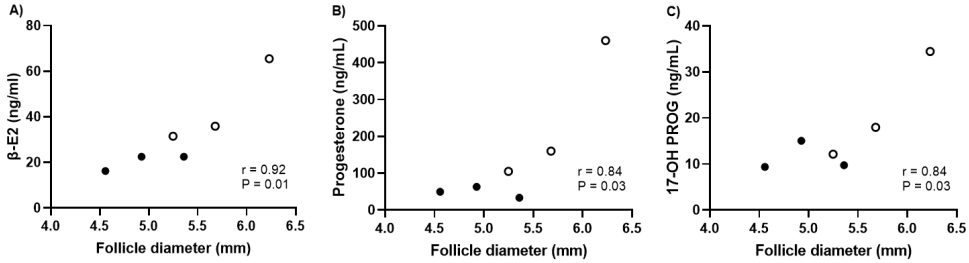

Figure 5. Average follicle diameter of the 10 largest follicles per ovary versus A) β-oestradiol, B) Progesterone and C) 17α-hydroxyprogesterone. Closed symbols represent pooled Duroc follicular fluid per replicate and open symbols represent Landrace.

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