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

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ABSTRACT

The Duroc sire line has a smaller litter size compared to the Landrace dam line and we have previously observed fewer surface follicles on Duroc ovaries one day after weaning. 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 distributions. However, no differences between breeds were observed after the final IVM period. The aim of this study was to assess subsequent in vitro embryo production (IVP) 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 follicle size of 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 sperm from both 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 broader for Duroc oocytes than for Landrace (407 ± 67% vs. 319 ± 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 sperm. Steroid hormone analysis of the follicular fluid showed differences in the pathways between breeds with a higher total level of estrogens ($P = 0.01$) and aromatase products/substrates ratio ($P < 0.01$) in Landrace compared to Duroc. In conclusion, results suggest that Duroc oocytes have a better in vitro oocyte developmental competence when cultured under the same in vitro conditions and breed differences in steroidogenesis were found in the early follicular phase.

1. Introduction

The efficiency of porcine in vitro embryo production (IVP) has considerably improved over the years [1], but is still lower compared to other domestic animal species. It is important to further improve IVP efficiency and embryo quality before in vitro embryo technologies can be implemented in the pig industry. In cattle, IVP outcomes were shown to vary for breeds with different reproductive performance [2,3]. Of the purebred pig breeds, the Norwegian Duroc sire line has been bred for production and meat quality traits and has a smaller litter size with an average of 9.2 total number of piglets born (TNB) compared to 13.8 TNB in the Norwegian Landrace dam line, which is selected for production and reproduction traits [4]. Although an earlier study on in vitro maturation reported no difference between breeds in the

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proportion of MII oocytes (90.1% for Duroc and 87.7% for Landrace) at the end of maturation, Duroc oocytes had a faster nuclear maturation and broader cumulus expansion at 20 h of maturation [5]. Based on these results, it was questioned whether in vitro embryo development subsequently would differ between the two breeds and whether this could be related to follicle development and follicle diameter on the ovaries.

The follicular fluid (FF) provides a microenvironment for the oocytes which, along with other factors, contain steroid hormones that are required for follicular development, reproductive function and fertility [6]. In antral follicles, theca cells respond to luteinizing hormone (LH) and convert cholesterol into androgens such as testosterone and androstenedione. The granulosa cells are in turn responsible for conversion of the androgens into estrogens such as 17β-estradiol and estrone by follicle stimulating hormone (FSH) induced aromatase (CYP19) [13]. During this follicular development the oocyte acquires developmental competency, which is defined as the ability to be fertilized and develop into a viable embryo [14]. In follicular fluid from the highly reproductive Meishan pig a higher estradiol concentration and increased aromatase activity in both granulosa and theca cells have been reported during the follicular phase compared to Large White pigs [7,8]. 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 [9,10]. On the contrary, others have shown higher levels of β-estradiol in FF from sows with larger follicles compared to sows with smaller follicles [11,12]. In addition, sows in the early follicular phase with a high percentage high-quality COCs showed higher concentrations of β-estradiol, progesterone, 19-norandrostenedione and α-testosterone in follicular fluid compared to low COC-quality sows, while cortisol concentration was lower [12]. 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. However, whether these differences in the steroid hormone concentrations at the beginning of follicular phase influence the efficiency of IVP in pigs is unknown.

The aim of this study was therefore to assess in vitro embryo development in the Duroc sire and Landrace dam line by fertilizing both Duroc and Landrace oocytes with sperm from a Duroc and Landrace boar, leading to four groups being compared: D x D, D x L, L x D, L x L. In addition, 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 were collected from 23 Norwegian Duroc and 36 Landrace sows (parity 1 and 2) from two separate Norsvin nucleus herds. The average weaning-to-service interval in Norwegian Duroc and Landrace herds 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, July 10, 2009 and Regulations for keeping pigs in Norway, February 18, 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. For each breed, data were collected in three replicates from June 2020 to June 2021. Landrace and Duroc sows were slaughtered on different days in the same week.

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 IVP 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 [13]. 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)

All sows were slaughtered 20–24 h after weaning at a commercial abattoir and the early follicular phase ovaries were collected and transported to the laboratory in 0.9% NaCl at 25–30 °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 ten largest follicles per ovary were measured 4 h after slaughter with a calliper rule, after which all 3–8 mm follicles were aspirated with an 18-gauge needle and 10 mL syringe. After aspiration, 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 dibutyryl-cAMP (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% CO_2 in air.

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

Both Duroc and Landrace oocyte were fertilized with cryopreserved sperm from a Duroc and a Landrace boar. The two boars were selected based on a previous IVP study with random ovaries, where similar fertilization, polyspermy, cleavage and blastocyst formation rates were obtained [14]. Frozen straws from each individual boar originated from the same ejaculate. Each 2.5 mL straw was thawed at 50 °C for 50 s [15] 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 500 g 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 5×10^5 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 3.0×10^4 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% CO_2 and 7% O_2. At day 4 of culture (fertilization day 0), PZM-5 medium was refreshed by replacing 250 μL of the medium with freshly 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. To assess total blastocyst cell number, blastocysts were fixed in 4% PFA at RT for 30 min, stained with 10 μg/mL Hoechst (H-33342, B2261, Sigma) and mounted in 6 μL fluorescence mounting medium (Dako, Glostrup, Denmark) under a coverslip. 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 410–480 nm emission filter.

2.7. Follicular fluid collection and steroid hormone analysis

During each replicate, follicular fluid (FF) was pooled from all 3–8 mm follicles aspirated per breed. To remove cellular debris, FF was centrifuged at 1,900 g for 30 min and supernatant was poured off. The follicular fluid samples were stored in 1.5 mL aliquots at −80 °C. For steroid analysis, samples were shipped on dry ice to the Department of Pharmacy at the University of Copenhagen. Follicular fluid steroids were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS) and steroids were extracted, purified and analysed [16]. 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-estradiol (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 3–8 mm follicles aspirated was not significantly different between the breeds (23.2 ± 7.0 for Landrace and 21.4 ± 6.5 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. Average follicle diameter was larger for Landrace with 5.7 ± 1.0 mm compared to 4.8 ± 1.0 mm for Duroc ($P < 0.0001$, Fig. 1).

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 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).

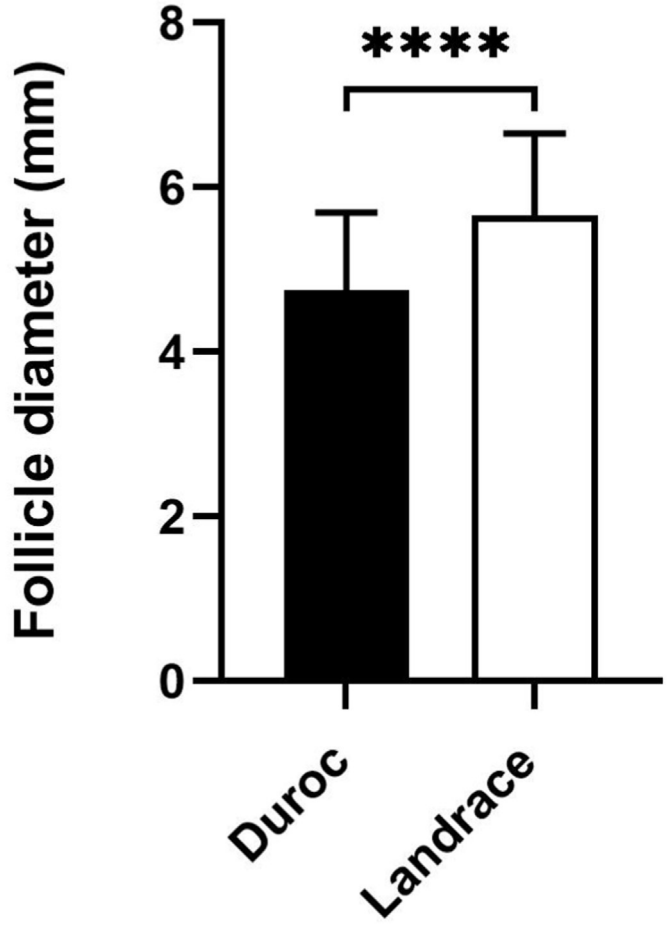

Fig. 1. Boxplot of average follicle diameter (mm) for the 10 largest surface follicles on 43 Duroc and 67 Landrace ovaries one day after weaning. ****P < 0.0001.

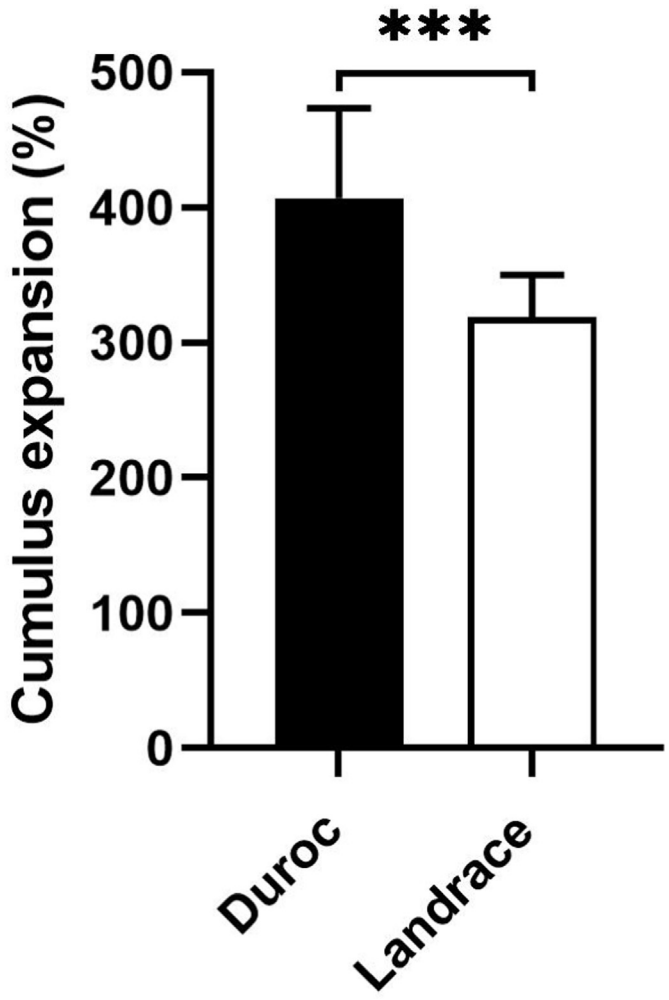

Fig. 2. Cumulus expansion (%) from 0 to 20 h of maturation for Duroc and Landrace cumulus-oocyte complexes (COCs). The cumulus expansion ratio was defined as the total COC area per well at 20 h divided by the total COC area per well at 0 h ***P < 0.001.

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 sperm (P = 0.02). Total blastocysts cell number was not significantly different between the groups.

3.5. Steroid hormones in follicular fluid

The levels of 14 steroid hormones were measured in follicular fluid for both breeds and the hormone pathways are shown in Fig. 3. No significant differences for the individual steroids were observed between the breeds, but a higher total level of estrogens (P = 0.01) was observed for Landrace compared to Duroc.

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

4. Discussion

In our previous study, cumulus expansion from 0 to 20 h of maturation was broader for Duroc oocytes, but the Duroc sows had a higher variation in parity (parity 1 to 6) compared to Landrace (parity 1 to 2) [5]. This could have affected the IVM outcomes and therefore only parity 1 and 2 animals were selected in the present study. Even so, a higher degree of cumulus expansion from 0 to 20 h of maturation was observed for Duroc COCs. Average follicle diameter of the ten largest follicles was 0.9 mm larger for Landrace ovaries compared to Duroc, and individual COC area at time of aspiration was additionally larger for Landrace COCs. This is in line with others who have reported a positive relationship between follicle size and a larger oocyte diameter with more layers of cumulus cells in porcine [11,17]. A broader cumulus expansion at the end of maturation has been reported for oocytes originating from larger preovulatory follicles [18]. This contradicts to results in the present study, but here the expansion could only be analysed at 20 h and this requires a cautious conclusion. Broader cumulus expansion was observed to reduce polyspermy [19] and induce higher in vitro fertilization and embryo development [20]. Present observations on higher cleavage and blastocyst rates in Duroc oocytes compared to Landrace may confirm the earlier observations and further studies on fertilization and polyspermy are of interest.

Clear differences in the follicular fluid steroid pathways were observed between the breeds at the early follicular phase, while oocytes in this phase are still growing and their granulosa cells only

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

Oocytes	Sperm	Zygotes analysed	Cleavage rate D2 (%)	Blastocyst rate D6 (%)	Blastocysts analysed	Total blastocyst cell number
Duroc	Duroc	125	66.1 ± 19.7[a]	14.4 ± 8.3[ab]	15	34.7 ± 15.8
Duroc	Landrace	99	71.6 ± 10.2[a]	24.2 ± 2.9[a]	14	34.9 ± 16.9
Landrace	Duroc	194	54.4 ± 13.6[b]	7.8 ± 9.6[b]	15	44.3 ± 20.4
Landrace	Landrace	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 ($P < 0.05$).

produce low levels of estradiol. A higher estradiol level in the higher reproductive pig breed during the late follicular phase has also been observed in another study [7]. Higher estrogen concentrations in Landrace could be related to an increased androgen substrate that was available and could be converted. Furthermore, the higher total level of estrogens 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 [12] and an increased aromatase activity was additionally observed in granulosa cells from the highly fecund Meishan pigs that had an increased estradiol level compared to Large White [10]. 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 endocrine 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 estradiol and testosterone for Duroc compared to Landrace [21]. Follicular fluid was in this study pooled from all 3–8 mm follicles aspirated and although average follicle size was larger for Landrace, it would be relevant to study steroid hormone levels according to different follicle sizes in both breeds.

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). 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 [22]. Besides steroid hormones, follicular fluid consist of several other components such as growth factors, interleukins, anti-apoptotic factors, proteins, peptides and amino-acid that affect oocyte developmental competence during maturation (reviewed by [23]). 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

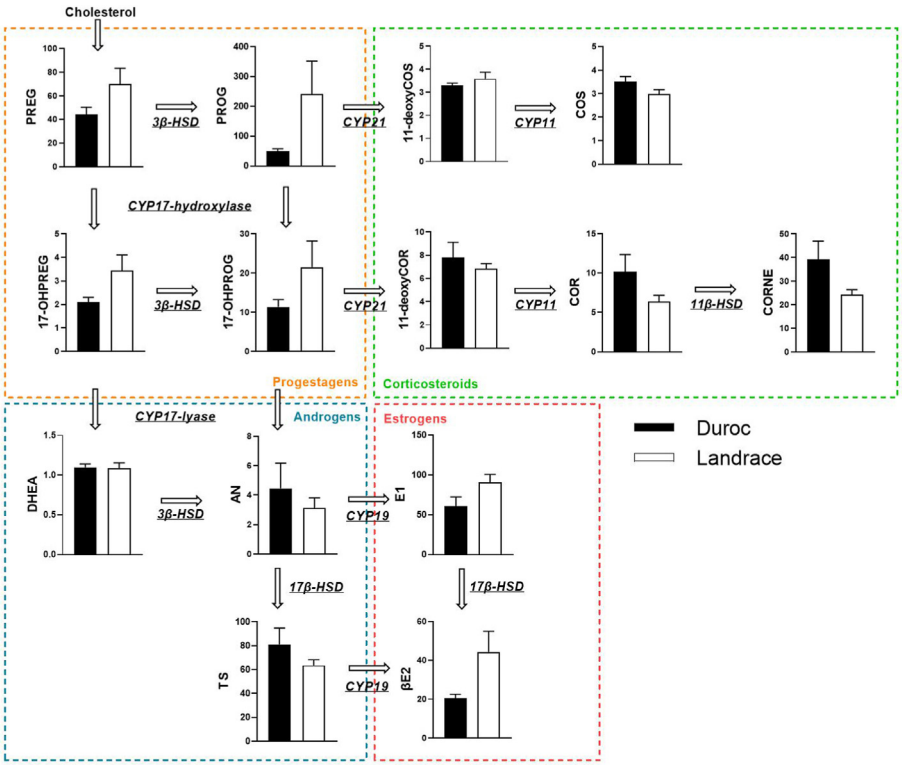

Fig. 3. Steroidogenic overview of the Duroc and Landrace follicular fluid collected in the early follicular phase one day after weaning. Steroid levels were measured in ng/ml.

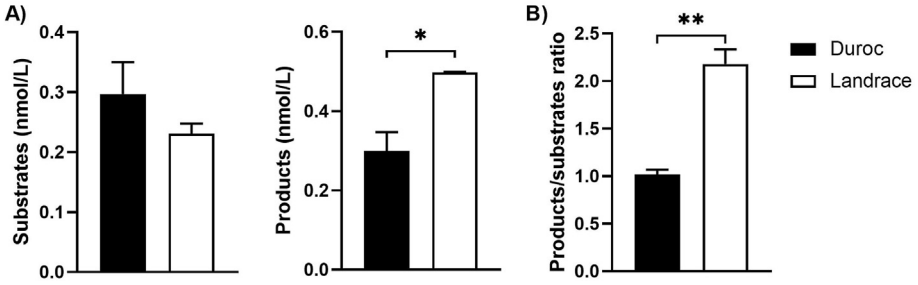

Fig. 4. A) Aromatase substrates (AN + TS) and products (E1 + βE2) and B) Aromatase products/substrates ratio in Duroc and Landrace follicular fluid collected in the early follicular phase.

responsiveness to gonadotropins compared to the breed with fewer follicles were shown. 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 [24]. Although pigs have multiple ovulations during an estrous cycle, further studies in follicular fluid components between Duroc and Landrace may provide insight into differences in oocyte developmental competence and how to improve IVP results. 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 [25].

In conclusion, the results suggest that Duroc oocytes have a better in vitro oocyte development competence compared to Landrace when cultured under the same in vitro conditions, as a broader cumulus expansion from 0 to 20 h of IVM and higher cleavage and blastocyst rates were obtained. Steroid hormone analysis of the follicular fluid showed differences in the pathways with a higher total level of estrogens and aromatase products/substrates ratio for Landrace, indicating higher endocrine 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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Data availability statement

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

CRediT authorship contribution statement

Reina Jochems: Conceptualization, Formal analysis, Investigation, Writing – original draft, Visualization. **Ann Helen Gaustad:** Conceptualization, Formal analysis, Writing – review & editing. **Bjarne Styrishave:** Formal analysis, Investigation, Writing – review & editing. **Louisa J. Zak:** Conceptualization, Writing – review & editing. **Irma C. Oskam:** Conceptualization, Writing – review & editing. **Eli Grindflek:** Conceptualization, Writing – review & editing. **Frøydis D. Myromslien:** Conceptualization, Writing – review & editing. **Elisabeth Kommisrud:** Conceptualization, Writing – review & editing. **Anette K. Krogenæs:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors have no conflict of interest to declare.

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